Exploring

Nitrogen Fixation and Assimilation in Symbiotic Pea Root Nodules by *in vivo* ¹⁵N NMR Spectroscopy

Anne Marie Scharff Ph.D. Thesis November 2001

Roskilde University Centre, Dept. of Life Sciences and Chemistry Risø National Laboratory, Plant Research Department Roskilde, Denmark

Preface

This Ph.D. project was carried out at the Plant Research Department at Risø National Laboratory under supervision of Lis Rosendahl and Iver Jakobsen and at the Department of Life Sciences and Chemistry at Roskilde University Centre under supervision of Poul Erik Hansen. The work was financially supported by grants from the Danish Research Agency and the Danish Research Foundation.

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The thesis is divided in four main parts: Part one contains a literature review and general theoretical introduction to the chosen subject. All my experimental work and obtained results are presented in part two, and the results are discussed in part three. Part four contains an article manuscript, which is in preparation for submission to Plant Physiology. The article manuscript contains a selection of the experimental work presented in part two in a more condensed form.

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Summary

Symbiotic nitrogen fixation, the process whereby nitrogen fixing bacteria enter into associations with plants, provides the major source of nitrogen for the biosphere. Symbiosis occurs between members of the plant family Leguminosac and soil bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium* (collectively called rhizobia). The prokaryotic partner of the symbiosis harbours the enzyme nitrogenase, which catalyses the convertion of atmospheric dinitrogen into ammonium, and thereby provides the host plant with fixed nitrogen in exchange for other nutrients and carbohydrates. The symbiosis occurs in specialised nodules that form on the roots of the plant and contain the nitrogen-fixing rhizobia.

In rhizobia-leguminous plant symbioses, the current model of nitrogen transfer from the symbiotic form of the bacterium, called a bacteroid, to the plant is that nitrogenase-generated ammonia diffuses across the bacteroid membrane and is assimilated into amino acids outside of the bacteroid. However, the transport of symbiotically fixed nitrogen across the membranes surrounding the bacteroid and the form in which this occurs has been a matter of controversy. Accurate estimation of the assimilation and translocation of fixed nitrogen in nodules would benefit from non-invasive and non-destructive measurements of the fate of the fixed nitrogen. Thus, the objective of the present Ph.D. study was to investigate nitrogen fixation and assimilation in ${}^{15}N_2$ fixing root nodules root by *in vivo* ${}^{15}N$ NMR spectroscopy.

Pea nodules were chosen as the model system and were used for the majority of experiments, but soybean and *Lotus* nodules were included in a few experiments. Specialised equipment and procedures for maintaining the nodules in a physiologically viable and ¹⁵N₂ fixing state while in the NMR tube have been developed and optimised in order to obtain informative *in vivo* ¹⁵N NMR spectra. Especially the oxygen supply to the root nodules is a critical factor in maintaining the high oxidative phosphorylation level needed to support nitrogen fixation. In vivo ³¹P NMR spectroscopy was used for monitoring the physiological state of the nodules.

A time course of *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing pea root nodules was recorded, which has not previously been reported. It was demonstrated that it is indeed possible to apply *in vivo* ¹⁵N NMR spectroscopy to the study of nitrogen fixation and assimilation in root nodules. By using NMR it was possible to observe directly the incorporation of ¹⁵N into living nodules, but detection was limited to ammonium and some of the more abundant amino acids because of the relatively low sensitivity of the method. In order to aid the interpretation of ¹⁵N NMR spectra, a new mass spectrometric technique, involving separation of amino acids by ion-pair reverse-phase liquid chromatography (IP-RPLC), was developed. This method made it possible to analyse the position of ¹⁵N in mono labelled glutamine and asparagine by using MS/MS and MS/MS/MS, respectively. The analysis could be done in a single experiment without any previous derivatisation procedures, which is a major simplification and improvement compared to GC-MS procedures, which are usually used for analysis of positional labelling.

Combining the results from *in vivo* NMR and IP-RPLC MS revealed that asparagine was ¹⁵N-labelled at both the amide and amino nitrogen, but the *in vivo* NOE factor of the asparagine amino group was presumably of an unfavourable size, which caused elimination of the signal in ¹⁵N spectra recorded with full decoupling. IP-RPLC MS analyses showed that y-aminobutyric acid (GABA) was ¹⁵N-labelled in considerable amounts, but ¹⁵N GABA was NMR invisible in living pea root nodules. This may be due to immobilisation of GABA in root nodules.

The major new information, which was generated by *in vivo* ¹⁵N NMR, is that a substantial pool of free ammonium is present in the metabolically active and intact symbiosis, and that glutamine is not ¹⁵N-labelled to any great extent at the amide nitrogen. The intracellular environment led to very unusual *in vivo* ¹⁵N ammonium chemical shifts, whereas no changes in the expected *in vivo* chemical shifts of amino acids were observed. It was therefore suggested that ammonium ions and amino acids are in different compartments. This could indicate that ammonium ions are located in the bacteroids, whereas the ¹⁵N-labelled glutamine/glutamate and asparagine are located in the plant cytoplasm.

Dansk resume

Biosfærens hovedkilde til kvælstof er den symbiotiske kvælstoffiksering, som finder sted, når kvælstoffikserende bakterier indgår i symbiose med planter. Symbiosen forekommer mellem medlemmer af bælgplantefamilien Leguminosae og jordbakterier af slægterne *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* og *Azorhizobium*, som samlet kaldes rhizobier. Symbiosens prokaryote partner indeholder enzymet nitrogenase, som katalyserer omdannelsen af atmosfærisk dinitrogen til ammonium. Det fikserede kvælstof eksporteres til værtsplanten som til gengæld forsyner rhizobierne med andre næringsstoffer og kulhydrater. Symbiosen foregår i specialiserede knolde, som dannes på plantens rødder og indeholder de kvælstoffikserende rhizobier i den symbiotiske bakterieform, som kaldes bakteroider.

Ifølge den aktuelle model for kvælstofoverførsel fra bakteroiden til planten diffunderer nitrogenase-dannet ammoniak over bakteroidmembranen og assimileres i aminosyrer udenfor bakteroiden. Der har imidlertid været nogen debat om transportmekanismen for symbiotisk fikseret kvælstof over membranerne der omgiver bakteroiden, og i hvilken molekylær form det foregår. Det ville derfor være nyttigt med non-invasive og non-destruktive målinger, der gør det muligt at følge den fikserede kvælstofs assimilering og translokation i rodknolde. Formålet med dette ph.d. projekt blev således at undersøge kvælstoffiksering og -assimilering i ¹⁵N₂-fikserende rodknolde ved hjælp af *in vivo* ¹⁵N NMR spektroskopi.

Ærterodknolde blev valgt som modelsystem og har været anvendt i størstedelen af eksperimenterne, men soja og *Lotus* rodknolde er blevet brugt i enkelte eksperimenter. Det var nødvendigt at udvikle og optimere specialiseret udstyr og procedurer for at kunne holde rodknoldene i en fysiologisk levende og ¹⁵N₂-fikserende tilstand i NMR røret, og for at kunne optage informative *in vivo* ¹⁵N NMR spektre. Specielt iltforsyningen til rodknoldene var en kritisk faktor for at kunne opretholde det høje oxidative fosforyleringsniveau, som er nødvendigt for at kvælstoffikseringen kan finde sted. *In vivo* ³¹P NMR spektroskopi blev brugt til at overvåge rodknoldenes fysiologiske tilstand.

Som den første kan jeg rapportere et tidsforløb af *in vivo* ¹⁵N NMR spektre af ¹⁵N₂-fikserende ærterodknolde. Jeg har demonstreret, at det er muligt at anvende *in vivo* ¹⁵N NMR spektroskopi i studiet af kvælstoffiksering og -assimilering i rodknolde. Ved hjælp af NMR var det således muligt direkte at observere indkorporeringen af ¹⁵N i levende rodknolde, men på grund af metodens relativt lave følsomhed er detektionen begrænset til ammonium og nogle af de mere hyppigt forekommende aminosyrer.

Med henblik på at støtte fortolkningen af ¹⁵N NMR spektrene blev der udviklet en ny massespektrometrisk teknik, som indbefattede separation af aminosyrerne ved ionpar omvendt fase væskekromatografi (IP-RPLC). Denne metode muliggjorde en analyse af ¹⁵N-positionen i enkeltmærket glutamin og asparagin ved hjælp af henholdsvis MS/MS og MS/MS/MS. Analysen kunne udføres i et enkelt eksperiment uden nogle forudgående derivatiseringsprocedurer, hvilket er en stor forenkling og forbedring sammenlignet med de GC-MS procedurer, der normalt anvendes til analyse af positionsmærkning.

Ved at kombinere resultaterne fra *in vivo* NMR og IP-RPLC MS kunne det ses, at asparagin var ¹⁵N-mærket i både amid- og aminogruppen, men *in vivo* NOE faktoren for asparagins aminogruppe havde formentlig en ufavorabel størrelse som bevirkede, at signalet blev elimineret i ¹⁵N spektre optaget med fuld proton dekobling. IP-RPLC MS analyser viste, at γ-aminosmørsyre (GABA) var højt ¹⁵N-mærket, men ¹⁵N GABA var NMR-usynligt i levende ærterodknolde. Dette foreslås at kunne skyldes immobilisering af GABA i rodknolde.

Den vigtigste nye information, som blev frembragt ved hjælp af *in vivo* ¹⁵N NMR, er tilstedeværelsen af en betragtelig pool af frit ammonium i den metabolsk aktive, intakte symbiose, og at glutamin ikke bliver ¹⁵N-mærket i amidgruppen i særlig høj grad. Det intracellulære miljø medførte et meget usædvanligt *in vivo* ¹⁵N ammonium kemisk skift, hvorimod der ikke kunne observeres nogle ændringer i aminosyrernes forventede kemiske skift *in vivo*. Jeg foreslår derfor, at ammonium ionerne og aminosyrerne er lokaliseret i forskellige kompartments. Dette kunne tyde på, at ammonium ionerne er i bakteroiderne, hvorimod den ¹⁵N-mærkede glutamin/glutamat og asparagin er lokaliseret i plantecytoplasma.

Abbreviations:

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Aap	Amino acid permease
AAT	Aspartate aminotransferase
ADH	Alanine dehydrogenase
Ala	Alanine
Amt	Ammonium transporter
ARA	Acetylene reduction assay
AS	Asparagine synthetase
Asp	Aspartate
Asn	Asparagine
BC	Bacteroid cytosol
BM	Bacteroid membrane
Bra	Branched-chain amino acid transporter
DW	Dry weight
FW	Fresh weight
GABA	y-aminobutyric acid
GAD	Glutamate a-decarboxylase
GC-MS	Gas chromatography mass spectrometry
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HMQC	Heteronuclear multiple quantum coherence
IP-RPLC	lon-pair reverse-phase liquid chromatography
Lb	Leghemoglobin
LCO	Lipo-chitin oligomer
MDH	Malate dehydrogenase
MDP	Methylene diphosphonic acid
ME	Malic enzyme
MES	2-(Morpholino)ethanesulfonic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
ΟΑΑ	Oxaloacetate
PBS	Peribacteroid space
PCCG	Phosphocholine-substituted B-1.3:1.6 cyclic glucan
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
ppm	parts per million
S/N ratio	Signal-to-noise ratio
SM	Symbiosome membrane
SS	Sucrose synthase
TCA	Tricarboxylic acid

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1 Legume-Rhizobium root nodules

Symbiosis occurs between members of the plant family Leguminosae and soil bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium* (collectively called rhizobia) (Taiz and Zeiger 1998) (Table 1). The prokaryotic partner of the symbiosis is able to convert atmospheric dinitrogen into ammonium and provide the host plant with fixed nitrogen in exchange for other nutrients and carbohydrates. The symbiosis occurs in specialised nodules that form on the roots of the plant and contain the nitrogen-fixing rhizobia.

