RESPIRATION AND NITROGEN FIXATION BY BACTEROIDS FROM SOYBEAN ROOT NODULES

SUBSTRATE TRANSPORT AND METABOLISM IN RELATION TO INTRACELLULAR CONDITIONS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AT THE

AUSTRALIAN NATIONAL UNIVERSITY

YOUZHONG LI

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DECLARATION

The research presented in this thesis is my own work unless otherwise stated elsewhere. The project was undertaken in the School of Biochemistry and Molecular Biology, Faculty of Science, at the Australian National University. The material presented in this thesis has not been submitted for any other degree.

Part of work on the bacteroid TCA cycle was completed by Dr Laura Green who was a post-doctoral fellow in Professor David A. Day’s research group.

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July 2002
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to have them missing me for such a long time.

The biggest debt I owe is to my father who passed away while I was studying here. I
dedicate this thesis to my father:

*Dear Dad, this is for you from your son*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ADH</td>
<td>Alanine dehydrogenase</td>
</tr>
<tr>
<td>AMT</td>
<td>Ammonia transport family</td>
</tr>
<tr>
<td>BNF</td>
<td>Biological nitrogen fixation</td>
</tr>
<tr>
<td>Bra</td>
<td>Branched-chain amino acid transporter</td>
</tr>
<tr>
<td>cMDH</td>
<td>Cytosolic MDH</td>
</tr>
<tr>
<td>cDNA</td>
<td>CopyDNA</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>Dct</td>
<td>Dicarboxylate transport system</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraactetic acid</td>
</tr>
<tr>
<td>GOGAT</td>
<td>Glutamine-oxoglutarate amino transferase</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>Lb</td>
<td>Leghaemoglobin</td>
</tr>
<tr>
<td>LbO₂</td>
<td>Oxy-leghaemoglobin</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>ME</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>Nicotine-adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>Nicotine-adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>neMDH</td>
<td>Nodule-enhanced MDH</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomole</td>
</tr>
<tr>
<td>PBM</td>
<td>Peribacteroid membrane</td>
</tr>
<tr>
<td>PBS</td>
<td>Peribacteroid space</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPC</td>
<td>Phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly-β-hydroxybutyrate</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl pyrrolidone</td>
</tr>
<tr>
<td>RB</td>
<td>Reaction buffer</td>
</tr>
<tr>
<td>SS</td>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>VHose</td>
<td>Vacuum connection hose</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>YMA</td>
<td>Yeast extract-mannitol agar medium</td>
</tr>
<tr>
<td>YMB</td>
<td>Yeast extract-mannitol medium</td>
</tr>
</tbody>
</table>
ABSTRACT

Bacteroids of *B. japonicum* from nodules of soybean roots were isolated using differential centrifugation (the standard bench method) and density gradient centrifugation methods (either sucrose- or Percoll-) under anaerobic conditions in which N\textsubscript{2} fixation was preserved. The relationships between N\textsubscript{2} fixation and respiration, O\textsubscript{2} supply, O\textsubscript{2} demand, substrate (mainly malate) transport and metabolism in bacteroids were investigated using the flow chamber system. In related experiments, the primary products of N\textsubscript{2} fixation which leave the bacteroids were investigated using a 15N-labelling technique in a closed shaken system and other biochemical methods.

In the flow chamber experiments, the rates at which O\textsubscript{2} was supplied to bacteroids in the chamber were varied by (a) changing the flow rate of reaction medium through the chamber; (b) by changing the [O\textsubscript{2} free] in the inflowing reaction medium by using either 3-5% (v/v) or 100% air in the gas mixture above the stirred reaction medium in two reservoir flasks; (c) by successively withdrawing bacteroids from the chamber, thus increasing the supply of O\textsubscript{2} per bacteroid to those remaining in the chamber. The results showed that the rate of O\textsubscript{2} supply regulates respiratory demand for O\textsubscript{2} by bacteroids rather than the O\textsubscript{2} concentration present in the reaction system. Respiration is always coupled to N\textsubscript{2} fixation.

Uptake of malate by bacteroids withdrawn from the flow chamber was measured under microaerobic conditions. Malate uptake by these N\textsubscript{2}-fixing bacteroids was lower than that by bacteroids isolated under aerobic conditions, which eliminate N\textsubscript{2} fixation of bacteroids, but is closely correlated with bacteroid respiration rates. When respiration was increased by an increase in O\textsubscript{2} supply, malate uptake by bacteroids was also increased. This suggested that transport of malate through the bacteroid membrane is also regulated by O\textsubscript{2} supply, but indirectly. Higher uptake by bacteroids under aerobic conditions was observed because respiration was enhanced by the high availability of
O₂, but the fast uptake of malate by bacteroids driven by the abnormal respiration rates may not reflect the reality of malate demand in vivo by bacteroids when N₂ fixation by bacteroids is fully coupled.

The results of ¹⁵N labelling experiments and other biochemical assays once again demonstrated that ammonia is the principal significant ¹⁵N labelled product of N₂ fixation accumulated during 30 min in shaken assays with 0.008-0.01 atm O₂. Alanine although sometimes found in low concentrations in the flow chamber reactions, was not labelled with ¹⁵N in shaken closed system experiments. No evidence could be obtained from the other biochemical assays, either. Therefore, it is concluded that these and earlier results were not due to contamination with host cytosolic enzymes as suggested by Waters et al. (Proc. Natl. Aca. Sci. 95, 1998, pp 12038-12042).

Malate transported into bacteroids is oxidized in a modified TCA cycle present in bacteroids. The results of flow chamber experiments with a sucA mutant (lacking α-ketoglutarate dehydrogenase) showed that respiratory demand for O₂ by the mutant bacteroids is regulated by O₂ supply in the same way as the wild-type. Despite differences in other symbiotic properties, rates of nitrogen fixation by the mutant bacteroids, based on the bacteroid dry weight, appeared to be the same as in the wild-type. Also N₂ fixation was closely coupled with respiration in the same manner in both mutant bacteroids and wild type bacteroids. These results and other supporting data, strongly support the conclusion that there is an alternative pathway of the TCA cycle in bacteroids, which enables the missing step in the mutant to be by-passed with sufficient activity to support metabolism of transported malate.
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1 INTRODUCTION

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen, which is a vital element because many nitrogen-containing compounds, such as protein, nucleic acids and other cellular constituents are essential to living systems. There is an abundant supply of nitrogen in the earth’s atmosphere--nearly 79% in the form of N\textsubscript{2} gas. However, N\textsubscript{2} is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. Therefore, nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life.

In order for nitrogen to be used for growth it must be converted, or fixed, from N\textsubscript{2} into more reactive forms of combined N such as ammonia (NH\textsubscript{3}), ammonium (NH\textsubscript{4}\textsuperscript{+}) or nitrate (NO\textsubscript{3}\textsuperscript{-}) ions. There are three processes which are mainly responsible for the conversion of N\textsubscript{2} into NH\textsubscript{4}\textsuperscript{+} or NO\textsubscript{3}\textsuperscript{-}: (1) atmospheric fixation by lightning which produces N-oxides that give rise to NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} ions; (2) industrial fixation produces both oxidized and reduced forms of combined N and (3) biological fixation (usually termed nitrogen fixation) by certain bacteria (alone or in a symbiotic relation with plants) produces reduced forms of N (e.g. NH\textsubscript{4}\textsuperscript{+}). N\textsubscript{2} fixed by abiological, natural processes, including lightning, combustion and volcanism accounts for about 10% of the annual fixation. However, little is known about its direct contribution to agricultural plant production. Man-made processes, mainly industrial ammonia production, contributes about 25% of the total annual fixation, whilst biological nitrogen fixation contributes about 65% of total annual fixation (Newton, 1999). Therefore, from an agricultural perspective, the processes of industrial and biological nitrogen fixation have the greatest potential impact on plant growth and crop yields.
Industrial fixation is a process, which is carried out under a great pressure, at very high temperature (600 °C) with the use of an iron-based catalyst, atmospheric nitrogen and molecular hydrogen. Ammonia formed in this way is used directly as fertilizer, or further processed to urea, ammonium sulphate ((NH₄)₂SO₄) or ammonium nitrate (NH₄NO₃). Industrial nitrogen fixation is an expensive process and consumes almost 1½% of total world fossil fuel used industrially, thus contributing to atmosphere CO₂. The use of industrially made nitrogen fertilizers also has further environmental consequences. Application of large quantities of nitrogen fertilizer, principally as ammonia or urea, to crops each year all around the world result in nitrogen run-off from agricultural land, constituting a major environmental problem; it pollutes ground waters and causes eutrophication of lakes and rivers.

A more environmentally friendly approach to supplying reduced nitrogen for plant growth is through the use of biological nitrogen fixation (BNF). Of the BNF processes, symbiotic nitrogen fixation is the most important agriculturally (Goodchild, 1977). Some bacteria living in a symbiotic relationship with plants of the legume family (e.g., soybean, alfalfa) are able to convert N₂ from the atmosphere to ammonia for use in the host plants; some other bacteria establish symbiotic relationships with plants other than legumes, to fix nitrogen (e.g., actinomycetes with alders). Some free-living bacteria also contribute significant quantities of combined N to natural ecosystems. Nitrogen fixation by the symbiotic association of legumes and rhizobia in root nodules contributes large amounts of combined N, not only to natural ecosystems but also to cropping systems. Estimates of N₂ fixed by various legumes alone in agriculture range from as low as 50 kg N ha⁻¹ annum⁻¹ to several hundred kg N ha⁻¹ annum⁻¹, which accounts for 25% of terrestrial N₂ fixation (Bergersen, 1982). This enables plants to grow in the absence of added nitrogen fertilizer and thus has obvious advantages for sustainable agriculture.

Since Lawes and Gilbert (1890) confirmed the announcement, made by Hellriegel and Wilfarth in 1888 (Schilling, 1988), demonstrating that atmospheric nitrogen was fixed
by nodulated peas and incorporated into the living material of plants, the importance of
the global role of nodulated legumes has been well recognized and research on the
principles and mechanisms of nitrogen fixation in various aspects, such as biochemistry,
cell biology and molecular biology, has been significantly advanced. These can be
briefly summarized as follows.

1.1 Nodules structure and function

Symbiotic nitrogen fixation is a result of a cooperative association formed between host
plant and soil bacteria. The most familiar examples of nitrogen fixing symbioses are the
root nodules of legumes (peas, beans, clovers, etc), which are formed on the roots of
legume plants after infection by soil bacteria, commonly known as rhizobia. There are 5
genera of these soil bacteria (*Rhizobium, Bradyrhizobium, Sinorhizobium,
Mesorhizobium and Azorhizobium*), of which *Rhizobium* species are common, but the
root nodules of soybean, chickpea and some other legumes are formed by infection with
*Bradyrhizobium* species. The interaction of rhizobia and legumes begins with signal
exchange and recognition of symbiotic partners, followed by attachment of the rhizobia
The root hair deforms, and the bacteria invade the plant by a newly formed infection
thread growing through it. Simultaneously, cortical cells are mitotically activated,
giving rise to the nodule primordium. Infection threads grow toward the primordium
and the bacteria are then released into the cytoplasm of the host cells, enclosed in a
plant-derived peribacteroid membrane (PBM). The nodule primordium then develops
into a mature nodule, while the bacteria differentiate into their endosymbiotic form, and
are known as the bacteroids (Bergersen, 1974; Goodchild, 1977) (Fig 1.1a, b). Bacteroids,
together with the surrounding PBMs, are called symbiosomes (Roth *et al.*, 1988). The number of bacteroids which are enclosed in each symbiosome varies from
one (e.g. peanut etc.) to many (e.g., soybean). Bacteroids synthesize nitrogenase and
other supporting enzymes while within the symbiosomes. They accomplish the reduction of atmospheric nitrogen (Bergersen, 1982; Bergersen & Goodchild, 1973; Newcomb, 1981), and then the reduced nitrogen is exported into the host plant (Sprent, 1980).

An important step in the understanding of the biochemistry of nitrogen fixation by bacteroids came with the initial isolation of the bacteroids anaerobically from the nodule breis (Bergersen & Turner, 1967). Using isolated bacteroids, Bergersen and Turner (1967) showed the capacity for $^{15}\text{N}_2$ fixation provided that low levels of O$_2$ were supplied during the reaction period. The addition of succinate, fumarate or pyruvate to bacteroids stimulated both N$_2$ fixation and H$_2$ evolution (Bergersen, 1982; Bergersen & Turner, 1967, 1968). That was an important finding in that it demonstrated that nitrogen fixation takes place in bacteroids and not in the peribacteroid membranes, as had been proposed earlier (Bergersen, 1962a).

1.2 Nitrogenase

Soon after Bortels (Bortels, 1930, 1937) discovered that molybdenum is essential for growth of N$_2$ dependent cultures of Azotobacter chroococcum, it was demonstrated that molybdenum can ease nitrogen deficiency symptoms and increase nitrogen contents of plants dependent on symbiotic N$_2$ fixation (Anderson, 1956; Evans et al., 1950); subsequently, it has thus been well established that molybdenum plays a very important role in most nitrogenases. It is now generally accepted that the commonest nitrogenases are composed of two important components: a Mo-Fe protein and an Fe protein (Thorneley & Lowe, 1984a, b) (respectively termed nitrogenase and nitrogenase reductase; Burris et al., 1980). The Mo-Fe protein has a molecular weight of about 200-240 kDa and is a tetramer composed of 2 different subunits giving a classical tetrahydral $\alpha_2\beta_2$ structure. The metal content of highly purified Mo-Fe protein varies
somewhat according to source and isolation procedures, but consensus values for Mo, Fe and acid labile sulphur are 1.3-2 atoms of Mo, about 30-32 atoms of Fe and 24-34 atoms of S (Israel et al., 1974; Lowe et al., 1985; Thorneley & Lowe, 1996). The molecular weight of each subunit is around 50-60 KDa, depending on methods used, (e.g., the α and β differ by about 4 KDa in nitrogenase from *Klebsiella pneumoniae* (Kennedy et al., 1976). Four discrete, cubane Fe₄S₄ clusters, are designated as P clusters and contain half of the total iron, the other half of the iron is contained in 2 moles of a metal cluster, designated as FeMo cofactor (FeMoco), each containing one Mo and half of the labile sulphur of the tetrameric protein (Stiefel & Cramer, 1985). The X-ray structure of nitrogenase from *A. vinelandii* showed that the contacts between the αβ-pairs are almost entirely between the β-subunits. The tetramer contains 30 Fe and 2 Mo atoms, which are distributed between two types of cluster, the “P” cluster (8 Fe, 7 S²⁻) and the FeMo-cofactor (7 Fe, 1 Mo, 9 S²⁻, 1 homocitrate). Each αβ-pair of subunits, which function independently of each other, therefore contains one P cluster and one FeMo-cofactor (Thorneley & Lowe, 1996). The P cluster together with FeMo-cofactor account for all functional iron and molybdenum atoms, and are responsible for the redox properties of the MoFe protein (Miller, 1991). The P cluster is likely to be involved in electron transfer between Fe protein and MoFe cofactor (Chan, 1993), while the MoFe cofactor is involved in substrate binding (Shah & Brill, 1977).

All purified Fe-proteins are dimers (α₂) of identical subunits with total molecular weights ranging from 58-72 KDa. They are now considered to contain one [4Fe-4S] cluster per dimer (Yates, 1992). It is assumed that these clusters are crucial to the Fe protein’s role, but how a single [4Fe-4S] cluster is bound between two identical subunits is not clear. The Fe protein has two binding sites for MgATP, one on each subunit and binds two MgATPs in both its oxidized and reduced states (Burgess, 1984; Yates, 1992). The Fe-protein is considered to function as a specific electron donor to the larger, MoFe protein, in a reaction that is coupled to the hydrolysis of ATP (Seefeldt et al., 1999).
Nitrogenase catalyses the reduction of N₂ to NH₃, as in the following equation:

\[ N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi \]

However, up to now, the mechanisms and reaction processes relating to nitrogenase activity are not very clear. Taking into account most of the observations made in experiments with isolated nitrogenase proteins and predicting accurately many observed features of N₂ fixation from the Kp1 (MoFe protein from *K. pneumoniae*) and Kp2 (Fe protein from *K. pneumoniae*) systems *in vitro*, Lowe and Thorneley (1984; see Burgess & Lowe 1996 and Lowe *et al.*, 1985) proposed a model, to describe this process in terms of enzyme kinetics. According to the model, the catalytic mechanism of nitrogenase contains two cycles, the Fe protein cycle (Fig 1.2a) and the MoFe protein cycle (Fig 1.2b). The reaction sequence starts from the reduced Fe protein being in its contracted MgATP conformation and is followed by formation of an active complex with the MoFe protein. In the next step, an electron is transferred from the Fe protein to the MoFe protein and 2 MgATP are concomitantly hydrolyzed to MgADPs and 2 Pis. Following MgATP hydrolysis, electron transfer and the release of inorganic phosphate, a complex that contains the oxidized Fe protein, two MgADPs and the reduced MoFe protein which has been reduced by one electron is formed. Next is the slow dissociation of the MoFe protein/Fe protein-MgATP complex in the rate-limiting step of the cycle, which is completed when the Fe protein is reduced by a nitrogenase electron donor and MgATP replaces MgADP. Following the first round of electron transfer, the free reduced MoFe protein can go on to form a complex with Fe protein Mg ATP and the cycle can be repeated over and over again.

In order to reduce N₂ to NH₃ and evolve H₂, the MoFe protein cycle (Fig. 1.2b) comprises 8 Fe protein cycles and transfers a total of 8 electrons and 8 protons to one-half of the MoFe protein. The reduction of N₂ in the scheme requires that MoFe protein
first undergoes 3 or 4 single electron reduction steps each requiring completion of one Fe protein cycle. N$_2$ can bind only to the MoFe protein (generally to the MoFe cofactor centre) either at the 3rd or 4th step (Fig 1.2b, E$_3$H$_3$ or E$_4$H$_4$) with displacement of a H$_2$ molecule. After the MoFe protein is further reduced and one NH$_3$ is released (probably at the oxidation state E$_5$), it returns to its highest oxidation state, E$_0$. The rate-limiting step of reactions in the cycle is the protein-protein dissociation of complex FeP$_{ox}$(MgADP)MoFeP$_{red}$ (Fig 1.2a) and not ATP hydrolysis, electron transfer or substrate reduction (Yates, 1992).

Another important feature of nitrogenase activity is H$_2$ evolution (Fig 1.2c), which is an indispensable function of the reduction of N$_2$ to 2NH$_3$. Sometimes, in the absence of N$_2$, nitrogenase activity in some experimental systems can be assessed by measurement of H$_2$ evolution (e.g. King & Layzell, 1991).

In addition to Mo-nitrogenase, it is now known also that some organisms (e.g. *A. chroococcum* and *A. vinelandii*) have alternative nitrogenase systems based on vanadium (V) and Fe or apparently on Fe alone. The alternative nitrogenases are broadly similar to Mo-nitrogenase in most physico-chemical properties and in their requirements for activity (Pau, 1991), but they differ from the Mo nitrogenases in their subunit structure because they have an additional $\delta$ subunit. There has been plenty of data on this protein so far, much of which has focused on the type of redox centres that the VFe proteins contain. How the presence of V changes the catalytic properties, compared with the more intensively studied Mo-nitrogenase, has also been studied (Eady, 1996; Pau, 1991).

As stated above, most of the knowledge about nitrogenase comes from studies with *K. pneumoniae, A. chroococcum* and *A. vinelandii*. From 1966, Evans and his colleagues (Koch *et al.*, 1967a) transferred this information to the study of the legume root nodule system and the activity of nitrogenase isolated from soybean bacteroids. Using the
C$_2$H$_2$-reduction technique, they reported nitrogenase activity in cell-free extracts of soybean bacteroids and recognized two nitrogenase components (Koch et al., 1967a, b). An important aspect of their work was the use of polyvinyl pyrrolidone (PVP) which aided in the removal of plant phenolics during preparation of bacteroids. However, the structure and biochemical properties of nitrogenase from legumes were not thoroughly studied until good yields of bacteroids could be harvested anaerobically (Israel et al., 1974; Whiting & Dilworth, 1974) to allow nitrogenase to be purified in its active state. Functional nitrogenase was purified by directly fractionating cell-free extracts anaerobically on DEAE cellulose columns followed by salt elution. Nitrogenase eluted from the columns as two components, the MoFe protein and the Fe protein. Both fractions were then further purified, to homogeneity, by anaerobic Sephadex gel chromatography (Israel et al., 1974; Whiting & Dilworth, 1974).

It was found that the biochemical properties and reaction sequences of the nitrogenase from legume nodule bacteroids is similar to the nitrogenase from *K. pneumoniae*, *A. chroococcum*, *Clostridium pasteurianium* and *A. vinelandii* (Bergersen et al., 1982; Burris et al., 1980; Kennedy et al., 1976; Turner & Bergersen, 1969; Whiting & Dilworth, 1974). The mechanisms of the reaction sequences relating nitrogenase are almost completely compatible with the current models of nitrogenase action (see above). It is commonly accepted that the organization of Mo-nitrogenase, encoded by 20 genes grouped in 8 operons in *K. pneumoniae*, is highly conserved in free-living and symbiotic diazotrophs (Fig. 1.3a). Of the 20 genes, 3 are responsible for the structure of the Mo-nitrogenase. They are *nifD* and *nifK* (for MoFe protein) and *nifH* (for Fe protein). The genes, *nifB*, *nifE* and *nifN* are required for synthesis of the FeMo-cofactor during the assembly of nitrogenase (Dean & Jacobson, 1992). The gene designated as *nifW* could participate in oxygen protection of the FeMo protein (Kim & Burgess, 1996). So far, it has been found that 10 *nif* genes of rhizobia are structurally homologous to those of *K. pneumoniae* (Arnold et al., 1988) and it is inferred that conserved *nif* genes play similar roles in *K. pneumoniae* and *Rhizobium meliloti*, *B.
japonicum, and A. caulinodans (see review by Fischer, 1994). In R. meliloti, the genes nifHDK coding the α and β subunits of MoFe protein and the homodimeric Fe protein are organized in an operon along with nifE, but nifHDK and nifE in A. caulinodans form two separate transcriptional units (Denefle et al., 1987; Masepohl et al., 1989), whereas in B. japonicum the nifH gene is located in a different operon, downstream of the nifDKENX operon (Fig 1.3b). In rhizobia, nifE, nifN and nifB also may be involved in synthesis of FeMoco of MoFe proteins. All the nif and fix genes of R. meliloti are located on a large plasmid (megaplasmid 1) but in B. japonicum and A. caulinodans they are located on the chromosome. The organization of these genes on the chromosome in B. japonicum has been characterized in 4 clusters (Fig. 1.3b) (reviewed by Fischer, 1994).

1.3 Leghaemoglobin

Leghaemoglobin (Lb), long known as a unique higher plant haemoglobin, exists at near mM concentration only in infected cells of legume root nodules and some other nodulated non-legumes (Appleby, 1992). However, genes coding for analogous haemoglobins are now known to be expressed in other plant tissues (Appleby, 1998). In legume nodules, the presence of Lb is closely correlated with N₂-fixing activity (Appleby, 1984). Since Kubo (1939, cited in Bergersen, 1982) first discovered that the long observed red pigment in legume nodules had spectral characteristics of a haemoglobin and Evans & Russel (1971) summarized evidence correlating the concentrations of this pigment in legume nodules with N₂-fixing effectiveness, a series of investigations on its chemical composition, amino acid sequence, kinetics of binding and release of O₂ and many other properties have been undertaken (Appleby, 1984; Bergersen, 1982).
1.3.1 Localization of Lb in legume nodules

It is now known that Lb is present principally in solution in the cytoplasm of infected nodule cells at a local concentration as high as 2-3 mM (Appleby, 1992; Bergersen, 1982), but whether it occurs also within peribacteroid membrane space (the space between the bacteroid surface and the peribacteroid membrane) has been controversial (Appleby, 1985; Bergersen, 1982; Bergersen & Appleby, 1981; Verma & Bal, 1976; Verma et al., 1978). Now it is generally accepted that Lb is confined to the host cytoplasm (Appleby, 1992).

1.3.2 Multiple components of Lb

Ellfolk (1972), using ammonium sulphate fractionation of nodule extracts from soybean, first separated and purified two stable major species and two minor species of Lb which he named Lba, Lbb, Lbc and Lbd. Later, using the isoelectric focusing technique, Fuchsman (1992) revealed that Lb contains four major molecular species Lba, Lbc1, Lbc2 and Lbc3 and four minor species of Lbb, Lbd1, Lbd2 and Lbd3. All the species were present in soybean nodules and their optical, ligand binding and redox properties are very similar (Appleby & Bergersen, 1980).

1.3.3 The optical spectra and oxygenation of Lb

Both crude extracts containing Lb (Kubo, 1939, cited in Bergersen, 1982) and purified Lb (Appleby & Bergersen, 1980) showed a similar optical spectrum when it was oxygenated, with two sharp optical absorption bands at 575 and 540 nm. When Lb is deoxygenated it only has one broad band at 557 nm. Further, only the oxygenated ferrous Lb has the former optical spectrum and the deoxygenated ferrous Lb has the latter. However, when Fe$^{2+}$ ion in Lb is oxidized to Fe$^{3+}$, Lb cannot be oxygenated and has a different optical absorption spectrum. The optical spectra of Lb are similar to...
those of animal haemoglobins and myoglobins and different from those of catalases, peroxidases or oxygenases. This indicates that the role of Fe$^{2+}$ Lb is reversible oxygen combination rather than being involved in redox or oxygen-activation functions (Appleby, 1992). Thus, its optical characteristics, together with kinetics of oxygenation/deoxygenation, can be used to measure free, dissolved O$_2$ in Lb solutions in physiological and biochemical studies of legume bacteroids (Appleby, 1992; Appleby & Bergersen, 1980) and other microaerobic bacteria (e.g. *K. pneumoniae*; Bergersen *et al.*, 1982). The technique enables measurements to be made at concentrations in the nanomolar range of dissolved O$_2$, i.e. at about 1% of that achieved by the best electrometric methods.

1.3.4 *Facilitation of O$_2$ flux to nitrogen fixing bacteroids by oxygenated Lb*

Studies on nitrogenase have shown that its Fe protein and MoFe protein components are inactivated by free O$_2$ (Robson & Postgate, 1980). Not only does O$_2$ cause rapid, irreversible oxidation of nitrogenase metal-S centres, it also represses the synthesis of nitrogenase proteins (Shaw, 1983). It is now generally accepted that leghaemoglobin plays a very important role in N$_2$-fixation in root nodules. The presence of Lb permits the rapid transport and distribution of O$_2$ throughout the nodules to the bacteroid surface at very low concentrations of free, dissolved O$_2$ that do not inactivate nitrogenase nor repress its synthesis. Leghaemoglobin facilitates the diffusive flux of O$_2$ to the vigorously respiring, phosphorylating and N$_2$-fixing *Rhizobium* bacteroids at very low concentrations of free, dissolved O$_2$ (Appleby, 1984). In this process, provided the leghaemoglobin is partially oxygenated, the very small diffusive flux of free, dissolved O$_2$ (present at sub-$\mu$M concentrations) is greatly augmented by the diffusion flux of oxyleghaemoglobin (present at near-mM concentrations) in the cytoplasm of the infected cells. At all points in the diffusion path, the concentration of free O$_2$ remains low because it is in equilibrium with the high-affinity, partially oxygenated Lb. Kinetics
study showed that Lb from soybean has a very high affinity for O₂, with a very rapid association constant and a slower (still moderately fast) dissociation constant:

\[
Lb + O_2 \xrightleftharpoons[k_1]{k_2} LbO_2
\]

In this reaction: \(k_1 = 120 \, \mu M \, sec^{-1}\), \(k_2 = 5.6 \, sec^{-1}\) and \(K_{eq} = k_2/k_1 = 47 \, nM\) (Gibson et al., 1989).

The facilitation of O₂ flux by Lb is also considered as one of possible protective mechanisms for nitrogenase (Gibson et al., 1989).

### 1.3.5 Purification and uses of Lb in experiments with gas phases

Leghaemoglobin is easily extracted from the nodules by crushing them in dilute buffers, filtering and centrifuging. The supernatants are then fractionally precipitated with (NH₄)₂SO₄ followed by column chromatography, reduction and oxygenation of the protein. In the extraction and purification, a large amount of nodules (either frozen or fresh or both) from soybean can be easily harvested and a fairly good yield of Lb can be obtained (ca 20 \(\mu\)moles [200g]⁻¹ nodules). Lb purified using the above method after oxygenation is remarkably resistant to autoxidation, provided good quality water, free of metallic ions is used. Thus, oxy-Lb (LbO₂) can be used in physiological and biochemical studies (Appleby & Bergersen, 1980). Also, good yields of LbO₂ have been obtained from other legumes, such as lupins, cowpeas etc., using similar methods (Bergersen, 1982).

As stated above, Lb has characteristic optical absorption spectra, the molar absorptivities of various peaks for ferrous, ferrous-oxy and ferric Lb in the visible range are known and the concentration of Lb may be estimated. However, because Lb is often partially oxygenated, or not fully reduced, greater accuracy is obtained for concentration measurements by using the spectrum of the reduced minus oxidized pyridine
haemochrome (Bergersen & Turner, 1973). Because its concentration can be calculated, Lb can be used routinely in physiological and biochemical experiments with suspensions of bacteroids prepared from nodules (Bergersen & Turner, 1990a), with suspensions of agar-grown bacteria (Bergersen et al., 1982) and with continuously cultured N₂-fixing bacteria (Bergersen et al., 1986). A good example of using purified Lb in the physiological experiments is that in which Bergersen and his colleagues measured N₂ fixation, O₂ consumption, uptake of substrates and ammonium production of soybean bacteroids under standardized conditions at low concentration of free dissolved O₂ in a flow-chamber apparatus which they developed (Bergersen & Turner, 1990a, 1993; Bergersen et al., 1982).

1.3.6 Diffusion of O₂, O₂ consumption and N₂ fixation in symbiosomes

It is a common knowledge that the nitrogenase enzyme is extremely sensitive to O₂ which causes irreversible damage to it (Robson & Postgate, 1980). On the other hand, both nitrogen fixing bacteroids and the infected cells of the host plant which supports nitrogen fixation, require ATP generated through oxidative phosphorylation. Thus it is very important to understand the mechanisms that legume nodules use to maintain precise control over the O₂ situation within them, i.e. how legume nodules regulate the O₂ flux to accommodate a high rate of respiration and at the same time maintain an appropriate low O₂ concentration in infected cells, to protect nitrogenase.

It has been suggested that the first level of oxygen protection is a thin layer designated as the barrier or boundary layer of cells located in the nodule inner cortex containing few gas-filled intercellular spaces (Minchin & Witty, 1990; Parsons & Day, 1990). This layer restricts O₂ from entering the inner cytoplasm from the surrounding atmosphere (Minchin & Witty, 1990; Parsons & Day, 1990) and allows a region of low [O₂] to be generated in the central zone of a nodule. The infected cells of nodule central tissue constitute a compact, intensive sink for O₂ (they contain more mitochondria than other
plant tissue (Millar et al., 1995) and thousands of bacteroids) (Bergersen, 1997a, b). Consequently, the high respiratory demand by both mitochondria and bacteroids of the infected cells generate very low concentrations of O₂ within them and thus, together with the diffusion barrier, confine [O₂] within a safe limit for nitrogenase activity (Robson & Postgate, 1980). Several simulation models have been developed and used to describe the profiles of [O₂], O₂ transport and nitrogen fixation functions in total nodule systems and in infected cells of nodule central tissue (Bergersen, 1994, 1996, 1999; Sheehy & Bergersen, 1986; Sheehy et al., 1987; Thumfort et al., 1994). These may be used to predict metabolic consequences of changes of applied conditions such as to ambient pO₂ or to substrate availability.

1.4 Regulation of nitrogen fixation by legume nodules

The regulation of biological nitrogen fixation in diazotrophic bacteria has attracted much research interest in recent years. So far, a body of evidence has been widely achieved at the genetic level from nitrogen fixing organisms, such as K. pneumoniae, A. chroococcum, A. vinelandii, R. meliloti and B. japonicum. The principal features of the transcription of \textit{nif} genes are highly conserved and the regulation of their expressions have been well documented (reviewed by Fischer, 1994 and Foussard et al., 1998). Because of space limitation, only the regulation of nitrogen fixation in \textit{B. japonicum} will be stressed in this section.

In \textit{B. japonicum}, N₂ fixation occurs only in the symbiotic state. In fact, even under stringently controlled, chemostat conditions only traces of nitrogenase activity have been detected in cultures in a few strains of \textit{Bradyrhizobium} spp. Therefore the following information is based on genetic analysis of mutants and constructs. In this organism, as in most of the nitrogen-fixing bacteria, expression of the genes involved in nitrogen fixation (\textit{nif} and \textit{fix}) is induced by low-oxygen concentrations. In this
induction, NifA (which belongs to the bacterial enhancer-binding protein family of transcriptional regulators that activate transcription from promoters recognized by the δ54-holoenzyme form of RNA polymerase) is believed to be the central regulator (Fischer, 1994; Kustu et al., 1989; Merrick, 1993; Morret & Segovia, 1993). NifA in *B. japonicum* is encoded in the *fixR-nifA* operon which responds to changes in atmospheric composition. Under anaerobic conditions in the root nodule or at low levels of oxygen in cultures, the *nifR-fixA* operon is induced (Thöny et al., 1989). However, its expression is strongly repressed to a lower level (1/5 of anaerobic) under aerobic conditions. Transcriptional analysis has shown that the induction of the expression of *fixR-nifA* operon by oxygen is dependent on two different overlapping promoters, *fixRp1* and *fixRp2*, which are dependent on the upstream activator sequence (UAS) (Borris et al., 1995, 1998). Recent studies have shown that UAS is activated by a newly found response regulator RegR (Bauer et al., 1998). Under aerobic conditions, RegR binds to the *fixR-nifA* UAS and activates transcription from the *fixRp2* promoter, leading to a low-level expression of *fixR-nifA* and a low level synthesis of NifA. If the environment is anaerobic, NifA activates its own expression (autoregulation) (Bauer et al., 1998) and then activates transcription of all of its target, *nif* and *fix* genes.

In *B. japonicum*, although *nifA* expression does not depend upon *fixLJ* as in *R. meliloti* and *A. caulinodans* (reviewed by Fischer, 1994; Foussard et al., 1998), FixLJ and FixK₂ are required for microaerobic induction of the *fixNOQP* genes, responsible for the synthesis of a high-affinity terminal oxidase (a distinct cytochrome oxidase) for respiration under microaerobic conditions (Hennecke, 1998; Preisig et al., 1996). Also *fixLJ* mutants of *B. japonicum* are unable to grow under anaerobic conditions with nitrate as the terminal electron acceptor. This shows the presence of other complex regulation in *B. japonicum*. 
1.5 Metabolite transport across symbiotic membranes of legume nodules

One of the most significant nutrient exchanges between two organisms of a symbiosis (e.g. soil bacteria and a higher plant) is fixed N\textsubscript{2} from the bacteria for a source of reduced carbon from the plant. The nature of this exchange is quite complicated and has attracted intensive research in recent years. The most comprehensive examination of the process has been between legume plants and nitrogen-fixing soil bacteria of the family Rhizobiaceae (rhizobia).

1.5.1 Carbon supply to bacteroids

During nodule development (see 1.1) and throughout the period of active nitrogen fixation, the symbiotic bacteria are dependent upon substrates supplied from the host cell in which they are embedded. During active N\textsubscript{2} fixation, an important substrate supplied to nodules is photosynthetically-produced sucrose (Kouchi & Yoneyama, 1986; Streeter, 1980, 1986), but this is not able to support bacteroid activity and is broken down via phosphoenolpyruvate (PEP) to C\textsubscript{4}-dicarboxylates, such as succinate and malate, which are readily used for energy production via bacteroid respiration (Bergersen, 1958; Bergersen & Turner, 1967; Day \textit{et al}., 1989; Streeter, 1987). The enhanced metabolism of sucrose to C\textsubscript{4}-carboxylates involves coordinated expression and control of three critical enzymes: sucrose synthase (SS), phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) (reviewed by Vance \textit{et al}., 1998) (Fig 1.4).

Sucrose synthase is an important enzyme in sucrose metabolism of root nodules. It predominately catalyses the initial step of sucrose hydrolysis (Day & Copeland, 1991). Evidence for SS roles in cleavage of sucrose in nodules are that SS is inhibited by glucose and has a high affinity for UDP, and that SS protein comprises a significant (0.5-1.0\%) proportion of nodule protein, and was among the first proteins to be
identified as a nodulin (nodule-enhanced protein) (Morell & Copeland, 1985). Recent
molecular characterization of sucrose synthase has been evaluated in legumes. Full-
length cDNAs of SS have been isolated from broad bean, alder and alfalfa (reviewed by
Vance et al., 1998). SS cDNA is highly expressed in effective nodules but is maintained
at background levels in ineffective nodules (Anthon & Emerich, 1990; Vance et al.,
1998), suggesting that SS expression in nodules is closely linked to nodule functioning.

Nodule PEPC provides oxaloacetate, which can be subsequently reduced to malate by
MDH. It is estimated that PEPC can provide as much as 30% of the C required for
nitrogen fixation and N assimilation (Maxwell et al., 1984). Both in vivo and in vitro
CO₂ fixation by PEPC is much higher in effective nodules than in ineffective nodules
(Egli et al., 1989; Rosendahl et al., 1990). A significant proportion of malate derived
from nodule CO₂ fixation by PEPC is immediately transported to bacteroids to support
nitrogenase (reviewed by Vance et al., 1998). Regulation of nodule PEPC is achieved at
both transcriptional and post-transcriptional levels (Chollet et al., 1996). In alfalfa, fava
bean and pea, PEPC activity, protein, and mRNA were enhanced several-fold during
effective nodule development although it is not directly coupled to nitrogenase. Studies
with alfalfa and soybean showed that PEPC can be phosphorylated in vitro. This
phosphorylation can reduce the sensitivity of PEPC to malate inhibition and allows the
synthesis of organic acids to continue even at a high concentration of malate in the
cytosol (reviewed by Vance et al., 1998). Pathirana et al. (1997) successfully isolated a
gene encoding the nodule enhanced PEPC, PEPC7. Immunogold and in situ
hybridisation studies later provided direct evidence that PEPC is expressed in the
parenchyma of effective nodules (reviewed by Vance et al., 1998).

Studies on MDH of alfalfa, lupin and pea have shown that its activity is correlated with
the increased amount of malate in root nodules. Kinetic analysis of the pea nodule-
enhanced cytosolic MDH showed that this enzyme had a higher affinity for oxaloacetate
and NADH than for malate and NAD⁺ (reviewed by Vance et al. 1998). It is suggested
that nodule-enhanced MDH (neMDH) favoured production of malate required for nodule metabolism (reviewed by Vance et al. 1998). Further, cDNAs and transcripts encoding different forms of MDH (neMDH and cytosolic MDH (cMDH)) have been characterized and the enzymes purified. The kinetics also have been analysed. The results showed that neMDH was more highly expressed in effective nodules than in any other tissues. Immunoblots confirmed that this expression was coincident with the respective MDH proteins (reviewed by Vance et al., 1998). Kinetic analysis of neMDH and cMDH indicated that the neMDH has a turnover rate and specificity constant that can account for forming enormous quantities of malate, as compared to cMDH (Miller, 1998; Vance et al., 1998).

C₄-dicarboxylates are oxidized within bacteroids, but the exact metabolic pathways involved in this process are not very clear. It is postulated that the oxidation of these acids involves the tricarboxylic acid (TCA) cycle. Enzyme activity and radiorespirometry have shown that the TCA cycle is present in B. japonicum (Keele et al., 1969; McKay et al., 1988) and all TCA cycle enzymes activities have been observed in several Rhizobium species (Finan et al., 1988, 1991; McKay et al., 1989; Saroso et al., 1986). Also, there is some genetic evidence showing the involvement of the TCA cycle and C₄-dicarboxylates in nitrogen fixation in bacteroids. For example, some mutants of R. meliloti exist which lack isocitrate dehydrogenase (McDermott & Kahn, 1992), α-ketoglutarate dehydrogenase (Duncan & Fraenkel, 1979) and succinate dehydrogenase (Gardiol, 1987), impairing their ability to undertake nitrogen fixation. However, mutants of B. japonicum lacking α-ketoglutarate dehydrogenase can grow with malate and succinate as their carbon sources and still fix nitrogen although fewer bacteroids occur in the nodules. This indicates that, at least part of the TCA cycle, can be by-passed in this case (Green & Emerich, 1997a, b; Green et al., 2000).

In R. leguminosarum, on the other hand, it is unlikely that there is an alternative pathway of the TCA cycle. In this rhizobium, the TCA cycle appears to be regulated in
other ways. Walshaw et al. (1997) showed that when the α-ketoglutarate dehydrogenase complex was blocked in a sucA mutant, this mutant formed ineffective nodules. However, it excreted large quantities of glutamate and α-ketoglutarate, resulting in a highly elevated intracellular concentration of glutamate, indicating that glutamate is not important carbon and energy source in *R. leguminosarum* bacteroids. Instead, glutamate appeared to be a sink for carbon and reductant. α-ketoglutarate fed into the intracellular glutamate pool, leading to glutamate excretion, resulting in an overflow pathway for regulation of the TCA cycle. These results are quite different from those observed with the *B. japonicum* sucA mutant (see above). In addition, PHB biosynthesis was thought to function in a similar way in *R. leguminosarum* to form a second overflow pathway to regulate carbon and redox balance of the TCA cycle, as activities of malate dehydrogenase and isocitrate dehydrogenase were unaffected in a PHB mutant (Walshaw et al., 1997).

Since the principle source of reduced carbon for bacteroids is \( \text{C}_4 \)-dicarboxylates, subsequent reduction through the TCA cycle requires a pathway to generate acetyl-CoA and an enzyme which operates the cycle by oxidizing dicarboxylates (malate + NAD(P) → pyruvate + CO\(_2\) + NAD(P)H). Studies have indicated that high levels of malic enzyme activity are present in bacteroids (Copeland et al., 1989a; Kimura & Tajima, 1989; McKay et al., 1988). During the operation of the TCA cycle, malic enzymes are thought responsible for oxidative decarboxylation of malate to pyruvate, with concomitant reduction of nicotinamide cofactor. Two forms of malic enzymes which require different coenzymes have been identified requiring NAD or NADP. In soybean bacteroids, NADP\(^+\) dependent malic enzyme (NADP-ME) has a much higher affinity for malate \( (K_m = 0.1 \pm 0.01 \text{ mM}) \) than NAD\(^+\)-dependent malic enzyme (NAD-ME; \( K_m = 1.9 \pm 0.4 \text{ mM} \)) and the former can be stimulated by ammonium (Copeland et al., 1989a). Thus it is considered that NADP-ME is of physiological importance for bacteroid metabolism in soybean. However, in alfalfa, Driscoll and Finan (1996) found that the *dme* mutant of *R. meliloti* (lacking NAD-dependent malic enzyme) failed to fix
nitrogen in nodules although nodules containing bacteroids were still induced (Driscoll & Finan, 1993, 1997). Both the NAD-dependent malic enzyme gene \((dme)\) and the NADP-dependent malic enzyme gene \((tme)\) of \(R.\) meliloti have been cloned and localized (Driscoll & Finan, 1993, 1997). The \(dme\) gene was expressed constitutively in both free-living cells and \(N_2\) fixing bacteroids, whereas the \(tme\) gene was repressed in bacteroids (Driscoll & Finan, 1997). These authors also demonstrated that the activity of the NAD-ME could be inhibited by acetyl-CoA, but the activity of NADP-ME could not. These results together support the hypothesis that in \(R.\) meliloti NAD-ME, together with pyruvate dehydrogenase, forms a pathway in which malate is converted to acetyl-CoA (Fig 1.5).

There is also some evidence that amino acids can be a source of carbon for bacteroids. Glutamate was reported to stimulate respiration of bacteroids (Bergersen & Turner, 1988, 1990b). Enzymes involved in proline metabolism are distributed in both the host and bacteroid cytoplasm, suggesting the possibility of active proline utilization by bacteroids (Kohl et al., 1988). In addition, glutamate, glutamine, glycine, serine, and most tryptophan auxotrophs of Rhizobium produce effective nodules (see review by Udvardi & Day, 1997), suggesting that amino acids may be supplied to bacteroids by their host plants and perhaps used as carbon sources. Firm evidence for this, however, is lacking.

1.5.2 Transport of carbon compounds across symbiotic membranes

In the infected cells of nodules, bacteroids are surrounded by the host-derived PBM (Fig.1.1a). This membrane separates the microsymbionts from the cytoplasm of the host cell and is not only a structural barrier between symbionts but also is a functional barrier. Thus the PBM, together with the bacteroid membrane must play very important roles in regulating the transport of metabolites between the symbiotic partners and in facilitating metabolic coupling between the two.
Numerous reports have shown that rhizobia contain a specific dicarboxylate transport system (Dct), which is responsible for the uptake of malate and succinate (reviewed by Jording et al., 1994). Ronson et al. (1981) first isolated a Dct- mutant that failed to grow on or transport succinate, fumarate or malate, and that formed nodules on red and white clover which were ineffective; revertant strains became effective again. Finan et al. (1983) reported similar results with pea (R. leguminosarum). The Dct system is energy-dependent because its activity is inhibited by uncouplers and metabolic poisons (Finan et al., 1981; Ou Yang et al., 1990). The Dct system has a high affinity for substrates, such as succinate, malate, and fumarate ($K_m$ values range from 2-15 $\mu$M) and is very active ($V_{max}$ from 10-80 nmol min$^{-1}$ [mg protein]$^{-1}$ in different genera) (reviewed by Udvardi & Day, 1997). Genetic studies show that there are 3 genes, which are related to the encoding of the Dct system ($dctA$, $dctB$ and $dctD$). The gene $dctA$ encodes for the dicarboxylate transporter, DctA, while $dctB$ and $dctD$, are considered to form a regulatory system that monitors the availability of dicarboxylate and interacts with the alternative sigma factor NtrA to regulate transcription of $dctA$ (Jording et al., 1994).

Isolated bacteroids may also transport amino acids (Dilworth & Glenn, 1981; Salminen & Streeter, 1987; Udvardi & Day, 1989). Transport studies with R. leguminosarum indicated that it has a general amino acid transport system, able to transport a wide range of L-amino acids, such as asparagine, aspartate, glutamate, glutamine, glycine, methionine and phenylalanine (Poole et al. 1985), with a very high affinity ($K_m$ 0.081 $\mu$M). Sequence analysis revealed that this system is a general amino acid permease ($aap$). Walshaw & Poole. (1996) recently have cloned the genes involved in this system and shown that it is an ATP binding cassette (ABC) transport protein consisting of four genes: $aapJQMP$, organised in one operon. AapJ is a periplasmic binding protein, AapQM are integral membrane proteins and Aap is the ABC subunit characteristic of this class of transporter (reviewed by Day et al., 2001; Poole & Allaway, 2000).
Previous studies showed that ABC transport systems of amino acids are usually very specific for a single amino acid or a related group of amino acids (Walshaw et al., 1997), however, the general amino acid permease of *R. leguminosarum* is unusual in that it can transport a wide range of L-amino acids, and therefore represents a new sub-class of amino acid transporter (Walshaw & Poole, 1996). Another controversial feature of the amino acid ABC transporter of *R. leguminosarum* is that it is bi-directional (Walshaw & Poole, 1996), being responsible for efflux in addition to uptake of solutes. It is generally accepted that ABC transporters only transport amino acids unidirectionally into cells. The net movement of solutes determined for an ABC transporter depends on the rates of uptake and efflux of solutes, reaching a plateau when both are saturated (Hosie et al., 2001).

Mutation of Aap did not eliminate glutamate uptake by *R. leguminosarum* and considerable uptake of aliphatic amino acids, such as alanine and leucine, also occurred. Consequently, another ABC transport system of amino acids was speculated to exist. Soon the evidence supporting this speculation was obtained. Hosie et al. (2001) reported that there is a second ABC transport system with high homology to the leucine, isoleucine and valine transporter (Liv) of *E. coli* and the equivalent branched-chain amino acid transporter (Bra) in Pseudomonas. This system is composed of one periplasmic-binding protein (BraC), two nucleotide-binding proteins (BraFG) and two integral membrane proteins (BraDE) (Hosie, et al., 2001). The genes encoding this system (braDEFGC) have been mutated. Double mutants of *aap* and *bra* are devoid of almost all detectable high-affinity amino acid uptake, including that of glutamate, alanine, leucine and histidine. This suggests that almost all high-affinity amino acid uptake in *R. leguminosarum* is regulated by two ABC transporters under most growth conditions. Aap and Bra are assumed not only to dominant high-affinity amino acid uptake but both are also capable of exchanging amino acid export by bacteroids (reviewed by Day et al., 2001).
*B. japonicum* bacteroids isolated from soybean nodules have been shown to transport glutamate, aspartate, and alanine in an energy-dependent manner (Udvardi & Day, 1997). Bacteroid amino acid transport in *B. japonicum* can be summarised as follows: 1) alanine is transported into bacteroids with a $K_m$ of 15 $\mu$M and a $V_{max}$ of 3 nmol min$^{-1}$ [mg protein]$^{-1}$; 2) glutamate and aspartate share a common carrier with a $K_m$ = 0.8 $\mu$M for glutamate and $K_m$ 27.5 $\mu$M for aspartate. This carrier can also transport glutamine and asparagine (Udvardi *et al.*, 1988a; Whitehead *et al.*, 1995). Aspartate can also be transported into *R. meliloti* via either the Dct system or a second as yet uncharacterised transport system (Watson *et al.*, 1993; reviewed by Day *et al.*, 2001). However, up to date, the transport mechanisms for those amino acids have not been as well investigated as for dicarboxylic acids.

A thorough examination of the carbon transport processes across the PBM began primarily with the development of a technique for large-scale isolation of intact and pure symbiosomes from legume nodules. Bergersen and Appleby (1981) first reported isolation of symbiosomes from soybean using low-speed centrifugation and continuous Percoll gradients. The yield of intact symbiosomes was not sufficient for the transport studies although it was sufficient for spectrophotometrical analysis of Lb and other components of symbiosome extracts. Later, Price *et al.* (1987) reported successful isolation of symbiosomes from soybean and so did Herrada *et al.* (1989) from French bean, using rate zonal centrifugation techniques. These techniques allowed numerous studies on the transport of compounds into symbiosomes (Day *et al.*, 1989, 1990; Herrada *et al.*, 1989; McRae *et al.*, 1989; Ou Yang & Day, 1992; Ou Yang *et al.*, 1990; Radyukina *et al.*, 1992; Rosendahl *et al.*, 1992; Szafrań & Kaaler, 1995; Udvardi *et al.*, 1991; Udvardi *et al.*, 1988b, 1990). To summarise, kinetic and inhibitor studies reported by these researchers demonstrated that the PBM has a carrier which preferentially transports malate and succinate into symbiosomes. The results are consistent with bacteroid transport studies showing dicarboxylates as the major carbon source for bacteroids (see above). It is assumed that dicarboxylates, once taken up, are metabolized
by the bacteroids. The mechanism of malate or succinate transport at the PBM is believed to be a passive process, driven by the membrane electrical potential ($\Delta \psi$) (Udvardi et al., 1991). Rates of malate transport across the PBM are deemed sufficient to meet the nitrogen fixation activity estimated from measurements with isolated bacteroids (Day et al., 1989). The dicarboxylate transporter on the PBM has yet to be identified but is believed to be distinct from the DctA protein on the bacteroid membrane (Day et al., 1990; Herrada et al., 1989; Udvardi et al., 1988b). The PBM specific protein, nodulin 26, has recently been suggested as a candidate dicarboxylate transporter due to its channel activity and ability to transport malate when isolated and inserted into a lipid bilayer (Weaver et al., 1994). However, more recent studies show that Nod 26 is an aquaporin (Tyerman et. al., 1999).

Up to date, there is no convincing evidence showing that the PBM can efficiently transport amino acids. But there are several models that postulate carbon and nitrogen exchange across the PBM via the movement of amino acids. These models are 1) a malate shuttle (Appels & Haaker, 1991); 2) a proline shuttle (Kohl et al., 1988) and 3) a malonamate shuttle (Kim & Chae, 1990). However, transport studies with isolated symbiosomes from a number of different legumes have not succeeded in identifying amino acid transporters on the PBM (reviewed by Udvardi & Day, 1997). But there has been reports of an aspartate carrier on pea PBM (Rudbeck et al., 1999) and alanine movement (for review, see Day et al., 2001).

1.5.3 Nitrogen fixation, transport of ammonia across the symbiotic membranes and the assimilation of ammonia

1.5.3.1 Nitrogen fixation

For many years it has been accepted that the product of nitrogen fixation by bacteroids within the cells of the central tissue in the root nodules of legumes is ammonia. The
most convincing evidence has come from the following experimental observations. (1) Ammonia was the earliest $^{15}$N-labelled product of N$_2$-fixation when detached root nodules of soybean (Glycine max Merr.; Bergersen, 1965) or serradella (Ornithopus sativa L.; Kennedy, 1966a, b) were incubated for short periods of time in atmospheres containing $^{15}$N$_2$. (2) With anaerobically-prepared soybean nodule bacteroids (the symbiotic form of B. japonicum), in microaerobic, shaken assays with $^{15}$N$_2$ in the gas phase, the principal product was $^{15}$N-ammonia (Bergersen & Turner, 1967). (3) Later, $^{15}$N-ammonia was the principal product of $^{15}$N$_2$ fixation by soybean bacteroids in an open, flow-reaction system in which a well-stirred suspension of soybean bacteroids was perfused with solutions containing dissolved O$_2$ and $^{15}$N$_2$; no $^{15}$N-labelled amino acids were detected (Bergersen & Turner, 1990a).

Recently, results from steady state $^{15}$N-labelling of nodulated roots of intact cowpea plants (Vigna unguiculata [L.] Walp. cv Vita 3 infected with Bradyrhizobium sp. strain CB756) showed that under conditions which maintain high rates of nitrogenase activity, $^{15}$N enrichment appears first in the amide group of glutamine, followed by the amino group of glutamine, ureide synthesis and transamination (Atkins & Thumfort, 2002). There was no evidence to support the idea that alanine (Waters et al., 1998) is a precursor of the amide group of glutamine or of the purine ring (Atkins & Thumfort, 2002). In addition to the evidence stated above, numerous other studies on biochemistry, physiology, cell biology and even molecular biology which relate to nitrogen fixation are almost completely based on the assumption that ammonia is the first product of nitrogen fixation to be assimilated in nodules. Nonetheless, this idea has been challenged recently (see 1.6).

1.5.3.2 Transport of fixed nitrogen across the symbiotic membranes

The ammonia produced by nitrogenase in bacteroids diffuses (Howitt et al., 1986; Jin et al., 1988; O'Hara et al., 1985) into the peribacteroid space (PBS), driven by its
concentration gradient and the expected $\Delta p$H across the bacteroid membrane (acidic in the PBS and alkaline inside bacteroids; Fig 1.6). The concentration gradient of ammonia is generated by the assimilation of NH$_3$ (see below) in the cytoplasm of infected cells, catalysed by a group of enzymes which are found to be very active in nodules and sufficient to maintain ammonia in the cytoplasm of host cells at very low concentrations (Streeter, 1989). The acidic pH in the PBS results from the ATPase activity associated with the PBM (Szafran & Kaaler, 1995; Udvardi & Day, 1989) and the bacteroid respiratory chain. This ATPase pumps protons into the PBS (Udvardi et al., 1991) and the bacteroid respiratory chain releases protons out of the bacteroid (O'Brian & Maier, 1989). Ammonia released by the bacteroids into the PBS is not readily re-absorbed by the bacteroids. The combination of elevated ammonium pools in the bacteroids (Kim & Chae, 1990) and the repression of the genes encoding the ammonium transport family (AMT) on the bacterial membrane, prevents the futile re-cycling of produced ammonium (Fig 1.6; reviewed by Udvardi and Day, 1997).

It has long been assumed that a transporter is required on the PBM for the movement of ammonium to the plant. Using the patch-clamp technique Tyerman et al. (1995) identified a voltage-gated monovalent cation channel on the PBM with a very high density (840-1000 channels per $\mu$m$^2$) and an apparent $K_m$ for NH$_4^+$ of about 30 mM. This channel is believed to be capable of transporting sufficient quantities of NH$_4^+$ across the PBM to meet the rates of ammonia production by bacteroids in vivo. NH$_3$ released by bacteroids is protonated once in the PBS which generated a high NH$_4^+$ concentration in the PBS. This, together with the negative voltage generated by the H$^+$-ATPase pump (-100 mV), would facilitate the channel and ensure the NH$_4^+$ efflux through the PBM (Tyerman et al., 1995). Using inside-out PBM vesicles of pea, Mouritzen & Rosendahl (1997) confirmed the results, showing that low-affinity NH$_4^+$ transporters are present on the PBM of legumes. More recently, a gene encoding a putative ammonium transport protein, $GmSAT1$, was isolated from a soybean nodule cDNA library (Kaiser et al., 1998). $GmSAT1$ is expressed primarily in the nodules and the protein has been localized
to the PBM. When expressed in yeast cells deficient in 2 of 3 plasma membrane ammonium transporters (MEP/AMT), GmSAT1 will complement growth on low ammonium concentrations and alter sensitivity to the toxic ammonium analogue methylammonium (Kaiser et al., 1998, 1999). However, GmSAT1 does not complement a triple yeast ammonium transport mutant (Marini et al., 2000) and therefore cannot itself be the ammonium channel. GmSAT1’s location on the PBM and putative interaction with plasma membrane ammonium transporters in yeast, suggested it may be involved in ammonium transport either directly in association with the AMT gene family or indirectly, possibly as a sensor-transcription factor (Day et al., 2001).

Localised pH differences from bacteroid to plant cytosol may help to coordinate and facilitate the exchange of malate and NH$_4^+$ between plant and bacteroids (Day et al., 1995). On the other hand, the exchange of malate and NH$_4^+$ across the PBM and bacteroid membranes may protect the PBS from excessive acidification (Brewin, 1991).

1.5.3.3 Assimilation of ammonia

The assimilation of ammonia released by bacteroids occurs in the cytoplasm of infected cells (Bergersen & Turner, 1967; Kennedy, 1966b). In all legumes, the first step of ammonia assimilation is catalysed by glutamine synthetase (GS), which converts ammonia to glutamine (Dunn & Klucas, 1973; Robertson et al., 1975). It is shown that glutamine synthetase is at very high levels in the nodule cytosol (estimated to be 2% of the cytosolic protein; McParland et al., 1976). Use of $^{13}$N$_2$ confirmed that glutamine is the first major product formed during ammonium assimilation (Meeks et al., 1978). Glutamine is then converted to glutamate by the glutamate synthase (GOGAT) reaction (Atkins, 1991; Cullimore & Bennett, 1988; Howitt & Gresshoff, 1985). Glutamate synthase activity is strongly correlated with ammonium synthesis by bacteroids (Atkins et al., 1984; Egli et al., 1989). In vivo labelling results also showed that NH$_4^+$ can be incorporated into glutamate via glutamate synthase (Ohyama & Kumazawa, 1980a, b;
Ta et al., 1986). Both glutamine synthetase and glutamate synthase have been purified and characterized (reviewed by Streeter, 1991). The assimilated nitrogen is translocated out of the legume nodules to other plant parts, but the form in which it is transported depends on the legume species. Temperate legumes export amides (principally glutamine and asparagine) whereas tropical legumes export largely ureides (Atkins, 1991; Schubert, 1986).

1.6 Recent challenge to ammonia as the primary product of nitrogen fixation

The long-accepted view that ammonia is the primary product of nitrogen fixation by bacteroids was challenged recently by Waters et al. (1998) who found that alanine, not ammonia, was the principal \(^{15}\)N-labelled product released from \(\text{N}_2\)-fixing soybean bacteroids. These bacteroids were purified on anaerobic sucrose density-gradients and were supplied with malate and shaken in a gas mixture containing \(^{15}\text{N}_2\) and 0.008 atm \(\text{O}_2\). The authors refuted previous findings based on the previous methods of bacteroid preparations which may have introduced contaminating cytosolic enzymes from the host tissue, causing ammonia release from the primary product, alanine. Allaway et al. (2000) subsequently showed that both \(^{15}\)N-alanine and \(^{15}\)N-ammonia were produced by bacteroids prepared from nodules of \(\text{Pisum}\) sp. on anaerobic Percoll density-gradients but that the proportion of these products was altered by the conditions applied. When conditions were optimized for nitrogen fixation, ammonia was the first and major product formed but alanine was formed at high bacteroid densities when ammonia accumulated (Allaway et al., 2000). These authors also used a mutant strain of \(\text{R. leguminosarum}\), defective in alanine catabolism to produce \(\text{N}_2\)-fixing nodules on peas. Such plants appeared to fix less \(\text{N}_2\), growing more slowly than plants nodulated by the wild type strain but the basis for this was not determined. Bacteroids from these nodules produced only ammonia in shaken assays. It was concluded that alanine was a
secondary product of N₂-fixing bacteroids; alanine amino-N arose from ammonia, derived from N₂ fixation, which accumulated in the experimental system used.

1.7 Aims of the project

A crucial feature of biological nitrogen fixation is that it requires a carefully controlled micro-aerobic environment. As stated above, nitrogenases are extremely sensitive to O₂ and become inactivated at quite low concentrations of the gas. On the other hand, nitrogen fixation requires large quantities of ATP whose synthesis via oxidative phosphorylation requires O₂. In other words, O₂ must be maintained at a low concentration near to the bacteroid. In the nodule, the required microaerobic conditions are maintained by combination of a physical O₂ barrier, provided by cells in the inner cortex of the nodule, and rapid respiration rates of the bacteroids and mitochondria within infected cells. O₂ diffusion inside the infected cells is enhanced by the production of large quantities of the O₂ carrier leghaemoglobin (see Bergersen, 1997a for a recent review).

A number of studies have provided knowledge of the types of carbon and nitrogen compounds exchanged between plant and bacteroid (see above), but these studies have been performed under aerobic conditions which inactivate nitrogenase and may alter other aspects of bacteroid metabolism. In particular, bacteroid respiratory demand increases dramatically at ambient concentrations and nitrogenase is inactivated. The previous work has been important, identifying a series of transport proteins on the PBM and bacteroid membrane. However, these studies do not allow determination of regulatory parameters under in planta conditions, nor the investigation of the interaction between PBM transporters and nitrogen fixation. It is important that the previous studies are placed in a physiological context and this project seeks to do that by isolating
bacteroids under anaerobic conditions which preserve nitrogen fixing activity, followed by detailed metabolite and transport assays during $N_2$ fixation.

The aims of this research project were to:

1. Isolate bacteroids from soybean nodules under anaerobic conditions and use the developed flow chamber techniques (Bergersen & Turner, 1990a) to monitor $N_2$ fixation, malate utilization and ammonium production simultaneously in isolated bacteroids.

2. Investigate the interaction between substrate delivery, nitrogen fixation and respiration rates, and ammonium efflux rates.

3. Use the flow chamber system to investigate alternative pathways of carbon metabolism in bacteroids.

4. Employ $^{15}$N labelling techniques and biochemical methods to re-investigate the nature of the $N_2$ fixation products which leave the bacteroids and the basis for disagreement about the roles of $NH_4^+$ versus alanine.
Figure 1.1a. The structure of infected cells of soybean nodules (Bergersen, 1994).
i, intercellular space; if, neighbouring infected cells; uf, neighbouring uninfected cells.
Figure 1.1b. The electron micrograph of an infected cell and neighbouring un-infected cells (Bergersen, 1994).

a, amyloplasts; b, bacteroids; i, intercellular space; m, mitochondria; uf, neighbouring uninfected cells.
Figure 1.2a. The Fe protein cycle of nitrogenase (Burgess & Low, 1996).
Cycle (a) and cycle (b) show reductant-dependent and -independent ATP hydrolysis.
Figure 1.2b. The MoFe protein cycle of nitrogenase (Burgess & Low, 1996). En-presents a functional half of the MoFe protein. Each dotted arrow represents a completed Fe protein cycle (Fig 1.2a) that transfers one electron from Fe protein to MoFe protein.
Enz0, 1, 2 ... correspond to E0, 1, 2... in Lowe-Thorneley Scheme

Figure 1.2c. The proposed scheme for hydrogen evolution, hydrogen binding and reduction at a Mo site, suggesting a role for homocitrate as the provider of a carboxylate leaving group (Burgess & Lowe, 1996).
Figure 1.3a. Organization of nif and fix gene clusters of K. pneumoniae (Elmerich, 1997).

Cluster I

Cluster II

Cluster III

Figure 1.3b. Organization of nif and fix gene clusters of B. japonicum (Fisher, 1994).
Figure 1.4. Carbon flow to bacteroids (Adapted from Vance & Heichel, 1991). Showing how C4-dicarboxylate, malate is formed and transported to bacteroids and the enzymes involved in the process.

- 1, sucrose synthase (SS);
- 2, phosphoenolpyruvate carboxylase (PEPC);
- 3, malate dehydrogenase (MDH);
- 4, malic enzyme;
- 5, alanine aminotransferase;
- 6, nitrogenase;
- 7, aspartate aminotransferase;
Figure 1.5. The TCA cycle and related metabolic pathways, showing that NAD-malic enzyme (DME), NADP-malic enzyme (TME) and pyruvate dehydrogenase (PDH) form a pathway in which malate is converted to acetyl-CoA (Driscoll & Finan, 1996). POD, pyruvate Pi dikinase; PYK, pyruvate kinase; ENO, enolase; PGM, phosphoglycerate mutase; PGK, 3-phosphoglycerate kinase; GAP, glyceraldehyde-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase; CIT, citrate synthetase; PCK, pyruvate carboxykinase; SDH, succinate dehydrogenase; FUM, fumarase; OAA, oxaloacetic acid; 2-P-G, 2-phosphoglycerate; 3-P-G, 3-phosphoglycerate; 1,3 dP-G, 1-3 diphosphoglycerate; G-3-P, glyceraldehyde-3-phosphate; CoA, coenzyme A.
Figure 1.6. Scheme summarizing the exchange of carbon and nitrogen thought to occur in soybean symbiosomes (Udvardi & Day, 1997).
1, PBM ATPase; 2, dicarboxylate carrier in bacteroid membrane; 3, PBM dicarboxylate carrier; 4, ammonium channel.
2 MATERIALS AND METHODS

2.1 Plants and nodules

Seeds of soybean (*Glycine max* (L.) Merr. cv Stevens) were obtained from commercial sources and seeds of cv Williams, used for determination of alanine dehydrogenase, were obtained from the Australian Tropical Crop Genetic Resource Centre, Bilouela, Qld. Seeds were inoculated at planting, with a peat-based culture of *B. japonicum* USDA110c (see below), and grown in pots (diam.: 24 cm; h: 28 cm) filled with river sand, in a naturally illuminated glasshouse. For the first four weeks, pots were supplied twice weekly with nutrient solution (500 ml pot⁻¹), free of combined nitrogen, containing 250 µM CaCl₂•2H₂O, 250 µM KCl, 125 µM KH₂PO₄, 125 µM K₂HPO₄, 500 µM MgSO₄•7H₂O and 25 µM FeEDTA (as commercial Iron Sequestrene) and 0.25 ml L⁻¹ of a solution of trace elements. The trace elements contained 18.1% (w/v) MnCl₂•4H₂O, 28.6% (w/v) H₃BO₃, 1.1% (w/v) ZnCl₂, 0.25% (w/v) NaMoO₄•2H₂O and 0.5% (w/v) CuCl•2H₂O (Gibson, 1980; Delves *et al*., 1986). From 4 weeks after planting until harvest, pots were supplied twice weekly with double strength of the above nutrient solution. Throughout, pots were watered three times daily with tap water. Nodules were picked from roots approximately 8 weeks after sowing. They were washed with tap water, blotted dry on paper towels for the preparation of bacteroids. Some nodules were collected and frozen and used for separate preparation of purified, unfractionated leghaemoglobin (see below).

Plant material and bacterial strains used for the investigation of the alternative tricarboxylic acid cycle (TCA) were prepared as follows.

Soybean plants (*Glycine max* L., cv. Stevens) were grown in modified Leonard jars (Green & Emerich, 1997b). Soybean seed was surface-sterilized prior to planting and inoculated with either wild type *B. japonicum* USDA110de or a *sucA* mutant derivative.
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(LSG184; Green & Emerich, 1997a). Plants were grown in a growth chamber with a 16-hour photoperiod and a day/night temperature of 27˚C/24˚C.

Bacterial materials used for analysis of endogenous alanine dehydrogenase were: B. japonicum strain USDA110c (the strain used in this laboratory for many years), strain USDA110de (kindly supplied by Prof D. W. Emerich, University of Missouri) and strain SU1014/1 (CB1809) (from the SUNFix Culture Collection, University of Sydney; R. leguminosarum bv viciae strains 3841 and RU1327 (aldA') were gifts from Prof. P. S. Poole, University of Reading. Cultures were maintained on yeast extract-mannitol agar (Dalton, 1980) and on Brown and Dilworth’s defined liquid medium (Dalton, 1980) but with 10 mM NH₄⁺ and succinate respectively as nitrogen and carbon sources (Allaway et al., 2000) when rhizobia were grown for preparation of cell-free extracts for determination of alanine dehydrogenase activity.

2.2 Chemicals

All chemicals, except where indicated, were of analytical grade purchased from Sigma.

2.3 Bacteroids

Suspensions of bacteroids were prepared anaerobically using the standard bench method or by density-gradient methods.

2.3.1 Preparation of bacteroids by the standard bench method

The standard bench method was adapted from Bergersen & Turner (1973, 1990a). Clean and dry nodules (30-50 g) picked as above and 70 ml breakage buffer containing
100 mM KH$_2$PO$_4$-KOH (pH 7.4), 0.2 M sucrose and 2 mM MgCl$_2$ were placed into a modified Sorvall Omnixer homogenizer (Fig. 2.1) which was connected to an argon gas system. The homogenizer, together with nodules, was well flushed with flowing argon (30 min) before nodules were homogenized (for 2 min). The homogenate was forced by argon pressure through a layer of Mira-cloth in the filter, by closing S1, opening the tap and adjusting S3. The filter was an Equip sterilizing filter (Sydney, NSW), modified by addition of a spacing ring to accommodate nodule debris and sealed with neoprene gaskets. It was flushed by flowing argon through S3 before the homogenate was passed through. The filtrate was collected from the receiver under flowing argon with an argon-flushed syringe (20 ml with a 10 cm-long canula) into argon-flushed 35 ml Beckman ultracentrifuge tubes capped with Spinco caps and centrifuged in a Ti 70 rotor in a Beckman ultracentrifuge at 3000 rpm (926 g) for 3 min at room temperature to remove coarse plant fragments. The supernatant was transferred by a syringe under flowing argon into different argon-flushed ultracentrifuge tubes and the homogenate was centrifuged as above at 7,500 rpm (5,790 g) for 10 min. The supernatant was collected from the centrifuge tubes under a flow of argon, frozen and stored for the preparation of leghaemoglobin (see below). The pelletted bacteroids were washed twice by resuspending the pellet in wash buffer (WB) containing 100 mM KH$_2$PO$_4$-KOH (pH 7.4), 2 mM MgCl$_2$ and 0.2 M sucrose and centrifuged at 7,500 rpm (5,790 g) in the Beckman (L8-M) ultracentrifuge at room temperature for 10 min. Bacteroids were finally resuspended mostly in the reaction buffer to be saved in the experiment, in a sealed and N$_2$-flushed tube. All the solutions used in the preparation were degassed under vacuum for 30 min and then stored under 1 atm of argon or N$_2$.

2.3.2 Preparation of bacteroids by density-gradient centrifugation
First, a crude bacteroid suspension was prepared in one of two ways: 1) nodules were disrupted anaerobically using the above homogenizing system; 2) nodules were fully disrupted using a Waring Blender under an argon-H₂ atmosphere in an anaerobic glove-box (Coy Laboratory Products Inc., Grass Lake, Michigan, USA) equipped with a gas analyzer. Debris was removed by centrifugation of the homogenate in a bench centrifuge, located in the same glove box, at 1000 g for 3 min. The bacteroids in the supernatant were pelleted by centrifugation in the same centrifuge at 3000 g for 10 min.

The bacteroid pellet, after removal of debris, was resuspended in degassed WB containing 50 mM MOPS-KOH buffer (pH 7.4), 2 mM (DL)-malate, 2 mM MgCl₂ and 0.2 M sucrose under a flow of argon and layered onto either (1) a degassed sucrose-density step gradient (adapted from Waters et al., 1998) containing 30% (10 ml), 40% (15 ml) and 57% (5 ml) (w/v) sucrose in WB (above) or (2) a degassed Percoll-density step gradient (adapted from Udvardi et al., 1988b) containing 40%(10 ml), 50% (15 ml) and 70% (5 ml) Percoll in WB. The gradients in sealed centrifuge tubes, prepared in the glove-box were centrifuged in the HB-4 SW rotor at 27,300 g (13,000 rpm) in a Sorvall centrifuge for 35 min at room temperature. The band on top of the 40/57% interface of the sucrose gradient was collected and loaded onto a second sucrose gradient comprising 30/40% sucrose after dilution with 2 volumes of WB. The pellet at the bottom of the centrifuge tubes was collected with a syringe in the glove-box and washed twice with WB. The band on top of the 50/70% interface of the Percoll gradients was collected and washed in the same way as above. Bacteroids were then resuspended in degassed reaction solution (see below) for flow chamber experiments and in degassed WB for closed shaken experiments.