Legume host plant	Rhizobium		
	Genus	Species	Biovariety
Pisum (pea)	Rhizobium	leguminosarum	viceae
Vicia (vetch)	Rhizobium	leguminosarum	viceae
Trifolium (clover)	Rhizobium	leguminosarum	trifolii
Phaseolus (French bean, common bean)	Rhizobium	leguminosarum	phaseoli
Medicago (alfalfa)	Sinorhizobium	meliloti	
Glycine (soybean)	Bradyrhizobium	japonicum	
Lotus (birdsfoot trefoil)	Mesorhizobium	loti	
Lupinus (lupine)	Mesorhizobium	loti	

 Table 1: Examples of root nodule symbiotic associations between legumes and rhizobla (modified from Hadri et al. 1998)

1.1 Establishment of the symbiosis

The formation of root nodules is initiated by a signalling cross talk involving both the host plant and the rhizobia (reviewed by Schultze and Kondorosi 1998) (Fig. 1). Rhizobia respond by positive chemotaxis to plant root exudates and move towards localised sites on the legume roots (reviewed by Rhijn and Vanderleyden 1995). The plant roots excrete host specific flavonoid signals, which activate the bacterial regulatory NodD. NodD iş. protein а transcription factor and induces expression of bacterial nod genes, which code for proteins involved



Fig. 1: Signal exchange in the *Rhizobium*-plant symbiosis. Flavonoids induce the rhizobial *nod* genes. This leads to the production of nodule-inducing (Nod) factors, lipochitooligosaccharides (LCOs) that are differently modified depending on the *Rhizobium* species (Modified from Schultze and Kondorosi 1998)

in the biosynthesis and excretion of Nod factors (reviewed by Denaric and Cullimore 1993; Cohn et al. 1998). The Nod factors are substituted lipo-chitin oligomers (LCO) and each rhizobial species produces a characteristic spectrum of Nod factors with specific substitutions that are important in determining host specificity (Schultze and Kondorosi 1998). The degree of host specificity varies tremendously among the rhizobia with some strains having a very narrow host range and others a very broad one (Rhijn and Vanderleyden 1995).

The Nod factors induce plant root hair curling, whereby rhizobia become enclosed in the small compartment formed by the curling. The next step is the formation of an infection thread through which the bacteria reach the root cortical tissue. Concurrent with infection, root cortical cells differentiate and start dividing, forming a distinct area within the cortex, called a nodule primordium, from which the nodule will develop.

The bacteria are internalised into the plant cells by an endocytotic process (Fig. 2), whereby the bacterium becomes surrounded by a plant derived membrane termed the symbiosome membrane (SM). After endocytosis the bacteria continue to divide and differentiate into the nitrogen-fixing bacteroid form (B). Differentiation involves the induction of genes necessary for the symbiotic state, the so-called *nif* and *fix* genes (see chapter 1.2). The SM enclosed bacteroids, called symbiosomes (see Figs. 2 and 3) (Roth *et al.* 1988), consist of bacteroid cytosol (BC) enclosed by a bacteroid double membrane (BM). The interface between the BM and the SM is called the peribacteroid space (PBS). Symbiosomes may be considered a tissue specific organelle in analogy with mitochondria and plastids in plant cells (Roth *et al.* 1988).





Fig. 2A: Schematic outline of the infection process, where bacteria enter the plant cell through an infection thread (IT) and an infection droplet (ID). See text for further description of the process (Modified from Brewin 1998). 2B: Electron micrograph of pea symbiosomes. Abbreviations: B, bacteroid; BM, bacteroid membrane; PBS, peribacteroid space; SM, sym-biosome membrane. Magnification: \times 6860 (Modified from Newcomb 1976).

The SM contains host-encoded ATP-dependent proton pumps, the orientation of which results in transfer of protons from the host cytoplasm to the PBS (Szafran and Haaker 1995). Consequently, an electrical as well as a pH gradient over the SM is generated (Fig. 3) (Udvardi *et al.* 1991; Ou Yang and Day 1992). In addition, the bacteroid respiratory electron transport chain, which is

localised on the bacteroid inner membrane and very active under nitrogen fixation, also results in protons being pumped into the PBS (O'Brian and Maier 1989). Electrical and pH gradients are concomitantly formed across the bacteroid inner membrane as well. The end result is acidification of the PBS. pH in the PBS has been estimated to be 1-2 pH units lower than pH in the plant cytosol (which is normally in the range 7.0-7.5) in an investigation of isolated symbiosomes, but the pH gradients in vivo will depend on the actual ATP-ase activity and bacterial respiration (Udvardi et al, 1991). The electrical and proton gradients across both the BM and SM provide a driving force for the transport of important metabolites, which will be described in chapter 2.1, 2.3 and 2.6.



Fig. 3: Generalised scheme of a symbiosome consisting of the bacteroid cytoplasm and bacteroid membrane (BM) surrounded by the host-derived symbiosome membrane (SM). The peribacteroid space (PBS) is acidified and electrical gradients are formed across the membranes by the action of H^{*}-pumping ATPases on the SM and the electron transport chains (ETC) on the BM (Modified from Day and Udvardi 1992).

1.2 Organisation and regulation of symbiosis specific genes

The complete genome of *S. meliloti*, the symbiont of alfalfa, has recently been sequenced and annotated and was reported to consist of three parts: a large 3.65 Mb chromosome and two megaplasmids, pSymA and pSymB, of 1.35 and 1.68 Mb, respectively (Galibert *et al.* 2001). In general, it has been found that a large part of the total DNA in rhizobia is present in the form of plasmids of variable number and size (reviewed by Hynes and Finan 1998). Many rhizobia contain one or more large plasmids, termed pSym, which carry most of the information needed for symbiosis, including the *nod* genes responsible for host specificity (cf. chapter 1.1) and the *nif* and *fix* genes involved in nitrogen fixation (reviewed by Kaminski *et al.* 1998; Galibert *et al.* 2001). The *nif* genes encode products, which are involved in the biosynthesis and assembly of the nitrogenase enzyme complex including the FeMo cofactor (see chapter 2.2). The precise functions of the *fix* gene products are largely unknown, apart from the fact that they are important for nitrogen fixation in symbiosis (Werner 1992).

The nifA gene product is a DNA binding regulatory protein required for the transcription of nif genes and some fix genes. The NifA protein is inactivated at high levels of oxygen and is furthermore thermolabile, and nitrogen fixation is thus regulated by O_2 (cf. chapter 1.4) and

temperature (Werner 1992). It has been shown that the low oxygen concentration of the nodule (see chapter 1.4) is the primary signal that elicits *nif* and *fix* gene expression (reviewed by Kaminski *et al.* 1998). On the other hand, nitrogenase synthesis in rhizobia is, with few exceptions, uncoupled from the nitrogen status of the bacteria. In this way, rhizobia can fix nitrogen in amounts well above those covering their own needs (reviewed by Kaminski *et al.* 1998).

Regulation of nitrogen assimilation in rhizobia (and other bacteria) is achieved at the genetic level by a central nitrogen regulatory (Ntr) system. The proteins NtrB and NtrC constitute a twocomponent regulatory system that responds to the nitrogen status of the ccll (Reitzer and Magasanik 1986). Under conditions of low nitrogen availability this system and a third protein NtrA mediate an activation of gene transcription of several genes involved in nitrogen assimilation in rhizobia including nitrate reductase, a high-affinity ammonium transporter (Amt, see chapter 2.3), and GS II (see chapter 2.4) (reviewed by Udvardi and Day 1997).

However, the Ntr system is generally not required for transcription of *nif* genes in rhizobia. Nitrogen fixation is, as described above, primarily regulated by oxygen concentration, whereas nitrogen assimilation is under nitrogen control, and this implies that the two processes can be uncoupled. This is what occurs in bacteroids where nitrogen fixation is assumed to occur without significant ammonium assimilation (cf. chapter 2.4). Biochemical and genetic evidence indicate that bacteroids are not nitrogen starved in nodules, and the ammonium assimilation pathway is thus not induced. This is consistent with the fact that mutations in the *ntr* genes that are required for induction of ammonium assimilating enzymes have little or no effect on the symbiotic performance of rhizobia (reviewed by Udvardi and Day 1997).

1.3 Nodule morphology

Legume nodule morphology can be divided in two main classes: indeterminate and determinate nodules (reviewed by Hadri *et al.* 1998; Bergersen 1982; Werner 1992; Hansen 1994) (Figs. 4 and 5). The tissue organisation is, however, similar in some respects. The nodules are composed of a central tissue where bacteroids are hosted, surrounded by several peripheral tissues (reviewed by Brewin 1991). The peripheral tissues include the nodule cortex, the endodermis and the nodule parenchyma, that harbours the nodule vascular bundles. The nodule parenchyma is also known as the inner cortex (Wycoff *et al.* 1998). The vascular system joins at the base of the nodule and enters the vascular system of the roots, thus facilitating the exchange of fixed nitrogen produced by the bacteroids for nutrients contributed by the plant.

Indeterminate nodules (Fig. 4) have persistent meristems, which lead to cylindrical-shaped structures that contain zones of cells with successive stages of development: meristem, infection, early symbiotic, late symbiotic and senescence (Vasse *et al.* 1990). Transcription of *nif* and *fix* genes (see chapter 1.2) starts in the early symbiotic zone (interzone II-III in Fig. 4), although the cells do not fix N₂ yet. Expression of leghemoglobin (see chapter 1.4) also starts in this zone.



Nitrogen fixation takes place exclusively in the late symbiotic zone (zone III in Fig. 4), where bacteroids are fully mature and differentiated. Temperate legumes such as pea, clover, alfalfa, lupine, *Lotus* and vetch form the indeterminate type of nodules. The bacteroids are grossly enlarged compared to free-living bacteria, and each symbiosome contains only a single bacteroid (Whitehead and Day 1997). Mature pea bacteroids become rod- or Y-shaped (cf. Fig. 2) and typical dimensions are $1 \times 3 \mu m$ (Dixon and Wheeler 1986). The vascular system is open at the meristem and nitrogen is exported from the nodule to the plant in the form of amides, principally the amino acids asparagine and glutamine.