2.4 Preparation of leghaemoglobin (Lb)

The methods were adapted from Appleby and Bergersen (1980).
2.4.1 Extraction from nodules and initial purification

Frozen nodules were thawed and macerated in 1-3 volumes of air equilibrated 0.1 M K-phosphate buffer (pH 7.4) containing 1mM EDTA, and then homogenized in a Waring blender or Sorvall Omnimixer for 2 min. The homogenate was filtered through a double layer of Mira-cloth (or cheesecloth or organdie). The nodule debris was discarded. The turbid red-brown filtrate, combined with the supernatants collected from bacteroid preparation, was clarified by centrifugation in a GSA rotor at 8,000 rpm (10,000 g) in a Sorvall centrifuge at 4°C for 20 min. The red-brown supernatant (volume measured) was brought to 55% saturation with ammonium sulphate (388g L⁻¹ at 0°C), adding the salt slowly with stirring. When dissolved, the mixture was gently stirred for another 15 min. Then the mixture was centrifuged as above for 30 min and the deposit discarded. The supernatant (volume measured) was brought to 80% saturation by adding 177 g L⁻¹ more of ammonium sulphate with stirring as above. The mixture was again centrifuged as above. The red precipitate of leghaemoglobin was dissolved in a minimum volume of 50 mM K-phosphate buffer (pH 7.4) containing 1 mM EDTA. The dissolved leghaemoglobin was dialyzed overnight against the same buffer, using pre-washed Visking dialysis tubing (washed first with boiled distilled water, then with fresh distilled water and stored in the 50 mM K-phosphate buffer). The next day, the solution was clarified by centrifugation as above for 30 min.

The Lb concentration of the clear red supernatant was assayed by the pyridine haemochromogen method (see below) and then concentrated to a desired concentration by pressure filtration over a Diaflo UM10 or other membrane with a 10,000 molecular weight exclusion limit. (At this stage these preparations contain 80-90% (Lb/protein) leghaemoglobin, which may have suffered 10-50% autoxidation).
The preparation was either used immediately for purification (below) or stored at -80 °C for later use.

2.4.2 Purification of unfractionated oxyleghaemoglobin

2.4.2.1 Preparation of Sephadex columns

Two separate columns of Sephadex G-75 (65 × 8 cm) and G-25 (30 × 5 cm) were prepared for purification of leghaemoglobin as follows. The G75 column was fitted with flow adapters, top and bottom (Fig 2.2a).

Both Sephadex G-75 and Sephadex G-25 were swollen and equilibrated with 50 mM K-phosphate buffer (pH 7.4) containing 1 mM EDTA, with stirring for 48 h at 4 °C in a cold room and the fines removed. Equilibrated Sephadexes were then degassed under vacuum for 4-5 h at room temperature before column packing.

With a lower flow adapter in place, the columns were filled with the K-phosphate buffer and the gels poured in, stirring with a long glass bar while settling. The upper adapter was fitted to the G75 column, ensuring that all air bubbles were removed, and the sample loading syringe and peristaltic pump tube connected to the lower flow adapter of each column. The effluent tube was positioned to give an aqueous head of 5-10 cm to each column. At least 2 column volumes of buffer were passed through each column before loading. When needed, the LKB Ultronak fraction collector was connected to the effluent line (Fig 2.2a). When the column was set up, any remaining air bubbles within the connecting lines were driven out by pumping (0.5-1.0 ml min⁻¹) the buffer back and forth.
2.4.2.2 Purification of Lb with the G75 column and oxygenation of Lb with the G25 column

About 50 ml of the dialysed preparation from above (2.4.1), containing 100-200 µmol of leghaemoglobin, was loaded onto the G75 column and eluted with the same K-phosphate buffer at the same flow as before loading.

A broad, turbid band of green-brown impurities was eluted first and discarded. The following clear, red fractions containing Lb were pooled and concentrated by pressure filtration over an Amicon UM 10 membrane to about 20-50 ml. The 20-50 ml of concentrate contained a mixture of ferrous-oxy and ferric-Lb, which was converted to ferrous-Lb by adding 4 molar equivalents (i.e. 4 mol for each mole of haem) of pure sodium dithionite, dissolved in a minimum volume of deaerated phosphate buffer (0.1 M, pH 7.4). The dithionite solution was added to the Lb under N₂ and aeration of the mixture was avoided, so as to prevent the formation of hydrogen peroxide with consequent degradation of the Lb. The mixture was loaded onto the G25 column (Fig 2.2b) to strip out unused dithionite and other salts, and the purple-red band eluted with the same column buffer. As the band passed into the air-saturated column, its leading edge became red as dithionite was removed and the Lb Fe²⁺ was oxygenated by dissolved O₂.

Lb in samples of effluent fractions was diluted with 50 mM K-phosphate buffer and the concentration of Lb was determined using the pyridine haemochromogen method (see below).

All fractions containing spectrally high-quality oxy-Lb (see below) were pooled and, when necessary, concentrated to approximately 2 mM over an Amicon UM10 membrane using compressed air, not N₂, to pressurize the filtration cell. The solution was then dispensed in aliquots of convenient volume, into small plastic tubes, frozen,
and stored at -80 °C. Preparations were stable for at least 1 year (Appleby & Bergersen, 1980).

2.4.2.3 Determination of concentration of Lb

The concentration of Lb (purified or assay solutions) was determined by the pyridine haemochrome method adapted from Appleby and Bergersen (1980). Haemochrome were produced by reacting a Lb solution with an equal volume of alkaline pyridine reagent containing 4.2 M pyridine in 0.2 M NaOH. The haemochrome was divided between two cuvettes, sample and reference. The reduced minus oxidized absorbance difference spectrum was measured at 600-500 nm against a reagent blank 2-5 min after adding a few crystals of sodium dithionite to the sample cuvette and stirring without aeration, to reduce the haemochrome; the contents of the reference cuvette were oxidized by adding a few crystals of potassium hexacyanoferrate (III).

The difference between absorbance at 556 and 539 nM (ΔA) was recorded and Lb concentration ([Lb]) was calculated using $\Delta_\varepsilon = 23.4 \times 10^3$ l mol$^{-1}$ cm$^{-1}$ (Bergersen & Turner, 1973). The calculation is as follows:

$$[Lb] = (A_{556}-A_{539}) \times 2D/23.4$$ (where D is the initial dilution)

2.4.2.4 Spectral quality of oxy-Lb (LbO$_2$)

The quality of preparation of oxy-Lb (LbO$_2$) was assessed with reference to the absorbance spectra of samples compared with Fig 2 of Appleby and Bergersen (1980). The α peak at 575-6 nm should be slightly higher than the β peak at 541 nm; there should be a negligible peak at 625-7 nm, which should not rise significantly during experiments.
2.5 Experimental systems

2.5.1 The flow chamber system

2.5.1.1 The flow reaction chamber and methods

The flow chamber, associated equipment and methods were as described previously (Bergersen & Turner, 1990a, b, 1992, 1993) and are illustrated in Fig 2.3a. The water-jacketed (30 °C), mechanically-stirred conical chamber (volume 12 ml; Fig 2.3b), is closed at its base by a bacteroid-retaining, microporous membrane filter (Amicon, pore size 0.45 µm), supported on a porous plastic disc. Beneath the disc is an annular collecting space connected to a spectrophotometer flow cuvette connected by stainless steel capillary (0.3 mm i.d.) tubing (Fig. 2.3a). Bacteroids in the chamber are perfused by reaction solution pumped from the reservoirs described below. Spent reaction solution emerging from the cuvette is collected as fractions for determination of NH₃ and other products in solution. NH₃ is considered to be the principal product of N₂ fixation by bacteroids in the chamber (Bergersen & Turner, 1990a, b). The apparatus incorporates facilities for recording automatically, at intervals of 1 min, values of ∆A (the differences between optical absorbances at 576, the α peak of oxy-leghaemoglobin and 560 nm, the trough between α and β peaks) of effluent solution and the time of the beginning and end of each effluent fraction collected for analysis. Values of ∆A are converted to concentrations of free, dissolved O₂ ([O₂ free]) and rates of consumption of O₂, having regard to (a) the ∆Aₜ values for effluent solution at t min, the ∆Aoxy value for fully-oxygenated (in air) reaction solution and ∆Aₜₜₜₜ, the value for reaction solution fully-deoxygenated and reduced with a few crystals of dithionite; (b) the concentrations of total Lb and free, dissolved O₂ in the reservoir reaction solution, (c) the kinetics of O₂-binding by soybean Lb (Kₑq = 0.047 µM O₂; Appleby, 1984), and (d) the flow rate
of solution through the chamber and the dry mass of bacteroids present there. At t min, the proportional oxygenation ($Y_t$) of Lb in the chamber is given by:

$$Y_t = \frac{\Delta A_t - \Delta A_{red}}{\Delta A_{oxy} - \Delta A_{red}}$$  \hspace{1cm} (1)

The concentration of free dissolved O$_2$ [$O_{2\text{,free}}$] ($C_t$, µM) at t min is given by:

$$C_t = [O_{2\text{,free}}] = \frac{Y_t \cdot K_{eq}}{1 - Y_t}$$  \hspace{1cm} (2)

The bacteroid respiration rate ($R_t$ nmol O$_2$ min$^{-1}$ [mg DW (dry weight)]$^{-1}$) is given by:

$$R_t = F(C_p(1 - Y_t) + (C_o - C_t))/Bdt$$  \hspace{1cm} (3)

In which $C_p$ is the [Lb] in the reaction solution, $C_o$ is the [$O_{2\text{,free}}$] (µM) in the inflowing reaction solution, $dt$ is the time interval (min) between successive measurements, $B$ is the dry mass of bacteroids in the chamber and $F$ is the flow rate of reaction solution through the chamber (ml min$^{-1}$).

The rate of N$_2$ fixation ($R_N$, nmol NH$_4^+$ min [mg DW]$^{-1}$) is given by:

$$R_N = F(C_{Nt} - C_{N0})/Bdt$$  \hspace{1cm} (4)

In which $C_{N0}$ is the concentration of NH$_3$-NH$_4^+$ (nmol ml$^{-1}$) in the inflowing medium and $C_{Nt}$ the concentration of NH$_3$-NH$_4^+$ in the effluent collected in time at $dt$ (min).

2.5.1.2 Reaction solutions

These were prepared in MQ deionized water and contained MOPS-KOH buffer (25 mM, pH 7.4), 2 mM MgCl$_2$, Na-(DL) malate (0.05 - 2 mM) and 10 to 15 ml of Lb
solution (prepared as above) per 300 ml. The solutions, in reservoir flasks (Fig 2.3a) connected to the reaction chamber by a peristaltically-pumped capillary line, were stirred at 23 °C for 1 h before use, in air (0.21 atm O₂) at 1 atm, (93.325–95.992 N m⁻² or 95.33-98.06 kPa pressure at the elevation of Canberra) or in closed reservoirs under atmospheres containing 0.125 or 0.25 atm O₂ in N₂. Concentrations of free, dissolved O₂ in these solutions were calculated from tables of solubility. Actual concentrations of Lb were measured by the pyridine hamocromogen method (2.4.2.3). Usually, the concentration ranged from 100-250 µM.

2.5.1.3 Experimental

The assembled flow chamber and connecting tubes were filled with N₂ before being completely filled with reaction solution which had been degassed under vacuum before storage under N₂. Bacteroids, prepared as above were finally resuspended under N₂ in reaction solution which had been degassed under vacuum; 0.5 to 1.0 ml of the suspension was injected slowly into the stirred chamber. When the chamber contents became visibly purplish (Lb partially deoxygenated), the reaction solution pump was started at a flow of about 0.5 ml min⁻¹ and data recording commenced. After several periods of different flow rates, if required, the flow was switched to a different reservoir and another series of flow rates were run.

2.5.2 Closed shaken assays

The system used for closed shaken assays was adapted from Waters et al. (1998) and Allaway et al. (2000).

Nitrogen fixation experiments were run in conical flasks (100 ml) closed with Suba seals and containing 9.0 ml of reaction solutions (2.5.1.2). After inserting the Suba seals, the flask contents were degassed under vacuum for 10 min with periodic
agitation, on a manifold fitted with hypodermic needle connections and an Hg manometer (Turner & Gibson, 1980). The flasks were flushed twice with argon and then filled with a gas mixture containing 0.008-0.02 atm of O\textsubscript{2} and N\textsubscript{2} to 1 atm (1 atm = 94.0-95.3 Kpa), from a screw-piston reservoir (Turner & Gibson, 1980). Flasks were brought to the reaction temperature (26 °C) before injecting 1.0 ml of bacteroid suspension containing 3-6 mg (DW) of bacteroids using a nitrogen-flushed syringe. The flasks were shaken at 100 or 150 rpm in a rotary shaker at 26 °C. Reactions were terminated by removing seals, admitting air to inactivate nitrogenase and immediately chilling on ice. The contents of the flasks were transferred to 12 ml Corex centrifuge tubes and then centrifuged at 0 °C and 10,000-12,000 g for 10 min to separate bacteroids from the reaction solution. Products of nitrogen fixation were measured (below) in the supernatant reaction solution.

### 2.6 Measurements of products of nitrogen fixation

#### 2.6.1 Measurement of ammonia

The colorimetric assay method for ammonia was adapted from Bergersen (1980). Assays used 12 ml glass scintillation vials. One ml samples of the fractions from the flow chamber experiments or supernatant from closed shaken experiments were used. For ammonia recovered by diffusion (2.6.3.4.4), the paper strip was immersed in 1 ml MQ H\textsubscript{2}O. Chaney-Marbach solution A (5 ml, see Bergersen, 1980) containing 50 g L\textsuperscript{-1} phenol and 0.25 g L\textsuperscript{-1} Na nitroprusside was added to the sample in an assay vial. The assay vial was left under room temperature for at least 30 min to allow NH\textsubscript{3} in the sample to be fully dispersed. Then 5 ml of Chaney-Marbach B containing 25g L\textsuperscript{-1} NaOH and 2.1 g L\textsuperscript{-1} Na hypochlorite was added to the vial. The amount of ammonia in the sample was determined by reading the optical absorbance at 625 nm after 30 min
color development. $\text{NH}_4^+$ (nmol) was calculated using the standards containing 0-151.4 µg of $\text{NH}_4^+$-N.

2.6.2 Measurement of alanine

2.6.2.1 Enzymatic assay of alanine

2.6.2.1.1 Sample preparation for the enzymatic determination of alanine

As the efflux fractions collected from the flow chamber experiments contained Lb which could interfere with the enzymatic determination of alanine, protein was removed as follows.

Samples (5-10 ml of each fraction) were placed into 12 ml centrifuge (Corex) tubes (Fractions of 1.5 ml or less were placed in microfuge tubes) and 10 µl of 60% perchloric acid were added for each ml of sample. Samples were then heated in a boiling water bath for 10 min and left in ice for 30 min. Each sample was neutralized by adding an equivalent amount of KOH followed by centrifuging in the microfuge (for small samples) for 10 min at 4 °C or (for large samples) in the SS34 rotor at 10,000 rpm in a Sorvall centrifuge for 10 min at 4 °C. The samples were always kept cold so that the precipitated salt did not redissolve. The supernatants were carefully taken for the following assay.

2.6.2.1.2 Enzymatic determination of alanine

The assay solution with a final volume of 1.5 ml per tube contained 450 µl of assay buffer containing 60 µM hydrazine, 75 µM glycine, 4 µM $\beta$-NAD, 0.5 units of alanine dehydrogenase (from $\text{Bacillus subtilis}$, Sigma) (except for “minus-enzyme” controls, see below) and 500 µl MQ water. Each assay used 500 µl of sample (or of standard in
flow chamber reaction buffer, see above). All the components were assembled in iced tubes before the assays were started by incubation at 37 °C for 1 h. Reduction of NAD was determined.

Because the background A₃₄₀ was different for each sample, a "minus-enzyme" control for each sample, including standards, was prepared, in which the alanine dehydrogenase was omitted. The absorbance of samples was read at 340 nm and the nmoles of alanine was calculated from a standard curve. Every assay was duplicated.

2.6.2.2 Amino acid analysis

2.6.2.2.1 Purification of amino acids from shaken assays

The method for the assays was modified from Redgwell (1980). Sephadex SP-C-25 (10 g, Pharmacia) was swollen and equilibrated in 2 × 500 ml 0.5 M (NH₄)₂SO₄ for 2 days. After filtration, the Sephadex was resuspended in 7% (v/v) formic acid several times over 8 h and finally stored in 1% formic acid. Four 5 ml glass syringes were used as columns. Each column contained 5 ml of the treated Sephadex gel. Before the samples were loaded, the columns were washed with 20 ml of 7% formic acid, followed by 20 ml MQ H₂O.

Samples (1 ml each) of the supernatants from a shaken experiment were pipetted onto the surface of columns (1 sample/column) and allowed to enter slowly into the gel, followed by successive washes with 0.5 ml H₂O until 20 ml had been collected. The eluates containing sugar and malate were discarded.

Next, amino acids were eluted from the Sephadex columns with 15 ml 0.2 M NH₄OH to yield the amino acid fraction. The first 9 ml of eluate contained no amino acids and was discarded; a further 3-4 ml of eluate was collected in ice and frozen until analyzed. The
Sephadex gel was recovered and retreated with \((\text{NH}_4)_2\text{SO}_4\) as above for preparation of new columns.

Each sample of 3-4 ml of column eluate was lyophilized, resuspended in 1.0 ml \(\text{H}_2\text{O}\) and then quantitatively transferred to an Eppendorf tube, lyophilized again and sent to Dr R. Parsons, University of Dundee for analysis by GC-MS (see Allaway et al., 2000).

The location of alanine in eluates from the Sephadex columns (above) was determined as follows: A standard alanine sample was prepared by adding 20 \(\mu\text{l}\) 100 mM alanine into 1 ml of reaction buffer (RB, as used in shaken experiments), making the final concentration in RB to 2 \(\mu\text{M}\) alanine or 2 nmol ml\(^{-1}\). A standard sample (1.0 ml) of this solution was loaded onto a Sephadex column and eluted as above with a precise volume of elutant \((V_{\text{total}})\). The eluate was collected drop by drop onto filter paper and dried in numbered squares. The total number of drops \((D_{\text{total}})\) was recorded. The filter paper was then sprayed with ninhydrin (0.1\% in acetone; preliminary colorimetric tests established that 1 \(\mu\text{mol drop}^{-1}\) of alanine was detectable by ninhydrin), dried and the color developed (Kennedy, 1966b). The numbers of drops \((D_b)\) of eluate in front of alanine and those containing alanine \((D_a)\) were recorded. The volume location of alanine in eluates was based on:

\[
\begin{align*}
V_{\text{total}}/D_{\text{total}} & = \text{volume (\(\mu\text{l}\))/drop (}\ V_d) \\
D_b \times V_d & = \text{volume of eluate before alanine (}\ V_b) \\
D_a \times V_d & = \text{volume of eluate containing alanine (}\ V_a)
\end{align*}
\]

2.6.2.2.2 Amino acid analysis

Alanine and other amino acids in samples from \(\text{N}_2\) fixation assays, were determined by HPLC analysis after derivitization, at the Nucleic Acid and Applied Protein Chemistry
Unit, the Department of Plant Science, Waite Campus, University of Adelaide or at the Ecosystems Research Group, Department of Botany, University of Western Australia.

2.6.3 Incorporation of $^{15}$N$_2$ into the products of nitrogen fixation

2.6.3.1 Preparation of $^{15}$N$_2$ gas

There were two $^{15}$N experiments, one in late summer of 2000 and one during winter of 2000. A fresh batch of $^{15}$N$_2$ was prepared for each. The preparation of $^{15}$N gas was based on the following reaction:

$$(\text{NH}_4)_2\text{SO}_4 + 2\text{NaOH} + 3\text{NaOBr} = \text{N}_2 + 3\text{NaBr} + 5\text{H}_2\text{O} + \text{Na}_2\text{SO}_4$$

In this reaction, 7.58 µmoles or 192 ml of N$_2$ was produced by oxidation of 1 g of ammonium sulphate at 20 °C at Canberra’s elevation.

The reagent, NaOBr, was prepared by (a) dissolving 100 g of NaOH in 133 ml of MQ water followed by cooling it in ice; (b) dissolving 16 ml of bromine in 140 ml of MQ water, cooling it in ice and (c) slowly adding 80 ml of (a) to (b) with stirring and continued cooling in ice. The solution was tightly capped in a bottle and stored in a refrigerator until used.

$^{15}$N$_2$ was produced by placing 3.0 g of ($^{15}$NH$_4$)$_2$SO$_4$, containing 59.8 atoms% $^{15}$N (Isotec Inc., Miamisburg, Ohio, USA), dissolved in a minimum volume of MQ water in the lower bulb (A) of the reaction apparatus shown in Fig 2.4 (also see Bergersen, 1980). As the oxidation by hypobromite usually produces impurities and seldom gives quantitative recoveries of N$_2$, further purification was undertaken using alkaline permanganate reagent containing 50 g L$^{-1}$ K-permanganate and 25 g L$^{-1}$ KOH followed
by 0.5 N sulphuric acid (Burris, 1976). The assembly of the apparatus used for $^{15}$N preparation is shown in Fig 2.4 and the detailed procedure of the preparation is described below.

Before the reaction was started, bulb B was disconnected from bulb A, but T2 at J2 was connected.

(1) 3.0 g of $(^{15}$NH$_4$)$_2$SO$_4$ was placed in A via J1 and dissolved in a minimum volume of MQ water. T1 was purged with argon and closed. Then J1 on bulb B was greased and seated onto bulb A.

(2) T3 and T4 were purged with argon through T3 and closed.

(3) 220 ml of alkaline hypobromite reagent was placed in bulb B and degassed for 15 min under vacuum through T3, T5 and T6 connected to the vacuum pump through the cold trap and vacuum (VHose 2). The cold trap protects the pump and the laboratory from the Br$_2$ vapor. Then the manifold/vacuum pump tap was closed to allow argon to fill bulb B to 1 atm via T4. This was determined with a manifold manometer connected through T4. Then T5 was closed and VHose 2 was disconnected. Next T3 was opened to allow argon to pass through the hypobromite at a few bubbles per min, while the next operation was completed.

(4) Bulb A was evacuated through T2, via VHose 1, T5 and the freezing trap to degas the solution in bulb A for 15 min, flushing with two changes of argon from the manifold and again B was evacuated. T2 then was closed.

(5) T1 was carefully opened to allow the hypobromite reagent to run slowly into bulb A. The flow was stopped before the upper surface of the hypobromite solution reached the opening of T1.
(6) When the effervescence in bulb A was completed, bulb B was evacuated through T3 and VHose 2 connected to vacuum pump via the cold trap. T3 was closed and T5 and VHose 1 were disconnected. T1 was slowly opened to draw the spent reagent back into B with care to avoid gas from A entering T1.

(7) J3 was opened and the spent reagent was aspirated out of bulb B. The bulb was then washed with two changes of MQ water before adding 150 ml of alkaline permanganate through T3, reconnecting J3, degassing the solution under vacuum through T3, VHose and T5, and finally bubbling argon through the solution (as in (3) above). Next the permanganate solution was drawn into B through T1 (as in (5) above). T1 was then closed and the bulb A was left overnight so that any lower oxides of N could be oxidized and absorbed.

(8) Next day, T3 was connected to the vacuum through VHose 2 and bulb B was evacuated. The spent permanganate solution was carefully withdrawn from A into B (as in (6) above). T1 was closed before the last of the solution was removed.

(9) J3 was opened and the spent permanganate solution was aspirated out. Bulb B was washed with several changes of water before adding 750 ml of 0.5N H₂SO₄ and degassing this solution under vacuum (as in (3) above) and bubbling with argon at 1 atm. Then T1 was opened, allowing the acid solution to run into A until a positive pressure was generated there. T1 was closed and the pressure of argon in bulb B was maintained at 1 atm. The ¹⁵N₂ gas was ready either to be used immediately or to be stored in the bulb A for later use.

2.6.3.2 Determination of O₂ contamination of ¹⁵N₂
The contamination of $^{15}\text{N}_2$ prepared as above by $\text{O}_2$ is not completely avoidable, but the $\text{O}_2$ content can be determined. A Rank $\text{O}_2$ electrode (Rank Bros., Cambridge, UK) was used for this purpose. The electrode chamber shown in Fig 2.5 was set up with 1.0 ml of MQ $\text{H}_2\text{O}$ in the bottom and air above. The electrode output was recorded as mV. The stirrer rate was set for the maximum, the gain of the measuring circuit and recorder were adjusted to give a 9.00 mV signal with the zero mV adjusted in all recorder ranges.

Tank argon was passed gently through P and allowed to egress around the capillary tube (a hypodermic #22 needle). When the output was diminished to ca 0.2 mV (still declining slowly), a sample of $^{15}\text{N}_2$ gas (see below) was added by syringe (10 ml) through P, flushing out the argon from the gas space.

A Suba-seal-stoppered 100 ml flask was evacuated on the manifold, and flushed twice with argon, before being filled to 1 atm with $^{15}\text{N}_2$ gas mix from the reservoir. An argon-flushed, gas-tight 20 ml syringe was inserted into the flask through the Suba seal. A sample of at least 10 ml was taken and passed slowly through the argon-flushed gas space of the electrode chamber. The equilibrium electrode reading was recorded.

After all measurements, all residual $\text{O}_2$ was removed, from the water in the electrode, by adding a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ and the zero $\text{O}_2$ electrode reading was recorded (Table 2.1).

The $\text{O}_2$ content of the $^{15}\text{N}_2$ in the example shown in Table 2.1, was calculated as 0.027 atm using a standard curve constructed from measurements made (as above) with gas samples of air in argon to give known pO$_2$ (atm).

For the closed shaken experiment, the gas phase of the shaken flasks was filled with argon and $^{15}\text{N}_2$ gas mixture prepared as follows.
2.6.3.3 Gas mixture for the use in closed, shaken experiments

The O2 electrode assay of the 15N2 preparation showed that 2.7% O2 was present (Table 2.1).

<table>
<thead>
<tr>
<th>Chamber Gas</th>
<th>Recorder Range (mV)</th>
<th>Equilibrium (mV)</th>
<th>pO2 (atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>air</td>
<td>0-10</td>
<td>9.00</td>
<td>0.21</td>
</tr>
<tr>
<td>Argon</td>
<td>0-2</td>
<td>0.21</td>
<td>0.004</td>
</tr>
<tr>
<td>15N</td>
<td>0-2</td>
<td>1.16</td>
<td>0.027</td>
</tr>
<tr>
<td>Na2S2O4 zero</td>
<td>0-2</td>
<td>0.025</td>
<td>0</td>
</tr>
</tbody>
</table>

For the closed shaken experiment, the 15N2 (about 800 ml at 1 atm-710 mm Hg in Canberra on the day of experiment) in bulb A prepared as above was transferred by displacement with degassed 0.5 N H2SO4, into the evacuated, argon flushed screw/piston reservoir (Fig 2 in Turner & Gibson, 1980 and shown in Fig 2.3a), with the piston set at about 1.5 L volume. The screw piston was adjusted so that the pressure in the reservoir was exactly 0.5 atm (i.e. 355 mm Hg), then tank argon was added to 1 atm (710 mm Hg).

<table>
<thead>
<tr>
<th>Chamber Gas</th>
<th>Recorder Range (mV)</th>
<th>Equilibrium (mV) (x)</th>
<th>pO2 (atm) (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0-10</td>
<td>9.05</td>
<td>0.21</td>
</tr>
<tr>
<td>argon (not zero yet)</td>
<td>0-2</td>
<td>0.27</td>
<td>0.0058</td>
</tr>
<tr>
<td>15N2/argon mix sample 1</td>
<td>0-2</td>
<td>0.38</td>
<td>0.0084</td>
</tr>
<tr>
<td>15N2/argon mix sample 2</td>
<td>0-2</td>
<td>0.38</td>
<td>0.0084</td>
</tr>
<tr>
<td>Na2S2O4 zero</td>
<td>0-2</td>
<td>0.02 (a)</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: In y = b (x-a), b = 0.0232558

This theoretically gave ca 1% O2, 50% argon and 49% 15N2. However, the actual percentage of each component of the gas mixture was determined using the O2 electrode (Fig 2.5). The readings are recorded in Table 2.2.
Atoms % $^{15}$N in the gas mixture was calculated as follows:

In the 800 ml of $^{15}$N gas prepared as above, 2% O$_2$ was detected. It is assumed that this came from contaminating air. The (NH$_4$)$_2$SO$_4$ from which the $^{15}$N was generated, contained 57.8 atoms % $^{15}$N (Isotech Inc.). This $^{15}$N would have been diluted by the contaminating air N$_2$ (0.3663 atoms % $^{15}$N).

In 800 ml of $^{15}$N as prepared, the air would amount to:

$$0.02 \times 800 \times 0.78/0.21 = 59.43 \text{ ml of N}_2 \text{ (air contains 78\% N}_2 \text{ \& 21\% O}_2)$$

So, the $^{15}$N in the gas prepared would be given by:

$$59.43 \times 0.3663/100 + (800-59.43) \times 57.8 = 428.26715 \text{ ml of } ^{15}\text{N}_2$$

This was in 800-(800 × 0.02) + 784 ml of N$_2$. So atoms % $^{15}$N in the gas would be:

$$100 \times (428.267/784) = 54.63$$

Thus, the gas mixture used in this experiment contained 0.84%O$_2$, 50% tank argon and 49% $^{15}$N (54.63 atoms %$^{15}$N).

### 2.6.3.4 Experimental system and preparation of samples for N analysis

#### 2.6.3.4.1 Experimental system

The closed, shaken $^{15}$N experiments used 100 ml Erlernyer flasks, closed with Suba seals. There were two series of flasks in each experiment, with 4 replicates in each.
Reaction solution (9.0 ml; see 2.5.1.2) was placed in each flask, the seals inserted and the contents degassed under vacuum and flushed with 2 changes of argon. Control flasks were filled with air and the $^{15}$N$_2$ series filled with the $^{15}$N$_2$ mixture prepared as above. At zero time, 1.0 ml of bacteroid suspension, prepared by the Percoll-density gradient method, was injected into each flask. The control flasks were opened to air and placed in ice. The $^{15}$N-containing flasks were placed on a rotary shaker at 100 rpm and 26 °C for 30 min, after which flasks were opened to air and placed on ice to terminate the reaction.

2.6.3.4.2 Collection of samples

After the experiment was stopped, 1 ml of the suspension in each control and $^{15}$N assay flask was taken for the analysis of total N in the sample. The rest of the suspension (9.0 ml) in each flask was removed to centrifugation tubes and bacteroids were separated from reaction solution by centrifugation (12,000 g for 10 min). Samples (2 × 1.0 ml) of supernatant were taken for the analysis of total N in supernatant. Samples (2 × 1.0 ml) of the supernatant were taken for amino acid N analysis. Another 2 aliquots were taken for analysis of NH$_3$-N. The pellets of bacteroids were resuspended in 5 ml MQ water and saved for the analysis of bacteroid N. See Fig 2.6 for the further details of sample distribution.

2.6.3.4.3 Preparation of samples

Samples for analysis of total N in bacteroid suspensions, supernatant, and the residue of supernatant after removal of NH$_3$-N, were digested as described by Bergersen (1980) and in Fig 2.6.

Kieldahl digestion of aliquots of samples was done in Quickfit Pyrex digestion tubes (diam.: 25; h: 210 mm) heated at 280-300 °C in temperature-controlled digestion blocks.
1.0 ml of digestion reagent, containing 100 g potassium sulphate (to elevated the boiling point) and 1 g metallic selenium in 1 L concentrated sulphuric acid (Bergersen, 1980) was added into each digestion tube. Digestion was continued for 3-4 h beyond clearing. When cool, the digests were made up to 10-25 ml with MQ water.

Diluted digests were then distilled into 1% boric/indicator solution containing 2% boric acid in MQ water, 5 ml L⁻¹ 0.1% methyl red (in 95% ethanol) and 20 ml L⁻¹ 0.1% bromocresol green (in 95% ethanol) in the receiving flasks using a Markham still (see Bergersen, 1980 for details). Distillates were acidified with 1 drop of 1N H₂SO₄ and concentrated to ca 1-2 ml by boiling on a hot plate, and then transferred to 10 ml scintillation vials. NH₃ in the samples was recovered by diffusion as below.

2.6.3.4.4 Collection of NH₃ by diffusion

The diffusion device consisted of a 12.5 ml scintillation vial closed with a rubber stopper bearing a nichrome wire hook on which was suspended a strip (10 x 3 mm) of Whatman filter paper moistened with 10% (v/v) sulphuric acid, which was calculated to be capable of absorbing up to 250 µg N in each sample.

Measured samples (1-5 ml and adjusted if necessary to be near-neutral) were placed in the dry vials. 1 ml of 20% (w/v) NaOH (or saturated borate buffer, pH 10) were slowly added to the liquid in the vial to bring the contents of the vials to alkaline (pH 10; phenolphthalein indicator was used when necessary). Then the vials were closed quickly with the hooked stoppers with suspended paper strips, without touching the sides. The diffusion vials were stood at room temperature for at least 16 h. For colorimetric analysis of NH₃, the paper strips were taken off the hooks with dry, clean forceps and dropped into an assay vial containing 1.00 ml of MQ H₂O. NH₃ was determined colorimetrically 1 h later (2.6.1). For ^1⁵N analysis, the strips were placed
with forceps in a tin boat and dried. When necessary, the standard carrier N (50 µl of a solution containing 0.118 g (NH₄)₂SO₄ in 25.0 ml of MQ H₂O, δ¹⁵N=-1.114‰ or 0.3659 atoms %) was added to the strips and dried in a desiccator.

2.6.3.4.5 Assessment of hydrolysis of alanine by alkali

The purpose of this assay is to clarify that any alanine, in the supernatant of the shaken experiments was not hydrolyzed during the process of NH₃ diffusion.

Alanine (containing 100 µg N) was added to 1 ml of reaction buffer (as used for shaken experiments). Any NH₃ produced was diffused as above onto filter paper strip, using sodium borate buffer (the same as above) to bring the pH in the diffusion vial to pH 10, or using 20% NaOH as a control of diffusion. 1 ml of reaction buffer without alanine was used as control of hydrolysis of alanine. The paper strips in the diffusion vials were taken out after 24 h of diffusion and dropped into an assay vial containing 1.0 ml MQ water. NH₃ was determined using the colorimetric assay (2.6.1).

2.6.3.5 Analysis of ¹⁵N incorporation into compounds

Total N in digested samples and ¹⁵N-NH₄⁺ recovered by diffusion (2.6.3.4.4) were analyzed using an ANCA SL stable isotope analytical system (Europa Scientific, UK) in the Division of Plant Industry, CSIRO, Canberra.

The presence of ¹⁵N-alanine in the soluble fraction was sought by purifying any amino acids present using chromatography in Sephadex (Pharmacia) SP-25 (see 2.6.2.2.1) and submitting them to GC-MS analysis after derivitization with N-(ter-Butyldimethylsilyl)-N-methyltrifluoroacetamide (Allaway et al., 2000). The amount of ¹⁵N incorporated into alanine was assessed from the ratios of the mass peaks at m/e 260 and 261 (the principal fragments of the derivative of alanine) as follows:
Chapter 2

Atoms % excess = \(100\left[\frac{R_s}{1+R_s} - \frac{R_c}{1+R_c}\right]\)

In which \(R_s\) and \(R_c\) are, respectively, the ratios mass 261/mass 260 for the mass spectral peaks of the samples (s) and the control (c). The calculation assumes that, apart from N, the isotopic ratios for all elements present in the fragment are the same in sample and control.