Determinate nodules, on the contrary, lack a persistent meristem and therefore remain spherical-shaped. The central tissue is characterised by containing only a single stage of plant and bacterial differentiation at any particular moment. Legumes of tropical origin such as soybean, common bean and French bean form determinate nodules. Bacteroids are only slightly larger than bacteria of the same strain grown in culture, and several bacteroids are contained within a single SM (Fig. 5) (Whitehead and Day 1997). Soybean symbiosomes are 2-5 µm in diameter and enclose 2-10 bacteroids



Fig. 5: Infected host cell from a determinate nodule (soybean) filled with bacteroids inside symbiosome membranes. Nearby are several smaller uninfected cells with large central vacuoles and amyloplasts abundant in starch (From Werner 1992).

(Day et al. 1989). The vascular system encircles the central tissue, and nitrogen is exported from the nodule as ureides: allantoin and allantoic acid.

The central tissue in determinate as well as indeterminate nodules contains both infected and uninfected cells and these have different metabolic roles (see chapter 2.1 and 2.5). The infected cell type is fully packed with bacteroids and has either a very small central vacuole (pea) or none at all (soybean) (Werner 1992). The plant cells also contain amyloplasts, which are sometimes termed proplastids or simply plastids because they do not always contain starch despite being highly differentiated (reviewed by Bergersen 1997). These double-membrane enclosed organelles are the sites of some of the ammonium assimilating reactions, which will be further described in chapter 2.5 and Fig. 11 (Atkins 1991; Robinson *et al.* 1994).

The volume of the central bacteroid-containing tissue and its duration in an intact state are the major determinants of the extent of N_2 fixation. With age and/or stress this tissue breaks down, from the base in indeterminate nodules and centrally in determinate nodules.

1.4 The oxygen dilemma

Oxygen has a prominent role in symbiosis as a signalling molecule in the early establishment of the symbiosis (cf. chapter 1.2), as a respiratory substrate and as an inhibitor of nitrogenase synthesis as well as activity. The nitrogenase complex (see chapter 2.2) inside the bacteroids is extremely sensitive to oxygen due to irreversible inactivation by O_2 . On the other hand, O_2 is required to support the highly active respiratory processes that take place aerobically in the plant and bacteroid compartments. In order to deal with this dilemma, nodules have developed structures and mechanisms that enables efficient delivery and dispersion of O_2 in the infected zone, while maintaining the free O_2 in this region at a concentration that ranges between 3 and 30 nM (Bergersen 1982; Witty *et al.* 1987) (Fig. 6). This concentration is about 4 or 5 orders of magnitude lower than the O_2 concentration in water or cell sap in equilibrium with air (ca. 250 μ M O_2).

The diffusion of O_2 into the central zone of nodules is, according to the dominant hypothesis, regulated by a physical barrier which is probably located in the inner cortical region (reviewed by Witty and Minchin 1990; Layzell 1998; Bergersen 1997). This barrier consists of layers of parenchyma cells, some of which (i.e. the boundary layer or gas diffusion barrier), have radially aligned cell walls and very few, small gas-filled intercellular spaces (Parsons and Day 1990). Water and/or glycoprotein may be deposited reversibly into intercellular spaces in this region or the cells may enlarge, thereby displacing the gas from the region to increase the length of the aqueous path for diffusion to the central zone. Because gas diffusion through water is 10^4 times slower than through air, the nodule permeability to O_2 as well as to other gasses would drop.

Legume nodules will vary their permeability to O_2 diffusion in response to a large number of treatments that alter the metabolic activity of the nodule (reviewed by Layzell 1998). When

nodules are exposed to an Ar/O_2 or C_2H_2 atmosphere, nitrogenase activity is inhibited within 10-40 minutes. This inhibition is associated with a decrease in the O_2 concentration of infected cells, and all of the initial nitrogenase activity can be recovered by increasing external pO_2 . This demonstrates that nitrogenase inhibition is due solely to a decrease in nodule O_2 permeability. Other treatments in which nitrogenase inhibition is only partially due to a decrease in nodule O_2 permeability include detopping, nodule excision, stem girdling, nitrate fertilisation and salt stress. In these cases increases in external pO_2 will only recover a portion of the initial nitrogenase activity. The remaining inhibition may be attributed to a reduction in the metabolic capacity of the nodule, possibly through a reduction in carbohydrate availability or glycolytic enzyme activity.

Once O_2 has crossed the cortical tissue it diffuses rapidly to the infected cells through a network of interconnected gas-filled intercellular spaces (Witty *et al.* 1987). When O_2 diffuses into the infected cell, it is rapidly and reversibly bound to leghemoglobin (Lb), which is a plant-derived, monomeric heme-protein produced only in the nodule. Lb has a key role in maintaining a high flux of oxygen

to support bacterial respiration, whilst at the same time maintaining the concentration of free O₂ at a low level. K_m for O_2 in the reaction $Lb + O_2 \leftrightarrow LbO_2$ is about 37 nM (Bergersen 1982), and Lb occurs in high concentrations in the plant cytosol of infected cells (reviewed by Appleby 1984 and 1992). In soybean the Lb concentration in the cytosol has been estimated to be ca. 3 mM (Bergersen 1982). Lb thus acts as an O₂ buffer in the nodules, ensuring the even partitioning of O₂ between the plant and bacteroid fractions of the nodules, and facilitates the diffusion of O₂ through the infected cells so that a high flux of O₂ occurs at a strictly controlled concentration to the rapidly respiring bacteroids.



Fig. 6: Predicted O_2 gradients across a legume nodule according to two different models. A new model by Thumfort *et al.* (1999) assumes a large intracellular O_2 gradient (solid line), whereas an earlier, more simple model assumes that all control of O_2 diffusion is located in the inner cortex (dashed line) (From Layzell 2000).

Another feature of the regulation of the free O_2 concentration inside the nodule is ascribed to the fact that the infected cells of nodule central tissue constitute a compact, intense sink for O_2 . Infected cells contain hundreds or even thousands of bacteroids and increased numbers of mitochondria compared with other plant tissues (Bergersen 1997). Furthermore, the mitochondria in the infected cells are distributed mainly around the periphery in close proximity to the cell wall

and intercellular air spaces (Bergersen 1982). Consequently O_2 will be consumed at a high rate in this region, which has been predicted to create an intracellular O_2 concentration gradient as outlined in Fig. 6 (Thumfort *et al.* 1999). The availability of O_2 may be a key-limiting factor of metabolism in the infected zone of nodules by restricting aerobic respiration and thereby ATP synthesis. Due to the mitochondrial clustering near the intercellular spaces, it has been suggested that the bacteroids rather than the mitochondria, would be the site of O_2 limited metabolism (Layzell 2000). Evidence to support this view was provided by recent results showing that a decrease in external pO_2 from 0.2 to 0.1 atm. was followed by a decrease in ATP levels in the bacteroid compartment but not in the plant compartment of intact, attached soybean nodules (Kuzma *et al.* 1999).

2 Carbon and nitrogen metabolism in root nodules

2.1 Carbon metabolism

Symbiotic nitrogen fixation has a high demand for carbon sources. Photosynthates are required to support nodule and bacteroid growth and maintenance. In nitrogen-fixing bacteroids, large amounts of carbon compounds are essential for the generation of ATP and reducing power needed for nitrogenase activity. Furthermore, carbon skeletons are required for assimilation of the fixed N_2 into amino acids. Carbon metabolism is thus intimately linked to the process of nitrogen fixation, and the outlines of carbon metabolism will be summarised in this chapter before presenting a more claborate introduction to nitrogen metabolism, which is central to my own experimental work.

The primary photosynthate translocated via the phloem from the shoot to the root nodules is sucrose as demonstrated by labelling studies (Reibach and Streeter 1983; Gordon *et al.* 1985). Sucrose is not taken up directly by the bacteroids, but is metabolised (Fig. 7) primarily via the action of sucrose synthase (SS) in the plant cytoplasm of uninfected cells in the infected zone of the nodule (reviewed by Gordon 1995; Day and Copeland 1991). SS is present at high levels in nodules and its activity correlate with nitrogenase activity (reviewed by Streeter 1991). The UDP-glucose, formed by SS activity, undergoes glycolysis, and phosphoenolpyruvate (PEP) is produced. PEP is carboxylated to form oxaloacetate by non-photosynthetic CO₂ fixation via the action of phosphoenolpyruvate carboxylase (PEPC) (reviewed by Kahn *et al.* 1998).

Oxaloacetate may either serve as carbon skeleton for the initial NH_4^+ -assimilatory reactions (see chapter 2.5) or it may enter the tricarboxylic acid (TCA) cycle (see below) or it may be further converted to malate by the action of malate dehydrogenase (MDH). MDH is present in extremely high levels of activity in nodule cytosol and is correlated with nitrogenase activity (Appels and Haaker 1988). The activity of PEPC is also very high in the plant cytosol of root nodules, and ¹⁴CO₂ labelling studies with intact nodules have demonstrated rapid and significant labelling of organic acids, particularly malate (Vance *et al.* 1983; Snapp and Vance 1986; Rosendahl *et al.* 1990; Salminen and Streeter 1992). Malate can be further reduced to form furnarate and succinate,

and these three organic acids accumulate to μ mol quantities per gram nodule fresh weight, while amounts of other TCA cycle acids are much less (reviewed by Vance and Heichel 1991).

The TCA cycle in the plant mitochondria does not appear to play a significant role in the synthesis of organic acids, as the activity of many of the TCA cycle enzymes is low (Kahn *et al.* 1998; Gordon 1992). However, Streeter (1991) suggested that the principal function of nodule



Fig. 7: A general scheme for the carbon metabolism taking place in the plant and bacteroid cytoplasm of root nodules (Modified from Kahn et al. 1998; Kaminski et al. 1998). Abbreviated metabolites are: PEP, phosphoenolpyruvate; OAA, oxaloacetate; 2-OX, 2-oxoglutarate; MAL, malate; FUM, fumarate; SUC, succinate; PYR, pyruvate; AcCoA, acetyl-coenzymeA; CIT, citrate; ISO, isocitrate; SUC-CoA, succinatecoenzymeA. Enzymes involved are within boxes: SS, sucrose synthase; PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; DctA, dicarboxylic acid transporter A; ME, malic enzyme; ODH, 2-oxoglutarate dehydrogenase. SM and BM are the symbiosome and bacteroid membranes, respectively.

mitochondria may be the production of 2-oxoglutarate, which is required as carbon skeleton in the initial steps of ammonium assimilation (see chapter 2.5). This can be achieved if only a part of the TCA cycle is operating leading to the net reaction acetyl-CoA + oxaloacetate \rightarrow 2-oxoglutarate + CO₂.

 C_4 dicarboxylic acids, especially malate and succinate, have been shown to be the favoured substrates *in vitro* for the support of nitrogen fixation by isolated bacteroids (reviewed by Streeter 1991). It is generally agreed that also in the intact symbiosis, C_4 dicarboxylic acids are the most probable substrates supplied to bacteroids (Day and Copeland 1991; Kaminski *et al.* 1998). This is supported by a large body of evidence including the fact that rhizobia mutants deficient for C_4 dicarboxylic acid transport (*dct*, see below) are uniformly unable to fix nitrogen when associated with their plant hosts (reviewed by Mitsch *et al.* 2000). Furthermore, label derived from ¹⁴CO₂ fixation in intact pea and soybean root nodules is rapidly incorporated into bacteroids in the form of malate and succinate (Rosendahl *et al.* 1990; Salminen and Streeter 1992).