2.6.3.6 Calculations used in measurements of the incorporation of \(^{15}\)N from \(^{15}\)N\(_2\) by bacteroids in the closed shaken experiments

The (NH\(_4\))\(_2\)SO\(_4\) standard used in closed shaken experiment is defined as ref. It has:

\[\delta^{15}\text{N} = -1.114 \, \text{‰} \text{ (wrt air N}_2\text{)}; \text{ atoms } %^{15}\text{N} = 0.3659\]

\[\text{Atoms } %^{15}\text{N} = 100\frac{R}{2 + R}\]

Therefore:

\[R_{\text{ref}} = 2 \left( \text{atoms } %^{15}\text{N}_{\text{ref}}/100 - \text{atoms } %^{15}\text{N}_{\text{ref}} \right)\]
\[= 0.007345\]

\[\delta^{15}\text{N} = 1000\left(\frac{R_{\text{sample}} - R_{\text{ref}}}{R_{\text{ref}}}\right)\text{‰}\]

And therefore:

\[
(\delta^{15}\text{N}_{\text{sample}} \times R_{\text{ref}})/1000 - R_{\text{ref}} = R_{\text{sample}}
\]

\[\text{atoms } %^{15}\text{N}_{\text{sample}} = 100 \frac{R_{\text{sample}}}{2 + R_{\text{sample}}}\]

\[\text{atoms } %^{15}\text{N excess} = \text{atoms } %^{15}\text{N}_{\text{sample (n)}} - \text{atoms } %^{15}\text{N}_{\text{sample (0)}}\]

In which the \(^{15}\text{N}_{\text{sample (n)}}\) sample was the one after incubation of bacteroids in the closed system; \(^{15}\text{N}_{\text{sample (0)}}\) was the one without \(^{15}\text{N}\), taken before the incubation started.
When carrier $\text{NH}_4^+$ was added to samples, the atoms % $^{15}\text{N}$ mass balance is calculated as:

$$c(a + b) = ax + bd$$

$$x = \frac{(c(a + b) - bd)}{a}$$

In which:

- $a = \mu g$ $\text{NH}_4^+$-$\text{N}$ in a sample before adding carrier
- $b = \mu g$ carrier $\text{NH}_4^+$-$\text{N}$ added per assay
- $c = \text{atoms} % ^{15}\text{N}$ measured in sample + carrier $\text{N}$
- $d = \text{atoms} % ^{15}\text{N}$ of carrier $\text{N}$
- $x = \text{atoms} % ^{15}\text{N}$ of original sample

2.7 Endogenous alanine dehydrogenase in cultured rhizobia

2.7.1 Culture of bacteria

2.7.1.1 Medium for stock culture and purity checks

Yeast Extract-Mannitol Medium (YMA) (Gibson, 1980), containing 1% mannitol, 0.05% $\text{K}_2\text{HPO}_4$, 0.02% $\text{MgSO}_4\cdot7\text{H}_2\text{O}$, 0.01% $\text{NaCl}$, 0.04% yeast extract (Difco or Oxoid). 1.5% agar (Difco) was used to prepare the stock agar cultures of bacteria ($B. japonicum$ USDA110c, 110de and SU1014/1 and $R. leguminosarum$ TY 3841 and RU1327).

To make this medium, all salts were dissolved in a minimum amount of MQ water and then the yeast extract was added and dispersed well by stirring before adding mannitol. The medium was distributed (when necessary) in 10 ml aliquots into 20 ml McCartney bottles or in 20 ml aliquots in 125 ml flasks, after adjustment to pH 7.0 and volume to 1 L. The flasks were plugged with cotton wool and the bottles were half way capped.
before they were autoclaved at 20 psi (121 °C) for 30 min. Upon removal from the autoclave, all bottles caps were tightly closed.

The McCartney (screw-capped) bottles were placed sloped while the agar cooled and set. For plate cultures, YMA was melted in a microwave oven and poured in 10 ml amounts into sterile Petri dishes and allowed to cool and set with the lids cracked open (to prevent collection of condensed water) in a sterile hood. All media were stored in a refrigerator until used.

2.7.1.2 Purity checking and preparation of stock cultures

The purity of each culture was checked by streaking received cultures on YMA and growth at 30 °C for 3-5 days until good clear single colonies were obtained which were free of contamination by other bacteria. Stock culture on YMA slopes were prepared from single colonies of each strain.

2.7.1.3 Preparation of bacteria for determination of alanine dehydrogenase

Preliminary experiments used broth cultures in YMB (YMA minus agar), grown in 125 ml conical flasks at 30 °C with shaking at 150 rpm for 4-5 days. The bacteria used for determination of alanine dehydrogenase were grown in defined liquid succinate medium (Poole et al., 1994; Brown & Dilworth, 1975) containing succinate and NH$_4^+$ as carbon and nitrogen sources, trace elements and vitamins. The constituents of the medium are given in Tables 2.3 & 2.4.

Before the medium was made, a 1000-fold strength solution of trace element stock and 100 ml of 10-fold vitamin were made. Then 1 ml of trace element stock solution was added into the vitamin solution.
To make the medium, basic chemicals were first dissolved in ca 800 ml H2O (FeCl3 was dissolved separately) and then the above vitamin solution (100 ml) containing trace elements was added to it. The solution was finally volumed to 1000 ml after pH was adjusted pH 7.0.

Table 2.3: Basic chemicals of succinate medium

<table>
<thead>
<tr>
<th>Basic Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>10</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO4·7H2O</td>
<td>2</td>
</tr>
<tr>
<td>CaCl2·2H2O</td>
<td>0.17</td>
</tr>
<tr>
<td>NaCl</td>
<td>3.4</td>
</tr>
<tr>
<td>MOPS buffer</td>
<td>20</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.4: Final concentration of trace elements and vitamins for the succinate medium.

<table>
<thead>
<tr>
<th>Trace elements</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl3</td>
<td>6.6</td>
</tr>
<tr>
<td>EDTA</td>
<td>15</td>
</tr>
<tr>
<td>ZnSO4·7H2O</td>
<td>0.16</td>
</tr>
<tr>
<td>NaMoO4</td>
<td>0.2</td>
</tr>
<tr>
<td>H3BO3</td>
<td>0.25</td>
</tr>
<tr>
<td>MnSO4·4H2O</td>
<td>0.02</td>
</tr>
<tr>
<td>CuSO4·5H2O</td>
<td>0.02</td>
</tr>
<tr>
<td>CoCl2·6H2O</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamins</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine-HCl</td>
<td>1</td>
</tr>
<tr>
<td>Ca-Pantothenate</td>
<td>2</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The succinate medium was distributed in 15 ml aliquots into 125 ml conical flasks plugged with cotton wool and autoclaved. Flasks were first inoculated from stock cultures and incubated at 30 °C with continuous shaking (150 rpm) in an incubator for 3-5 days.
Each liquid culture from 3 flasks was transferred to sets of 3-5 sterile flasks of the same medium under sterile conditions and again incubated as above for 3-5 days until the bacteria were harvested. This step produced sufficient, uniform bacteria for preparation of cell free extracts.

2.7.2 Determination of alanine dehydrogenase

The method was modified from Smith and Emerich (1993a, b).

2.7.2.1 Preparation of cell-free enzyme extracts

Bacteria cultured as above were collected in 12 ml Corex centrifuge tubes and centrifuged in an SS34 rotor at 12,000 g, 4 °C in a Sorvall centrifuge for 30 min. Supernatants were discarded and the pellets were resuspended in 5 ml of breakage buffer containing 25 mM TES-NaOH buffer (pH 7.5), 50 mM KCl, 5 mM MgSO$_4$ and 5 mM DTT and placed in ice.

Bacteroids prepared anaerobically using the standard bench method for flow chamber experiments or in samples recovered from flow chamber experiments (2.8.2) were centrifuged in the SS34 rotor at 12,000 g in a Sorvall centrifuge for 10 min. Pellets were resuspended in the above breakage buffer (5 ml) and kept in ice.

Suspensions of cultured bacteria and bacteroids prepared as above were slowly passed twice through a precooled French Press (American Instrument Co., Inc, USA) at a pressure of 16,000 psi. The cell homogenates were collected into 12-ml Corex tubes and centrifuged in a SS34 rotor at 12,000 g and 5 °C in Sorvall centrifuge for 1 h. The supernatants, crude soluble enzyme extracts, were collected and kept on ice until assay of activities.
2.7.2.2 Enzyme assay

The principal of the assay:

\[
\text{NADH} + \text{NH}_4^+ + \text{Pyruvate} \rightleftharpoons L\text{-alanine} + \text{NAD} + \text{H}_2\text{O}
\]

Specific activity (amination) of alanine dehydrogenase was measured by the change in absorbance at 340 nm due to oxidation of NADH. The assay mixture contained 100 mM TAPS buffer-NaOH (pH 8.5), 2 mM DTT, 160 μM NADH, 2 mM pyruvate and 70 mM NH$_4$Cl. Each assay was started by adding the crude enzyme extract (20-40 μl). For 5-10 min the rate of change of absorbance at 340 nm was recorded. The kinetics (amination) of alanine dehydrogenase were measured in the same buffer except that the concentration of pyruvate or NH$_4$Cl was varied.

Enzyme activity was calculated using the following formula:

\[
\text{Enzyme activity (nmols NADH oxidized min}^{-1} \ [\text{mg protein}]) = \frac{((\Delta A_{340}/\Delta t) \cdot V \times 10^6)/\varepsilon_{mM} \cdot v \cdot P}{t}
\]

In which \( t = \text{min} \); \( V = \text{reaction volume (ml)} \); \( v = \text{sample volume (ml)} \); \( P = \text{mg protein/}v \) and \( \varepsilon_{mM} = 6.22 \times 10^3 \).

2.7.3 Detection of alanine degrading enzymes adhering to bacteroids

Bacteroids prepared using the bench method were suspended in the reaction buffer used for flow chamber experiments and centrifuged at 12,000 g for 10 min. The activity of alanine dehydrogenase in the deaminating direction (2.7.2.2) was examined by testing for the reduction of NAD using the same reaction buffer used for endogenous alanine dehydrogenase assay, but using NAD and omitting pyruvate and NH$_4$+ (see 2.7.2).
2.8 Malate uptake

2.8.1 Malate uptake by bacteroids as prepared using the bench method

Bacteroids prepared using the bench method were resuspended in the same wash buffer used in bacteroid preparation and kept anaerobically at room temperature. 16 ml of wash buffer containing 100 µM oxy-Lb (LbO$_2$) was degassed under vacuum and divided into two. One was transferred by argon-flushed syringe into a capped, argon-flushed tube (0.9 ml tube$^{-1}$) and used for the microaerobic assay of malate uptake (the O$_2$ for bacteroid respiration being supplied from LbO$_2$); the other was shaken in air for 30 min and later distributed into another tube (also 0.9 ml tube$^{-1}$) and used for the aerobic assay of uptake. Each assay was started by taking 100 µl of bacteroids from each tube and adding them to 3 ml of reaction solution, degassed under argon or in air, in capped reaction tubes. The reaction solution contained 25 mM MOPS-KOH (pH 7.4), 50 µM $[^{14}$C]-malate, 100 µM LbO$_2$ and 2 mM MgCl$_2$. The silicon-oil filtration technique (Ou Yang et al., 1990; Udvardi et al., 1988b) was employed to determine the malate uptake. For time courses, at 1, 3, 5 and 7 min after the reaction started, 150 µl of the reaction mixture was taken out of the reaction tube and layered above 100 µl of silicon-oil (AR 200, Wacker Chemie, Munich, Germany), which separated the suspension from the bottom layer which contained 10 µl of 6 N HClO$_4$ in a 400 µl microfuge tube. The reaction was terminated by centrifuging the bacteroids through the silicon oil into the acid in a Beckman 152 microfuge at full speed (10,000 g) for 20 sec. The tip of the microfuge tube was cut off just above the acid layer and placed in a 1.5 ml Eppendorf tube and the bacteroid pellet was dispersed by vortexing after adding 1 ml scintillation fluid, and the radioactivity due to $^{14}$C was counted by liquid scintillation spectrometry (Beckman, LS 2800).
For effects of malate concentration, the reactions used 10–700 µM $^{14}$C-malate and were terminated after 5 min, 10 sec or, for the time courses, varying periods of time.

2.8.2 Malate uptake by bacteroids from the flow chamber

Samples of bacteroids were collected from the chamber into glass syringes by first inserting the #22 needle of a N$_2$-flushed syringe through the free injection port (Fig 2.3b) whilst maintaining the medium flow, closing off the effluent port tap (Fig 2.3a, T4) and easing the plunger of the syringe as the full medium flow displaced chamber content into the syringe. Usually 2.0 ml was collected at a time.

After each sample was taken, it was necessary to adjust the flow rate, in order to maintain constant conditions in the chamber. The flow rate was 1.00 ml min$^{-1}$, before taking the first sample.

Table 2.5. The calculation of flow rate after 2.0 ml of bacteroids was removed from the flow chamber at each sampling (a = 2.0).

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Bacteroids removed (mg)</th>
<th>Bacteroids remaining (mg)</th>
<th>New flow rate (ml/min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>$a/12 \times b = 0.1667b$</td>
<td>$(1-0.1667)b = 0.8333b$</td>
<td>1.20</td>
</tr>
<tr>
<td>Sample 2</td>
<td>$a/12 \times 0.8333b = 0.1389b$</td>
<td>$(1-0.1389)b = 0.6944b$</td>
<td>1.44</td>
</tr>
<tr>
<td>Sample 3</td>
<td>$a/12 \times 0.6944b = 0.1157b$</td>
<td>$(1-0.1157)b = 0.5787b$</td>
<td>1.73</td>
</tr>
<tr>
<td>Sample 4</td>
<td>$a/12 \times 0.5787b = 0.0964b$</td>
<td>$(1-0.0964)b = 0.4823b$</td>
<td>2.07</td>
</tr>
<tr>
<td>Sample 5</td>
<td>$a/12 \times 0.4823b = 0.0804b$</td>
<td>$(1-0.0804)b = 0.4019b$</td>
<td>2.50</td>
</tr>
</tbody>
</table>

* To maintain constant flow rate/bacteroids. The initial flow rate was 1.00 ml min$^{-1}$. The new flow rate would be different from experiment to experiment.

Table 2.5 is an example to illustrate the calculation of the weight of bacteroids remaining in the chamber after collection of 5 serial samples, each of $a$ ml. The chamber volume is 12.0 ml. The initial bacteroid weight is $b$ mg.
The time courses of malate uptake and effects of malate concentration were measured by the same silicon-oil techniques as above (2.8.1). The concentration curves were determined by varying malate concentrations in each reaction tube.

2.8.3 Malic enzyme (ME) and malate dehydrogenase (MDH) assays

The method for these assays was adapted from Copeland et al. (1989a).

The principle of the assay:

\[
\begin{align*}
\text{Malate} & \xrightarrow{\text{ME}} \text{OAA (Pyruvate)} \\
& \xleftarrow{\text{MDH}} \text{Malate}
\end{align*}
\]

Bacteroids were prepared using the bench method and incubated in the flow chamber for 80 min before they were taken out of the chamber with an argon-flushed syringe. A batch of bacteroids as prepared and stored anaerobically in ice was used as control.

Each sample of bacteroids was transferred into argon-flushed centrifuge tubes and centrifuged at 12,000 g for 10 min. Pellets were resuspended in 2-3 times their volume of breakage buffer containing 25 mM TES buffer-NaOH (pH 7.5), 5 mM KCl, 5 mM MgSO\(_4\)•7 H\(_2\)O and 5 mM DTT and transferred into bead cuvettes under an argon stream. They were broken using a glass bead beater (Mini-Beadbeater, Biospec, OK, USA) at a speed of 4200 rpm for at least 5 periods of 1 min, cooling on ice between treatments. The homogenate was centrifuged in a Ti 80 rotor at 150,000 g (40,000 rpm), 5 ºC for 30 min in an Optima LE 8K ultracentrifuge (Beckman Instruments, Palo, Alto, CA, USA) to remove cellular debris and unbroken cells. The supernatant, the crude soluble enzyme extract, was kept in ice until used for enzyme assays. The bacteroids,
after preparation or after taking out of the flow chamber and during breakage were always kept on ice whenever it was possible.

Some bacteroids as prepared were kept in air for at least 30 min before they were broken as above and then used for a comparison experiment, in which the activity of ME and MDH of those bacteroids was compared to that of anaerobic bacteroids.

The activities of ME and MDH were measured by the change in absorbance at 340 nm due to reduction of NAD or NADP or oxidation of NADH. For assay of NAD-ME, the reaction solution contained 50 mM HEPES-KOH (pH 7.3), 6 mM malate, 20 mM KCl, 2 mM MnCl$_2$•4H$_2$O, 0.75 mM EDTA and 1 mM NAD and for NADP-ME, the 1 mM NAD of the mixture was replaced by 1 mM NADP and the pH was adjusted to pH 8.3. For the assay of MDH, the reaction solution contained 50 mM HEPES-KOH (pH 7.0), 1 mM MgSO$_4$, 10 mM oxaloacetic acid and 0.014 mM NADH. Each assay was started by adding 5–100 µl of the crude enzyme extract into 2 ml of reaction mixture. Changes in absorbance at 340 nm were recorded for at least 5 min. The activity of enzymes was calculated as for the alanine dehydrogenase assay (see 2.7.2.2).

2.9 Determination of protein and bacteroid dry weight

Protein concentration in extracts was estimated by the method of Lowry et al. (1951). Bacteroid dry weight was determined after drying the centrifuged bacterial pellets in tared Corex tubes (triplicated) at 80 °C to constant weight, after the bacteroids were washed with wash buffer (once) and MQ water (twice) by centrifugation (12,000 g, 10 min) and resuspension.
2.10 Evaluation of data

All data were evaluated by standard statistical methods (Moroney, 1954; Fisher & Yates, 1953; Kendall, 1969). Where data from several experiments were pooled, an analysis of variance was performed, based on Brockwell (1980).
Figure 2.1. Apparatus used for the bench method of preparing bacteroids and purification by fractional centrifugation.
Figure 2.2a. The Sephadex (G75) column for purification of leghaemoglobin.
The column was eluted from the top with K-phosphate buffer (2.4.2.2). A broad, turbid band of green-brown impurities in the front was discarded and the following clear, red fractions containing leghaemoglobin was collected with a fraction collector (right-hand bottom).
Figure 2.2b. The air-saturated Sephadex (G25) column for the oxygenation of leghaemoglobin. The fractions collected from the G75 column (Fig 2.2a) was mixed with dithionite under N₂. The mixture was loaded onto the top of the column. The column was eluted with the same K-phosphate buffer (Fig 2.2a). As the band passed into the air-saturated column, its leading edge became red as dithionite was removed and the Lb Fe³⁺ was oxygenated by the dissolved O₂.
Figure 2.3a. Flow Chamber
Figure 2.3b. Cross section of the flow chamber (see Fig2.3a)
A, the main block; B, annular water jacket, sealed by a cylindrical tube of Perspex cemented to the main block and bearing inlet and outlet tubes; C, stainless steel stirrer and shaft; D, stainless steel bearing, housing a bronze bush and O-ring shaft seal; E, injection port (one or two); G, lower block; H, porous disc; I, annular collecting space for effluent solution; K, microporous membrane filter disc; M, O-ring seal between the two chamber block; L, outlet tube from collecting annulus (Bergersen & Turner 1990).
Figure 2.4. Assembly for the preparation of $^{15}$N-nitrogen.  
A & B: reservoirs; J1-J3: outlets of the reservoirs; T1-T6, taps; VHose 1 & 2: vacuum connections.
Figure 2.5. The electrode chamber used for the determination of O$_2$ contained in $^{15}$N$_2$ gas.
1.0 ml of each assay was taken into digest tubes and digested after the addition of digestion acid, digest made to 25 ml with H₂O. 3 ml of digest distilled. Each distillate volumed to 10 ml with MQ H₂O. NH₃ from 8 ml of the distillate collected by diffusion. Atoms % ¹⁵N in NH₃ analyzed.

9.0 ml of the balance centrifuged at 12,000g for 10 min

Supernatant

Bacteroid pellet

Total N in supernatant: 1 ml of supernatants placed in digestion tubes and digested after adding 1 ml digestion acid. Digests made to 10 ml with H₂O. The 10 ml each digest distilled. Distillates made to 10 ml and 5 ml of each digest diffused to collect NH₃. Atoms % ¹⁵N in NH₃ analyzed.

Supernatant volumed and used for following assays:
1. NH₃ in 2 ml of the supernatant collected by diffusion and estimated colorimetrically.
2. NH₃ in second 2 ml collected and atoms % ¹⁵N in NH₃ analyzed.
3. Third 2 ml lyophilized after addition of internal standard (10 µl of nor-leucine per ml). Atoms % ¹⁵N in amino acid analyzed by GC-MS.

Bacteroids resuspended in 1-5 ml of MQ H₂O and transferred quantitatively to digestion tubes and digested after addition of digestion acid. Digests made to 25 ml with H₂O. 3 ml of each digest distilled and NH₃ collected by diffusion. Atoms % ¹⁵N in NH₃ analyzed.

Residues after NH₃ removal was digested. Digests made to 10 ml. 8 ml of each digest distilled. Each distillate made to 20 ml. NH₃ in 10 ml of the diluted distillate was collected by diffusion. Atoms % ¹⁵N in NH₃ analyzed.

Figure 2.6. The illustration of preparation of samples for ¹⁵N experiments.
3 REGULATION OF BACTEROID RESPIRATION BY OXYGEN SUPPLY

3.1 Introduction

Rates of N\textsubscript{2} fixation by bacteroids from soybean root nodules have been found to be directly correlated with bacteroid respiration rates (Bergersen, 1997b), and are assumed to be governed by the concentration of free dissolved O\textsubscript{2} ([O\textsubscript{2}free]) to which they are exposed (Bergersen, 1999; Thumfort \textit{et al.}, 1994). These assumptions were supported by the early studies with bacteroids of \textit{B. japonicum} (strain CB1809), anaerobically isolated from root nodules of soybean and assayed in a flow-through chamber (Bergersen & Turner, 1990a). However, under certain experimental conditions, when energy-yielding substrates were not limiting, respiration of bacteroids was regulated by the rate of supply of O\textsubscript{2} rather than by the concentration of O\textsubscript{2} \textit{per se} (Bergersen & Turner, 1990a, 1993). When no exogenous substrates were supplied, utilization of endogenous reserves by bacteroids was also regulated by the rate of O\textsubscript{2} supply (Bergersen & Turner, 1992), which in turn regulated bacteroid respiration. For example, in one flow chamber experiment, consumption of O\textsubscript{2} by bacteroids was regulated by the flow of reaction solution and its O\textsubscript{2} content (free dissolved O\textsubscript{2} plus O\textsubscript{2} from LbO\textsubscript{2}). Bacteroids seemed to possess the capacity to vary their demand for O\textsubscript{2} when the conditions in the flow chamber were changed. If the flow rate increased, the O\textsubscript{2} demand increased as a result of the increase in respiration but the O\textsubscript{2} concentration in the chamber remained unchanged (Bergersen & Turner, 1990a). Recent preliminary research (Holtzapffel & Bergersen, 1998) with bacteroids of \textit{B. japonicum} (strain USDA110c) indicated that when the rates of supply of O\textsubscript{2} and malate were increased by raising the flow rate, respiration and N\textsubscript{2} fixation increased dramatically and [O\textsubscript{2}] fell. A further increase in flow rate again increased the rate of O\textsubscript{2} consumption but [O\textsubscript{2}] remained below the level of the initial steady state. This suggested that the increase in
flow rate had increased the bacteroid O₂-demand (Holtzapffel & Bergersen, 1998). The research described in this chapter was undertaken to extend these findings by investigating further the relationship between N₂ fixation, respiration and the regulation of respiration by O₂.

### 3.2 Experimental

Experiments with the flow chamber system (2.5.1) used bacteroids of *B. japonicum* (USDA110c) from soybean nodules (cv. Stevens), prepared anaerobically by differential centrifugation by the standard bench method (2.3.1) or by density-gradient methods (2.3.2). The rates at which O₂ was supplied to bacteroids in the chamber were varied by (a) changing the flow rate of reaction medium through the chamber; (b) by changing the [O₂ free] in the inflowing reaction medium by using either 3-5% (v/v) or 100% air in the gas mixture above the stirred reaction medium in two reservoir flasks (A and B; Fig 2.3a); or (c) by successively withdrawing bacteroids from the chamber, thus increasing the supply of O₂ to the remaining bacteroids in the chamber.

The time courses of changes in ∆A of Lb were recorded, [O₂ free] and rates of O₂ consumption calculated (2.5.1.2), and, during steady states, samples of effluent solution were collected for determination of NH₃/NH₄⁺ (2.6.1) as a measure of N₂ fixation.

### 3.3 Results

Bacteroids prepared by the “standard bench method” (involving simple differential centrifugation) were used first so that results could be compared directly with those obtained previously by Bergersen and Turner (1990a). Changes in [O₂ free], bacteroid respiration and N₂ fixation during a typical flow chamber experiment are shown in Fig 3.1. When a flow rate (0.5 ml min⁻¹) of reaction solution (Solution A) containing 2 mM
malate, 62.8 μM oxyleghaemoglobin and low [O₂ free] (60 μM) was initially supplied to the bacteroids, bacteroid respiration was low, remaining under 1 nmol O₂ min⁻¹ [mg DW]⁻¹. [O₂ free] declined slowly and continued to decline to approximately 20 nM, even after the first change of the flow rate (Fig 3.1 a and Table 3.1). When the flow rates were increased from about 0.5 to 0.75 and subsequently to 0.99 ml min⁻¹ (at 47 min and 84 min, respectively), the rate of supply of O₂ and substrate (malate) was increased by approximately 1.5-fold and then to 2-fold. Simultaneously, [O₂ free] in the chamber decreased progressively to near steady states at 20.2 and 11.5 nM O₂. Despite this decrease in free oxygen, the rate of respiration increased from 0.97 to 1.61, and then to 2.23 nmol O₂ min⁻¹ [mg DW]⁻¹ (Fig 3.1b, Table 3.1). Concomitantly, N₂-fixation increased from 0.64 to 0.82, and then to 0.98 nmols min⁻¹ [mg DW]⁻¹ (Fig 3.1 c, Table 3.1). When O₂ supply to the bacteroids was increased further by changing the reaction solution from 60 μM [O₂ free] (A) to 240 μM [O₂ free] (B), at the same flow rate, respiration increased by 156%, from 2.23 to 5.74 nmol O₂ min⁻¹ [mg DW]⁻¹. [O₂ free], on the other hand, first increased and then declined to the level before the reaction solution was changed (Fig 3.1a). When O₂ supply was increased further by progressively increasing the flow rate of solution B, respiration increased sequentially from 5.74 to 8.48, to 11.18, and finally to 17.90 nmol O₂ min⁻¹ [mg DW]⁻¹ (Table 3.1). During this time, [O₂ free] decreased from 13 to 6 nM until the upper limit of respiration rate was approached, at which point [O₂ free] rose to almost 10 nM (e.g., at the flow of 3.1 ml min⁻¹; Fig 3.1a, Table 3.1). N₂ fixation followed the increases in respiration, rising from 1.07, to 1.76, then to 2.31, and finally to 4.69 nmols NH₃ min⁻¹ mg⁻¹ (Table 3.1).

In the course of the change of flow rate from the beginning to the end of the experiments shown in Fig 3.1 and Table 3.1, O₂ supply was increased from 58.6 to 940 nmol min⁻¹ (an increase of approximately 16-fold), and respiration increased from 0.97 to 17.90 nmol O₂ min⁻¹ [mg DW]⁻¹, which was an 18.4-fold increase. Yet [O₂ free] declined from 35 to 9.8 nM. The overall increase in the rate of N₂ fixation during the
course of the experiment was 7.4-fold from 0.64 to 4.68 nmol NH₃ min⁻¹ [mg DW]⁻¹ (Table 3.1).

Similar results were achieved when an air-saturated reaction solution (containing 253.3 µM O₂) was supplied throughout to bacteroids (Table 3.2). In this experiment, the supply of O₂ (via flow rate) was increased from 144.4 to 550 nmol min⁻¹, an increase of about 3.8-fold. [O₂ free] decreased from 47.5 nM at 15 min to 10.6 nM at 151 min. Respiration, although much lower than in Table 3.1 because of a greater bacteroid concentration, increased from 0.22 to 1.51 nmol O₂ min⁻¹ [mg DW]⁻¹ (ca 4.8-fold), while N₂ fixation increased about 2.6-fold (Table 3.2).

The effects of increased flow rate were independent of bacteroid preparation methods. Similar declines in [O₂ free] and increase in respiration and in N₂ fixation occurred with bacteroids prepared using either sucrose gradients (Table 3.3) or Percoll gradients (Table 3.4). In the experiment with bacteroids prepared by sucrose gradients, O₂ supply was increased by up to 13.2-fold either by changing flow rate (with a total increase of about 5-fold) or by changing the reaction solution from A (containing low O₂, 117.7 µM) to B (containing high O₂, 296.7 µM). [O₂ free] dropped from 33.1 nM at 25 min to 7.3 nM at 152 min before it began to rise when the upper limit of respiration was approached. The respiration increased more than 15-fold and N₂-fixation increased 3.5 fold (Table 3.3) during these changes. The experiment with bacteroids prepared with Percoll gradients was performed slightly differently, but with similar results to the experiment above. These bacteroids were supplied with reaction solution A containing low O₂ (68.18 µM) for 88 min, during which time O₂ supply was increased from 38.18 to 114.68 nmol min⁻¹ by changing flow rate twice from 0.56, to 0.90, and then to 1.68 ml min⁻¹. Subsequently, the bacteroids were supplied with reaction solution B containing high O₂ (295.8 µM) for 100 min, during which time O₂ supply was increased 4.13-fold due to the change of solution and the flow rate was increased from 1.68 to 2.38 (less than 2-fold). [O₂ free] decreased first from 24.3 nM at 20 min to 5 nM at 87
min, then increased concomitantly with the change in reaction solution, but decreased after flow rate was again increased (until the upper limit of respiration was reached). N₂ fixation followed these changes in respiration. Respiration increased along with the increase in O₂ supply to the bacteroids, no matter how the reaction solution changed (Table 3.4).

In a different flow-chamber experiment with bacteroids prepared by the bench method, after an initial increase in flow rate, O₂ supply to bacteroids was further increased by the progressive removal of bacteroids from the flow chamber whilst maintaining constant flow rates. Here, respiration increased after each sample was taken (Fig 3.2, Table 3.5). At the beginning of the experiment, the initial flow rate was 0.51 ml min⁻¹, the O₂ supply was 167.3 nmol min⁻¹, [O₂ free] was 54.6 nM, and respiration was maintained at the low level of 0.56 nmol O₂ min⁻¹ [mg DW]⁻¹. When the flow rate was increased to 0.82 ml min⁻¹, O₂ supply was increased from 169 to 269 nmol O₂ min⁻¹, [O₂ free] declined quickly to 35.3 nM and respiration increased to 0.84 nmol O₂ min⁻¹ [mg DW]⁻¹. When the O₂ supply was increased by reducing the bacteroids in the chamber rather than by increasing flow rate at 12 min, [O₂ free] declined further as the O₂ supply per unit bacteroid increased; when more bacteroids were progressively removed from the chamber at 35, 76 and 101 min after the flow rate was adjusted to 1 ml min⁻¹, respiration increased after each removal of bacteroids (Fig 3.2b, Table 3.5), whereas [O₂ free] was almost unchanged (Fig 3.2a, Table 3.5).

### 3.4 Discussion

N₂ fixation in bacteroids is a process which requires large amounts of energy (ATP and reductant). This energy is produced by bacteroid respiration, which is dependent on the supply of O₂ to the bacteroids. In the inner nodule tissue, bacteroid respiration is often limited to a certain extent by the availability of O₂ (Bergersen, 1962b). O₂ penetrates
into the central tissue in response to the respiratory demand of the inner nodule tissue (Witty et al., 1986). The penetration of O₂ further into the inner nodule is restricted by a layer of cells, the so-called boundary cell layer, in the inner cortex. The boundary layer is a tightly packed set of cells with very few gas-filled intercellular spaces. This cellular structure is thought to impede O₂ diffusion and reduce [O₂ free] in the central tissue of nodules (Witty et al., 1986; Parsons & Day, 1990; Witty & Minchin, 1998; Bergersen, 1997b, 1999). It is commonly assumed, when constructing simulations of nodule function, that bacteroid respiration rates are governed by the [O₂ free] to which they are exposed (Thumfort et al., 1994; Bergersen, 1999). The flow chamber data presented here, together with the results reported by Holtzapffel & Bergersen (1998) and previous other work with bacteroids of *B japonicum* (CB1809) (Bergersen & Turner, 1990a, 1993), show that bacteroid respiration rate depends on the rate of O₂ delivery, rather than [O₂ free] per se. This was confirmed in independent experiments presented here, in which the rate of O₂ delivery was varied by several different means.

The data presented in Figure 3.1 and Table 3.1 show that when bacteroids prepared by the standard bench method were incubated in the flow chamber, with adequate supplies of malate and O₂, the initial respiration rate was low, and N₂ fixation was limited to 0.6 nmol NH₃ min⁻¹ [mg DW]⁻¹ and the equilibrium level of [O₂ free] was 35 nM and then declined (Table 3.1). At this stage, substrate supply was not the limiting factor of respiration rate because even if the malate supplied to the bacteroids was not sufficient, bacteroids could utilize endogenous stores of poly-β-hydroxybutyrate (PHB) (Bergersen & Turner, 1993). Rather, it appears that respiration was limited by the low rate of O₂ supply. When the supply of O₂ was increased by progressively increasing flow rate, respiration also increased while [O₂ free] continued to decline. Eventually respiration reached its maximum, at which point [O₂ free] began to rise (at 3.1 ml min⁻¹; Fig 3.1a, Table 3.1, Table 3.3 & Table 3.4). Over the entire experiment (Table 3.1), O₂ supply was increased 16-fold through the increase of the flow rate and change in dissolved O₂ in the supply of medium, respiration increased 18.4-fold. [O₂ free], on the other hand,
declined, from 35 to 9.8 nM, indicating that the more O₂ was available, the more of it was used (Fig 3.1, Table 3.1). Clearly, in flow chamber experiments with USDA110c bacteroids, respiration is regulated by the rate of O₂ supply, rather than by the [O₂ free] prevailing in the stirred chamber.

In the other flow chamber experiments using bacteroids prepared by density gradients (either sucrose- or Percoll-), the changes in the activities of bacteroids during the experiments were similar. N₂-fixation and respiration of these bacteroids also increased along with the increase of O₂ supply via the increase of flow rate. A comparison of the activities of those bacteroids drawn from Table 3.1, 3.3 and 3.4, for similar increase of flow is presented in Table 3.6.

Also, comparable results were achieved when the O₂ supply per bacteroid was increased by withdrawing bacteroids from the chamber, rather than by increasing the flow rate. This indicated that no matter how the O₂ supply was increased, it regulated the respiration of bacteroids. At no time in these experiments, was respiration rate correlated with the [O₂ free].

In previous work with bacteroids of *B. japonicum* strain CB1809 (Bergersen & Turner, 1993), respiration rates were found to be related to [O₂ free] in a Michaelis-Menten fashion. The reason for this important difference between studies is not clear but could be due to bacterial strain differences. Respiration by USDA110c was more susceptible to changes in the O₂ supply. From the data presented here (Fig. 3.1-3.2 and Table 3.1-3.5), calculations show that malate was supplied at rates in excess of bacteroid requirements for respiration. In the experiments with CB1809 bacteroids, no exogenous substrate was supplied during the initial period at low flow rate and during this period the bacteroids showed regulation of endogenous O₂ demand in response to changes in O₂ supply, by mobilizing poly-β–hydroxybutyrate (PHB) utilization (Bergersen & Turner, 1992). PHB reserves appear to be much greater or more easily mobilized in
CB1809 bacteroids and may contribute to respiration even when exogenous substrate is supplied. Thus there may be several mechanisms for regulating bacteroid respiration in response to changes in supply of O$_2$. The relationship between substrate supply and respiratory demand is explored in the next chapter.
Figure 3.1a. Data from a flow-chamber experiment, showing the change in $[O_{2\text{free}}]$ (▲) in the chamber due to the change in flow rate (─).
Solution A contained 60 µM $[O_{2\text{free}}]$ and solution B 240 µM $[O_{2\text{free}}]$. Both solution contained 2 mM malate and 26.8 µM LbO$_2$. 
Figure 3.1b. The data from the same flow-chamber experiment as in Fig 3.1a, showing the change of respiration in the course of incubation of bacteroids in the flow chamber.
Figure 3.1c. Data from the same flow-chamber experiment as in Fig 3.1a, showing the production of ammonia by bacteroids during the incubation in the chamber.
Figure 3.2a. Flow rate and $[\text{O}_2\text{ free}]$ of bacteroids in the flow chamber experiment, showing that $[\text{O}_2\text{ free}]$ declined first and then remained constant when O$_2$ supply to bacteroids was increased by taking samples out of the chamber at 11, 35, 76 and 101 min.