Import of C₄ dicarboxylic acids into bacteroids has been demonstrated to involve two different active transporters: One is of plant origin and is located on the SM (Day *et al.* 1989; Udvardi *et al.* 1988a). It functions as an uniporter for monovalent dicarboxylic acids with high affinity for malate and succinate and a smaller affinity for oxaloacetate, fumarate and 2-oxoglutarate and allows movement of the anions down their electrical and concentration gradient across the SM into the PBS (Udvardi *et al.* 1988a; Ou Yang *et al.* 1990). The second transporter, the dctA gene product of rhizobia, is a single protein carrier embedded in the inner membrane of the bacteroid (reviewed by Jording *et al.* 1994) and appears to be a proton/dicarboxylic acid symporter that is driven by the proton gradient across the bacteroid inner membrane (Udvardi and Day 1997). The gene products of dctB and dctD are involved in the regulation of the expression of dctA (Jording *et al.* 1994). The Dct system has a high affinity for the substrates malate, succinate and fumarate (reviewed by Udvardi and Day 1997).

The SM as well as the BM are, on the other hand, relatively impermeable to sugars such as glucose, which further supports the view that C_4 dicarboxylic acids and not sugars are the dominant carbon source made available to the bacteroid (Udvardi and Day 1997).

The TCA cycle is the dominant metabolic pathway by which C_4 dicarboxylic acids are oxidised in bacteroids (Kahn *et al.* 1998). To maintain the TCA cycle with C_4 dicarboxylic acids as the sole carbon source, a pathway to generate acetyl-CoA is essential. Several such enzymatic pathways exist, but it is believed that oxidative decarboxylation of malate to pyruvate catalysed by malic enzyme (ME), and concomitant reduction of a nicotinamide cofactor is the dominant means by which the TCA cycle is maintained (Mitsch *et al.* 2000). Oxaloacetate and 2-oxoglutarate are TCA intermediates and are thus present in the bacteroid cytosol to provide carbon skeletons for the initial NH₄⁺-assimilatory reactions (see chapter 2.4).

There has been speculation that amino acids may also play a role in carbon and/or energy supply to the bacteroids (reviewed by Udvardi and Day 1997) and this will be described further in chapter 2.7.

2.2 N₂ is fixed by the nitrogenase enzyme complex

Reduction of atmospheric N₂ into NH₃ is catalysed by the bacteroid enzyme nitrogenase, which is a 2:1 complex of two components: the Fe and the MoFe protein, respectively (Fig. 8) (reviewed by Howard and Rees 2000; Kaminski *et al.* 1998; Smith 2000; Orme-Johnson 1992). The Fe protein (also called dinitrogenase reductase) is a homodimer of about 60 kDa that contains a single Fe₄S₄ cluster. The MoFe protein (also known as dinitrogenase) is an $\alpha_2\beta_2$ heterotetramer of about 220 kDa containing 4 Fe₄S₄ clusters (the so-called P-clusters), and a cofactor with Fe and Mo, which is the catalytic site for N₂ reduction.



Fig. 8: The reaction catalysed by nitrogenase. Ferredoxin reduces the Fe protein. Binding and hydrolysis of ATP to the Fe protein is thought to cause a conformational change of the Fe protein, which facilitates the redox reactions. The Fe protein reduces the MoFe protein and the MoFe protein reduces the N_2 . It is possible for two Fe proteins to bind to the MoFe protein at one time, but only one is shown for clarity. The darker arrows trace the flow of electrons (Modified from Taiz and Zeiger 1998; Dixon and Wheeler 1986).

The reaction sequence (reviewed by Howard and Rees 2000; Orme-Johnson 1992; Werner 1992) (Fig. 8) starts by reduction of the Fe protein by the low-potential electron donor ferredoxin. Electrons are transferred, one at a time, from the Fe protein to the MoFe protein in a process that involves MgATP hydrolysis. The cycle repeats until enough electrons have been provided for the complete reduction of the N₂ substrate. The overall reaction catalysed by nitrogenase is:

$$N_2 + 16 MgATP + 8 e^i + 8 H^* \rightarrow 2 NH_3 + 16 MgADP + 16 P_i + H_2$$
 (Eq. 1)

This chemical equation reflects one of the main features of biological nitrogen fixation: its high energetic cost due to the high stability of the N_2 triple bond. Two ATP molecules are hydrolysed at

every electron transferred from the Fe protein to the MoFe protein. In addition N₂ reduction is always accompanied by the concomitant reduction of at least two protons to evolve H₂, thus adding to the energetic cost of the process (cf. chapter 8). In the absence of other reducible substrates, the total electron flux through nitrogenase is funnelled into hydrogen production (Hadfield and Bulen 1969). Metabolism of dicarboxylic acids inside bacteroids through the TCA cycle (see chapter 2.1) and subsequent oxidative phosphorylation leads to the production of H⁺, electrons, and ATP required for the nitrogen fixation process (reviewed by Streeter 1991).

A second, major feature of nitrogenase is its oxygen-lability, mainly contributed by the Fe protein component, which is irreversibly inactivated by O_2 with typical half decay times of 30 to 45 seconds (Dixon and Wheeler 1986).

The product of nitrogenase exists as an equilibrium distribution of two forms, the acid ammonium (NH_4^+) and the base ammonia (NH_3) , which are readily inconvertible. An acid-base equilibrium distribution is determined by pH, and as the pK_a of ammonium is 9.23 at 20 °C (Bates and Pinching 1950), ammonium will be the dominating species at all physiologically relevant pH values. According to the Henderson-Hasselbach equation, the ammonia concentration is for example only 1% and 0.01% of the ammonium concentration at an intracellular pH of 7.23 and pH 5.23, respectively. In the following, the term ammonium or NH₄⁺ will mean an equilibrium distribution of ammonium and ammonia as determined by the actual pH, unless otherwise stressed.

The free ammonium concentration in the bacteroid cytoplasm is not known due to experimental obstacles, but an estimate has been published for bacteroids in soybean nodules (Streeter 1989). The ammonium content in isolated bacteroids was estimated to be 1.20 ± 0.08 and 2.00 ± 0.08 µmol g⁻¹ FW nodules in two different experiments. It was assumed that 1 g FW nodules contained 0.15 g bacteroids, and that bacteroids consisted of 90% water. Based on these assumptions and a mean bacteroid ammonium content of 1.6 µmol g⁻¹ FW nodule, the bacteroid ammonium concentration was roughly approximated to be 12 mM.

2.3 Transport of ammonium/ammonia across bacteroid and symbiosome membranes

Free-living N₂-fixing rhizobia transport ammonium in two ways (reviewed by Day *et al.* 2001). A high-affinity ammonium transporter (Amt) allows rapid uptake from low concentrations of ammonium in the surrounding medium (Glenn and Dilworth 1984; Howitt *et al.* 1986) and thus ensures recycling of ammonium lost from the cell and scavenging of exogenous ammonium. The second mechanism involves NH₃ leaving and entering the cell by simple diffusion across the membranes. At high external concentrations of ammonium, the Amt system is repressed (see chapter 1.2), and diffusion of NH₃ provides the cell with sufficient nitrogen for growth (Howitt *et al.* 1986).

Bacteroids isolated from N_2 -fixing nodules, on the other hand, do not express the Amt system, and ammonium transport is thought to occur solely by simple diffusion of NH₃ down a concentration gradient out of the cell and into the PBS (Fig. 9) (Glenn and Dilworth 1984; Howitt *et al.* 1986). As pH in the PBS is presumed to be lower than in the bacteroid compartment (cf. chapter 1.3), the acid-base equilibrium will be displaced even more in the direction of ammonium, and this enhances

the NH_3 concentration gradient across the BM – the so-called acid trap (Udvardi and Day 1997). Ammonium will not be reuptaken by the bacteroid because the Amt system is suppressed.

Movement of ammonium ions from the PBS into the plant cytoplasm requires a transport mechanism across the SM, and an ammonium channel has recently been characterised in soybean (Tyerman *et al.* 1995) and in pea (Mouritsen and Rosendahl 1997) (Fig. 9). The soybean ammonium transporter was identified by patch-clamp studies of isolated symbiosomes and was shown to be a voltage-gated monovalent cation channel. This channel opens to allow ammonium efflux from the symbiosome when a membrane potential (negative on the plant side) is generated across the SM, which is likely to occur *in vivo* (see chapter



Fig. 9: Ammonium transport in symbiosomes. Ammonia produced in the bacteroid diffuses into the peribacteroid space (PBS), where it is protonated (trapped) in the acidic environment. Suppression of rhizobial Amt prevents cycling of ammonium back into the bacteroid. Movement of ammonium ions into the plant cytoplasm requires the operation of a channel. Ammonia assimilation into amino acids also occurs, and these may also be provided to the plant (see chapter 2.4) (Modified from Day et al. 2001).

1.3). It is capable of transporting NH_4^+ across the SM at rates that are adequate to account for estimates of N₂ fixation rates in symbiosomes *in vivo*, and at low and physiologically relevant concentrations (10-20 mM) it shows a preference for NH_4^+ (Tyerman *et al.* 1995). A presumably similar voltage-driven channel capable of carrying out ammonium transport from the bacteroid side across the SM was identified in pea root nodules (Mouritsen and Rosendahl 1997). Uptake of the ammonium analogue ¹⁴C-methylamine into energised bacteroid-side-out SM vesicles when a membrane potential was generated across the membrane was demonstrated in this study. The uptake mechanism appeared to have a high capacity for transport, and saturation was not observed in the studied concentration range of 25 μ M to 150 mM methylamine.

An attempt has been made to isolate a cDNA from soybean encoding the SM ammonium channel using functional complementation of yeast (Kaiser et. al. 1998). A single cDNA termed GmSAT1 (Glycine max symbiotic ammonium transport) yielded transcripts that were able to complement an ammonium transport defect in a yeast mutant (Kaiser et al. 1998). However, further analysis has indicated that the GmSAT1 protein is not itself a channel, but that it merely interacts with

endogenous Mep ammonium transporters in yeast, in a way, which seems to enable ammonium uptake (Marini et al. 2000).

Besides the ammonium transport across the SM described above, it has also been suggested that diffusion of NH_3 across the SM, either via simple diffusion or facilitated via a channel, might play a role in the translocation of nitrogen (reviewed by Day *et al.* 2001). Passive diffusion of NH_3 across the SM could be adequate to support measured rates of ammonium assimilation, provided that a steep concentration gradient was maintained by rapid removal of ammonium in the host cell by the enzyme glutamine synthase (see chapter 2.5) (Udvardi and Day 1990). However, these ideas remain to be investigated in more detail.

2.4 Nitrogen assimilation in bacteroids

Until recently it has been generally agreed that *Rhizobium* bacteroids do not assimilate ammonium to any great extent during symbiosis. The paradigm has been that fixed nitrogen is secreted exclusively as ammonium, which is then assimilated into amino acids in the plant cytosol (reviewed by Udvardi and Day 1997; Kaminski *et al.* 1998; Kahn *et al.* 1985; Streeter 1991). However, more recent results challenge this view and suggest a possible involvement of amino acids as the form of fixed nitrogen delivered from the bacteroid to the plant (reviewed by Poole and Allaway 2000; Day *et al.* 2001). At present, consensus has not been reached as to whether substantial nitrogen assimilation takes place in the nodule bacteroid compartment, and in the following, many of the apparently contradictory results will be presented and discussed.

Early studies with anaerobically prepared nodule bacteroids showed that fixation of ${}^{15}N_2$ occurred in the bacteroids and that ${}^{15}NH_4$ ^{*} was the main product excreted into the medium (Bergersen and Turner 1967). Ammonium accounted for 94-95% of the total fixed ${}^{15}N$, which led to the conclusion that no significant nitrogen assimilation took place in the bacteroids. In accordance with this, many laboratories have subsequently found that the appropriate ammonium assimilatory enzymes were repressed in symbiotic bacteroids (Brown and Dilworth 1975; Kurtz *et al.* 1975; Werner *et al.* 1980).

In whole nodules, the primary assimilatory products were shown to be glutamate and glutamine (Kennedy 1966), and subsequent studies examined the localisation of assimilatory enzyme activity in nodules. The concerted action of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT), catalyse the incorporation of NH_4^+ into glutamine and glutamate by the combined reactions shown in Fig. 12. This pathway is known to be the main route of ammonium assimilation in free-living rhizobia, when the external ammonium concentration is low (Nagatani *et al.* 1971; Brown and Dilworth 1975; Kondorosi *et al.* 1977). Glutamate dehydrogenase (GDH) catalyses the incorporation of NH_4^+ into glutamate (Fig. 12) at high ammonium concentrations (Nagatani *et al.* 1971; Brown and Dilworth 1975).

It is unclear whether GDH plays a significant role in bacteroid nitrogen assimilation. Substantial bacteroid GDH activity was reported by Brown and Dilworth (1975) to be present in bacteroids from many different host plants, whereas Miller *et al.* (1991) demonstrated high GDH activity levels only in *R. meliloti* bacteroids compared to other species. Kurz *et al.* (1975) found low GDH activity levels in *R. leguminosarum* bacteroids throughout the entire 13-weeks growth period of the plant.

GOGAT activities in isolated bacteroids were demonstrated to be very low and insufficient to account for the level of nitrogen fixation and assimilation observed for the intact nodule (Kurz *et al.* 1975; Brown and Dilworth 1975). Some GS activity was present in the bacteroids, but its activity was not correlated to nitrogenase activity during plant development, and the low level of GOGAT precluded the operation of the GS-GOGAT cycle (Kurz *et al.* 1975). Additional evidence in support of the view that the bacteroid GS-GOGAT pathway is not important in the assimilation of fixed nitrogen in the nodule is provided by Kondorosi *et al.* (1977). In this study it was showed that a *Rhizobium* GOGAT mutant, which was unable to assimilate ammonium and grow in the free-living state without supply of glutamate, was still fully effective in symbiosis.

The role of bacteroid GS during symbiosis is not well understood, but it seems that a low level of GS activity could be required for symbiosis. This is suggested by several observations that rhizobia mutants lacking GS are severely impaired in nodule infection and/or nitrogen fixation (Donald and Ludwig 1984; Moreno et al. 1991). Rhizobia and bradyrhizobia possess at least two different GSs, GS I and II, which are encoded by non-homologous genes (reviewed by Kaminski et al. 1998). The regulation of the gene encoding GS I is unknown apart from the fact that it is only slightly affected by nitrogen. The expression of GS II is under *ntr* control and thus turned off during bacteroid differentiation (see chapter 1.2) (Kaminski et al. 1998).

Data from several *in vitro* studies with bacteroids and symbiosomes have suggested that some amino acid synthesis does occur in the bacteroid cytoplasm under microaerobic conditions. Pea and soybean bacteroids supplied *in vitro* with dicarboxylic acids were found to excrete alanine and aspartate (Appels and Haaker 1991; Kouchi *et al.* 1991), and symbiosomes and bacteroids fed with ¹⁴C-malate or -succinate synthesised, and in some cases excreted, ¹⁴C labelled amino acids, primarily alanine, aspartate and glutamate (Salminen and Streeter 1987; Kouchi *et al.* 1991; Miller *et al.* 1991; Rosendahl *et al.* 1992).

It has been confirmed that bacteroids also synthesise amino acids in the intact symbiosis. Substantial amounts of ¹⁴C-glutamate, ¹⁴C-aspartate and ¹⁴C-alanine were found in bacteroids after exposure of whole pea and soybean nodules to ¹⁴CO₂ (Rosendahl *et al.* 1990; Salminen and Streeter 1992).

Salminen and Streeter (1987 and 1990) found substantial and rapid synthesis of ¹⁴C-glutamate in soybean bacteroids after supply with ¹⁴C-dicarboxylic acids and this was attributed to channelling of 2-oxoglutarate to glutamate because of inhibition of 2-oxoglutarate dehydrogenase of the TCA

cycle (cf. Fig. 7) due to an increased NADH/NAD^{*} ratio under microaerobic conditions. The labelling of the large glutamate pool turned over slowly, suggesting a low rate of catabolism (Salminen and Streeter 1990). Assays of the enzyme glutamate α -decarboxylase (GAD) that catalyses the conversion of glutamate to γ -aminobutyric acid (GABA) (cf. Fig. 12) indicated that the enzyme was absent from the six tested strains of *B. japonicum*, and consistent with this no ¹⁴C labelling could be detected in the GABA pool (Salminen and Streeter 1990).

GAD activity has, however, later been demonstrated to be present in isolated *R. meliloti* bacteroids, and so has all other enzymes of the so-called GABA shunt (see chapter 2.5 and Fig. 13) (Fitzmaurice and O'Gara 1991; Miller *et al.* 1991). *R. meliloti* mutants defective in GAD have been reported to posses low nitrogenase activity, and alfalfa plants inoculated with the mutant were reduced in shoot dry weight (Fitzmaurice and O'Gara 1988). Whether GABA plays a role in bacteroid nitrogen metabolism is, however, still a matter of debate, and it may turn out to be strain specific. Miller *et al.* (1991) examined isolated bacteroids from different host plants and found very low levels of GABA in bacteroids from pea, bean, soybean and lupine nodules, whereas *R. meliloti* bacteroids from alfalfa contained substantial amounts of GABA. The GABA accumulation was primarily observed when bacteroids were isolated anacrobically, and when bacteria were incubated in the presence of 4% oxygen GABA concentrations declined, but remained much higher than in all other analysed microsymbionts. GABA was not exported from isolated bacteroids under any incubation conditions.

Recently there has been some evidence that substantial alanine synthesis takes place inside the bacteroids and that alanine may even be the form of fixed nitrogen exported by the bacteroid to the plant cell (Waters et al. 1998; Allaway et al. 2000) (see Fig. 10). As mentioned above, it has previously been observed that soybean and pea bacteroids and symbiosomes excreted alanine (Salminen and Strecter 1987; Appels and Haaker 1991; Rosendahl et al. 1992), but in the study by Waters et al. (1998) alanine was found to be the only excreted com-



Fig. 10: A general scheme for the possible assimilation processes taking place in the bacteroid and transfer of symbiotically fixed nitrogen through the bacteroid membrane (BM), peribacteroid space (PBS) and symbiosome membrane (SM). Enzymes involved are within boxes: MDH, malate dehydrogenase; ME, malic enzyme; N₂ase, nitrogenase; ADH, alanine dehydrogenase; GDH glutamate dehydrogenase; AAT, aspartate dehydrogenase. Circles represent identified membrane transport mechanisms, whereas dotted lines indicate transport by passive diffusion (Modified from Day *et al.* 2001).

pound that increased reproducibly and linearly with time from purified soybean bacteroids incubated under nitrogen-fixing conditions. ${}^{15}N_2$ labelling experiments demonstrated that the recently formed ammonium from nitrogenase was incorporated into alanine, which was then transported out of the bacteroid. When incubated with ${}^{15}N_2$ the excreted alanine was 98 atom% excess ${}^{15}N$. A further unusual feature of this work was that no significant ammonium excretion could be observed and ${}^{15}N$ -ammonium labelling was typically less than 0.06 atom% excess.

The excreted alanine was found to be formed via the reaction of ammonium with pyruvate, catalysed by NADH-dependent alanine dehydrogenase (ADH) (Waters *et al.* 1998; Emerich *et al.* 2000; Allaway *et al.* 2000) (cf. Fig. 10). *B. japonicum* ADH has previously been found to be an extremely powerful ammonium assimilatory enzyme unlike most ADHs, which have catabolic roles (reviewed by Emerich *et al.* 2000). Waters *et al.* (1998) proposed that N₂ reduction by nitrogenase and ammonium assimilation by ADH were so tightly coupled that the newly formed ammonium was never released into the cell ammonium pool. In agreement with the catalytic mechanism, the amount of ¹⁵N in ammonium within the bacteroid under conditions in which alanine excretion occurred was at the limit of detection suggesting that little free ammonium existed within the microsymbiont. When exogenous ammonium was added to bacteroids under N₂-fixing conditions, alanine excretion increased implying that the formation of ammonium from nitrogenase was limiting rather than the synthesis of alanine.

Waters et al. (1998) suggested that the reason for the lack of consistency with earlier investigations, where bacteroids have been found to excrete predominantly ammonium, is that most bacteroid preparations have been contaminated with plant enzymes capable of liberating ammonium by degrading excreted alanine and other excreted compounds. Furthermore, the choice of oxygen tension during bacteroid incubation in different studies was suggested as another reason why alanine excretion has not been observed previously, as the oxygen tension was shown to strongly influence the amount of excretion of alanine (Waters et al. 1998).

An impressive, joint biochemical and genetic study by Allaway *et al.* (2000) pursued the controversial results regarding bacteroid alanine synthesis. It was confirmed that alanine was also synthesised at high rates in pea bacteroids, but only when bacteroids were incubated at high densities under conditions that allowed accumulation of ammonium in the external medium. When bacteroids were incubated under low to moderate densities, only ammonium could be detected as a secretion product. The fact that the precise partitioning of ammonium to alanine could be altered so dramatically by the *in vitro* assay conditions, may explain many of the contradictions in the earlier literature. The whole cell K_m for alanine synthesis was 3.2 mM NH₄⁺, which was similar to the K_m of ADH (5.1 mM) (Allaway *et al.* 2000).