▲: respiration; ─: flow rate. Arrows show the sampling time.
Figure 3.2b. Flow rate and respiration of bacteroids in the flow chamber experiment, showing that respiration increased after each sample was taken out of the chamber at 11, 35, 76 and 101 min. ▲: respiration; ─: flow rate.
Table 3.1. Data from steady states in the flow chamber experiment illustrated in Fig 3.1. The bacteroids (50 mg DW in the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained 62.8 µM oxyleghaemoglobin and either 60 µM (A) or 240 µM (B) [O₂ free]. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min⁻¹)</th>
<th>Reaction solution</th>
<th>O₂ supply* (nmol min⁻¹)</th>
<th>[O₂ free] (µM)</th>
<th>n</th>
<th>Respiration (nmol O₂ min⁻¹ mg⁻¹)</th>
<th>N₂ fixation (nmol NH₃ min⁻¹ mg⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>31 - 42</td>
<td>0.478</td>
<td>A</td>
<td>58.57</td>
<td>0.0350 (0.0437)</td>
<td>12</td>
<td>0.971 (0.044)</td>
<td>0.637 (0.017)</td>
<td>2</td>
</tr>
<tr>
<td>47 - 75</td>
<td>0.747</td>
<td>A</td>
<td>91.54</td>
<td>0.0202 (0.0025)</td>
<td>29</td>
<td>1.610 (0.099)</td>
<td>0.818 (0.092)</td>
<td>7</td>
</tr>
<tr>
<td>84 - 95</td>
<td>0.991</td>
<td>A</td>
<td>121.45</td>
<td>0.0115 (0.0011)</td>
<td>11</td>
<td>2.231 (0.205)</td>
<td>0.978 (0.110)</td>
<td>4</td>
</tr>
<tr>
<td>99 - 113</td>
<td>0.991</td>
<td>B</td>
<td>300.02</td>
<td>0.0129 (0.0009)</td>
<td>15</td>
<td>5.741 (0.150)</td>
<td>1.077 (0.083)</td>
<td>4</td>
</tr>
<tr>
<td>119 - 124</td>
<td>1.441</td>
<td>B</td>
<td>436.33</td>
<td>0.0062 (0.0003)</td>
<td>16</td>
<td>8.483 (0.020)</td>
<td>1.762 (0.277)</td>
<td>7</td>
</tr>
<tr>
<td>140 - 147</td>
<td>1.899</td>
<td>B</td>
<td>575.02</td>
<td>0.0067 (0.0006)</td>
<td>18</td>
<td>11.177 (0.491)</td>
<td>2.306 (0.193)</td>
<td>7</td>
</tr>
<tr>
<td>162 - 172</td>
<td>3.105</td>
<td>B</td>
<td>940.19</td>
<td>0.00981 (0.0002)</td>
<td>11</td>
<td>17.902 (0.711)</td>
<td>4.685 (0.549)</td>
<td>8</td>
</tr>
</tbody>
</table>

*: Free dissolved O₂ plus O₂ in oxyleghaemoglobin.
Table 3.2. Data from steady states in a representative flow chamber experiment. The bacteroids (85.5 mg DW in the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained oxyleghaemoglobin 253.3 µM [O<sub>2</sub> free]. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>O&lt;sub&gt;2&lt;/sub&gt; supply* (nmol min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>[O&lt;sub&gt;2&lt;/sub&gt; free]** (µM)</th>
<th>n</th>
<th>Respiration (nmol O&lt;sub&gt;2&lt;/sub&gt; min&lt;sup&gt;-1&lt;/sup&gt; [mg DW]&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>N&lt;sub&gt;2&lt;/sub&gt; fixation (nmol NH&lt;sub&gt;3&lt;/sub&gt; min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-74</td>
<td>0.492</td>
<td>144.39</td>
<td>0.0475-0.0252</td>
<td>60</td>
<td>0.221 (0.0165)</td>
<td>0.090</td>
<td>1</td>
</tr>
<tr>
<td>75-104</td>
<td>0.953</td>
<td>279.68</td>
<td>0.0209-0.0202</td>
<td>30</td>
<td>0.454 (0.0490)</td>
<td>0.142</td>
<td>1</td>
</tr>
<tr>
<td>105-136</td>
<td>1.493</td>
<td>438.15</td>
<td>0.0195-0.0168</td>
<td>32</td>
<td>0.741 (0.0519)</td>
<td>0.147</td>
<td>1</td>
</tr>
<tr>
<td>137-151</td>
<td>1.874</td>
<td>549.96</td>
<td>0.0132-0.0106</td>
<td>15</td>
<td>1.057 (0.1492)</td>
<td>0.233</td>
<td>1</td>
</tr>
</tbody>
</table>

*: Free dissolved O<sub>2</sub> plus O<sub>2</sub> in oxyleghaemoglobin.

**: The range of [O<sub>2</sub> free] in each time interval.
Table 3.3. Data from a flow chamber experiment using bacteroids prepared on a sucrose gradient.
The bacteroids (69.75 mg DW in the chamber) were prepared by sucrose-density gradients. The substrate was 2 mM malate; the reaction solution contained 57.7 µM oxyleghaemoglobin and 60 µM (A; total O2 = 57.7 + 60 = 117.7) or 239 µM (B; total O2 = 57.7 + 239 = 296.7) [O2 free]. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min⁻¹)</th>
<th>Reaction solution</th>
<th>O2 supply* (nmol min⁻¹)</th>
<th>[O2 free]** (µM)</th>
<th>n</th>
<th>Respiration (nmol O2 min⁻¹ [mg DW]⁻¹)</th>
<th>N2 fixation (nmol NH3 min⁻¹ mg⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-51</td>
<td>0.465</td>
<td>A</td>
<td>54.41</td>
<td>0.0331-0.0279</td>
<td>27</td>
<td>0.640 (0.0197)</td>
<td>0.227 (0.007)</td>
<td>3</td>
</tr>
<tr>
<td>52-75</td>
<td>0.724</td>
<td>A</td>
<td>84.71</td>
<td>0.0239-0.0190</td>
<td>24</td>
<td>1.066 (0.0807)</td>
<td>0.280 (0.025)</td>
<td>3</td>
</tr>
<tr>
<td>76-98</td>
<td>0.916</td>
<td>A</td>
<td>107.17</td>
<td>0.0173-0.0384</td>
<td>23</td>
<td>1.243 (0.2494)</td>
<td>0.302 (0.072)</td>
<td>3</td>
</tr>
<tr>
<td>99-128</td>
<td>0.916</td>
<td>B</td>
<td>271.14</td>
<td>0.0388-0.0177</td>
<td>30</td>
<td>3.790 (0.2469)</td>
<td>0.448 (0.099)</td>
<td>3</td>
</tr>
<tr>
<td>129-151</td>
<td>1.435</td>
<td>B</td>
<td>424.76</td>
<td>0.0068-0.0065</td>
<td>23</td>
<td>5.908 (0.4365)</td>
<td>0.412 (0.076)</td>
<td>6</td>
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<tr>
<td>152-171</td>
<td>1.863</td>
<td>B</td>
<td>551.45</td>
<td>0.0073-0.0088</td>
<td>20</td>
<td>7.574 (0.7244)</td>
<td>0.596 (0.048)</td>
<td>3</td>
</tr>
<tr>
<td>172-184</td>
<td>2.428</td>
<td>B</td>
<td>718.69</td>
<td>0.0086-0.0170</td>
<td>13</td>
<td>9.852 (0.7849)</td>
<td>1.382 (0.182)</td>
<td>4</td>
</tr>
</tbody>
</table>

*: Free dissolved O2 plus O2 in oxyleghaemoglobin.
**: The range of [O2 free] in each time interval.
Table 3.4. Data from a flow chamber experiment using bacteroids prepared on a Percoll gradient.
The bacteroids (71.1 mg DW in the chamber) were prepared by Percoll-density gradients. The substrate was 2 mM malate; the reaction solution contained 55.5 µM oxyleghaemoglobin and 12.68 µM (A; total O₂ = 55.5 + 12.68 = 68.18) or 240.3 µM (B; total O₂ = 55.5 + 240 = 295.5) [O₂ free]. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min⁻¹)</th>
<th>Reaction solution</th>
<th>O₂ supply* (nmol min⁻¹)</th>
<th>n</th>
<th>[O₂ free]** (µM)</th>
<th>Respiration (nmol O₂ min⁻¹ [mg DW]⁻¹)</th>
<th>N₂ fixation (nmol NH₃ min⁻¹ mg⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-47</td>
<td>0.560</td>
<td>A</td>
<td>38.18</td>
<td>28</td>
<td>0.0243-0.0102</td>
<td>0.459 (0.0296)</td>
<td>0.290</td>
<td>1</td>
</tr>
<tr>
<td>48-72</td>
<td>0.900</td>
<td>A</td>
<td>61.36</td>
<td>23</td>
<td>0.0076-0.0068</td>
<td>0.784 (0.0630)</td>
<td>0.352</td>
<td>1</td>
</tr>
<tr>
<td>73-87</td>
<td>1.682</td>
<td>A</td>
<td>114.68</td>
<td>15</td>
<td>0.0050-0.0052</td>
<td>1.517 (0.1517)</td>
<td>0.690</td>
<td>1</td>
</tr>
<tr>
<td>88-127</td>
<td>0.577</td>
<td>B</td>
<td>170.68</td>
<td>40</td>
<td>0.0088-0.0093</td>
<td>2.208 (0.4434)</td>
<td>0.281</td>
<td>1</td>
</tr>
<tr>
<td>128-153</td>
<td>0.953</td>
<td>B</td>
<td>281.90</td>
<td>26</td>
<td>0.0078-0.0059</td>
<td>3.889 (0.0607)</td>
<td>0.440</td>
<td>1</td>
</tr>
<tr>
<td>154-170</td>
<td>1.948</td>
<td>B</td>
<td>576.22</td>
<td>17</td>
<td>0.0059-0.0078</td>
<td>7.873 (0.1087)</td>
<td>1.010</td>
<td>1</td>
</tr>
<tr>
<td>171-195</td>
<td>2.387</td>
<td>B</td>
<td>706.07</td>
<td>25</td>
<td>0.0075-0.0088</td>
<td>9.624 (0.1482)</td>
<td>0.813</td>
<td>1</td>
</tr>
</tbody>
</table>

*: Free dissolved O₂ plus O₂ in oxyleghaemoglobin.
**: The range of [O₂ free] in each time interval.
Table 3.5. Data from steady states in the flow chamber experiment illustrated in Fig 3.2.
The bacteroids (78.9 mg DW in the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained 91 µM oxyleghaemoglobin and 237 µM \([O_2\text{ free}]\). At each removal, 2 ml of the chamber contents were removed and replaced with the same volume of fresh reaction solution. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min(^{-1}))</th>
<th>Bacteroids DW (mg ml(^{-1}))</th>
<th>(\text{O}_2) supply* (nmol min(^{-1}))</th>
<th>([O_2\text{ free}]) (µM)</th>
<th>n</th>
<th>Respiration (nmol O(_2) min(^{-1}) [mg DW](^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7</td>
<td>0.510</td>
<td>23.013</td>
<td>167.28</td>
<td>0.0546 (0.0069)</td>
<td>7</td>
<td>0.556 (0.0288)</td>
</tr>
<tr>
<td>8-11</td>
<td>0.820</td>
<td>23.013</td>
<td>268.96</td>
<td>0.0353 (0.0017)</td>
<td>4</td>
<td>0.842 (0.1119)</td>
</tr>
<tr>
<td>16-34</td>
<td>1.000</td>
<td>19.177</td>
<td>269.96</td>
<td>0.0123 (0.0055)</td>
<td>19</td>
<td>1.158 (0.0448)</td>
</tr>
<tr>
<td>38-75</td>
<td>1.000</td>
<td>15.981</td>
<td>328</td>
<td>0.0045 (0.0005)</td>
<td>38</td>
<td>1.165 (0.0258)</td>
</tr>
<tr>
<td>80-100</td>
<td>1.000</td>
<td>13.317</td>
<td>328</td>
<td>0.0042 (0.0002)</td>
<td>13</td>
<td>1.670 (0.0593)</td>
</tr>
<tr>
<td>105-117</td>
<td>1.000</td>
<td>11.098</td>
<td>328</td>
<td>0.0045 (0.0003)</td>
<td>13</td>
<td>2.002 (0.0673)</td>
</tr>
</tbody>
</table>

*: Free dissolved O\(_2\) plus O\(_2\) in oxyleghaemoglobin.
### Table 3.6. Comparison of the activities of bacteroids prepared using different methods.
The comparison was based on the data presented in Table 3.1, 3.3 & 3.4.

<table>
<thead>
<tr>
<th>Bacteroid preparation</th>
<th>Time intervals</th>
<th>Increase of the activities</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Flow (T1-T2)</td>
<td>net inc. *</td>
<td>fold</td>
<td>O₂ Supply**</td>
<td>net inc.</td>
</tr>
<tr>
<td>Bench method</td>
<td>31-147</td>
<td>1.41</td>
<td>3.97</td>
<td>516.45</td>
<td>9.82</td>
<td>10.21</td>
</tr>
<tr>
<td>Sucrose-density</td>
<td>25-171</td>
<td>1.40</td>
<td>4.00</td>
<td>497.04</td>
<td>10.14</td>
<td>6.93</td>
</tr>
<tr>
<td>gradients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percoll-density</td>
<td>20-170</td>
<td>1.39</td>
<td>3.48</td>
<td>538.04</td>
<td>15.09</td>
<td>7.82</td>
</tr>
<tr>
<td>gradients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Net increment;  
**: Free dissolved O₂ plus O₂ in oxyleghaemoglobin.
4 RELATIONSHIP BETWEEN MALATE TRANSPORT AND RESPIRATORY DEMAND IN ISOLATED BACTEROIDS

4.1 Introduction

During N₂ fixation in legumes, nodules are supplied with sucrose (Kouchi & Yoneyama, 1986; Streeter, 1980, 1986). In nodule tissues, sucrose is then broken down to C₄-dicarboxylates, such as succinate and malate, which are readily used for energy production via bacteroid respiration (Bergersen, 1958; Bergersen & Turner, 1967; Day et al., 1989; Streeter, 1987) and to support N₂ fixation (Bergersen & Turner, 1967). It was found that these acids are present in significant amounts in nodules (Stumpf & Burris, 1979) and that the transport and metabolism of C₄-dicarboxylates in bacteroids is essential if the nodules are to be symbiotically functional (Finan et al., 1981). Later, transporters were found on the bacteroid and symbiosome membranes, which are responsible for C₄-carboxylate transport (Finan et al., 1981; Jording et al., 1994; Ou Yang et al., 1990; Ronson et al., 1981; Udvardi et al., 1988b; Udvardi & Day, 1997). However, these studies had been performed under aerobic conditions which inactivate nitrogenase and may alter other aspects of bacteroid metabolism. Notably, in aerobic conditions, bacteroid respiratory demand increases dramatically and nitrogenase is inactivated. The previous work has been important since it identified a series of transport proteins on the PBM and bacteroid membrane. However, these studies do not allow determination of regulatory parameters under in planta conditions, nor the investigation of the interaction between symbiotic transporters and nitrogen fixation. The work described in this chapter aimed to place previous studies with bacteroids in a physiological context by isolating bacteroids under anaerobic conditions which preserve nitrogen fixing activity, followed by measurements of malate transport and metabolism during N₂ fixation.
4.2 Experimental

In preliminary experiments on malate uptake, bacteroids were prepared using the standard bench method and resuspended in WB containing 100 µM LbO₂ degassed under vacuum (2.8.1). Aerobic bacteroids were obtained by shaking part of this suspension in air for at least 30 min. Two different assay conditions were designed: 1) aerobic reaction solutions containing 100 µM LbO₂ in air; 2) microaerobic assays performed in the same reaction solutions but degassed quickly under vacuum and sealed in argon-flushed tubes (O₂ for bacteroid respiration came from the LbO₂). The uptake of ¹⁴C-malate was determined essentially as described by Udvardi et al. (1988) and Ou Yang et al. (1990) (2.8.1).

Malate uptake by bacteroids from the flow chamber reactions was measured using the method 2 above. Samples (2-5 ml) were withdrawn from the flow chamber into argon-flushed syringes at certain time intervals (2.8.2).

Activities of malic enzyme and malate dehydrogenase were determined as described by Copeland et al. (1989a). Bacteroids prepared using the standard bench method were incubated in the flow chamber for certain times and samples withdrawn with argon-flushed syringes as above. The effluents were pelleted by centrifuging at 12,000 g for 10 min before they were resuspended in breakage buffer and broken using a glass bead beater (2.8.3). The homogenates were centrifuged as described in 2.8.3 and the activity of ME and MDH were measured and calculated by the change in absorbance at 340 nm due to reduction of NAD or NADP or oxidation of NADH (2.8.3). Bacteroids prepared using the same method were directly used as controls or kept in air at least for 30 min and used for comparison experiments (2.8.3).
4.3 Results

4.3.1 Malate uptake by bacteroids isolated anaerobically using the standard bench method

The results of initial experiments showed that malate uptake by anaerobically isolated bacteroids continued steadily for 5-9 min (Fig 4.1). The initial rate of uptake by anaerobically isolated bacteroids which were subsequently assayed under microaerobic conditions was the lowest. The greatest initial rate of uptake was found with bacteroids which were isolated anaerobically, incubated under aerobic conditions for some time, and then assayed aerobically, but the rate of uptake was not sustained, declining after 5 min (Fig 4.1).

4.3.2 Malate uptake by flow-chamber bacteroids

Bacteroids used in this work came from samples taken during two separate flow chamber experiments, one of which is illustrated in Fig 3.2a. The uptake of malate by these bacteroids was measured microaerobically as described above. The results are presented in Fig 4.2 and Table 4.2. Table 4.1 shows the data for conditions prevailing in the flow chamber prior to sampling for one of the two experiments. The first sample for uptake measurement was taken out of the flow chamber when the first steady state was approached after the first increase of flow, in which the \([O_2\text{ free}]\) had declined. The initial rate of malate uptake by those bacteroids remained low, only about 0.7 nmol min\(^{-1}\) [mg DW]\(^{-1}\) (Table 4.2). In the second steady state sampled, the O\(_2\) supply per bacteroid had been increased due to removal of bacteroids from the flow chamber (Table 4.1, 4.2), the \([O_2\text{ free}]\) had decreased to about 8 nM and respiration increased to 1.4 nmol O\(_2\) min\(^{-1}\) [mg DW]\(^{-1}\) (Table 4.2). The uptake of malate by the bacteroids, drawn from the chamber during this steady state, was higher, with an initial rate of 2.3 nmol min\(^{-1}\) [mg DW]\(^{-1}\)
The greatest uptake of malate occurred in the bacteroids obtained from the fourth steady state, with an initial rate of 3.9 nmol malate min\(^{-1}\) [mg DW]\(^{-1}\) (Table 4.2), although this declined after 5 min (Fig 4.2). Bacteroid respiration in this steady state was also the highest, but [O\(_2\) \text{free}] remained unchanged (Table 4.1, 4.2). The uptake by bacteroids from the third steady state was intermediate between that of the bacteroids from the second and fourth steady states (Fig 4.2; Table 4.2). Respiration in this steady state also was intermediate between the second and fourth steady state (Table 4.1, 4.2).

### 4.3.3 The effect of flow chamber conditions on the kinetics of malate uptake

The relationships between concentrations of \(^{14}\text{C}\)-malate and rates of uptake by bacteroids withdrawn from the flow chamber or stored anaerobically for the same period were measured after steady states in the chamber had been established (>80 min from the start of the experiment). There were 2 similar experiments of 82 or 85 min duration.

Bacteroids in 5.0 ml of flow chamber buffer were withdrawn from the flow chamber. In the 2 experiments, steady state O\(_2\) supply was 353 and 315 nmol O\(_2\) min\(^{-1}\), respectively; [O\(_2\) \text{free}] was 3.0 nM in both; respiration, 1.4 and 1.6 nmol O\(_2\) min [mg DW]\(^{-1}\) and N\(_2\) fixation 0.2 nmol NH\(_3\) min\(^{-1}\) [mg DW]\(^{-1}\) in both. Data from the two experiments were combined and submitted to analysis of variance. The malate uptake data are shown in Fig 4.3a & b. The data show that the \(V_{\text{max}}\) of malate uptake was much greater for bacteroids taken from the flow chamber than those that had been kept under anaerobic conditions for the same time (10 vs. 2.6 nmol [mg DW]\(^{-1}\)). However, the apparent \(K_{m\text{s}}\) for the two sets of bacteroids were not significantly different (0.8 and 0.6 mM respectively; Fig 4.3b).
4.3.4 *The effect of chloramphenicol (CAP) on malate uptake by the flow chamber bacteroids*

As bacteroids were incubated in the flow chamber for some time before malate uptake was measured, the increased uptake observed may have been due to the synthesis of more transporter protein during the incubation of bacteroids in the chamber. This would, effectively, increase the apparent $V_{\text{max}}$ of uptake. To test for this possibility, an inhibitor of protein synthesis, chloramphenicol (CAP), was employed in the malate uptake experiments with the bacteroids from the flow chamber. The flow chamber experiments were performed in the same way as for the time courses of malate uptake by the flow chamber bacteroids (Fig 4.2; Table 4.2) except that CAP (200 $\mu$g ml$^{-1}$) was included in the chamber buffer. The concentration of CAP used was based on studies with free-living *B. japonicum* cultures. It was found that CAP at 150 $\mu$g ml$^{-1}$ completely inhibited growth of strain USDA110c in yeast extract-mannitol (2.7.1.1). Bacteroids were withdrawn from the flow chamber after 15, 37, 73 and 102 min. The data from steady states in one of the two similar flow chamber experiments are presented in Table 4.3. The time courses of malate uptake are illustrated in Fig 4.4.

In the presence of CAP, the rate of uptake by the bacteroids increased with increased O$_2$ demand as noted previously (Table 4.1). However, after the third steady state, the uptake declined (Fig 4.4). The results suggest that CAP may have affected the turnover of carrier proteins when the bacteroids were incubated in the flow chamber for more than 1 h, but did not prevent the increase in transport due to increased O$_2$ supply before 1 h. This indicates that synthesis of new malate transport protein was not the primary reason for the initial response of malate uptake to respiratory demand but that sustained exposure to CAP may have prevented synthesis of new transporters in the normal protein turnover process.
4.3.5 *The effect of incubation time on the kinetics of malate uptake*

Initial work showed that uptake of $^{14}$C-malate by bacteroids was linear between 1 and 5 min (Fig 4.1). Consequently, kinetics of malate uptake by the flow chamber bacteroids were determined by incubating the bacteroids in reaction solution (2.8.1) containing $[^{14}$C]-malate for 5 min. However, Fig 4.1 indicates also that there may have been higher rates between 0 and 1 min. Assay times of 5 min may allow metabolism to influence uptake. Thus, comparative experiments were performed to determine whether the kinetics of uptake were affected by the duration of malate uptake assays. The bacteroids used for these experiments were prepared in air using the homogenizing system of the standard bench method (see 2.3.1). All four experiments were pooled and the means had very similar standard deviations (max 1.4 nmol malate min$^{-1}$ [mg DW]$^{-1}$) (Fig 4.5). Results showed that the apparent $K_m$ for the uptake assayed at different times were indeed different. When the uptake was assayed for 5 min, the apparent $K_m$ was 1.9 mM, but over 10 sec, it was 0.1 mM. The apparent $V_{max}$ was also different, 42.6 nmols min$^{-1}$ [mg DW]$^{-1}$ in the 5 min assay and 5.7 nmol min$^{-1}$ [mg DW]$^{-1}$ in the 10 sec assay.

Because of these results, the kinetics of malate uptake by the flow chamber bacteroids were again determined using 10 sec assay times.

Bacteroids prepared using the standard bench method were injected into the flow chamber and steady states established. The samples for malate uptake measurements were withdrawn from the chamber 80 min after the injection, when a steady state was well established. Malate uptake at various concentrations of malate was measured as above and the $K_m$s of uptake were determined. There were two similar flow chamber experiments and the relevant chamber conditions are presented in Table 4.4. During the steady states of the two experiments, O$_2$ supply was respectively, 324 and 355 nmol min$^{-1}$, [O$_2$ free], 7 nM and 6 nM, respiration, 2.0 and 2.2 nmol O$_2$ min [mg DW]$^{-1}$ and nitrogen fixation rates were the same at 0.3 nmol NH$_4^+$ min$^{-1}$ [mg DW]$^{-1}$ (Table 4.4).
The apparent $K_m$ of uptake for the bacteroids withdrawn from this steady state, measured using 10 sec incubation, was 52 µM (Fig 4.6), compared with 0.8 mM in 5 min assays of flow chamber bacteroids (Fig 4.3; see 4.3.3). These results suggest that at the longer assay times, metabolism of malate played a significant role and biased the kinetic parameters. Enzymes of malate metabolism were, therefore, investigated under the same conditions.

4.3.6 The determination of activities of malic enzyme and MDH

The bacteroids prepared using the standard bench method were injected into the flow chamber and one sample for the measurements of malic enzymes (NAD-ME and NADP-ME) and MDH was withdrawn 80 min after injection of bacteroids into the chamber. The flow chamber experiment (Table 4.5) was similar to that used for the determination of kinetics of malate uptake (4.3.5; also see Table 4.4). Enzyme activities were determined as described in 2.8.3. The bacteroids as prepared were used as control. The results showed that, with 252 nmol min$^{-1}$ of O$_2$ supply, 3.2 nmol O$_2$ min$^{-1}$ [mg DW]$^{-1}$ of respiration and 0.2 nmol NH$_4^+$ min$^{-1}$ [mg DW]$^{-1}$ of nitrogen fixation, the activities of NAD-ME in the bacteroids withdrawn from the flow chamber were higher than those in the bacteroids as prepared, but those of NADP-ME remained almost unchanged. MDH activity was lower in the flow chamber bacteroids than in the controls.

4.4 Discussion

N$_2$ fixation in vivo is supported by bacteroid respiration coupled with the consumption of substrates (e.g. malate; Bergersen & Turner, 1990a; Udvardi et al., 1988b) provided by the host plant. A number of studies have shown that current photosynthate is translocated into nodules as sucrose (Lowrie & Wheeler, 1975; Kouchi & Nakaji,
Chapter 4

1985). $^{13}$C- and $^{14}$C labelling studies demonstrated that sucrose is converted to C₄-dicarboxylic acids, succinate and malate (Reibach & Streeter, 1983, Romanov et al., 1985; Kouchi and Yoneyama, 1986). Thus, the potential role of dicarboxylic acids in the formation of effective legume-Rhizobium symbioses has been intensively investigated (Ronson et al., 1981; Finan et al. 1983; Rosendahl et al., 1991). Evidence from those authors demonstrated that a supply of C₄-dicarboxylic acids to bacteroids is essential for nitrogen fixation. As the bacteroids within the symbiosome are physically separated by the plant-derived symbiosome membrane (PBM), the C₄-dicarboxylates must be transported through the PBM and then the bacteroid membrane before effectively supporting the metabolic activities of bacteroids. Under aerobic assay conditions, Udvardi et al. (1988b) and Ou Yang et al. (1990) demonstrated that both the PBM and bacteroid membrane of soybean nodules possess an energy-dependent transporter mediating a rapid influx of dicarboxylates, mainly malate and succinate. This report triggered a series of intensive studies focused on the biochemistry, physiology and molecular biology of the transport system. The present work, using anaerobically isolated bacteroids, reinvestigated the manner of malate uptake, trying to place the previous studies in a physiological context. Bacteroids were prepared anaerobically, thus preserving nitrogen fixation activities, and uptake was measured in microaerobic conditions, close to those prevailing in vivo in nodule cells.

The results of the preliminary experiment (Fig 4.1) demonstrated that the rate of malate uptake by bacteroids not exposed to air was relatively slow but remained constant for 7 min. The rate of uptake by aerobically treated bacteroids under aerobic conditions of uptake similar to that used formerly (Udvardi et al., 1990) was high initially but declined after 5 min (Fig 4.1). This suggests that the mechanism of malate uptake is complex. Under aerobic conditions of preparation and assay, nitrogenase was inactivated, N₂ fixation ceased, and malate uptake and metabolism by the bacteroids benefited by diversion of reductant and the high respiration rate which results from the high availability of O₂. After 5 min the uptake of malate may have slowed because of
the accumulation of malate in the bacteroids. Microaerobic conditions, on the other hand, may not be favourable for respiration to support a high initial uptake but may favour longer-term uptake. Microaerobic conditions are closer to the physiological conditions experienced by bacteroids in nodules. Under these conditions, the [O$_2$ free] prevailing near bacteroids may not be high enough to support a high rate of respiration and malate transport. It is known that bacteroids are capable of persistent respiration at low O$_2$ concentrations without addition of exogenous energy-yielding substrates (Bergersen and Turner, 1990a) and that supply of malate interacts with the accumulation and utilization of the endogenous reserves of poly-$\beta$-hydroxybutyrate (Bergersen & Turner 1990b). It now appears that malate transport must be considered as a regulatory parameter.

The results of malate uptake by the bacteroids from the flow chamber showed that concomitantly with an increase in bacteroid respiration rates immediately preceding sampling (Table 4.1), the rate of transport of [$^{14}$C]-malate into bacteroids also increased (Fig 4.2; Table 4.2). For example, with an increase of bacteroid respiration in the chamber from 2.1 to 3.0 nmol O$_2$ min$^{-1}$ [mg DW]$^{-1}$ due to the increase of O$_2$ supply per bacteroid (Table 4.1), the rate of uptake of malate by bacteroids increased (Table 4.2) by a total of almost 7-fold, between the first and fourth samples. The rate of malate supply to bacteroids in the chamber (40 nmol min$^{-1}$ [mg DW]$^{-1}$) at a flow of 1 ml min$^{-1}$, Table 4.1) greatly exceeded the rate of O$_2$ consumption (2.1 nmol min$^{-1}$ [mg DW]$^{-1}$ in the same conditions (Table 4.1). It is unlikely, therefore, that the increase in malate uptake resulted simply from an increase in malate supply to the chamber; this was certainly not the case in the standard uptake assays of Fig 4.1. Nor was this simply a case of a stronger sink for malate (resulting from increased respiration) stimulating uptake, because (1) significant differences in rate of uptake were recorded when aerobically and anaerobically prepared bacteroids were assayed at the same oxygen concentration (Fig 4.1; Table 4.2); (2) with successive increases in bacteroid respiration preceding each sampling (Table 4.3), when malate uptake was measured under standard
conditions in which respiratory rates were uniform and malate concentrations were not limiting, specific rates of malate uptake also increased (Table 4.2). These results suggest that rates of malate uptake may be tightly coupled with rates of bacteroid respiration and thus with N\textsubscript{2} fixation. Further, the respiratory demand during the period preceding the transport assays seemed to feed-back and affect the uptake of malate during the standard uptake assays. Since the $V_{\text{max}}$ but not $K_m$ of uptake increased upon exposure to higher oxygen supply under constant pre-incubation and assay conditions (Fig 4.3), it appears that more malate carrier proteins were engaged following the increase in respiratory demand, but the small and late effect of chloramphenicol (Fig 4.4) indicates that this was probably not the result of synthesis of new carrier proteins during this initial phase. Instead, the respiratory demand seemed to feed-back and affect the uptake of malate directly. The nature of this conditioning process is not known but it suggests that both organic acid transport and metabolism in the bacteroid are controlled by a common factor. Pyridine nucleotide redox poise is a likely candidate for this factor.

Under aerobic conditions, usually high uptake of malate by bacteroids could be observed (Udvardi et al. 1988b; Fig 4.1) because respiration at this stage is enhanced due to the high availability of O\textsubscript{2}. However, nitrogen fixation of bacteroids is eliminated by the aerobic conditions. Thus the fast uptake of malate by bacteroids driven by the abnormally high respiration rates at ambient oxygen concentrations, may not reflect the reality of malate demand in vivo by bacteroids when nitrogen fixation by bacteroids is fully coupled.

There has been some evidence which shows that malate transported into bacteroids is metabolised via the TCA cycle. Bacteroids have been shown to contain tricarboxylic acid cycle enzymes (Finan et al., 1988, 1991; McKay et al., 1989; Saroso et al., 1986; reviewed by Streeter, 1991). Further evidence has shown that prior to operation of the TCA cycle, two forms of malic enzymes, NAD-ME and NADP-ME are thought to be responsible for oxidative decarboxylation of malate to pyruvate which is then oxidized.
via pyruvate dehydrogenase to form acetyl-CoA (see Fig 1.5), which is essential to the TCA cycle (Copeland et al., 1989b; Driscoll & Finan, 1997). Metabolism of malate via the TCA cycle also requires synthesis of equimolar amounts of OAA, which is the product of the oxidation of malate via MDH (reviewed by Vance et al. 1998 & by Day & Copeland, 1991). Day and Copeland (1991) assumed that between NAD-ME and NADP-ME, the former is more important for ATP production during nitrogen fixation by soybean bacteroids because NADP-ME has a higher $K_m$ for malate and only becomes active when substrate is abundant. The results of measurements of malic enzyme activities obtained from the present work showed that NAD-ME activity is closely related to bacteroid respiration rates. When $O_2$ demand by bacteroids in the flow chamber was enhanced by $O_2$ supply, malate uptake increased. To accommodate the increased substrate entering the bacteroids and to sustain the high rate of respiration, the activity of NAD-ME also increased. This finding supports the above assumption (Day & Copeland, 1991). It is also possible that the increase in NAD-ME activity influenced the rate of malate uptake by the bacteroids. However, since CAP did not prevent the increase in malate transport then the observed increase in NAD-ME was not the result of protein synthesis. Further studies are needed to determine if in fact NAD-ME is the main avenue for malate oxidation in the flow chamber.

Usually, in vivo, the infected central zone of nodules is subjected to very low $O_2$ tensions and the metabolism of substrate is limited by $O_2$ supply rather than by carbon supply (McDermott et al., 1989; also see above). Thus large quantities of PHB are accumulated (Goodchild & Bergersen, 1966; Wong & Evans, 1971). A significant amount of acetyl-CoA is stored in PHB when substrate is oxidized under microaerobic conditions in excess of the capacity of respiration. If the TCA cycle does not operate fully, it also appears that acetyl-CoA and NAD(P)H are partitioned between PHB synthesis and nitrogen fixation (reviewed by Day & Copeland, 1991). What regulates the balance between the PHB and TCA cycles and respiration has not yet been elucidated. It seems likely that in the flow chamber experiments, $O_2$ supply regulates the
O₂ demand. When O₂ supply is increased, respiration of bacteroids increases and malate uptake is also increased. High demand for O₂ is supported by transported substrate, malate or succinate, which is subsequently oxidized by NAD-ME to acetyl-CoA. Acetyl-CoA readily supports the TCA cycle and consequent respiration thus produces sufficient ATP to support nitrogen fixation and malate uptake - a cycle of dependencies.
Figure 4.1. The time course of $[^{14}C]$-malate uptake by bacteroids prepared using the standard bench method (LSD = 14.0 nmol malate [mg DW]$^{-1}$; p<0.05).

Bacteroids prepared in this way were divided into two parts. One was maintained anaerobically under argon (○, ▲); the other was exposed to air, in a rotary shaker at 100 rpm at room temperature for at least 30 min (●, △).

Two assay conditions were designated: aerobic (△, ▲) and microaerobic (○, ●).
Figure 4.2. Time courses of microaerobic $[^{14}C]$-malate uptake by bacteroids prepared using the standard bench method and withdrawn from the flow chamber after 12 ($\Delta$), 37 ($\blacktriangle$), 74 ($\bigcirc$) and 102 ($\bullet$) min (The data presented here are pooled from 2 similar experiments, LSD = 6.4 nmol malate [mg DW]$^{-1}$; p<0.05).
Chapter 4

Figure 4.3. Kinetics of malate uptake by bacteroids as prepared and after a prolonged steady state in the flow chamber bacteroids (Data are means from two similar experiments; LSD = 0.37 nmol min\(^{-1}\) [mg DW]\(^{-1}\); \(p<0.05\)). Bacteroids were prepared using the bench method and injected into the flow chamber. When the steady state had been established for 1 h, 5.0 ml of the chamber contents was removed and the uptake of malate by the bacteroids measured. During the steady state, the respiration was 1.4 nmol O\(_2\) min\(^{-1}\) [mg]\(^{-1}\) and O\(_2\) supply 352 nmol min\(^{-1}\). The incubation time of bacteroids with reaction solution containing different concentration of malate was 5 min. Bacteroids as prepared and stored anaerobically for 82-85 min were used as control.

- The flow chamber bacteroids; ○: Bacteroids as prepared.

a. The relationship between [malate] and uptake after 5 min.
b. Lineweaver-Burk plot from which the \(K_m\)s of uptake were determined.
Figure 4.4. Time courses of malate uptake by flow chamber bacteroids in the presence of chloramphenicol (CAP) (Data are the average of two similar experiments. LSD = 5.7 nmol malate [mg DW]^{-1}; p<0.05).

The concentration of CAP was 200 µg ml^{-1}. The bacteroids, prepared using standard bench method were injected into the flow chamber (see Table 4.3 for the detailed data) and taken out of the chamber after 14 (●), 37 (○), 71 (△) and 101 min (▲). Malate uptake was measured using the silicon-centrifugation technique.
Figure 4.5. Effect of assay time on kinetics of malate uptake by aerobically-prepared bacteroids (The data are pooled from two similar experiments. LSD = 1.1 nmol min$^{-1}$ [mg DW]$^{-1}$; p<0.05).

a. Concentration curve: ●, 5 min of assay time; ○, 10 sec of assay time.

b. Lineweaver-Burk plot of the concentration curves. Symbols are the same as in a.
Figure 4.6. The kinetics of malate uptake.
Bacteroids were drawn from the flow chamber after 80 min. The assay time for uptake was 10 second (The data are pooled from two similar experiments. LSD = 1.01 nmol min\(^{-1}\) [mg DW]\(^{-1}\); \(p < 0.05\)).

a. The concentration curve.

b. The Lineweaver-Burk plot from which the \(K_m\) were determined.
Table 4.1. Data from steady states of a typical flow chamber experiment used for malate uptake studies.

The bacteroids (164.5 mg DW injected initially into the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained 96 µM oxyleghaemoglobin and 227 µM [O₂ free]. Samples were withdrawn from the chamber after 12, 37, 74 and 102 min. At each removal, 2 ml of the chamber contents were removed and replaced with the same volume of fresh reaction solution. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min⁻¹)</th>
<th>Bacteroids DW (mg ml⁻¹)</th>
<th>O₂ supply* (nmol min⁻¹)</th>
<th>[O₂ free] (µM)</th>
<th>n</th>
<th>Respiration (nmol O₂ min⁻¹ [mg DW]⁻¹)</th>
<th>N₂ Fixation (nmol NH₃ min⁻¹ [mg DW]⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-9</td>
<td>0.500</td>
<td>13.71</td>
<td>161.50</td>
<td>0.0425 (0.0129)</td>
<td>7</td>
<td>1.033 (0.1495)</td>
<td>nd**</td>
<td>4</td>
</tr>
<tr>
<td>10-12</td>
<td>0.911</td>
<td>13.71</td>
<td>294.25</td>
<td>0.0136 (0.0014)</td>
<td>4</td>
<td>2.086 (0.4649)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>16-37</td>
<td>0.911</td>
<td>11.60</td>
<td>294.25</td>
<td>0.0083 (0.0022)</td>
<td>22</td>
<td>2.071 (0.0930)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>41-74</td>
<td>0.911</td>
<td>9.81</td>
<td>294.25</td>
<td>0.0052 (0.0007)</td>
<td>34</td>
<td>2.449 (0.0717)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>77-102</td>
<td>0.911</td>
<td>8.30</td>
<td>294.25</td>
<td>0.0055 (0.0006)</td>
<td>26</td>
<td>3.024 (0.1606)</td>
<td>0.248 (0.060)</td>
<td>4</td>
</tr>
<tr>
<td>105-111</td>
<td>0.963</td>
<td>7.02</td>
<td>311.05</td>
<td>0.0059 (0.0010)</td>
<td>7</td>
<td>3.578 (0.4230)</td>
<td>0.284 (0.007)</td>
<td>2</td>
</tr>
<tr>
<td>112-120</td>
<td>1.461</td>
<td>7.02</td>
<td>471.91</td>
<td>0.0060 (0.0004)</td>
<td>9</td>
<td>5.362 (0.1165)</td>
<td>0.533 (0.072)</td>
<td>2</td>
</tr>
</tbody>
</table>

* : Free dissolved O₂ plus O₂ in oxyleghaemoglobin.
**: Not determined.
Table 4.2. Steady state values of [O$_2$ free] and rates of bacteroid respiration in relation to measurements of uptake of $^{14}$C-malate by samples of bacteroids withdrawn from flow chamber experiments and assayed in standard microaerobic assays.

There were two separate experiments (one of which is illustrated in Figure 3.2a), in which the bacteroids in the flow chamber were supplied with 50 $\mu$M malate. Tabulated data are means and standard deviation (in brackets in the table) for the 10 min preceding each sampling. Malate uptake was measured using the silicon-oil filtration technique as described in 2.8.1. Data for duplicate malate uptake assays in two experiments were pooled and submitted to analysis of variance.

<table>
<thead>
<tr>
<th>Sample time (min)</th>
<th>[O$_2$ free] (µM)</th>
<th>Respiration (nmol O$_2$ min$^{-1}$ [mg DW]$^{-1}$)</th>
<th>$^{14}$C-malate uptake (nmol min$^{-1}$ [mg DW]$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.034 (0.0020)</td>
<td>0.91 (0.13)</td>
<td>0.68*</td>
</tr>
<tr>
<td></td>
<td>0.011 (0.0400)</td>
<td>1.48 (0.28)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>0.008 (0.0010)</td>
<td>1.39 (0.04)</td>
<td>2.29 *</td>
</tr>
<tr>
<td></td>
<td>0.007 (0.0006)</td>
<td>1.53 (0.09)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>0.005 (0.0002)</td>
<td>1.62 (0.04)</td>
<td>2.92*</td>
</tr>
<tr>
<td></td>
<td>0.005 (0.0003)</td>
<td>1.82 (0.05)</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>0.006 (0.0003)</td>
<td>1.91 (0.02)</td>
<td>3.93*</td>
</tr>
<tr>
<td></td>
<td>0.006 (0.0010)</td>
<td>2.18 (0.13)</td>
<td></td>
</tr>
</tbody>
</table>

* Analysis of variance showed that, despite small but significant differences between the two experiments, malate uptake increased significantly (p<0.001) with each increase in bacteroid flow chamber respiration.
Table 4.3. Data from steady states in one of the flow chamber experiments used in Fig 4.4.
The bacteroids (169.4 mg DW injected initially into the chamber) were prepared by the standard bench method. The reaction solution contained 2 mM malate, CAM (200 µg ml\(^{-1}\)), 103.8 µM oxyleghaemoglobin and 226.5 µM [O\(_2\) free]. At each removal, 3 ml of the chamber contents were removed and replaced with the same volume of fresh reaction solution. Samples (3 ml each) of chamber contents were removed after 15, 37, 73 and 102 min. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Time intervals (min)</th>
<th>Flow (ml min(^{-1}))</th>
<th>Bacteroids DW (mg ml(^{-1}))</th>
<th>O(_2) supply* (nmol min(^{-1}))</th>
<th>[O(_2) free] (µM)</th>
<th>n</th>
<th>Respiration (nmol O(_2) min(^{-1}) [mg DW](^{-1}))</th>
<th>N(_2) Fixation (nmol NH(_3) min(^{-1}) [mg DW](^{-1}))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>0.460</td>
<td>14.12</td>
<td>151.94</td>
<td>0.0102 (0.0014)</td>
<td>10</td>
<td>0.909 (0.0229)</td>
<td>nd**</td>
<td></td>
</tr>
<tr>
<td>10-15</td>
<td>0.910</td>
<td>14.12</td>
<td>300.57</td>
<td>0.0077 (0.0004)</td>
<td>6</td>
<td>1.738 (0.0613)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>19-37</td>
<td>0.989</td>
<td>11.94</td>
<td>326.67</td>
<td>0.0059 (0.0007)</td>
<td>19</td>
<td>2.207 (0.1091)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>40-73</td>
<td>0.911</td>
<td>9.81</td>
<td>326.67</td>
<td>0.0056 (0.0002)</td>
<td>34</td>
<td>2.450 (0.6017)</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>76-102</td>
<td>0.911</td>
<td>8.30</td>
<td>326.67</td>
<td>0.0062 (0.0001)</td>
<td>27</td>
<td>3.066 (0.0599)</td>
<td>0.226 (0.032)</td>
<td>5</td>
</tr>
<tr>
<td>105-111</td>
<td>0.963</td>
<td>7.02</td>
<td>326.67</td>
<td>0.0068 (0.0002)</td>
<td>7</td>
<td>3.613 (0.0837)</td>
<td>0.202</td>
<td>1</td>
</tr>
<tr>
<td>112-121</td>
<td>1.461</td>
<td>7.02</td>
<td>534.76</td>
<td>0.0075 (0.0004)</td>
<td>10</td>
<td>5.8558 (0.1097)</td>
<td>0.288 (0.122)</td>
<td>2</td>
</tr>
</tbody>
</table>

*: Free dissolved O\(_2\) plus O\(_2\) in oxyleghaemoglobin.
**: Not determined.
Table 4.4. Data from the steady state in the flow chamber of two similar experiments for the determination of the kinetics of malate uptake. The bacteroids (182.1 [Experiment 1] and 190.8 [Experiment 2] mg DW injected initially into the chamber) were prepared by the standard bench method. The substrate was 2 mM malate; the reaction solution contained 82.1 (Experiment 2: 111.1) µM oxyleghaemoglobin and 242 (Experiment 2: 229.7) µM [O₂ free]. At each removal, 4 ml of the chamber contents were removed and replaced with the same volume of fresh reaction solution. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Flow (ml min⁻¹)</th>
<th>Bacteroids DW (mg ml⁻¹)</th>
<th>O₂ supply* (nmol min⁻¹)</th>
<th>[O₂ free] (µM)</th>
<th>n</th>
<th>Respiration (nmol O₂ min⁻¹ [mg DW]⁻¹)</th>
<th>N₂ Fixation (nmol NH₃ min⁻¹ [mg DW]⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.000</td>
<td>12.90</td>
<td>324.10</td>
<td>0.0065 (0.0006)</td>
<td>51</td>
<td>2.032 (0.066)</td>
<td>0.324 (0.117)</td>
<td>7</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.044</td>
<td>13.44</td>
<td>355.80</td>
<td>0.0064 (0.0002)</td>
<td>44</td>
<td>2.184 (0.170)</td>
<td>0.258 (0.46)</td>
<td>5</td>
</tr>
</tbody>
</table>

* : Free dissolved O₂ plus O₂ in oxyleghaemoglobin.
Table 4.5. The determination of the activities of malic enzymes and MDH.

a. The flow chamber data.

Bacteroids were prepared using the standard bench method and incubated in the flow chamber. Totally, 107.07 mg DW of the bacteroids was injected in the chamber. Two samples were withdrawn from the flow chamber after 12 and 80 min respectively. The latter was used for the determination of enzymes. The substrate was 2 mM malate; the reaction solution contained 63.25 μM oxyleghaemoglobin and 237 μM [O₂ free]. O₂ was supplied at 0.84 ml min⁻¹ of reaction solution. At each time, 4 ml of the chamber contents were removed and replaced with the same volume of fresh reaction solution. “n” gives the number of observations in each steady state. Values in parenthesis are standard deviation.

<table>
<thead>
<tr>
<th>Bacteroid DW (mg ml⁻¹)</th>
<th>O₂ supply (nmol min⁻¹)</th>
<th>[O₂ free]* (μM)</th>
<th>Respiration (nmol O₂ min⁻¹ [mg DW]⁻¹)</th>
<th>n</th>
<th>N₂ Fixation (nmol NH₃ min⁻¹ [mg DW]⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>252.51</td>
<td>0.0062 (0.0018)</td>
<td>3.162 (0.440)</td>
<td>54</td>
<td>0.207 (0.137)</td>
<td>12</td>
</tr>
</tbody>
</table>

*: Free dissolved O₂ plus O₂ in oxyleghaemoglobin.

b. The activities of the enzymes.

The samples were transferred under flow of argon into argon-flushed centrifuge tubes and centrifuged at 12,000 g for 10 min. The pellets were resuspended in breakage buffer in liquid N₂ and immediately frozen and stored at –80 °C. Next day, a cell-free extract was prepared using a glass bead beater (Biospec, OK, USA) and the activities were determined (see 2.8.3).

<table>
<thead>
<tr>
<th>Bacteroids</th>
<th>Specific Activities of Enzymes (nmol NDA(P) or NAD(H) min⁻¹ [mg protein]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD-ME</td>
</tr>
<tr>
<td>As prepared</td>
<td>64.93</td>
</tr>
<tr>
<td>From steady state</td>
<td>94.20</td>
</tr>
</tbody>
</table>
5 REASSESSMENT OF THE PRODUCT OF NITROGEN FIXATION

5.1 Introduction

For many years it has been accepted that the fixation of atmospheric N\textsubscript{2} by the root nodules of legumes involved the production of ammonia by symbiotically diazotrophic bacteria (bacteroids) within the cells of the central tissue of the nodules. It was recognised that, due to the pK of 9.26 of the ammonia-ammonium equilibrium, at physiological pH, only a very small proportion of this nitrogen existed as NH\textsubscript{3}. The NH\textsubscript{4}\textsuperscript{+} ion would not diffuse across biological membranes, giving rise to concerns about the mechanism by which fixed N left bacteroids to be assimilated in the host cell (eg. Kahn \textit{et al.}, 1985). Subsequently an ammonium channel was identified on the peribacteroid membrane (PBM) (Tyerman \textit{et al.}, 1995) and the accepted interpretation persisted. That is, the acidic nature of the symbiosome space acts as an acid trap to form NH\textsubscript{4}\textsuperscript{+} ions thereby creating a gradient for diffusion of NH\textsubscript{3} out of the bacteroids, with the NH\textsubscript{4}\textsuperscript{+} subsequently transported to the plant via the PBM channel.

The basis for this interpretation was substantially due to the following experimental observations. (1) Ammonia was the earliest $^{15}$N-labelled product of N\textsubscript{2}-fixation when detached root nodules of soybean (\textit{Glycine max} Merr; Bergersen, 1965) or serradella (\textit{Ornithopus sativa} L.; Kennedy, 1966a, b) were incubated for short periods of time in atmospheres containing $^{15}$N\textsubscript{2}. (2) With anaerobically-prepared soybean nodule bacteroids (the symbiotic form of \textit{Bradyrhizobium japonicum}), in microaerobic, shaken assays with $^{15}$N\textsubscript{2} in the gas phase, the principal product was $^{15}$N-ammonia (Bergersen & Turner, 1967). (3) Later, $^{15}$N-ammonia was the principal product of $^{15}$N\textsubscript{2} fixation by soybean bacteroids in an open, flow-reaction system in which a well-stirred suspension
of soybean bacteroids was perfused with solutions containing dissolved O₂ and ¹⁵N₂; no ¹⁵N-labelled amino acids were detected (Bergersen & Turner, 1990a).

The long-accepted view was challenged by Waters et al. (1998) who reported that alanine, not ammonia, was the principal ¹⁵N-labelled product when N₂-fixing soybean bacteroids, purified on anaerobic sucrose density-gradients, were supplied with malate and shaken in a gas mixture containing ¹⁵N₂ and 0.008 atm O₂. These authors concluded also that the earlier results arose from the use of bacteroid preparations that were contaminated with cytosolic enzymes from the host tissue, which released ammonia from the primary product, alanine. Allaway et al. (2000) subsequently showed that both ¹⁵N-alanine and ¹⁵N-ammonia were produced by bacteroids prepared from nodules of Pisum sp. on anaerobic Percoll density-gradients but that the proportion of these products was altered by the conditions applied. When conditions were optimised for nitrogen fixation, ammonia was the first and major product formed but alanine was formed at high bacteroid densities when ammonia accumulated (Allaway et al., 2000). These authors also used a mutant strain of R. leguminosarum, defective in alanine catabolism, to produce N₂-fixing nodules on peas. Although plants fixed N₂ at normal rates, they grew more slowly than plants nodulated by the wild type, but the basis for this was not determined. Bacteroids from these nodules produced only ammonia in shaken assays. It was concluded that alanine was a secondary product of N₂-fixing bacteroids, arising from ammonia, derived from N₂ fixation, which accumulated in the experimental system used. More recently, Atkins & Thumfort (2002) have reported that assimilation of ¹⁵N₂ by nodulated roots of cowpea produced ¹⁵N-labelled products which showed no evidence that alanine was a precursor of the amide groups of glutamine or of the purine ring of ureides (the major N₂-fixation product translocated from the nodules).

The present work intended to reinvestigate the nature of the N₂ fixation products which leave the bacteroids and to verify the results of Waters et al. (1998) using density-gradient-purified soybean bacteroids, ¹⁵N labelling techniques and other biochemical
methods, but the verification was not achieved. Instead, ammonia was the sole significant $^{15}$N-labelled product of $^{15}$N$_2$ fixation accumulated during 30 min in shaken assays with 0.008-0.01 atm O$_2$. Alanine, although sometimes found in low concentrations in flow chamber reactions, was not labelled with $^{15}$N in shaken, closed system experiments. It is concluded that these and earlier results (Bergersen & Turner, 1967, 1990a) were not due to contamination with host cytosolic enzymes as suggested by Waters et al. (1998) and that ammonia is the principle product of N$_2$-fixation in soybean.

5.2 Experimental

5.2.1 Bacteria

*B. japonicum* strain USDA110c is the strain used in this laboratory for many years (to identify the strain from the other strains in the work presented in this chapter, it is designated as “USDA110c”). Strain USDA110de was kindly supplied by Prof. D. W. Emerich, University of Missouri and strain SU1014/1 (CB1809) by the SUNFix Culture Collection, University of Sydney. *Rhizobium leguminosarum* bv *viciae* strains 3841 and RU1327 (*aldA*) were gifts from Prof. P. S. Poole, University of Reading. Cultures were maintained on yeast extract-mannitol agar (Dalton, 1980) and on Brown and Dilworth’s defined liquid medium (Dalton, 1980) but with 10 mM NH$_4^+$ and succinate respectively as nitrogen and carbon sources (Allaway *et al.*, 2000) when rhizobia were grown for preparation of cell-free extracts for determination of alanine dehydrogenase activity.

5.2.2 Nodules and bacteroid suspensions

Nodules aged about 35 days were picked from roots of soybean (*Glycine max* Merr. cv Stevens and sometimes for comparison, cv Williams) inoculated at planting with *B.*
japonicum strain USDA110c and grown in pots of sand. Growing conditions are as described in 2.1. The nodules were washed in tap water, drained and blotted dry with paper towels and used immediately for preparation of bacteroid suspensions: (1) by the standard method of anaerobic homogenisation in phosphate buffer as described in 2.3.1, or (2) by homogenisation in the same solution under argon in an anaerobic glove box, followed by separation and washing of bacteroids by anaerobic centrifugation on Percoll (Pharmacia) density gradients as described in 2.3.2. Finally, the bacteroids were dispersed in reaction solution and an aliquot saved for determination of dry weight after centrifugation and washing with distilled water as described in 2.8.

5.2.3 Experimental systems

Experiments in closed systems were as described in 2.5.2 and in the flow chamber system were as described in 2.5.1. Products of nitrogen fixation in supernatants of reaction solution or in effluent of the flow chamber were measured as described in 2.6.

All the other methods used for the research work presented in this chapter were detailed in 2.6 and 2.7.

5.3 Results and discussion

5.3.1 Determination of alanine and NH3 production by bacteroids under various conditions

5.3.1.1 Flow chamber experiments

Waters et al. (1998) stressed the need to use gradient-purified bacteroids which earlier work had shown to be free of host cytosolic enzyme activity and that the critical pO2 for
alanine production by density gradient-purified bacteroids in their shaken, closed system was 0.008 atm. Therefore, alanine production by gradient purified bacteroids and by bacteroids prepared by differential centrifugation, in flow chamber reactions at low steady state $[O_2]$ was closely investigated.

Bacteroids prepared using either density-gradient methods or the standard bench method were injected into the flow chamber in separate experiments. The effluent fractions during steady states were collected and $NH_4^+$ was measured colorimetrically as described in 2.6.2. Alanine was determined using the enzymatic method described in 2.6.2.1.2. The flow chamber conditions were similar to those used for the investigation of the regulation of $O_2$ demand by $O_2$ supply in chapter 2 and illustrated in Fig 5.1a. As noted earlier $NH_4^+$ was readily detected (Fig 5.1b; Table 5.3; also see Fig 3.1), but in most cases alanine was not detectable. Occasionally, a small amount of alanine could be measured enzymatically (Fig 5.1b). Alanine production was not improved in any systematic pattern when the density-gradient-purified bacteroids were used in the chamber (Table 5.1).

Allaway et al. (2000) suggested that failure to detect significant alanine in flow chamber effluents (Bergersen & Turner, 1990a) could have been due to continuous removal of ammonia from the flow chamber, thus preventing $NH_3$ reaching a concentration needed for significant alanine formation. To address this issue, the effluents from the flow chamber collected during steady states were purified and concentrated using Sephadex ion-exchange columns (2.6.2.2.1). The amino acids in eluates from these columns were analysed by HPLC (see 2.6.2.2.2). The results are presented in Table 5.1 in comparison with the $NH_4^+$ results in the corresponding experiments. It has been shown that $N_2$ fixation into $NH_3$ by bacteroids in the flow chamber system depends on respiration rates (see chapter 3 and Bergersen, 1997a). Sucrose-density gradient purified bacteroids, as used by Waters et al. (1998), generally had lower rates of respiration and $NH_3$ production but yielded no more alanine than bacteroids prepared by other methods.
Percoll-gradient purified bacteroids were more active in respiration and N₂ fixation than sucrose gradient bacteroids and produced alanine at up to 19% of rates of NH₃ production, whilst bacteroids prepared by the differential centrifugation method, although producing more NH₃ per unit of respiration, produced only a little alanine at rates unrelated to respiration (Table 5.1).

5.3.1.2 Closed shaken experiments

As noted above, some alanine was present in the soluble fractions from flow chamber experiments, but only appeared at a very low level and was unlikely to be related to bacteroid respiration. On the other hand, Allaway et al. (2000) suggested that the flow chamber is not suitable for detecting alanine because the continuous removal of effluent from the chamber prevented the NH₃ reaching a concentration needed for significant alanine formation. Thus a closed shaken system, similar to those used by Waters et al. (1998) and Allaway et al. (2000), was employed and various experiments with it were performed to seek the right conditions under which the N₂ fixation is conserved and alanine formation can be detected. These experiments are summarized in Table 5.2.

Under some circumstances, some alanine was found in supernatants from centrifuged bacteroid suspensions taken from closed, shaken flasks, after concentration and purification using Sephadex columns (Table 5.3). However, it is unlikely that alanine production was directly due to N₂ fixation because similar concentrations of alanine were found in 1 h assays with Percoll gradient bacteroids in air (in which nitrogenase activity would have been destroyed) and at a pO₂ of 0.008 atm at which production of alanine by soybean bacteroids was reported to be optimal (Waters et al., 1998). In this experiment, alanine comprised respectively 72% and 90% of the total of ten amino acids detected (Table 5.3). However alanine was always produced at much lower rates than NH₃ (Fig 5.2). When up to 5 mM NH₄⁺ was supplied in such experiments, the rates of alanine production doubled but remained much lower than NH₃ production with no
added $\text{NH}_4^+$ (Table 5.4). It was often found that bacteroid suspensions contained significant amounts of alanine (and ammonia) upon isolation (Fig 5.2). This may arise during the isolation procedure. Experiments with extracts from bacteroids prepared by Percoll density gradient and other methods (below) indicated that alanine dehydrogenase was present internally in the bacteroid preparations (below) but produced only limited amounts of alanine in the medium from exogenous malate and $\text{NH}_4^+$. This suggests that *B. japonicum* USDA110c, maintained in our laboratory, has little potential for alanine formation under nitrogen fixing conditions.

N$_2$ fixation into NH$_3$ by these bacteroids in shaken assays continued for 30 - 40 min, declining gradually thereafter (Fig 5.2), but in the flow chamber, steady rates were sustained easily for several successive periods of $>20$ min.

### 5.3.2 Incorporation of $^{15}$N$_2$ into the products of N$_2$ fixation

There were two similar $^{15}$N$_2$ experiments with Percoll-gradient purified bacteroids shaken in stoppered flasks using conditions designed to be close to those reported to produce $^{15}$N-alanine (Allaway *et al*., 2000; Waters *et al*., 1998). These experiments were conducted 6 months apart; the results of the first (bacteroids from summer-grown nodules) are presented (Table 5.5). The data for analyses of the total N of the experimental system indicated that digestion of the morpholino-moiety of the MOPS buffer may have been incomplete. However, this was a constant error and did not affect calculation of the N-weighted $^{15}$N balance in which a matrix of determinations of $^{15}$N-labelled fractions and no-$^{15}$N$_2$ controls was used. Also, as noted elsewhere (Bergersen & Turner, 1967; Waters *et al*., 1998) there was a substantial background of endogenous ammonia in these reaction mixtures (perhaps including ammonia absorbed in H$_2$SO$_4$ on the untreated filter paper strips onto which the $^{15}$N-ammonia was diffused). These factors contributed to the relatively low $^{15}$N enrichment of the soluble ammonia (1.2 atoms % excess; Table 5.5), compared with the enrichment of the $^{15}$N$_2$ supplied (54.6
atoms % $^{15}$N). The analysis accounted for 79.2% and 75.4%, respectively, of the total N and the $^{15}$N, which was fixed. About 25% of the $^{15}$N, which was fixed, was incorporated in the bacteroids (similar to previous reports; Bergersen and Turner, 1967, 1990a). The soluble fraction after removal of the bacteroids contained $>50\%$ of the fixed $^{15}$N, 93% of which was $^{15}$N-NH$_3$ with enrichment of 1.2 atoms % excess. The residue of the soluble fraction, after removal of the NH$_3$, where any amino acids would have been located, contained only 6% of the total $^{15}$N excess.

The second experiment, using winter-grown nodules, produced almost identical distribution and enrichment of fixed $^{15}$N but the total amount of $^{15}$N fixed was 25% lower, perhaps reflecting lower rates of fixation during winter. In both experiments alanine was present in the soluble fraction but the GC-MS analysis revealed no significant excess of $^{15}$N in flasks containing bacteroids exposed to $^{15}$N$_2$ for 30 min, compared with control flasks (Table 5.6). Values of R for the standard (Sigma) alanine were lower than those for the control (no $^{15}$N$_2$) alanine. This may have been due to different isotopic composition of any of the elements in the Sigma alanine, but the m/e signals were free of signals from other chemicals which, at very low concentration, may alter the m/e signals of control or enriched experimental alanine. Additionally, the data for pure alanine were obtained at slightly higher concentration than for control and experimental alanine. In all cases, alanine was the most significant compound derivitized. It should be noted that, had the standard alanine (Sigma; as used by Waters et al., 1998) been used as the control for natural $^{15}$N abundance, the alanine from the reactions would have appeared to have been enriched with $^{15}$N (0.88 atoms % excess).

Allaway et al., (2000) used bacteroids from pea root nodules to show that alanine was synthesized from the soluble pool of NH$_3$/NH$_4$$^+$ in shaken assays. In my experiments, although the NH$_3$/NH$_4$$^+$ had by far the greatest atoms % excess $^{15}$N of any fraction (Table 5.5), it was only 1.2 atoms % excess. This may have contributed to the failure to measure significant $^{15}$N-alanine by the relatively insensitive GC-MS method (Table
5.6). Nevertheless, Table 5.5 shows that there was little room in the $^{15}$N balance of the soluble fraction for $^{15}$N alanine. In this experiment, an increment of 2.0 (± 0.8) µg NH$_3$-N was measured between zero time and 30 min samples. Therefore it was possible to calculate that newly fixed NH$_3$/NH$_4^+$ had 22.8 (95 % confidence limits 1.8 - 43) atoms % excess $^{15}$N. This rather imprecise estimate reflects the analytical difficulty of determining small increments of NH$_3$-N.

5.3.3 Detection of alanine degrading enzymes

5.3.3.1 Detection of alanine degrading enzymes adhering to bacteroids

There was speculation that the earlier results which showed NH$_3$ is the primary product of N$_2$ fixation, arose from the use of bacteroid preparations that were contaminated with cytosolic enzymes from the host tissue, which released ammonia from the primary product, alanine (Waters et al., 1998). If it is the case, it is quite possible that the supernatant of the bacteroid suspension may contain enzymes which have the capacity to deaminate alanine. The work presented in this section is to determine the activity of alanine dehydrogenase (ADH) which adhered to bacteroids prepared by differential centrifugation or by density gradient purification. Bacteroids after preparation by either Percoll-density gradient or by the fractional centrifugation in the bench method were resuspended in 1 ml WB for 1 h and centrifuged at 12,000 g for 10 min. The activity of ADH was determined using the method as described in 2.7.2. The results showed that the solution in which the bacteroids had been suspended did not have any capacity to deaminate alanine, suggesting that the supernatant of washed bacteroids was not contaminated by ADH. In other tests, although there was a slow production of NH$_3$ (<5 nmols NH$_3$ mg$^{-1}$ h$^{-1}$) from bacteroids prepared by fractional centrifugation, this was not stimulated in the presence of alanine concentrations up to 10 mM.
Obviously, contamination of bacteroids with enzymes degrading alanine was not the cause of failure to detect alanine as a major product of nitrogen fixation in the present work (Tables 5.5, 5.6) or in previous work (Bergersen & Turner, 1967; 1990a). This finding suggests that the density gradient methods, which take longer and result in bacteroids with impaired metabolic properties (Table 5.1) may not be necessary.

5.3.3.2  *Endogenous alanine dehydrogenase (ADH)*

This work was undertaken to test whether the failure to detect $^{15}$N-alanine as a product of $^{15}$N$_2$ fixation (Table 5.1; Bergersen & Turner, 1990a) may have been due to the absence of ADH activity from the bacteria used, either constitutively or due to differences in symbiotic expression. Therefore, a comparison was made between cultures, some of which had known ADH phenotypes, from a variety of sources. All were grown in succinate medium (Allaway *et al*., 2000), which is known to promote expression of *aldA* in *R. leguminosarum* (P. S. Poole, priv. com). Also, a comparison was made between bacteroids from nodules on cv. Williams and cv. Stevens, in case there may have been a difference between *aldA* expression in the two varieties when nodulated by the same strain of *B. japonicum* (cf. Stripf & Werner, 1978).

ADH activities were determined in cell-free extracts prepared by disruption of suspensions in a French press (2.7.2). Liquid cultures of various strains of rhizobia (2.7.1) were harvested in mid-log phase by centrifugation and resuspended in breakage buffer. Bacteroids were prepared using Percoll density gradients, as used in the $^{15}$N experiment (Table 5.5) and also resuspended in breakage buffer. Enzyme extracts were prepared as described in 2.7.2.1. ADH activity was estimated at 25 ºC according to Allaway *et al*., (2000) and Smith & Emerich (1993a, b) (2.7.2). The apparent $K_m$ values for pyruvate and NH$_4^+$ were determined.
5.3.3.2.1 ADH activity in bacteroids

An unpurified, soluble extract of the bacteroid preparation, as used in the $^{15}$N experiment (Table 5.5) contained endogenous ADH activity (> 600 nmols NADH min$^{-1}$ [mg protein]$^{-1}$ in the aminating direction), with an apparent $K_m$ of 0.9 mM and 7 mM for pyruvate and NH$_4^+$ respectively (Table 5.7). This activity was greater than that of extracts of the same strain grown in succinate medium. These results indicated clearly that the enzyme activity had not been lost in the symbiotic state or during the isolation of bacteroids, and that the failure to detect substantial alanine excretion from N$_2$-fixing bacteroids did not result from such loss.

5.3.3.2.2 ADH activities in extracts of cultured B. japonicum strains

The results of ADH activities in extracts of cultured B. japonicum strains are presented in Table 5.7. It is clear that in all strains in which ADH was known and in the strains used in my work (USDA110c) or previously (USDA110de; Waters et al., 1998; CB1809, Bergersen & Turner, 1990a), ADH was present. The aldA mutant strain (RU1327; Allaway et al., 2000) had negligible activity. USDA110c bacteroids from nodules of cv Stevens had high ADH activity when freshly prepared (although those from winter-grown plants had lower activity, causing differences between experiments; Table 5.7). After storage at -70 °C for several months, bacteroids prepared as for the $^{15}$N experiment (Table 5.5), yielded cell-free extracts with ADH activity and kinetic values (data not shown) similar to those in Table 5.7. Bacteroids prepared from USDA110c nodules on cv Stevens and cv Williams both had active ADH (Table 5.7). These results show that it is most unlikely that the data of Table 5.5 or of Bergersen & Turner (1990a) were due to defects in expression of aldA or to lack of ADH activity in the bacteroids.
5.4 General discussion

It has long been accepted that ammonia is the principal product of N₂ fixation by the bacteroids of *B. japonicum*. This had not been questioned until Waters *et al.* (1998) presented data showing alanine rather than ammonia is the principal nitrogen-containing compound released from N₂ fixing bacteroids of *B. japonicum* when the bacteroids were isolated from soybean nodules using sucrose density-gradient procedures. These authors proposed that the bacteroids which were used in earlier work (Bergersen & Turner, 1967; 1990a) were significantly contaminated by cytosolic enzymes. These contaminants may have degraded alanine, to produce ammonia, thus preventing identification of alanine as a major product of N₂-fxation. However, the results obtained from the present work clearly showed that NH₃/NH₄⁺ was the principal product from soybean bacteroids, prepared by various procedures, when assayed in solution in a flow chamber under N₂ fixation conditions. Small quantities of alanine were also produced, reaching 20% of the NH₃/NH₄ in some conditions.

Some ^1⁵^N was assimilated into Percoll gradient-purified bacteroids when they were shaken with ^1⁵^N₂ and 0.008 atm O₂. Under these conditions, ^1⁵^N-ammonium accounted for 93% of the ^1⁵^N fixed into the soluble fraction. This fraction contained no measurable ^1⁵^N-alanine. Neither these bacteroids nor those prepared by the previously used differential centrifugation method, when incubated with exogenous alanine under non-N₂-fixing conditions, gave rise to NH₃ from alanine. Therefore, contamination of bacteroid preparations with enzymes of plant cytosolic origin and capable of producing NH₃ from alanine, cannot explain the failure to detect ^1⁵^N-alanine (as proposed by Waters *et al.*, 1998). It seems that Waters *et al.* (1998) may not have used exactly the same procedure as the other workers and so bacteroid preparations as used in my research, were tested for the presence of such contamination.
Smith & Emerich (1993a, b) purified the ADH from *B. japonicum* bacteroids and demonstrated that this enzyme possessed a Theorell-Chance mechanism, favoring alanine formation with a rapid-equilibrium addition of ammonium. And Waters *et al.* (1998) pointed out that this enzyme could efficiently assimilate nitrogenase-generated ammonia into alanine. Thus a question may arise from my failure to detect alanine excretion from the bacteroids. Could it be possible that ADH was damaged and the activity lost? To address this issue, cell-free extracts of the bacteroids as used in the $^{15}$N experiments were prepared and the ADH activity measured. The results showed that the bacteroids contained ADH and were able to produce alanine from pyruvate and NH$_4^+$ (Table 5.7). Other experiments with ADH in extracts of cultured rhizobia also showed clearly that the enzyme activity was present (Table 5.7), indicating that the failure to detect substantial alanine excretion from N$_2$-fixing bacteroids did not result from the loss of ADH activity in the symbiotic state or during the isolation of bacteroids.

It can be concluded that ammonia is the principle product of N$_2$-fixation by bacteroids from soybean nodules under a range of different conditions *ex planta*, including those used by Waters *et al.* (1998). The reason for this difference between the two laboratories could not be determined from our experiments. The possibility remains, that sucrose density gradient-purified bacteroids (Waters *et al.*, 1998), in closed shaken assays, differ from those purified on Percoll density gradients, but this is highly unlikely.
Figure 5.1a. Data from a flow chamber experiment, showing metabolic conditions of the flow chamber in which the excretion of NH$_4^+$ and alanine by bacteroids prepared using the standard bench method was determined.

A. $[O_2_{free}]$ (Δ) in the chamber due to the change in flow rate (−).

B. Respiration (▲) change due to the change in flow rate (−).
Figure 5.1b. The excretion of NH$_4^+$ (filled bars) and alanine (open bars) by bacteroids prepared using the standard bench method during different steady states in the flow chamber experiment shown in Fig 5.1a.
Table 5.1. Examples of bacteroid activities during steady states in flow chamber experiments.
Bacteroids were prepared anaerobically by the standard bench method or by Percoll density gradients. Variations in respiration rates (O₂ consumption) were achieved by changing rates of flow through the reaction chamber of media containing different concentrations of dissolved O₂. In the flow chamber (vol. 12 ml) there were 72 and 96 mg (DW) of bacteroids, respectively, prepared by the two methods. Effluents were purified and concentrated using Sephadex columns (2.6.2.2.1) and alanine was determined by HPLC (2.6.2.2.2) and NH₄⁺ by colorimetric method (2.6.1).