The importance of alanine synthesis in planta was examined by identifying and mutating the rhizobial gene for alanine dehydrogenase (aldA) and subsequently inoculating plants with the mutant. The mutant bacteroids fixed nitrogen and secreted ammonium at normal rates but did not synthesise alanine, and host plants were 20% reduced in biomass compared with those inoculated

with the wild type (Allaway et al. 2000). Overall these data suggest that in planta alanine synthesis by AldA has a significant effect on the efficiency of nitrogen export, but cannot be the sole nitrogen secretion product from bacteroids.

2.5 Nitrogen assimilation in the plant

The assimilation of ammonium in the plant cytoplasm is, as opposed to assimilation processes taking place in the bacteroid, considered to be fairly well understood. The activity of nitrogen assimilatory enzymes is strongly enhanced in nodule tissues as compared to roots, and induction of nodule-specific genes encoding isoforms of nitrogen assimilating enzymes occurs (reviewed by Atkins 1991; Cullimore end Bennett 1992; Vance 2000). Several time course studies have shown that the rise in nodule nitrogenase activity and leghemoglobin concentration at the onset of nitrogen fixation is paralleled by a dramatic increase in the specific activities of nitrogen assimilatory enzymes in the plant cytoplasm fraction. Especially the activities of GS and GOGAT have been demonstrated to increase, but also aspartate aminotransferase (AAT) and asparagine synthetase (AS) (see Fig. 12), (reviewed by Boland *et al.* 1980; Streeter 1991).

The first step in the assimilation of ammonium into organic compounds occurs via the so-called GS-GOGAT cycle (Figs. 11 and 12) (reviewed by Robertson and Farnden 1980). Ammonium is

incorporated into the amide group of glutamine in an ATPdependent reaction catalysed by GS. The enzyme GOGAT subsequently catalyses the formation of two molecules of glutamate from a molecule of glutamine and a molecule of 2oxoglutarate in an NADH dependent process. Glutamate serves as the central nitrogen metabolite in the plant nodule cells for the synthesis of the other amino acids, nucleic acids and other nitrogen containing compounds.



Fig. 11: A general scheme for the assimilation of symbiotically fixed nitrogen into amino acids in the plant cytoplasm of indeterminate legume root nodules. The enzymes involved are within boxes: GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; AS, asparagine synthetase (Modified from Vance 2000).

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Glutamine synthetase (GS):<br/>glutamate + NH4* + ATP \rightarrow glutamine + ADP + PiGlutamate synthase (previously glutamine: 2-oxoglutarate aminotransferase) (GOGAT):<br/>2-oxoglutarate + glutamine + NADH \rightarrow 2 glutamate + NAD*Net GS-GOGAT cycle:<br/>2-oxoglutarate + NH4* + ATP + NADH \rightarrow glutamate + ADP + Pi + NAD*Glutamate dehydrogenase (GDH):<br/>2-oxoglutarate + NH4* + NAD(P)H + H* \leftrightarrow glutamate + NAD(P)* + H2OAspartate amino transferase (AAT):<br/>oxaloacetate + glutamate \leftrightarrow aspartate + 2-oxoglutarateAsparagine synthetase (AS):<br/>aspartate + glutamine + ATP \rightarrow asparagine + glutamate + AMP + PP;Glutamate c-decarboxylase (GAD):<br/>glutamate + H* \rightarrow y-aminobutyric acid (GABA) + CO2
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Fig. 12: Enzyme catalysed amino acid synthesis and catabolism (Modified from Boland et al. 1980, Temple et al. 1998; Salminen and Streeter 1990).

GS is located predominantly in the cytoplasm of infected cells, whereas GOGAT occurs in the plastids and possibly also to some extent in the cytoplasm (Shelp and Atkins 1984; Vance 2000) (cf. Fig. 11). It has been shown that a nodule-specific form of GS is expressed only in infected cells (Forde *et al.* 1989). GS occurs as numerous isozymes (reviewed by Vance 2000) and is present in very large amounts in the host cytoplasm of nodules. It has been estimated that, in soybean nodules, the enzyme comprises 2% of the soluble protein in the cytosol (McParland *et al.* 1976). The apparent K_m values for ammonium of host GSs are in the order of 0.2 mM (McCormack *et al.* 1982; Cullimore *et al.* 1983), and the cytosol thus constitutes a massive sink for free ammonium coming from bacteroids. Streeter (1989) estimated that the ammonium concentration in the soybean nodule cytosol was essentially nil, which seems reasonable in view of the presence of large amounts of GS with a low K_m for ammonium.

Prior to 1970 it was generally assumed that ammonium was assimilated in the plant cytoplasm by the direct amination of 2-oxoglutarate to produce glutamate in a single reaction catalysed by GDH (cf. Fig. 12). However, the plant enzyme's high K_m for ammonium (> 1 mM) suggested that GDH did not have a major role in nitrogen assimilation, and this was confirmed by a large body of evidence pointing at the GS-GOGAT cycle as the primary nitrogen assimilating pathway (reviewed by Atkins 1991; Temple *et al.* 1998).

After the primary assimilation of ammonium into glutamine and glutamate, nitrogen is subsequently rapidly incorporated into aspartate and asparagine. Aspartate is formed directly by the amination of oxaloacetate with glutamate as the amino group donor in a reversible reaction catalysed by AAT (Figs. 11 and 12). Nodules contain two AAT isozymes of which AAT-1 is expressed also in other parts of the plant, whereas AAT-2 is highly expressed in root nodules with low expression in other tissues (reviewed by Cullimore and Bennett 1992; Vance 2000). In situ hybridisation and immunogold localisation studies have demonstrated that AAT-2 is fairly limited to plastids of infected cells in nodules, while AAT-1 transcripts were expressed in uninfected cells, nodule vascular bundles and nodule parenchyma (Vance et al. 1994; Yoshioka et al. 1999). This indicates that only AAT-2 plays a principal role in assimilation of recently fixed nitrogen.

Asparagine is formed through amidation of aspartate with mainly glutamine as the amide group donor (Figs. 11 and 12) (Ta et al. 1986; Snapp and Vance 1986). This reaction is ATP-consuming and catalysed by AS, which is localised in the nodule plant cytosol (Cullimore and Bennett 1992; Vance 2000). Synthesis of asparagine also occurs by direct incorporation of free ammonium into aspartate, and this reaction has been estimated to account for 35% of the asparagine biosynthesis in alfalfa nodules (Ta et al. 1986). In *E. coli* both the glutamine and the ammonium dependent AS have been characterised, but in plants the production of the AS that catalyses synthesis of asparagine from ammonium and aspartate has not been established (reviewed by Vance 2000).

Asparagine, in particular, and to some extent also glutamine are the most important nitrogen transport forms in indeterminate nodules (cf. chapter 1.3) and are thus the end products of nitrogen assimilation in this type of nodules. In determinate nodules, on the other hand, purines are synthesised in the infected cells from glutamine and glycine and exported to neighbouring uninfected cells, where ureides are synthesised in a process that involves oxidative degradation of purines (reviewed by Werner 1992). The ureides, allantoin and allantoic acid, are the prevalent form in which nitrogen is exported to the xylem in determinate nodules (cf. chapter 1.3).

Root nodule nitrogen metabolism also includes the synthesis (see Figs. 12 and 13) and accumulation of γ -aminobutyric acid (GABA), which is present in relatively high concentrations in nodules of all legumes tested (Larher *et al.* 1983; Ta *et al.* 1986; reviewed by Vance and Heichel 1991). The concentration of GABA in the plant cytosolic fraction of alfalfa nodules was found to be 50times higher than in the bacteroids (Ta *et al.* 1986). GABA is not usually incorporated into proteins and the role of GABA in plants is still unclear.

A number of functions for GABA in nodules has been suggested; among these a function in cytoplasmic pH regulation during microaerobic and aerobic conditions (Reid *et al.* 1985). The enzyme GAD that catalyses the H⁺-consuming α -decarboxylation of glutamate leading to GABA synthesis (cf. Fig. 12) has a sharp pH optimum around 5.8, and its activity will thus be stimulated if pH declines from the normal physiological level (reviewed by Bown and Shelp 1997). This has been confirmed by *in vivo* NMR studies of carrot cells, where it was demonstrated that an increase

in cytosolic pH preceded GABA accumulation (Carroll et al. 1994). GAD activity increased with reduced pH and declined as pH recovered.

It has also been suggested that synthesis of GABA in nodules provides an alternative route for succinate biosynthesis if/when 2-oxoglutarate dehydrogenase of the TCA cycle is inhibited under the oxygen-limiting conditions inside the nodule (Kahn et al. 1985; McDermott et al. 1989). This so-called GABA shunt is illustrated in Fig. 13. An accompanying feature of the GABA shunt is the concomitant of alanine production through transamination of pyruvate by the action of GABA transaminase.



Fig. 13: The GABA shunt constitutes an alternative route for succinate biosynthesis from 2-oxoglutarate. Krebs cycle = TCA cycle. Enzymes are indicated in italics: GDH, glutamate dehydrogenase; GAD, glutamate α -decarboxylase; GABA-T, GABA transaminase; SSADH, succinic semialdehyde dehydrogenase (Modified from Bown and Shelp 1997).

A third suggestion for a role for GABA in plants is the hypothesis that GABA is a temporary N store (reviewed by Shelp *et al.* 1999). It has been shown that elevated glutamate levels, caused by for instance inhibition of glutamine synthesis or reduced protein synthesis, stimulate GABA synthesis. Some experimental evidence indicates that the locations of GABA production and accumulation are not identical, and that accumulated GABA is sequestered within organelles (reviewed by Shelp *et al.* 1999).

Recent results suggest that alanine may be the form in which at least a part of the nitrogen is delivered to the plant (cf. chapter 2.4) (Waters *et al.* 1998; Allaway *et al.* 2000), but the metabolic fate of alanine after it leaves the bacteroid is not known. The current understanding of nitrogen assimilation in the plant cytoplasm, as it has been described in this chapter, does not yet provide any explanation how alanine can be a starting point in the plant assimilation process. A model by Waters and Emerich (2000) that attempts to integrate alanine in the well-known nitrogen assimilating pathways will be described in chapter 2.7.

2.6 Amino acid transport across bacteroid and symbiosome membranes

Several transport studies with isolated bacteroids from many different host plants confirm that bacteroids are capable of accumulating certain amino acids from the external medium quite rapidly (Glenn and Dilworth 1981; Udvardi *et al.* 1988b; Herrada *et al.* 1989; Salminen and Streeter 1987, 1990; Jin *et al.* 1990; Whitehead *et al.* 1998). An indication that amino acids may be transported across the BM *in planta* comes from the observation that some rhizobial amino acid auxotrophs are able to establish nitrogen-fixing symbioses, which suggests that the necessary amino acids are supplied to bacteroids by their host plants (reviewed by Udvardi and Day 1997).

A general amino acid permease (Aap) that transports a wide range of L-amino acids including glutamate, aspartate, proline, histidine and aliphatic amino acids with very high affinity (K_m for glutamate 81 nM) has been described in free-living *R. leguminosarum* (Poole *et al.* 1985; Walshaw *et al.* 1997a and b). This permease system has been cloned and was shown to consist of four proteins forming an ATP-binding casette (ABC) transporter (Walshaw and Poole 1996). It has been suggested that Aap is either bidirectional or regulates another efflux channel, because amino acids can be taken up as well as excreted by free-living *R. leguminosarum* bacteria (Walshaw and Poole 1996).

A second ABC transport system, the so-called branched-chain amino acid transporter (Bra) has also been cloned in *R. leguminosarum*, and double mutants in *aap* and *bra* are devoid of almost all detectable high-affinity amino acid uptake (reviewed by Day *et al.* 2001). In addition to these dedicated amino acid transporters, it has been demonstrated that aspartate can be transported by the Dct system (see chapter 2.1), albeit with low affinity (Reid *et al.* 1996; Watson *et al.* 1993). However, under most growth conditions the Aap and Bra appear to dominate high-affinity amino acid uptake in free-living bacteria and both are capable of exchanging amino acids.

The precise mechanisms by which amino acids are transported across BM in symbiosis is not known, but exchange of intracellular amino acids from bacteroids is likely to occur via the general amino acid transport systems, Aap and Bra (Day *et al.* 2001). The results by Waters *et al.* (1998) (see chapter 2.4) demonstrate that alanine is exported from the bacteroid and thus that some carrier mediated transport mechanism exists, and it is possible that specific export systems for amino acids such as alanine may bee induced in symbiosis (Day *et al.* 2001).

The SM is, as opposed to the BM, considered to be rather impermeable to amino acids. Transport studies with isolated symbiosomes from a number of different legumes have, in the majority of cases, failed to identify amino acid transporters on the SM (Udvardi *et al.* 1988b; Herrada *et al.* 1989; Udvardi *et al.* 1990; Ou Yang and Day 1992; Whitehead *et al.* 1998). However, a notable exception is provided by Rudbeck *et al.* (1999), who demonstrated that aspartate could be transported from the bacteroid to the plant side of the SM (cf. Fig. 10) by the operation of a proton-aspartate symporter on energised pea SM membrane vesicles. Furthermore, it has been suggested that substantial amino acid concentration gradients may exist *in vivo*, and that amino acids may

diffuse down the gradients across the SM, perhaps via the well-known aquaporin, Nodulin 26 (Udvardi and Day 1997; Day et al. 2001; Rivers et al. 1997).

Despite the general view that the SM is highly impermeable to amino acids, there has been several observations of isolated symbiosomes secreting amino acids like alanine and aspartate at substantial rates (cf. chapter 2.4) (Udvardi *et al.* 1990; Rosendahl *et al.* 1992). However, further work is needed before firm conclusions can be drawn about *in vivo* amino acid transport out of symbiosomes and transport mechanisms remain to be identified.

Given the apparent lack of transporters on the SM for amino acids, the presence of high-affinity amino acid transporters on the BM is surprising and needs an explanation. In free-living *B. japonicum* glutamate transport is induced when external substrate concentrations are very low and repressed when glutamate is abundant (Whitehead *et al.* 1995). The persistence of amino acid transporters in bacteroids may, therefore, simply reflect the restricted availability of substrates from the plant. In this situation transporters may assist bacteroids in taking up amino acids from a very low concentration pool in the PBS. Slow movement of amino acids across the SM followed by rapid uptake into the bacteroid via high-affinity transporters, may allow plant amino acids to supplement fixed nitrogen in the bacteroid for biosynthetic purposes.

2.7 Nutrient exchange cycles

Several models that integrate carbon and nitrogen exchange via shuttles and cycles involving uptake and efflux of amino acids across the SM and BM have been proposed (Kahn et al. 1985; Kouchi et al. 1991; Waters and Emerich 2000; Rosendahl et al. 2001). All of the models are hypothetical and based on a mixture of solid experimental data and more or less speculative assumptions, and the suggestions may thus inspire further investigation. I have chosen to present a selection of the models in order to illustrate the many possible extensions and alternatives to the more mainstream metabolic pathways described in the previous chapters 2.4, 2.5 and 2.6.

Kahn et al. (1985) were the first to propose a simple nutrient exchange model in which nitrogenase-generated ammonium was assimilated into a carbon skeleton in the plant cytoplasm and subsequently carried across the BM to serve as a reduced carbon source (see Fig. 14). In that way, supply of reduced carbon for bacteroid metabolism would require bacteroid nitrogen fixation, and thus urge the bacteroid to fix nitrogen continuously. Glutamate and aspartate were suggested



Fig. 14: Model of nutrient exchange between plant and bacteroid across the symbiosome membrane (SM) and bacteroid membrane (BM) as proposed by Kahn *et al.* (1985). (Modified from Udvardi and Kahn 1992).

as candidates to participate in membrane transversing shuttles.

A more elaborate version of the model was also presented by Kahn *et al.* (1985) (see Fig. 15) inspired by the malate-aspartate shuttle that operates in mitochondria (Meijer and VanDam 1974). It is a simple mechanism requiring only two enzymes (AAT and MDH) and involving the exchange of glutamate for aspartate and malate for 2-oxoglutarate across the membrane barrier. The net effect of the malate/aspartate shuttle would be the transport of reducing equivalents (NADH) across SM and BM without transport of nitrogen and carbon. A number of laboratories have provided evidence in favour of a malate/aspartate shuttle, but it is still not clear whether it operates in the legume-*Rhizobium* symbiotic interface (Udvardi and Kahn 1992).

Work in both R. leguminosarum (Appels and Haaker 1991; Rosendahl et al. 1992) and B. japonicum (Streeter and Salminen 1990) suggests that bacteroids would be willing players in such a scheme since aspartate and 2-oxoglutarate was released when isolated bacteroids were incubated in the presence of malate and glutamate. AAT has been proposed to catalyse the formation of oxaloacetate from aspartate in the suggested malate/aspartate shuttle, and this role of AAT has been supported by results showing that exogenously supplied ¹⁴C-aspartate to alfalfa nodules was rapidly metabolised to malate, succinate and fumarate (Snapp and Vance 1986). Rastogi and Watson (1991) found that an S. meliloti



Fig. 15: The malate-aspartate shuttle as it may exist in legume root nodules. AAT, aspartate amino transferase; MDH, malate dehydrogenase (Modified from Udvardi and Kahn 1992).

aatA mutant, which seems to be unable to generate a high level of AAT activity, forms ineffective nodules (fix) with its host plant alfalfa, and this has also been interpreted as an indication that an exchange of organic acids and amino acids might play a role in symbiotic metabolism (Kahn *et al.* 1998).

The major argument against the operation of a malate/aspartate shuttle is the impermeability of the SM to glutamate (cf. chapter 2.6) (Udvardi *et al.* 1988b). A lack of formal shuttle mechanisms is also indicated by the fact that a mutation of the *aap* genes encoding the amino acid permease

described in chapter 2.6, prevented growth on glutamate as a carbon source in free-living *R. leguminosarum*, but did not have any major effect on symbiotic nitrogen fixation (Walshaw *et al.* 1997a).

Kouchi et al. (1991) also suggested involvement of glutamate as a possible carbon source for bacteroids in nodules and carried out studies of glutamate uptake and metabolism in isolated *B. japonicum* bacteroids and symbiosomes. The results suggested that the major pathway of glutamate utilisation in bacteroids was transamination to form aspartate, but it was additionally suggested that the GAD catalysed GABA pathway (cf. Fig. 13) was partly responsible for the catabolism of glutamate. The utilisation of glutamate as a carbon source was severely inhibited in isolated symbiosomes as compared to isolated bacteroids, but at rather high, though still physiologically relevant, glutamate concentrations, considerable glutamate utilisation was observed, indicating that the SM may not work as a barrier *in vivo* (Kouchi et al. 1991).

Waters and Emerich (2000) have proposed an alanine-pyruvate nutrient exchange cycle (see Fig. 16) that takes into account the recent findings of alanine as the primary nitrogen transport compound from bacteroids (cf. chapter 2.4). The overall net metabolite transfer of the cycle is the movement of one molecule of ammonium from the bacteroid to the plant in exchange for one molecule of CO₂ from the plant to the bacteroid.



Fig. 16: Proposed alanine-pyruvate exchange cycle. The broken vertical lines represent the symbiosome and bacteroid membranes, SM and BM, respectively. The open arrows represent the transport of malate and alanine across the membranes. Abbreviated metabolites are: 2-OG: 2-oxoglutarate, PEP: phosphoenol pyruvate, OAA: oxaloacetate. (Modified from Waters and Emerich 2000).

In this model malate is transported into the bacteroid (cf. chapter 2.1), decarboxylated into pyruvate and synthesised into alanine. Alanine is assumed to be transported out of the symbiosome, into the cytosol of infected plant cells, where ammonium is transferred to another carrier such as 2oxoglutarate to form glutamate, which can then be used to synthesise other nitrogen containing compounds. The pyruvate, which is generated from alanine transamination, presents a problem to the model, because pyruvate is not readily oxidised by plant mitochondria. However, if puruvate could be converted into phosphoenol pyruvate (PEP), it could subsequently be recycled back to malate via the enzymes PEP carboxylase and MDH (cf. chapter 2.1) in the infected plant cells. The enzymes for conversion of pyruvate to PEP or to oxaloacetate (OAA) are known in plants but remain to be identified in root nodules, and the solidity of the model is therefore not known at the moment. The transport of alanine across the SM is also a weak point of the model, but it is suggested that alanine alternatively could be deaminated within the PBS to liberate ammonium, which could be transported out of the symbiosome by ammonium transporters on the SM (see chapter 2.3).

Rosendahl *et al.* (2001) proposed the operation of transamination cycles including processes taking place in the symbiotic interface between the bacteroid and the plant as well as in the bacteroid cytoplasm (see Fig. 17). It was hypothesised that these processes may constitute a component of the transfer of nitrogen between the symbionts and possibly also a mechanism whereby the bacteroid TCA cycle may be regulated during symbiotic nitrogen fixation. This model encompasses previous findings that aspartate under certain circumstances is the amino group donor

for synthesis of glutamate from 2-oxoglutarate via AAT activity in bacteroids (Reid et al. 1996) and that glutamate may enter the PBS via a transporter present on the BM (Walshaw et al. 1997). In addition the model includes the recently identified aspartate transporter on the SM (see chapter 2.6) (Rudbeck et al. 1999), observations of high AAT activity in the PBS (Rosendahl et al. 2001) and preliminary results indicating the presence of AAT in the PBS (Simonsen 1998).



Fig. 17: Proposed model for operation of a transamination cycle (Modified from Simonsen 1998). AAT, aspartate aminotransferase; Asp, aspartate; 2-OX, 2-oxoglutarate; Glu, glutamate; OAA, oxaloacetate; Dc, dicarboxylic acid. 1, amino acid permease; 2 and 3, dicarboxylic acid transporters; 4, aspartate transporter.

3 In vivo NMR spectroscopy

3.1 Fundamental principles of nuclear magnetic resonance (NMR)

A few important principles of NMR will be summarised in the following to provide a suitable background for the later presentation and discussion of *in vivo* NMR experiments on root nodules. A detailed introduction to NMR as well as its application to plant-microbe symbioses and plant systems in general can be found in various textbooks and recent reviews on which the present chapter is also based (Sanders and Hunter 1993; Pfeffer and Shachar-Hill 1996; Bligny and Douce 2001; Köckenberger 2001; Pfeffer *et al.* 2001; Ratcliffe and Shachar-Hill 2001).