<table>
<thead>
<tr>
<th>Bacteroid Preparation</th>
<th>[O₂ free] (nM)</th>
<th>Bacteroid activities (nmol min⁻¹ [mg DW]⁻¹)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂ consumption</td>
<td>NH₃ production</td>
<td>Alanine production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differential Centrifugation</td>
<td>7</td>
<td>0.94</td>
<td>0.87</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.62</td>
<td>1.18</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.52</td>
<td>2.09</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Percoll Density gradients</td>
<td>5</td>
<td>1.48</td>
<td>0.69</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.33</td>
<td>0.28</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.05</td>
<td>1.01</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2. A summary of 13 different experiments on NH$_4^+$ and alanine production by bacteroids prepared by different methods in closed shaken assays. Flasks (150 ml, closed with Suba-seals) contained reaction solution (2.5.2). Substrate was 2 mM malate. Final volume after bacteroids suspension was injected was 10 ml and final density of bacteroids were as presented in the table. Some of the experiments were repeated once or twice.

<table>
<thead>
<tr>
<th>Expt No</th>
<th>Bacteroid preparation</th>
<th>O$_2$ in gas phase of assay vial</th>
<th>Bacteroid Density (mg DW ml$^{-1}$)</th>
<th>Other treatments</th>
<th>Assay time</th>
<th>N$_2$ Fixation (nmol [mg DW]$^{-1}$)</th>
<th>NH$_4^+$</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bench method</td>
<td>0.008 atm</td>
<td>11.4</td>
<td></td>
<td>1h</td>
<td>9.3</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Bench method</td>
<td>0.008 atm</td>
<td>8.6</td>
<td>2 mM succinate</td>
<td>1h</td>
<td>No change with succinate</td>
<td>No change with succinate</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Bench method</td>
<td>0.004-0.04 atm</td>
<td>8.6</td>
<td></td>
<td>1h</td>
<td>No change with O$_2$</td>
<td>No change with O$_2$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Sucrose density gradients</td>
<td>0.004-1 atm</td>
<td>9.5</td>
<td>Time course 0, 20, 40, 60 min</td>
<td>92.34, 100.66, 115.05, 118.45; no change with O$_2$</td>
<td>4.3, 13.54, 11.41, 10.98; no change with O$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sucrose density gradients</td>
<td>0.004-0.016 atm</td>
<td>11.1</td>
<td>Time course 0, 20, 40, 60 min</td>
<td>No change with O$_2$; increased with time</td>
<td>No change with time or O$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Percoll density gradients</td>
<td>0.05 atm</td>
<td>3.2</td>
<td>5 mM NH$_4^+$</td>
<td>Time course 0, 30, 60, 90 min</td>
<td>Not determined</td>
<td>No change with time; doubled with 5 mM NH$_4^+$, see Table 5.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Percoll density gradients</td>
<td>0.1 &amp; 0.2 atm</td>
<td>Low: 3.0 High: 6.0</td>
<td>Time course 0, 30, 60, 90 min</td>
<td>No change with O$_2$ or density; increased with time.</td>
<td>No change with time, O$_2$ or density</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Percoll density gradients</td>
<td>0.1 &amp; 0.2 atm</td>
<td>Low: 2.8 High: 5.6</td>
<td>100 &amp; 150 rpm shaking</td>
<td>Time course 0, 10, 20, 30 min</td>
<td>No change with O$_2$, density or shaking; increased with time.</td>
<td>No change with time, O$_2$, density or shaking</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Percoll density gradients</td>
<td>0.038 atm</td>
<td>Low: 0.9 High: 3.8</td>
<td>150 rpm shaking</td>
<td>Time course 0, 15, 30, 45 min</td>
<td>No change with density; increased with time.</td>
<td>No change with time or density</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Percoll density gradients</td>
<td>0.038 atm</td>
<td>3.8</td>
<td>150 rpm shaking; 2 &amp; 5 mM NH$_4^+$</td>
<td>Time course 0, 15, 30, 45 min</td>
<td>Not determined</td>
<td>Increased with 5 mM NH$_4^+$ spiking as above</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Percoll density gradients</td>
<td>0.008 atm</td>
<td>Low: 1.8 High: 7.0</td>
<td>100 &amp; 150 rpm shaking</td>
<td>Time course 0, 15, 30, 45 min</td>
<td>No change with density or shaking; increased with time.</td>
<td>No change with time, density or shaking</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Percoll density gradients</td>
<td>0.008 &amp; 0.02 atm</td>
<td>Low: 1.0 High: 3.9</td>
<td>100 rpm shaking</td>
<td>Time course 0, 15, 30, 45, 60 min</td>
<td>Increased with time or high O$_2$/low density</td>
<td>No change with time or O$_2$</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Percoll density gradients</td>
<td>0.008 &amp; 0.02 atm</td>
<td>Low: 0.9 High: 3.5</td>
<td>100 rpm shaking</td>
<td>Time course 0, 15, 30, 45, 60 min</td>
<td>Increased with time or low O$_2$/low density</td>
<td>No change with time or O$_2$</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.3. Amino acids (nmol [mg DW]⁻¹) present in supernatants from reactions in closed systems with N₂-fixing bacteroids prepared by two anaerobic methods and shaken for 1 h with 0.008 atm O₂ in N₂ or with air in the gas space.

<table>
<thead>
<tr>
<th>Bacteroid preparation</th>
<th>Differential centrifugation</th>
<th>Percoll density gradient</th>
<th>Percoll density gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg bacteroid assay⁻¹</td>
<td>3.6</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Gas phase</td>
<td>0.008 atm O₂</td>
<td>0.008 atm O₂</td>
<td>air</td>
</tr>
<tr>
<td>Glutamate</td>
<td>16.9</td>
<td>nd*</td>
<td>nd</td>
</tr>
<tr>
<td>Serine</td>
<td>2.5</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Alanine</td>
<td>23.0</td>
<td>13.4</td>
<td>13.1</td>
</tr>
<tr>
<td>Valine</td>
<td>2.2</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>nd</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.2</td>
<td>nd</td>
<td>0.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>nd</td>
<td>nd</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*: nd = not detectable
Table 5.4. Alanine production by bacteroids in a closed shaken experiment in which NH$_4^+$ was added exogenously. In the experiment, bacteroid dry weight was 0.885 mg ml$^{-1}$. [O$_2$] was 0.02 atm. Alanine was determined by HPLC (2.6.2.2.2) and NH$_4^+$ was determined by the colorimetric method (2.6.1).

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>Alanine Produced (nmols [mg DW]$^{-1}$)</th>
<th>NH$_4^+$ produced (nmols [mg DW]$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5 mM NH$_4^+$ spiked</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>1.85</td>
</tr>
<tr>
<td>30</td>
<td>1.27</td>
<td>4.18</td>
</tr>
<tr>
<td>45</td>
<td>1.87</td>
<td>5.91</td>
</tr>
<tr>
<td>60</td>
<td>2.77</td>
<td>3.93</td>
</tr>
</tbody>
</table>
Table 5.5. Incorporation of $^{15}$N from $^{15}$N$_2$ (54.6 atoms % $^{15}$N) by USDA110c bacteroids in 30 min at 26 °C in flasks (100 ml) containing 0.008 atm O$_2$, shaken at 100 rpm.

<table>
<thead>
<tr>
<th>Fraction Analysed</th>
<th>µg N assay$^{-1}$*</th>
<th>Atoms % $^{15}$N Excess*</th>
<th>$^{15}$N excess (ng assay$^{-1}$)</th>
<th>(% recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total N</td>
<td>7344 ± 382</td>
<td>0.0130 ± 0.0010</td>
<td>958 ± 71</td>
<td>100</td>
</tr>
<tr>
<td>Bacteroid total N</td>
<td>4141 ± 813</td>
<td>0.0059 ± 0.0016</td>
<td>243 ± 122</td>
<td>25.4</td>
</tr>
<tr>
<td>Soluble total N**</td>
<td>1676 ± 153</td>
<td>0.0287 ± 0.0016</td>
<td>481 ± 41</td>
<td>50.2</td>
</tr>
<tr>
<td>Soluble NH$_3$-N†</td>
<td>36 ± 0.8</td>
<td>1.2336 ± 0.0347</td>
<td>448 ± 23</td>
<td>46.8</td>
</tr>
<tr>
<td>Soluble residue</td>
<td>1061 ± 84</td>
<td>0.0053 ± 0.0007</td>
<td>57 ± 13</td>
<td>5.9</td>
</tr>
</tbody>
</table>

*: Means ± sem  
**: After centrifuging bacteroids  
†: Recovered from soluble fraction by diffusion  
††: After removal of NH$_3$
Table 5.6. Data for GC-MS analysis of the \( N\)-(tert-Butyldiemethylsilyl)-\( N \)-methyl-trifluoroacetamide derivatives of alanine from (a) standard alanine (Sigma); (b) control: purified from supernatants of bacteroid reactions in assay flasks in air, stopped in ice at zero time; and (c) purified from soluble fractions of 30 min reactions with \( ^{15} \text{N}_2 \). Data for the same experiment presented in Table 5.1. R is the ratio (ion-current counts at m/e 261)/(ion-current counts at m/e 260).

<table>
<thead>
<tr>
<th>Sample Analysed</th>
<th>R ((\pm \text{sem}))</th>
<th>n</th>
<th>Atoms % excess (^{15}\text{N}) ((\pm \text{sem}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Std. alanine</td>
<td>0.22956 ± 0.00028</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>b. Control</td>
<td>0.24293 ± 0.00252</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>c. Reactions with (^{15}\text{N}_2)</td>
<td>0.24326 ± 0.00044</td>
<td>4</td>
<td>0.00076 ± 0.00053</td>
</tr>
</tbody>
</table>
Table 5.7. Activities of ADH in the amination direction in cell-free extracts of strains of *B. japonicum* grown in succinate broth and in bacteroids of strain USDA110c prepared from fresh nodules on cv. Stevens or cv Williams.

<table>
<thead>
<tr>
<th>Source</th>
<th>Activity (nmol NADH min⁻¹ [mg protein]⁻¹)*</th>
<th>K_m (pyruvate) (mM)*</th>
<th>K_m(NH₄⁺) (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. japonicum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA110c</td>
<td>25.4 ± 0.2</td>
<td>0.29 ± 0.06</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>USDA110de</td>
<td>30.6 ± 2.5</td>
<td>0.39 ± 0.01</td>
<td>2.9 ± 0.05</td>
</tr>
<tr>
<td>SU1014(CB1809)</td>
<td>132.7 ± 3.5</td>
<td>1.2 ± 0.07</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> bv <em>viciae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TY3841 (<em>aldA⁺</em>)</td>
<td>18.8 ± 0.2</td>
<td>0.84 ± 0.04</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>RU1327 (<em>aldA⁻</em>)</td>
<td>0.3 ± 2.6</td>
<td>Nd†</td>
<td>Nd†</td>
</tr>
<tr>
<td><strong>Bacteroids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA110c on cv. Forrest</td>
<td>633 ± 108</td>
<td>0.9 ± 0.2</td>
<td>6.8 ± 0.9</td>
</tr>
<tr>
<td>USDA110c on cv. Williams</td>
<td>154 ± 6</td>
<td>1.3 ± 0.1</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Average and range of two separate experiments
† Not determined
Figure 5.2. Production of NH$_3$ and alanine by USDA110c bacteroids in microaerobic shaken assays.  
A. Bacteroids prepared by differential centrifugation, with 0.02 atm. O$_2$ in the gas phase.  
B. Bacteroids prepared on a Percoll density gradient, with 0.008 atm. O$_2$ in the gas phase.  
(●) NH$_3$; (▲) alanine.
6 THE TCA CYCLE OF BACTEROIDS

6.1 Introduction

The C4-dicarboxylates, malate and succinate, support vigorous respiration by bacteroids isolated from legume root nodules and are considered to be the principal carbon sources which the plant supplies to bacteroids (Bergersen, 1958; Bergersen & Turner, 1967; Day et al., 1989; Streeter, 1987; Rosendahl et al., 1991). These dicarboxylates are oxidized within bacteroids. However, the exact metabolic pathways involved in this process are not very clear. Studies of enzyme activity and radiorespirometry have shown that enzymes of the TCA cycle are present in B. japonicum (Keele et al., 1969; McKay et al., 1988) and this cycle is generally considered to generate energy from respiration. Also, it is reasonable to assume that there may be other pathways which supply energy from malate and succinate. During the differentiation of rhizobia into N2-fixing bacteroids, they must adjust their metabolic functions so that they can maintain aerobic metabolism under low O2 concentrations. For example, during symbiosis, bacteroids express a terminal oxidase with a very high affinity for oxygen (Hennecke, 1998; Preisig et al., 1996). Additional adjustments to central metabolism are presumably required to optimize the flow of ATP and reductant to nitrogenase (Day & Copeland, 1991; Dunn, 1998; McDermott et al., 1989). Inhibition of the TCA cycle may compromise the supply of ATP and reductant to nitrogenase since the main carbon sources are the dicarboxylic acids, malate and succinate (Rosendahl et al., 1991). Of particular concern is the potential for excess reduced pyridine nucleotides to inhibit several enzymes in the TCA cycle, one of which is α-ketoglutarate dehydrogenase which is particularly sensitive to inhibition by high ratio of NADH to NAD (Salminen & Streeter, 1990). Because of this sensitivity, several groups suggested that bacteroids may use an alternative pathway around this step of the TCA cycle (Day & Copeland, 1991; Dunn, 1998; McDermott et al., 1989). The γ-aminobutyrate (GABA) shunt has
received particular attention as a potential bypass, because bacteroids express some of the necessary enzymes (reviewed by Poole & Allaway, 2000). However, the first enzyme of this pathway, glutamate decarboxylase, is usually present at very low levels in rhizobia (Jin et al., 1990; Kouchi et al., 1991; Miller et al., 1991; Salminen & Streeter, 1990), raising the question of whether the GABA shunt would be active enough to compensate for inhibition of α-ketoglutarate dehydrogenase. Using a sucA mutant of B. japonicum that lacks α-ketoglutarate dehydrogenase, Green and Emerich (1997a, b) demonstrated that the mutation of sucA has no effect on other TCA cycle enzymes and the mutant can grow on malate as its sole carbon source and form fully functional bacteroids in symbiosis with soybean. This indicates that the α-ketoglutarate dehydrogenase step of TCA can be by-passed and that a novel α-ketoglutarate-decarboxylating activity is present in B. japonicum. This decarboxylase activity, along with succinate semialdehyde dehydrogenase, may form an alternative pathway and allow the TCA to function under conditions that would otherwise inhibit its operation.

In this chapter, Dr L. S. Green and I cooperated and directly measured oxygen consumption and ammonia excretion by the bacteroids of the sucA mutant (strain LSG184), using the flow chamber system and comparing the results with those from wild type bacteroids. We confirmed that bacteroids formed by the sucA mutant displayed wild type rates of respiration and N₂ fixation, despite the absence of α-ketoglutarate dehydrogenase, and further demonstrated that there is an alternative pathway for α-ketoglutarate metabolism. The characterization of this pathway was not my work but it is discussed in relation to my findings.
6.2 Experimental

6.2.1 Plant material and bacterial strains

Soybean plants (*Glycine max* L., cv. Stevens) were grown, under bacterologically-controlled conditions, in modified Leonard jars (Green and Emerich, 1997b). Soybean seed was surface-sterilized prior to planting and inoculated with either wild type *B. japonicum* USDA110de or a *sucA* mutant derivative (LSG184; Green & Emerich 1997a). Plants were grown in a growth chamber with a 16-hour photoperiod and a day/night temperature of 27°C/24°C.

6.2.2 Flow chamber experiments

Nodules were harvested 5 weeks after inoculation and bacteroids purified anaerobically by differential centrifugation in the standard bench method (2.3.2). Bacteroids (20-60 mg dry weight) were incubated in a flow chamber (2.5.1; Bergersen and Turner, 1990a, b) and perfused with buffer containing 40-65 µM soybean leghaemoglobin as an oxygen carrier and 0.5 mM DL-malate as carbon source (2.5.1.2). Analytical methods and calculations were performed essentially as described (2.5.1.1; 2.6.1). In brief, oxygenation of effluent leghaemoglobin was monitored spectrophotometrically and used to calculate the oxygen concentration in the flow chamber and the respiration rate of the bacteroids. Fractions of the flow chamber effluent were collected and assayed for the presence of ammonia (the principal products of N₂ fixation in flow chamber experiments with soybean bacteroids, Bergersen & Turner, 1990a; section 2.6.1), and the results used to calculate the rate of N₂ fixation.

Culture supernatants were analyzed by HPLC and enzymatic methods for products of malate metabolism and whole cell CO₂ evolution rates were measured as described by Green *et al.* (2000). Cell-free extracts of cultured bacteria were analyzed for the
presence and activities of glutamate decarboxylase, α-ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase as described in Green et al. (2000). The results of these measurements were used to construct a modified TCA cycle and are discussed in this chapter.

6.3 Results and discussion

Previous work had shown that nodules formed by the sucA mutant, although containing a reduced number of bacteroids, had some nitrogenase (acetylene reduction) activity (Green & Emerich, 1997b). To measure N₂ fixation ability of the mutant more directly, we assayed isolated mutant bacteroids in the flow chamber system (2.5.1). The reaction buffer (2.5.2.1) in these experiments contained 40-65 µM leghaemoglobin and 0.5 mM Na-(DL) malate as a carbon source. Bacteroids were prepared using the standard bench method. A total of 6 experiments were performed, 4 experiments with the sucA mutant and 2 experiments with the wild type. A typical experiment using the mutant bacteroids (Fig 6.1) showed that, at each step of the increase in flow rate, [O₂ free] in the chamber fell (Fig 6.1b) and respiration (Fig 6.1d) and N₂ fixation (Fig 6.1f) increased concomitantly, indicating that the mutant bacteroids were able to maintain low oxygen levels in the flow chamber, and achieve N₂ fixation and respiration rates comparable to the wild type (Fig 6.1a, c, e). [O₂ free] increased (Fig 6.1b) in the same manner as wild type (Fig 3.1a) when the O₂ supply reached the limit of respiration, where [O₂ free] rose in response to a further increase in flow (Fig 6.1b between 130 and 150 min).

To test the dependence of the sucA mutant bacteroids on an exogenous carbon source, the malate was omitted from the reaction buffer in one experiment. Under these conditions, the mutant was unable to achieve a respiratory rate sufficient to maintain a low O₂ concentration in the flow chamber and very little N₂ fixation was observed
This result indicated that N$_2$ fixation by the $sucA$ mutant depended on catabolism of exogenous malate rather than on internal reserves.

As a further test of the symbiotic function of the mutant bacteroids, we examined the relationship between respiration rate and N$_2$ fixation, a measure of metabolic efficiency (Bergersen, 1997a). Steady-state N$_2$-fixation rates were plotted against the accompanying respiration rates from four separate experiments, two using wild-type bacteroids and two using $sucA$ mutant bacteroids (Fig 6.2). As expected, for both wild type and mutant, increases in respiration rate and, consequently, the supply of ATP, were accompanied by increases in the rate of N$_2$ fixation. Wild type and mutant values fell along the same curve, providing further evidence that the $sucA$ mutant bacteroids were functioning normally, when exogenous malate was supplied.

In the experiments with wild type, both USDA110de and USDA110c, there was often an instant increase in respiration and a simultaneous decrease in [O$_2$ free] in the chamber when the flow rate was increased (Fig 6.1b & 6.3A). The same changes also occurred in the experiment with $sucA$ mutant bacteroids (Fig 6.1d & 6.3B), indicating that the mutant can behave in the same way as wild type bacteroids do in response to the change in the rate of O$_2$ and substrate supply to the chamber.

In planta, the O$_2$ supply is always limited in infected cells. Any artificial increase in [O$_2$] in the gas phase in which nodules are located, triggers a rapid decline in [O$_2$] within infected cells (reviewed by Vance and Heichel, 1991). It was assumed that soybean nodules contain a regulatory mechanism which maintains a stable oxygen concentration inside nodules to allow nitrogenase to function, and there is evidence for a variable diffusion barrier in the nodule inner cortex (e.g. Witty & Minchin, 1998). My results (Fig 6.1, 6.3 and Chapter 3) suggest there is a possible supplementary physiological mechanism which could support this regulation. Increased O$_2$ supply
would adjust bacteroid respiration so that nodule oxygen concentration and \( N_2 \) fixation are maintained at stable levels (Weisz & Sinclair, 1987). It is demonstrated that soybean nodules adjust their internal \( O_2 \) concentration in response to increased external \( pO_2 \) within minutes (Bergersen, 1962a; Pankhurst & Sprent; 1975; Ralston & Imsande, 1982; reviewed by Vance & Heichel, 1991). It seems likely that in bacteroids this adjustment in respiration proceeds more rapidly (Fig 6.3). The \( suca \) mutant has exactly the same regulation of respiration in response to the \( O_2 \) change despite the modified TCA cycle. Further, the relationship between \( N_2 \) fixation and bacteroid respiration was not changed in the \( suca \) mutant (Fig 6.2).

### 6.4 General discussion

The results of this study provide further evidence that \( B. japonicum \) possesses a functional bypass around the \( \alpha \)-ketoglutarate dehydrogenase step of the TCA cycle. Flow chamber experiments showed that \( suca \) mutant bacteroids were not impaired by the loss of \( \alpha \)-ketoglutarate dehydrogenase, even when relying on exogenous malate to support \( N_2 \) fixation. The mutant was able to grow on malate as its sole carbon source, without excreting stoichiometric amounts of organic acid metabolites of malate. Furthermore, the mutant could decarboxylate exogenously supplied \( \alpha \)-ketoglutarate and glutamate at rates comparable to the wild type. Together these results strongly suggest that \( B. japonicum \) has an alternative to the conventional TCA cycle for the catabolism of dicarboxylic acids.

#### 6.4.1 Respiration, \( N_2 \) fixation and oxygen supply and demand

Plants inoculated with LSG184 produced small pale nodules, with low nitrogenase activity, contained fewer bacteroids than USDA110de nodules (Green & Emerich, 1997a) and appeared nitrogen deficient. Nevertheless, mutant bacteroids were isolated
in sufficient quantity to use in flow chamber experiments which showed that the *sucA*
mutant was not impaired by the loss of α-ketoglutarate dehydrogenase even when
relying on exogenous malate to support N2 fixation.

The work presented in chapter 3, together with other previous work, showed that
bacteroid respiration rate depends on the rate of O2 supply to bacteroids rather than [O2
free] (see chapter 3; Bergersen & Turner 1990a, 1993; Holtzapffel & Bergersen, 1998).
When the O2 supply to bacteroids was increased in the flow chamber, respiration and N2
fixation increased and [O2 free] in the chamber decreased simultaneously. This
phenomenon was present in all the wild type bacteroids of USDA110c and
USDA110de, no matter how the bacteroids were prepared, e.g. by density gradients or
differential centrifugation (Fig 3.1a-b; 2a-b; Table 3.1-5; Fig 6.1a,c, e), indicating that
O2 supply regulates the O2 demand by the respiration and N2 fixation activities of
bacteroids. In the experiments with the *sucA* mutant bacteroids, it is also shown that the
mutant was able to achieve the same rates of respiration and N2 fixation and to maintain
the same levels of O2 in the flow chamber as the wild type bacteroids despite the normal
TCA cycle being blocked due to the lack of α-ketoglutarate dehydrogenase (Fig 6.1b, d,
f). The O2 supply to those bacteroids also regulates the O2 demand of the mutant
bacteroids. The analysis of co-relationship between respiration rate and N2 fixation
showed that increased respiration rate was closely accompanied by N2 fixation in both
wild type and mutant bacteroids (Fig 6.2). This showed that the metabolic activities of
both wild type and mutant bacteroids have the same metabolic efficiency (Bergersen
1997a). Fig 6.3 shows that the *sucA* mutant was able to quickly respond to increase in
O2 supply (flow rate) with increased respiration in the same way that the wild type does.
The mutant also has a mechanism to regulate O2 concentration in response to a change
in the metabolic conditions, e.g. the O2 supply to bacteroids was changed by the
increase in flow rate. Nevertheless, all the data show that there is an alternative pathway
replacing the normal TCA cycle in *B. japonicum* bacteroids. The activity of this modified TCA cycle is sufficient to support nitrogenase activity at the usual rates.

*In vivo*, O₂ limitation of bacteroids inside the soybean nodules is likely to lead to an increase in NADH/NAD ratios in the bacteroids and thus to significant inhibition of α-ketoglutarate dehydrogenase (Salminen & Streeter, 1990; reviewed by Poole & Allaway, 2000), resulting in the normal TCA cycle no longer being fully functional. Usually, the TCA cycle performs two functions in organisms, energy production and the provision of biosynthetic precursors. The loss of α-ketoglutarate dehydrogenase in bacteroids would affect these functions, especially energy production. Subsequently, N₂ fixation activity, which needs a high-energy supply, would be affected. To maintain carbon flux and energy supply under oxygen-limiting conditions, bacteroids could theoretically use alternative metabolic pathways to bypass the inhibited reactions of the TCA cycle. The results of flow chamber experiments, presented above, showed that the rate of respiration and N₂ fixation by *sucA* bacteroids were equivalent, on a bacteroid basis, to the wild type. This clearly indicates that bacteroids were indeed supplied sufficient energy to fully function in terms of N₂ fixation although the TCA cycle is blocked. This implies that there is an alternative pathway fulfilling the energy production role of the TCA cycle.

### 6.4.2 Evidence from biochemistry and other metabolic aspects

In culture, the *sucA* mutant (LSG184 grew more slowly than the wild type (USDA110de) but nevertheless, within 48 h, LSG184 consumed all of the malate and attained the same cell dry weight as the USDA110de (Green *et al.*, 2000). Yet LSG184 excreted little α-ketoglutarate and glutamate, indicating further that the mutant had an alternative means, subsequently shown to be succinic semialdehyde (Green *et al.*, 2000), for catabolizing α-ketoglutarate and glutamate. In contrast to these results, *R.*
leguminosarum sucA mutant secrete large quantities of glutamate and α–ketoglutarate and form ineffective nodules unable to fix N₂ (Walshaw et al., 1997), apparently lacking the alternative pathway.

The GABA shunt is the usual α-ketoglutarate dehydrogenase bypass pathway proposed for the rhizobia. In this pathway, α-ketoglutarate is first converted to glutamate, which is then decarboxylated to GABA by glutamate decarboxylase. GABA is then transaminated to form succinic semialdehyde, which is oxidized via succinic semialdehyde dehydrogenase to succinate, and thereby rejoins the TCA cycle. All bacteroids examined so far have had succinic semialdehyde dehydrogenase activity comparable to other TCA cycle enzymes (25-34 nmols min⁻¹ [mg protein]⁻¹; Jin et al., 1990; Fitzmaurice & O’Gara, 1991; Kouchi et al., 1991; Miller et al., 1991). In contrast, the amount of glutamate decarboxylase activity found in bacteroids has generally been much lower (0-1.4 nmols min⁻¹ [mg protein]⁻¹; Jin et al., 1990; Salminen & Streeter, 1990; Kouchi et al., 1991; Miller et al., 1991). An exception is one study that found high levels of glutamate decarboxylase activity in Sinorhizobium meliloti (Fitzmaurice & O’Gara, 1991). This group also isolated a mutant strain in which reduced levels of succinic semialdehyde dehydrogenase were correlated with an inability to grow on glutamate and reduced rates of N₂ fixation (Fitzmaurice & O’Gara, 1993). This finding, although not yet corroborated by genetic evidence from other rhizobia, has sustained interest in the GABA shunt as a potentially important bypass pathway during symbiosis.

Although B. japonicum has the other two enzymes required for operation of the GABA shunt, the amount of glutamate decarboxylase found is very low (Kouchi et al., 1991; Salminen & Streeter, 1990; Green & Emerich, 1997a; Green et al., 2000), suggesting that the pathway is not very active. A CoA-independent α-ketoglutarate decarboxylase activity that is capable of forming succinic semialdehyde directly from α-ketoglutarate, has been found in B. japonicum (Green & Elmerich, 1997a). The activity requires
thiamine pyrophosphate but not CoA or pyridine nucleotides, and is thus distinct from α-ketoglutarate dehydrogenase (Green & Elmerich, 1997a). The results suggest that wild-type *B. japonicum* can decarboxylate α-ketoglutarate via two different enzymes, one the canonical α-ketoglutarate dehydrogenase, and the other a CoA- and NAD(P)-independent activity.

It can be proposed that the CoA-independent α-ketoglutarate decarboxylase activity in *B. japonicum*, along with succinic semialdehyde dehydrogenase, forms a functional bypass around the α-ketoglutarate dehydrogenase step of the TCA cycle (Fig 6.4). The phenotype of the sucA mutant implies that this bypass can support TCA cycle activity sufficient for growth on malate and malate-dependent N₂ fixation. Such a pathway operates as part of a modified TCA cycle in *Euglena gracilis* mitochondria (Shigeoka et al., 1986), and has also been proposed, purely on the basis of genome sequence analysis, for bacteria that lack the structural genes for α-ketoglutarate dehydrogenase (Cordwell, 1999).

Two enzymes have been described that catalyze the conversion of α-ketoglutarate to succinic semialdehyde: *E. gracilis* α-ketoglutarate decarboxylase (EC 4.1.1.71; Shigeoka et al., 1986) and a bifunctional protein encoded by menD (Palaniappan et al., 1992). The latter enzyme is part of the menaquinone biosynthetic pathway, and the succinic semialdehyde produced by decarboxylation of α-ketoglutarate would normally be consumed by a second activity on the enzyme to form 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC). However, α-ketoglutarate decarboxylation can occur independently of SHCHC synthesis, and succinic semialdehyde is then released from the enzyme (Palaniappan et al., 1992). Since the gene encoding *E. gracilis* α-ketoglutarate decarboxylase has not yet been cloned, it is not known whether it is related to menD. However both proteins are 62,000 Da in size and are homomultimers in their active form (Shigeoka & Nakano, 1991; Meganathan, 1996).
Whether the \textit{B. japonicum} activity is encoded by a homolog of \textit{menD} or by a unique gene must await further study.

A potential advantage of having an alternative pathway for \(\alpha\)-ketoglutarate catabolism is that succinate semialdehyde dehydrogenase, unlike \(\alpha\)-ketoglutarate dehydrogenase, can reduce NADP instead of NAD. Theoretically, operation of the bypass could facilitate continued flux of carbon through the TCA cycle under conditions, such as oxygen-limitation, that cause buildup of excess NADH and feedback inhibition of \(\alpha\)-ketoglutarate dehydrogenase. Drawbacks of using the bypass instead of \(\alpha\)-ketoglutarate dehydrogenase would be the need to synthesize succinyl-CoA from succinate, and the loss of the substrate level phosphorylation catalyzed by succinyl-CoA synthetase. These disadvantages may account for the \textit{sucA} mutant’s slower than normal growth on malate (Green & Emerich, 1997a), and delayed-nodulation phenotype (Green & Emerich, 1997a, b). For wild-type \textit{B. japonicum}, having both pathways for \(\alpha\)-ketoglutarate catabolism may allow greater flexibility in partitioning the reductant generated by the TCA cycle in response to changing metabolic demands.

Two other rhizobia, \textit{Mesorhizobium loti} and \textit{Rhizobium leguminosarum}, also showed CoA-independent \(\alpha\)-ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase activity (Green \textit{et al.}, 2000), suggesting that the bypass we have proposed for \textit{B. japonicum} is distributed widely among the rhizobia. The \(\alpha\)-ketoglutarate decarboxylase/succinic semialdehyde dehydrogenase bypass may be a general adaptation for catabolizing dicarboxylic acids under the microaerobic conditions inside legume nodules. Indeed, one study showed that succinic semialdehyde dehydrogenase activity was required for optimal N\(_2\) fixation in \textit{Sinorhizobium meliloti} (Fitzmaurice & O’Gara, 1993). Structural genes for succinic semialdehyde dehydrogenase have been found on the symbiotic plasmid of NGR234 (Frieberg \textit{et al.}, 1997), and in the symbiotic island of \textit{M. loti} (Sullivan \textit{et al.}, 2000), suggesting a
symbiotic function. Whether an \( \alpha \)-ketoglutarate decarboxylase/succinic semialdehyde dehydrogenase bypass is required for symbiotic \( N_2 \) fixation must await the analysis of mutants specifically defective in this pathway.
Figure 6.1. Respiration and N₂ fixation by bacteroids in response to flow of medium through the flow chamber.

Data for the wild type (USDA110de, a, c, e) compared to the mutant sucA (LSG184, b, d, f).

Bacteroids were prepared from 5-week old nodules using the standard bench method. Two different reaction solutions were used in these experiments. Reaction solution A was equilibrated with 50% air-50% N₂ (10% O₂), and solution B was equilibrated with air (20% O₂).

Symbol “∆” and “─” in a and b represent the [O₂ free] and the flow rate, respectively.
Figure 6.2. Relationship between steady-state rates of respiration and nitrogen fixation by bacteroids of wild type (USDA110de) and sucA (LSG184). Bacteroids were prepared using the standard bench method. Respiration was determined using the flow chamber (2.5.1) and N₂ fixation was measured colorimetrically (see 2.6.1). Average respiration and N₂ fixation rates were calculated for each steady state attained in four experiments. Each point represents a single steady state.
Figure 6.3. Effect of increase in flow rate (—) on the time course of [O$_2$ free] (∆) and bacteroid respiration (▲) in two of the experiments shown in Fig 6.2. Bacteroids were prepared as in Fig 6.2. The flow chamber experiments were performed as described in 2.5.1. The reaction solutions were the same as used in the experiments as described in Fig 6.1. A. Wild type-USDA110de. B. suca mutant-LSG184.
Figure 6.4. Proposed pathways for \( \alpha \)-ketoglutarate catabolism in *B. japonicum* (Green *et al.*, 2000).

KGDH, \( \alpha \)-ketoglutarate dehydrogenase; KDC, \( \alpha \)-ketoglutarate decarboxylase; SSDH, succinic semialdehyde dehydrogenase; SSA, succinic semialdehyde; GABA, \( \gamma \)-aminobutyrate; GAT, \( \gamma \)-aminobutyrate aminotransferase; GDC, glutamate decarboxylase.
7 GENERAL DISCUSSION

7.1 Introduction

Symbiotic nitrogen fixation takes place in legume root nodules induced and formed when specific soil bacteria from genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* or *Azorhizobium* invade the roots of the host plant (Brewin, 1991; Mylona, *et al.*, 1995; Werner, 1992). During the development of the resulting symbiosis, the bacteria differentiate from a free-living to the symbiotic form, the bacteroids. Concomitantly, bacteroids are hosted within a special intracellular environment and become dependent on the host plant for their sustaining nutrients. As the intracellular bacteroid population grows, the intracellular concentration of O$_2$ declines. This O$_2$ limiting environment has both advantages and disadvantages. Most importantly, it protects the O$_2$-labile nitrogenase now being synthesized in bacteroids, from the effects of atmospheric O$_2$. However, it also restricts the ability of bacteroids to metabolize carbohydrate and fix N$_2$ (Layzell, 2000) because O$_2$ is required by bacteroid respiration which must provide a large amount of ATP for N$_2$ fixation. While adapting to this specialized environment, the bacteria undergo extensive adjustments to their metabolic activities, gene expression, physiological functions (including nitrogen and carbon metabolism), respiration, metabolite transport, and many other biochemical and genetic aspects (Werner, 1992).

One of the most important adjustments is that bacteroids have developed regulatory mechanisms controlling the O$_2$ levels in their environment, so that, on one hand, the nitrogenase activities are preserved, and on the other hand, metabolic activities supporting nitrogen fixation are optimized. Simultaneously, the host tissues of the legume nodules also play a very important role in developing this regulatory mechanism. The inner cortical tissues of nodules provide a partial barrier (Minchin &
Witty, 1990) or a boundary layer (Parsons & Day, 1990) to the diffusion of gases through an inner cortical zone. As a result, the O_2 concentration declines sharply across this diffusion barrier (Tjepkema & Yocom, 1974; Witty, 1991), the internal pO_2 being approximately 10^{-4}-10^{-5} of the O_2 concentration in nodule cortex in equilibrium with air (250-260 µM) (Layzell & Hunt, 1990; Thumfort et al., 1994; Keith et al., 1998). Mathematical models of gas diffusion in nodules (Sheehy et al., 1985, 1987; Sheehy & Bergersen, 1986; Hunt et al., 1988; Denison, 1992; Moloney & Layzell, 1993, Thumfort et al., 1994; Bergersen, 1994, 1996) are consistent with the presence of a variable barrier in the cortex, which functions to maintain an extremely low O_2 concentration throughout the entire central infected zone of the nodule. Under these conditions of restricted O_2 supply, the flux of O_2 to bacteroids for respiration within symbiosomes is enhanced (facilitated) by the presence in the cytoplasm of infected cells, of high concentrations of the O_2-binding protein, leghaemoglobin. Leghaemoglobin is located in the cytosol of the host cells in which the symbiosomes are located. Leghaemoglobin, at mM concentrations, is 10-20% saturated with O_2 at the low concentrations of free O_2 prevailing in the cytosol of infected nodule cells. A flux of oxyleghaemoglobin to the symbiosome surface greatly augments the flux of free, dissolved O_2 (Appleby 1984, 1994). In addition, the infected cells of the nodule central tissue, with their thousands of bacteroids and abundant mitochondria (Millar et al., 1995), constitute a compact, intense sink for O_2 and play an important role in maintaining a low level of free O_2 in the central infected zone (Bergersen, 1997b). All the above factors together regulate the provision of adequate fluxes at low concentrations of O_2 dissolved in the cytoplasm of infected nodule cells. It is important to understand how this regulated O_2 flux feeds back to regulate the metabolism (especially respiration) which supports N_2 fixation.

My work presented in the previous chapters of this thesis focuses on the O_2 regulation of respiration, the transport and metabolism of energy-providing substrate into
bacteroids, and nitrogen fixation in relation to bacteroid respiration. In this chapter these aspects will be discussed further in relation to the work of others.

7.2 Oxygen regulation of O2 demand by bacteroid respiration & terminal oxidases of bacteroids

In order to cope with the low O2 regime within soybean nodule cells, the bacteroid has developed different respiratory chains terminating with oxidases of different affinities for O2 (Appleby, 1984; Preisig et al., 1993). One of the oxidases, the cbb3 cytochrome complex, has a very high affinity for O2 (\(K_m < 10 \text{ nM O}_2\)). Consequently, there is efficient facilitated O2 transfer from oxygenated leghaemoglobin (\(K_m = 46 \text{ nM O}_2\)) in the nodule cell cytoplasm to the respiratory complex in the bacteroids (Bergersen, 1982; Bergersen & Turner, 1990a; O’Brian & Maier, 1989).

Preisig et al., (1996) showed that respiration by membranes prepared from anaerobically-grown, wild-type *B. japonicum* 110 spc4 respired with an app\(K_m\) of 19 nM O2. This resulted from the operation of a branched respiratory pathway terminating in oxidases of diverse affinities for O2: the high-affinity cbb3-type oxidase (\(K_m = 7 \text{ nM O}_2\)) and another oxidase (or a mixture of oxidases) of lower affinity (app\(K_m = 56.2 \text{ nM O}_2\)). In aerobically-grown bacteria of the same strain, respiration is largely mediated by an aa3 terminal oxidase (\(K_m = 56 \text{ nM O}_2\)) which is significantly down-regulated in the symbiotic form of these bacteria, the bacteroids (Gabel & Meier 1993, Keister & Marsh, 1990), but there may be some residual activity. In steady states in flow chamber reactions with *B. japonicum* bacteroids, it was first reported that the respiratory system appeared to have a very high affinity for O2 (app\(K' \approx 4.9 \text{ nM: Bergersen & Turner 1990a})). Later Bergersen & Turner (1992) noted that the earlier report may have been in error because of limited availability of C-substrate and that when only respiration rates which clearly were not substrate-limited were used, all data points fell on or close to
single plotted lines of $v^{-1}$ versus $s^{-1}$, whose slopes and intercepts gave an $appK_m$ of 20-25 nM O$_2$. This result has been repeated subsequently (Bergersen 1997a). Preisig et al. (1996) were surprised that Bergersen and Turner (1993) did not detect steady state respiration via the $cbb3$-type oxidase. They attributed the failure to detect respiration via a high-affinity oxidase with a $K_m$ of only 7 nM O$_2$ in the flow chamber experiments, to the inclusion of data from [O$_2$] of only $> 8$ nM O$_2$. Bergersen (priv. comm.) has shown that the earlier results (Bergersen, 1997b) could be due to respiration via 2 of the oxidases described by Preisig et al. (1993). In the present work (Chapter 3), it is argued that regulation of respiration involves the high affinity oxidase. In Fig 7.1, it is shown that the bacteroid respiration with $appK_m$ of 20-25 nM O$_2$ observed by Bergersen (1997a) could have been due to respiration catalyzed simultaneously by the two terminal oxidase systems described by Preisig et al. (1996). It should also be borne in mind that the flow chamber measurements with intact bacteroids represent respiration by the entire electron transport chain, fed electrons by more than one pathway of carbon metabolism. This contrasts with the much simpler membrane vesicles used by Preisig et al. (1996).

7.2.1 Changes in O$_2$-demand and bacteroid oxidases

The data in chapter 3 show that O$_2$-demand and transport of malate into USDA110c bacteroids in flow chamber reactions, increase in response to increased O$_2$-supply. Consequently, concentrations of dissolved O$_2$ in the chamber are maintained near to the $K_m$ of the high affinity, $cbb3$-type terminal oxidase described by Preisig et al. (1996). The data from those experiments have been examined to test the possibility that such increased O$_2$-demand may have been due to increased $V_{max}$ of the high affinity terminal oxidase system and the possible consequences of such an increase have been examined using the simulated nodule infected cell model (Bergersen 1996, 1999), modified to include two bacteroid terminal oxidases of different affinity for O$_2$. 


Observed steady-state respiration rates from two series of experiments were used here. The data obtained were fitted to curves calculated for the oxidases described by Preisig *et al.* (1996), according to the following calculation (Segal 1975):

$$\text{Respiration observed} = v_{\text{observed}} = s \cdot V_{\text{max} (1)}/(K_m (1) + s) + s \cdot V_{\text{max} (2)}/(K_m (2) + s)$$

(1)

In which $K_m (1) = 7\text{nM } \text{O}_2$ and $K_m (2) = 56.2\text{ nM } \text{O}_2$ (Preisig *et al.*, 1996) and $s = [\text{O}_2\text{free}]$.

$$V_{\text{max (total)}} = V_{\text{max (1)}} + V_{\text{max (2)}}$$

(2)

$V_{\text{max (observed)}}$ was obtained from linear regression data of double reciprocal plots of $s$ versus $v_{\text{observed}}$.

The degree of engagement of the two oxidases was estimated by varying $V_{\text{max (1)}}$ and $V_{\text{max (2)}}$ reciprocally, whilst maintaining $V_{\text{max (total)}}$ constant and equal to $V_{\text{max (observed)}}$, until the best fit was obtained between observed respiration rates and the sum ($v_{\text{total}}$) of the respiration rates for each of the oxidases calculated separately.

### 7.2.2 Confirmation of bacterial terminal oxidases of different O$_2$-affinity

Specific steady respiration rates of USDA110c bacteroids in flow chamber experiments were generally greater than those of CB1809 bacteroids reported previously but it was easier to obtain steady respiration rates related to [O$_2$] with CB1809 because respiration was less affected by O$_2$-supply (Fig 3.1b; Table 3.1). However, the N$_2$ fixation rates were similar for both strains at similar respiration rates. Data fitted to curves relating observed steady state respiration rates of CB1809 bacteroids obtained from Fig 1 of
Bergersen (1997a) to curves for the respective \( K_m \) values of the two terminal oxidase systems are shown in Fig. 7.1. The best fit between the observed data and the curve for the sum of the two respiratory systems was obtained when \( V_{\text{max}} (1) \) was 6.5 nmol O\(_2\) min\(^{-1}\) [mg DW]\(^{-1}\) and \( V_{\text{max}} (2) \) was 18.1 nmol O\(_2\) min\(^{-1}\) [mg DW]\(^{-1}\) to give \( V_{\text{max}} \) (total) of 24.6 nmol O\(_2\) min\(^{-1}\) mg\(^{-1}\), compared with \( V_{\text{max}} \) (observed) of 23.6 nmol O\(_2\) min\(^{-1}\) mg\(^{-1}\) and apparent \( K_m \) (observed) of 29.5 nM O\(_2\). These observed values are similar to those reported previously for steady state bacteroid respiration in flow chamber reactions (Bergersen, 1997a; Bergersen & Turner, 1992). The observed data points in Fig. 7.1, when plotted as \( v^{-1} \) versus \( s^{-1} \), yielded a straight regression line with a very highly significant correlation coefficient (\( R^2 = 0.98 \)) indicating the difficulty of discerning the presence of multiple oxidases in such plots (cf. Segal, 1975). Preisig et al. (1996) implied that such difficulty was responsible for earlier failures to detect in bacteroids the presence of respiration via such a high-affinity oxidase as described for the \( cbb_3 \)-type oxidase in \( B. japonicum \). These authors also refer to the difficulty posed by autoxidation of leghaemoglobin when estimates of oxidase affinity are made from data derived from deoxygenation of oxyleghaemoglobin. It is emphasized that no such autoxidation occurred in the flow-chamber experimental system used here or in earlier experiments (e.g. Bergersen 1997a,b) (Fig 7.2).

### 7.2.3 Changes in O\(_2\)-demand related to changes in \( V_{\text{max}} (1) \)

The data presented in chapter 3 suggested that the \( V_{\text{max}} (1) \) of the \( cbb_3 \)-type cytochrome oxidase of \( B. japonicum \), may be variable, thus explaining increased demand for O\(_2\) in response to increases in rate of supply of O\(_2\) to respiring bacteroids. In Table 7.1, data from steady states in one of the flow chamber experiments have been used to calculate the possible magnitude of such changes. It was assumed that the increased O\(_2\)-demand was due entirely to a change in \( V_{\text{max}} (1) \) because the increased demand occurred at [O\(_2\)] near to \( K_m (1) \) (Table 3.1), where the activity of oxidase 2 was very low; \( V_{\text{max}} (2) \) remained...
fixed at the initial value (2.52 nmol O$_2$ min$^{-1}$ mg$^{-1}$). Table 7.1 (column 5) indicates the maximum range of the increase in $V_{\text{max}}$ in this experiment. The data show that, whilst the supply of O$_2$ increased 16-fold, O$_2$ consumption increased 18.5-fold in response to a very large increase in $V_{\text{max}}$ which caused [O$_2$] to fall from 35 to <10 nM (Table 7.1).

7.2.4 Simulated consequences of changes in bacteroid O$_2$-demand during symbiosis

Using the simulated nodule cell described earlier (Bergersen 1996, 1999), and now modified to include the two bacteroid oxidases considered here, it was found that varying the respective $V_{\text{max}}$ values by identical increments had different effects. Predictably, increasing $V_{\text{max}}$ from 1.08 to 3.25 x $10^{-7}$ moles O$_2$ s$^{-1}$ g$^{-1}$ caused increased gradients of deoxygenation of leghaemoglobin and consequently, lowered [O$_2$], due to increased bacteroid respiration across the cell; N$_2$ fixation increased. However, due to increased gradients of [O$_2$] across the outermost layers of the mitochondrial zone, mitochondrial respiration declined. Such a decrease could cause diminished cytosolic concentrations of ATP used for cellular processes such as solute transport and thus diminish symbiotic activity in vivo. Such diminutions in nodule activity in response to increased availability of O$_2$ have been attributed to increased diffusion resistance in the nodule inner cortex (Witty et al., 1986). My simulation supports the possibility that there is a contributory effect of bacteroid metabolism. Effects of increases in $V_{\text{max}}$ (2), (although in the same sense as changes in respiration via oxidase 1), were of much smaller magnitude because, over most of the distance from the cell surface to the cell centre, [O$_2$] was in a range in which respiration via oxidase 2 was low and little influenced by the small changes in [O$_2$] occurring there (Table 7.2). The simulation assumed that the two oxidases were equally well-coupled to N$_2$ fixation (i.e. for every increase of one unit in respiration, N$_2$ fixation increased by 0.25 units; Bergersen 1997a). There is some evidence that coupling of bacteroid respiration to N$_2$ fixation may be very low for oxidase 2 (Bergersen & Turner 1975, Bergersen, 1997b; cf. Preisig et
al., 1996). If this is so, increasing respiration through oxidase 2 would not increase N₂ fixation significantly but in some circumstances could reduce it (e.g. Fig. 2 in Bergersen 1997a). In an extreme interpretation, oxidase 2 could be regarded as being uncoupled from N₂ fixation (i.e. could be a ‘protective’ oxidase; Appleby, 1984). It would be possible to explore a more realistic simulation of the effects of changes in $V_{max}$ of the respective oxidases by using estimates of the degree of variable coupling of the bacteroid terminal oxidases to N₂ fixation (estimated from the data of Bergersen 1997a,b), but this has not been done.

This section has shown that bacteroid terminal oxidases of different kinetic properties are active in B. japonicum strain CB1809 and it has been shown, using the data from strain USDA110c (presented in Chapter 3) in a simulation model, that differential functions could be expressed through kinetic changes, particularly in the $V_{max}$ of the high affinity $cbb_3$-type oxidase. This is one of the special adjustments that bacteroids developed to cope with the low O₂ concentration in the environment in which they are enclosed.

### 7.3 Carbon metabolism in relation to O₂ supply and respiration

As stated in the introduction of this chapter, after the invasion of bacteria into the host legume plant, they adapt to the intracellular environment, changing into the symbiotic form, the N₂-fixing bacteroids. They are now completely dependent on the host plant for their energy-providing carbon supply. The host plant provides the nodules with sucrose (Kouchi & Yoneyama, 1986; Streeter, 1980, 1986) from photosynthesis and products of sucrose catabolism are transported across the PBM to be used by bacteroids. It has been demonstrated that sucrose is broken down via phosphoenolpyruvate (PEP) to C₄-dicarboxalates, malate and succinate, by the three important enzymes: sucrose synthase...
(SS), phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) (Fig 1.4; reviewed by Vance et al., 1998). Malate and succinate are readily used by bacteroids to support their nitrogen fixation via respiration (Bergersen, 1958; Bergersen & Turner 1967; Day et al., 1989; Streeter, 1987). *Rhizobium* or *Bradyrhizobium* mutants unable to take up malate and succinate, form ineffective nodules (Ronson et al., 1981; Finan et al., 1983; Vance & Heichel, 1991).

A specific dicarboxylate transport system (Dct), which is responsible for the uptake of malate and succinate in both free-living rhizobia and bacteroids has been reported (Ronson, et al., 1981; Finan et al., 1981, 1983; reviewed by Jording et al., 1994) and its biochemical properties and genetic regulation has been investigated (reviewed by Udvardi & Day, 1997; Jording et al., 1994).

As bacteroids enter the special environment where the [O₂] is extremely limited, the bacteroid respiration is depressed (see above). The transport of C₄-dicarboxylates across the symbiotic membranes (which is driven by ATP hydrolysis) will be restricted, leading to energy limitation and lower N₂ fixation. It is found that N₂ fixation can be increased by 20–40% by doubling the atmospheric pO₂ (Werner, 1991). This could mean that metabolite transport is also regulated by the O₂ supply to the infected cells and could involve both the PBM and bacteroid transport systems. However, previous work (Udvardi et al., 1988b; Ou Yang et al., 1990) on C₄-carboxylate transport did not seriously take this factor into account. The bacteroids used by those researchers were mainly prepared aerobically and the transport activity was measured in air. Bacteroid metabolic activities may have been altered and O₂ regulation of the transport not preserved because the conditions are too far away from what bacteroids are experiencing in the infected cells.
7.3.1 \textit{O}_2 \text{ supply regulates malate transport across the bacteroid membrane}

The present work first demonstrated that the C\textsubscript{4}-carboxylate, malate is taken up by anaerobically prepared bacteroids under microaerobic conditions (Fig 4.1) although the rate of uptake was relatively slower but remained constant longer than that reported by former workers (Udvardi et al., 1988b). However, the difference in malate uptake by bacteroids between the present work and previous work suggests that the O\textsubscript{2} status present during the isolation procedure and provided to the assay process is important. Aerobic conditions during preparation and assay inactivated nitrogenase, N\textsubscript{2} fixation ceased and malate uptake by the bacteroids was compensated by diversion of reductant and the high respiration which results from the high availability of O\textsubscript{2}. Usually, this would not last long as the metabolic balance in cells is broken down. Microaerobic conditions are closer to the physiological conditions existing in nodules, under which the [O\textsubscript{2} free] prevailing near bacteroids may be just sufficient to support a long-term malate uptake. This suggests that malate uptake by bacteroids is controlled by O\textsubscript{2}.

Further experiments with bacteroids withdrawn from the flow chamber showed that maximum rates of malate uptake by bacteroids increased with an increase in respiration as a result of the increase of O\textsubscript{2} supply (Fig 4.2; Table 4.2). It is unlikely that the increase of malate uptake by bacteroids resulted simply from an increase in malate supply to the chamber because the malate concentration (2 mM) was always well above the limiting threshold (Bergersen, 1993). Nor was this simply a case of a stronger sink for malate (resulting from increased respiration) stimulating uptake, because (1) significant differences in rate of uptake were recorded when aerobically and anaerobically prepared bacteroids were assayed at the same oxygen concentration (Fig 4.1; Table 4.2); and (2) with successive increases in bacteroid respiration preceding each sampling (Table 4.3), when malate uptake was measured under standard conditions in which respiratory rates were uniform and malate concentrations were not limiting,
specific rates of malate uptake also increased (Table 4.3). The other evidence from the employment of CAP as a protein synthesis inhibitor showed that enhanced malate uptake is not due to the synthesis of new carrier protein but to increased utilization of existing carriers. These results suggest that rates of malate uptake may be directly affected by bacteroid respiration but are indirectly controlled by O₂ supply to the bacteroids. The nature of this process is not known yet but it suggests that both organic acid transport and metabolism in the bacteroid are controlled by a common factor. Pyridine nucleotide redox poise is a likely candidate for this factor.

7.3.2 Malate metabolism in N₂ fixing bacteroids

Malate transported into bacteroids is oxidized, but the exact metabolic pathway involved in this process is not clear. There is some evidence showing that malate is likely metabolized via the TCA cycle, as all enzymes necessary for the operation of this cycle have been found to be present in bacteroids (Finan et al., 1988, 1991; McKay et al., 1989; Streeter, 1991). Two forms of malic enzymes, NAD-ME and NADP-ME, which are thought to be responsible for the oxidative decarboxylation of malate to pyruvate, have been found. This decarboxylation functions prior to the TCA cycle to form acetyl-CoA which is essential for the operation of the TCA cycle (Driscoll & Finan, 1997). However, as all the metabolic activities within bacteroids are placed under O₂-limiting conditions, it is reasonable to assume that the full operation of the TCA cycle cannot be entirely preserved. At least the TCA cycle is modified to adapt to the low-O₂ environment. For example, the O₂ limitation inside of nodules can lead to increased NADH/NAD ratios, which consequently causes significant inhibition of α-ketoglutarate dehydrogenase, implying that the respiration via the TCA cycle may in part be limited or blocked at certain steps (Salminen & Streeter, 1990; reviewed by Poole & Allaway, 2000). As a result, several groups suggested that bacteroids may use an alternative pathway to by-pass the restricted step of the TCA cycle (Day &
Copeland, 1991; Dunn, 1998; McDermott et al., 1989). Possibilities are the GABA shunt (Salminen & Streeter, 1990), and the glyoxylate cycle (Kornberg, 1966). Using a sucA mutant of *B. japonicum* which is α-ketoglutarate dehydrogenase-deficient, Green & Emerich, (1997a, b) demonstrated that the α-ketoglutarate dehydrogenase step of the TCA cycle can be by-passed and that the mutant can grow on malate to form fully functional (in terms of N₂-fixation) bacteroids. The present work, using the flow chamber and other biochemical analysis, gathered some more evidence from the comparison between wild-type and the sucA mutant to support the hypothesis that a novel alternative pathway, rather than the GABA shunt or glyoxylate cycle, exists in bacteroids. Bacteroids of the sucA mutant use this pathway to compensate for the loss of α-ketoglutarate dehydrogenase.

The flow chamber results obtained with the sucA mutant showed that respiratory demand for O₂ by the mutant bacteroids is regulated by O₂ supply in the same way as the wild-type (Fig 6.1). Rates of nitrogen fixation by the mutant bacteroids, based on the bacteroid dry weight, appeared to be the same as that by the wild-type and nitrogen fixation was closely coupled with respiration in the same manner in both mutant and wild type bacteroids (Fig 6.2). Normally, the TCA cycle performs two important roles in organisms, energy production and the provision of biosynthetic precursors. The loss of α-ketoglutarate dehydrogenase in bacteroids would cause energy deficiency, thus affecting N₂ fixation, which needs a large amount of energy supply. The results clearly indicated that this is not the case. Rather, the bacteroids, although present in small numbers in small nodules, were fully functioning in terms of respiration and N₂ fixation. These results, together with the results reported by former researchers (Green & Emerich, 1997a, b), also strongly support that there is an alternative pathway, which can lead to fully functional bacteroids (Chapter 5).
The results of measurements of NAD-ME and NADP-ME activities obtained from the present work showed that NAD-ME activity is closely related to bacteroid respiration rates. When O₂ demand by bacteroids in the flow chamber was enhanced by O₂ supply, the malate uptake increased and concomitantly the NAD-ME activity increased. Malic enzymes are considered to be responsible for the pyruvate decarboxylation to form acetyl-CoA, which is essential for the TCA cycle. Actually, between these two enzymes, NAD-ME is considered more important for ATP production in bacteroids (Day & Copeland, 1991). The results strongly support these assumptions. Malic enzymes, especially NAD-ME, play a very important role in the metabolism of malate through the TCA cycle even if bacteroids use the alternative TCA cycle. It can be assumed that malate is oxidized by NAD-ME to form acetyl-CoA and then acetyl-CoA is ready to support the alternative TCA cycle.

It can be concluded that under O₂-limiting conditions in nodules, bacteroids use malate as the carbon source and malate is transported under regulation by the O₂ supply. The transported malate is metabolized, with the participation of NAD-ME or sometimes NADP-ME, through the TCA cycle which is modified to by-pass the restriction at α-ketoglutarate dehydrogenase step of the normal TCA cycle imposed by high NADH/NAD⁺ ratios. These are very important adjustments which bacteroids make to respond to the O₂-limiting environment in the nodules.

7.4 Confirmation of the product of nitrogen fixation

In the symbiosis of legume and rhizobia, one of the most important metabolic events happening is that bacteroids fix atmospheric N₂ to reduced nitrogen, which is readily used by the host plant. Based on a variety of experimental observations, Bergersen (1965), Kennedy (1966a, b) and Bergersen & Turner (1967) suggested that the principal product of nitrogen fixation is ammonia. Ammonia is subsequently converted to NH₄⁺
and is transported through the PBM to the host plant (Tyerman et al., 1995). This has been well accepted for many years. Many other studies related to nitrogen fixation based on this assumption have been undertaken so far. However, this long-accepted view was challenged by Waters et al. (1998) who claimed that alanine, rather than ammonia, was the principal $^{15}\text{N}$-labelled product when N-fixing soybean bacteroids, purified on anaerobic sucrose density-gradients, were supplied with malate and shaken in a gas mixture containing $^{15}\text{N}_2$ and 0.008 atm O$_2$. The present work carefully reassessed the products of N$_2$ fixation using both the closed system (2.5.2) similar to that employed by Waters et al. (1998) and the flow chamber system employed by Bergersen & Turner (1985, 1990a, b) (2.5.1). Bacteroids used in the work were prepared using density-gradients (both sucrose and Percoll) (2.3.2) and the standard bench method (2.3.1). The O$_2$ concentration and bacteroid density were varied in those assay systems.

The results of colorimetric assay of NH$_4^+$ showed that the NH$_3$ is the main product excreted by bacteroids. Under some circumstances, some alanine was found in the effluent of the flow chamber and in the supernatants from the centrifugation of bacteroids suspension collected from the closed, shaken flasks, after concentration and purification. However, none of them showed that alanine is the principal product of N$_2$ fixation because there was little difference between the concentration of alanine in 1 h assays with Percoll gradient bacteroids in air and at a pO$_2$ of 0.008 atm (at which production of alanine was reported to be optimized; see details in chapter 5). Further, the amount of alanine produced in the flow chamber was usually very low compared with the amount of NH$_3$ produced. In the $^{15}\text{N}$ incorporation experiments with Percoll-gradient purified bacteroids, using conditions designed to simulate those of Waters et al. (1998), the most abundance of $^{15}\text{N}$ was still found in NH$_3$/NH$_4^+$. Thus there is no doubt that N$_2$ was fixed by bacteroids principally into NH$_3$. 


Allaway et al. (2000) reported that both $^{15}$N-alanine and $^{15}$N-ammonia were produced by bacteroids prepared from nodules of *Pisum* sp. on anaerobic Percoll density-gradients but the proportion of these products was altered by the conditions applied. The authors showed that when conditions were kept at low-moderate bacteroid density in a closed assay system, ammonia is the first and major product formed. As the bacteroid density was increased, alanine synthesis began at high rates resulting from accumulation of $\text{NH}_4^+$ (formed from N$_2$ fixation). Using $^{15}$N$_2$, both the NH$_3$ and alanine pools became highly labelled, consistent with NH$_3$ being the direct precursor of alanine. However, in my work, the results showed no sign of this. Alanine was always present at very low levels and no $^{15}$N labelling could be found. Even if the bacteroid density was varied, alanine was still not significantly detectable. The substrate supplied to the bacteroids in these experiments was not limiting even when the bacteroid density increased. Therefore the only significant change to bacteroids when their density in the assay systems was increased was that the O$_2$ supply to each bacteroid decreased. That is, at high density, O$_2$ partitioning among the bacteroids was reduced and respiration became limited by low O$_2$. As a result, the nitrogen fixation decreased and less NH$_3$ was produced. This sheds little light on how the alanine produced in the system of Allaway et al. (2000) is immediately related to the alanine production in my assay systems. The only possible explanation of the differences between Allaway et al. (2000) and my results is that the symbioses formed between different legumes and their rhizobia may have different regulatory mechanisms. For example, soybean bacteroids may be more tightly regulated by oxygen supply than their pea counterparts.

Waters et al. (1998) proposed that, because of the contamination of bacteroid suspensions with cytosolic enzymes from the host tissue, the earlier work failed to detect alanine from bacteroid. These contaminants were proposed to deaminate alanine and ammonia was consequently produced. The present work was unable to detect any activity able to deaminate alanine and adhering to the outside of bacteroids prepared
either by Percoll-density gradients or by differential centrifugation in the bench method. Therefore, my failure to detect alanine from the N₂ fixing bacteroid assays cannot be attributed to the contamination of bacteroids with enzymes of plant origin degrading the alanine. Also, it is unlikely that the earlier work showing NH₃ as the principal primary product was due to such a cause.

Waters et al. (1998) indicated that ADH could efficiently assimilate nitrogenase-generated ammonia into alanine. Therefore, the failure to detect the alanine excretion from my N₂-fixing bacteroids may have been due to the loss of or damage to bacteroid ADH. However, the results of measurements of ADH in cell-free extracts from the bacteroid and free-living rhizobia clearly showed that this is not the case. The bacteroids contained ADH activity and were able to form alanine from pyruvate and NH₄⁺ (Table 5.7).

The results obtained in the present work clearly demonstrated that ammonia, rather than alanine, is the principal product of N₂ fixation in soybean bacteroids. These results are consistent with the role of O₂ in bacteroid respiration and nitrogen fixation. O₂ regulates bacteroid respiration and nitrogen fixation is always coupled with respiration. Within certain limits, the nitrogenase activity is well preserved by low O₂ tension generated by the high respiration rates of bacteroids, using malate as carbon source. The high respiration provides sufficient energy to support substrate transport and N₂ fixation. Under the regulation by O₂, these metabolic activities, including N₂ fixation are maintained effectively.

Bacteroids isolated from the N₂-fixing nodules do not express the high affinity ammonia transporter (Amt) although these transporters are present in free-living rhizobia (reviewed by Day et al., 2001). Thus, it seems that NH₃ moves across the bacteroid membrane by simple diffusion, driven by the acidity of the peribacteroid space.
Subsequently, it is transported through the PBM in the cytoplasm of infected cells (Tyerman et al., 1995) and assimilated there by GS and GOGAT for the host plant to use (reviewed by Streeter, 1991). Using GC-MS, Thumfort and Atkins (2001) recently demonstrated that assimilation of $^{15}\text{N}_2$ by nodulated roots of cowpea produced $^{15}\text{N}$-labelled products which showed no evidence that alanine was a precursor of the amide groups of glutamine or of the purine ring of ureides (the major N$_2$-fixation product translocated from nodules in this legume).

7.5 Conclusions

In summary, the results presented in the thesis demonstrated that:

1). Respiration of bacteroids from soybean nodules is regulated by the O$_2$ supply.

2). The C$_4$-dicarboxylic acid, malate is a carbon source which is transported to support N$_2$ fixation by bacteroids via respiration. Malate transport, as an energy-dependent process, is regulated by O$_2$ supply to bacteroids via respiration.

3). There is a modified TCA cycle which is responsible for metabolism of the transported malate in soybean bacteroids.

4). The principal product of N$_2$ fixation by bacteroids is NH$_3$/NH$_4^+$. No evidence was obtained for a role for alanine as a primary product.

7.6 Future work
C₄-dicarboxylates, malate or succinate, once transported into bacteroids, are readily used to support N₂ fixation via respiration in bacteroids. However, malate and succinate must be transported through the PBM which encloses the bacteroids and bacteroid membrane before they are utilized by the bacteroids.

Using a technique for a large-scale isolation of intact and pure symbiosomes from soybean nodules (Bergersen & Appleby, 1981; Price et al., 1987), Udvardi et al. (1988) first demonstrated that the PBM from soybean nodules possesses a carrier which preferentially transports malate and succinate into symbiosomes. The mechanism of malate or succinate uptake by symbiosomes through the PBM is believed to be a passive process, driven by the membrane electrical potential (Δψ) (Udvardi et al., 1991). Rates of this transport are thought to be sufficient to meet the nitrogen fixation activity estimated from measurements with isolated bacteroids (Day et al., 1989). Unfortunately, the measurements of malate transport through the PBM were performed using aerobically isolated symbiosomes. *In vivo*, the environment which an infected cell provides is also O₂-limiting but it is impossible to determine whether the O₂ tension matters in relation to malate transport and if, as seen from my results, how the process is regulated by O₂. In order to investigate this mechanism, the large-scale anaerobic preparation of symbiosomes without significant contamination by free bacteroids is needed. The methods to be used to monitor N₂ fixation, malate transport and utilization and respiration need to be developed. The flow chamber system used in the present work might be able to be modified for use for this purpose.
Figure 7.1. Steady state respiration of bacteroids of *B. japonicum* strain CB1809 from soybean nodules in flow chamber experiments (Bergersen, unpublished results), showing how the observed respiration is the sum of that by two respiratory terminal oxidase systems of different affinity for O₂. Calculated respiration via the high-affinity *cbb₃*-type oxidase 1 with $K_m^{(1)} = 7$ nM O₂ and $V_{max}^{(1)} = 6.5$ nmol O₂ min⁻¹ [mg DW]⁻¹ (thin continuous line) and via the low-affinity oxidase system 2 with app $K_m^{(2)} = 56.2$ nM O₂ and $V_{max}^{(2)} = 18.1$ nmol O₂ min⁻¹ [mg DW]⁻¹ (dashed line); the sum of these two respirations is shown by the thick continuous line. Observed respiration rates are indicated by ▲.
Figure 7.2. Deoxygenation of oxyleghaemoglobin by CB1809 bacteroids in a closed cuvette over a 30 min period (Bergersen, private communication).

a. Spectra of deoxygenation. Curve 1 is a zero time wavelength scan and curve 9 the superimposed scan at 20 and 30 min. The sharply-defined isosbestic points indicate that the only reaction observed is deoxygenation. Also, there is no evidence of oxidation in the development of absorbance at 625-650 nm.

b. The time course of changes in absorbance at the \( \alpha \) peak (76 nm) and the trough (562 nm) between the \( \alpha \) and \( \beta \) peaks of oxyleghaemoglobin.
Table 7.1. Respiration of USDA110c bacteroids during steady states in a flow chamber experiment in which supply of O$_2$ was progressively increased.
The first column shows the time interval for each steady state measurement. It is assumed that $V_{max}$
remains throughout at 2.52 nmol O$_2$ min$^{-1}$ [mg DW]$^{-1}$, the value calculated for the first steady
state. Data in columns 1 to 4 were extracted from Table 3.1. Data in column 5 were calculated as:

$$V_{max(t)} = \{v \cdot \left[ s \cdot V_{max(2)}/(K_m(2) + s) \right] \cdot \left[ (K_m(t) + s)/s \right] \}$$

(from Expression 1)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>O$_2$ supply (nmols min$^{-1}$)</th>
<th>s (nM O$_2$)</th>
<th>Bacteroid Respiration (nmols O$_2$ min$^{-1}$ [mg DW]$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$v$</td>
</tr>
<tr>
<td>31 - 42</td>
<td>58.6</td>
<td>35.0</td>
<td>0.97</td>
</tr>
<tr>
<td>47 - 75</td>
<td>91.5</td>
<td>20.2</td>
<td>1.61</td>
</tr>
<tr>
<td>84 - 95</td>
<td>121.5</td>
<td>11.5</td>
<td>2.23</td>
</tr>
<tr>
<td>99 - 113</td>
<td>300.0</td>
<td>12.9</td>
<td>5.74</td>
</tr>
<tr>
<td>119 - 124</td>
<td>436.3</td>
<td>8.2</td>
<td>8.48</td>
</tr>
<tr>
<td>140 - 147</td>
<td>475.0</td>
<td>6.7</td>
<td>11.18</td>
</tr>
<tr>
<td>162 - 172</td>
<td>940.2</td>
<td>9.8</td>
<td>17.90</td>
</tr>
</tbody>
</table>
Table 7.2. Effects of varying $V_{\text{max}}(1)$ and $V_{\text{max}}(2)$ of two bacteroid terminal oxidase systems on activities of a simulated soybean nodule cell.

The computer simulation model (modified from that described by Bergersen 1996, 1999) was operated repeatedly after each change in $V_{\text{max}}(1)$ and $V_{\text{max}}(2)$, until constant results were obtained. The values for each $Y_{\text{min}}$ was varied independently so that $Y_{\text{max}}$ was always the same (i.e. so that the relative oxygenation of leghaemoglobin and thus the concentration of free O$_2$, immediately adjacent to the gas-filled intercellular spaces), was constant throughout.

<table>
<thead>
<tr>
<th>Bacteroid respiration (moles O$_2$ s$^{-1}$.g$^{-1}$)</th>
<th>Relative Lb oxygenation*</th>
<th>Activity cell$^1$ (moles s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}(1)$</td>
<td>$V_{\text{max}}(2)$</td>
<td>$Y_{\text{min}}$</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>1.08x 10$^{-7}$ †</td>
<td>3.01 x 10$^{-7}$ †</td>
<td>0.200</td>
</tr>
<tr>
<td>2.51 x 10$^{-7}$</td>
<td>3.01 x 10$^{-7}$</td>
<td>0.125</td>
</tr>
<tr>
<td>3.25 x 10$^{-7}$</td>
<td>3.01 x 10$^{-7}$</td>
<td>0.028</td>
</tr>
<tr>
<td>1.08 x 10$^{-7}$</td>
<td>4.44 x 10$^{-7}$</td>
<td>0.175</td>
</tr>
<tr>
<td>1.08 x 10$^{-7}$</td>
<td>5.18 x 10$^{-7}$</td>
<td>0.165</td>
</tr>
</tbody>
</table>

*: The relative oxygenation of cytosolic leghaemoglobin (Lb) at the centre ($Y_{\text{min}}$), immediately beneath the gas-filled intercellular spaces ($Y_{\text{max}}$) and the volume-averaged oxygenation ($Y_{\text{av}}$) along the diffusion path from the intercellular space to the centre of the cell. The concentration of free, dissolved O$_2$ in the host cytoplasm is related to the equilibrium constant for binding of O$_2$ by leghaemoglobin ($K_{eq} = 4.6 \times 10^{-5}$ moles O$_2$ m$^{-3}$) as follows: [O$_2$] = $K_{eq}$ $Y(1 – Y)$ moles O$_2$ m$^{-3}$.

†: The values for $V_{\text{max}}$ which gave the best fit to the observed bacteroid respiration in Fig. 7.1. For both oxidases, the increments of $V_{\text{max}}$ applied in successive lines were 1.43 x 10$^{-7}$ and 0.74 x 10$^{-7}$ moles O$_2$ s$^{-1}$.g$^{-1}$. 

†: The values for $V_{\text{max}}$ which gave the best fit to the observed bacteroid respiration in Fig. 7.1. For both oxidases, the increments of $V_{\text{max}}$ applied in successive lines were 1.43 x 10$^{-7}$ and 0.74 x 10$^{-7}$ moles O$_2$ s$^{-1}$.g$^{-1}$. 

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