A number of nuclei, among these the biologically relevant ¹H, ¹³C, ¹⁵N, ¹⁷O, ¹⁹F and ³¹P, possess a magnetic moment and angular momentum (nuclear spin). The magnetic dipole axes of the nuclei are usually randomly ordered. However, when exposed to a magnetic field (B_0), this field interacts with the magnetic moments of the nuclei (see Fig. 18). A new thermal equilibrium is achieved in which the population of nuclei with the magnetic dipole axes aligned parallel with the external field is slightly bigger than the population of the nuclei with anti-parallel aligned axes. These two spin states have different energies and are separated by an amount, ΔE , which is field dependent:

$$\Delta \mathbf{E} = h \gamma \mathbf{B}_0 / 2\pi \qquad (\mathbf{E} \mathbf{q}, 2)$$

where y is the magnetogyric ratio of the nucleus (see Table 2).

The ratio of the populations of the lower and the higher energy state (N_{α} and N_{β} , respectively) is given by the Boltzmann distribution:

$$N_{\alpha}/N_{\beta} = \exp(-\Delta E/kT)$$
 (Eq. 3)

A weak net magnetisation of the sample, which can be represented by a vector M_0 , results from this unequal population distribution. The sample magnetisation can be measured through the induction of a voltage signal in a coil surrounding the sample after its manipulation by an appropriate radio-frequency (r.f.) pulse.



Fig. 18: Schematic representation of the nuclear resonance phenomenon. The sample magnetisation M_0 arises from the uneven distribution of the nuclear spin ensemble between two different states with their axes either aligned parallel or anti-parallel to the axis of the main magnetic field B_0 . The magnetic dipoles precess (here indicated as vectors) around the main field axis with the Larmor frequency ω . After the application of a $\pi/2$ radio frequency pulse, the original distribution is shifted away from the thermodynamic equilibrium, and a phase coherence is established between the precessing dipoles. The result is a sample magnetisation ML, precessing in the transverse plane with a frequency ω , and the detection of this precession forms the basis of an NMR signal. The spin ensemble returns to the original distribution between the two states through T_1 relaxation. The loss of phase coherence is described by T_2 relaxation. Both processes occur simultaneously but are depicted separately in the diagram for clarity (From Köckenberger 2001).
One of the most fundamental principles underlying NMR is the proportionality between the resonance frequency (the Larmor frequency, ω) and the magnetic field that acts on the nuclei:

$$\omega = \gamma B_0 \qquad (Eq. 4)$$

The identification of the chemical nature of a compound by its resonance lines is based on this principle. Each nucleus within a molecule experiences a slightly different magnetic field because the main magnetic field is shielded by surrounding electron clouds. Thus, the effective magnetic field acting on a given nucleus depends on the chemical nature of the group and the chemical environment in which it is bound. Because of differences in shielding each nucleus will absorb energy at a slightly different frequency. For instance, the nitrogen nuclei in the amide and amino groups, respectively, of glutamine molecules can be identified by their different resonance frequencies due to differences in the local shielding of the magnetic field. The separation of the resonance frequencies from an arbitrarily chosen reference is called the chemical shift (δ) , which is calculated on the basis on the relationship:

$$\delta = 10^{6} (v_{i} - v_{r}) / v_{r}$$
 (Eq. 5)

where v_i and v_s are the resonance frequencies of the compound of interest and the reference compound, respectively. δ , which is independent of the field strength of the magnet, is a unitless number that is expressed in parts per million (ppm). The result of an NMR analysis is a spectrum, that is, a plot of intensity against energy in which each signal occurs at a characteristic chemical shift.

Nucleus	Magnetogyric ratio, y	Natural abundance
	(10' rad T' s')	(%)
'H	26.752	99.98
ъс	6.728	1.11
¹⁵ N	-2.712	0.365
¹⁹ F	25.181	100
3 ¹ P	10.839	100

 Table 2: Properties of some biologically relevant nuclei with spin 1/2 (Modified from Berger et al. 1997).

3.2 T₁ and T₂ relaxation

After the thermal equilibrium is perturbed by the initial r.f. pulse, the nuclei start to return to the initial state by relaxation (cf. Fig. 18). The initial population distribution between the different states can be restored only by an exchange of energy between the exited nuclei and their environment. This is a first order process with a time constant T_1 for an identical ensemble of nuclei. In the classical mechanical treatment, T_1 relaxation (also known as longitudinal or spin-

lattice relaxation) represents the recovery of the bulk magnetisation vector in the direction of the main magnetic field.

There are continuous fluctuations of the local magnetic fields due to rotational and translational molecular motion, which causes a decay of the average sample magnetisation vector in the transverse plane by a first order process with a time constant T_2 . T_2 relaxation is also known as transverse or spin-spin relaxation.

The mechanistic explanations of both longitudinal and transverse relaxation rates include a variety of physical processes, such as inter- and intramolecular dipole-dipole interactions between two nuclei.

The experimental or apparent T_2 time constant is called T_2^* and may be shorter (but never longer) than the true T_2 because of local magnetic field inhomogeneity. The inhomogeneity of the magnetic field in plants stems mainly from the presence of small air-filled intercellular spaces (reviewed by Köckenberger 2001). Air and water have different magnetic susceptibility when exposed to a magnetic field, which can result in strong internal magnetic field gradients at the interface between water and air. This will shorten the experimental transverse relaxation time constant T_2^* and this effect becomes more pronounced with increased magnetic field strength. Shortening of T_2^* can also be caused by such diverse factors as interactions with paramagnetic ions and molecules, reduced molecular motility and exchange effects.

A shortened T_2^* will lead to line broadening of signals in an NMR spectrum. The resonance line width, $\Delta v_{1/2}$ (the full width at half the height of the peak), and T_2^* are related by:

$$\Delta v_{1/2} = (\pi T_2^*)^{-1}$$
 (Eq. 6)

In general the broader the signal, the more difficult it is to define its shape and extent and the greater the chance of overlap with neighbouring signals. However, identifying the cause of the line broadening leads to information about the intracellular environment of the ion or the molecule that gives the NMR signal. In extreme cases line broadening can render a signal undetectable and this too may be informative. Nuclei that are present in a sample without being detected in a simple NMR experiment, are said to be NMR-invisible. This often occurs for large molecules e.g. high molecular weight polyphosphate or for small molecules that bind to large molecules such as ADP binding to proteins. Interaction with paramagnetic compounds can also greatly reduce T_2^* and lead to NMR-invisibility. Different compartments in a cell may have different relaxation properties.

3.3 Signal-to-noise ratio

The signal in an NMR experiment is a small electromotive force that is induced in the detection coil by the precession of the sample magnetisation in the transverse plane. The signal is modulated

down in frequency, digitised and sent to a computer where it is converted into a spectrum by a mathematical procedure called a Fourier transformation. However, from the coil to digitisation there are a number of sources for experimental noise that impair the detection sensitivity (Hoult and Richards 1976). The signal-to-noise (S/N) ratio is approximately proportional to the square of the applied magnetic field ($B_0^{7/4}$) and to the number of nuclei within the sample.

The natural abundance of a magnetic isotope of interest (cf. Table 2) is one of the crucial factors to determine whether the number of nuclei within a biological sample is large enough to exceed the detection threshold. ³¹P is 100% naturally abundant so therefore it is favourable to observe ³¹P metabolites by NMR. ¹⁵N is, however, only present at 0.365% of a natural population and furthermore has a low magnetogyric ratio (cf. Table 2) which leads to rather low sensitivity of ¹⁵N NMR. ¹⁵N metabolites are thus not easily detected in an unenriched sample. However, this can be exploited to advantage because the distribution of label from a given ¹⁵N labelled substrate, which is administered to the biological system under study, can be followed through a metabolic pathway by *in vivo* ¹⁵N NMR.

A common way to increase the S/N ratio is signal averaging by the addition of many single scans or transients. In a spectrum resulting from the summation of *n* transients, the signals will be *n* times bigger that they are in a single spectrum. The noise will, however, increase by only \sqrt{n} and so the ratio of S/N amplitude will increase by \sqrt{n} . Increasing the S/N ratio by signal averaging therefore rapidly leads to very long experimental times.

Application of the nuclear Overhauser effect (NOE) is a means to enhance signal intensities. NOE is a change in the intensity of a spectroscopic signal caused by the continuous saturation of a transition of another nearby nucleus during the NMR experiment. The nuclei interact through space and need not be covalently bound. The saturation of the transition of one of the nuclei will cause a new population distribution to develop as relaxation occurs. Dipole-dipole relaxation will lead to depopulation of the upper level and thereby an increased population difference, which will cause intensity enhancement of the signal from the observed nucleus. It is particularly interesting for the insensitive ¹⁵N nucleus, where the proton induced NOE may yield a large increase of the signal intensities. The NOE factor (η) is a fractional increase determined by the magnetogyric ratios of the saturated and the observed nucleus, γ_A and γ_X , respectively:

$$\eta = \gamma_A / 2 \gamma_X \qquad (Eq. 7)$$

In practice the effective NOE factor, η_{obs} , may be smaller than the theoretical value as a result of molecular or experimental factors. The NOE factor, η , gives the maximal fractional increase in a signal due to the NOE, and the resulting total intensity of the enhanced X-signal is given by:

$$I = (1 + \eta) I_0$$
 (Eq. 8)

where I_0 is the original intensity. The maximum proton-induced η for ¹⁵N is according to Eq. 7 about -4.9 as the γ -values of ¹H and ¹⁵N differ by a factor of nearly 10 (see Table 2). As the ¹⁵N magnetogyric ratio is negative the NOE factor will also be negative. This will give improved signal-to-noise only if η_{obs} is so large and negative that the net signal is inverted but more intense than in the absence of NOE. However, in the case where the dipole-dipole contribution is less than 100%, it is possible for η_{obs} to be close to -1, leading to a complete loss of signal (cf. Eq. 8).

4 Concluding remarks and objectives

Carbon and nitrogen metabolism in legume-*Rhizobium* root nodules has been studied with intermittent intensity since the 1960s. However, the complex interwoven metabolic network is still not fully understood, as described in chapter 2 and illustrated by the many different metabolic schemes presented in chapter 2.7.

The form of fixed nitrogen exported by the bacteroid to the plant cell is still a matter of debate, and the recent controversial and remarkable results by Waters *et al.* (1998) and Allaway *et al.* (2000) (cf. chapter 2.4) mandate new investigations of nodule nitrogen metabolism. The degree of partitioning to alanine and antmonium by different rhizobia species is unknown, as are the effects of different environmental conditions and AldA expression levels on alanine synthesis. Alanine may be a major secretion product of bacteroids, but its metabolic fate after it leaves the bacteroid is still unknown. Likewise, transport systems for alanine as well as other amino acids on the SM remain to be identified.

The size of the free ammonium pool in the bacteroid cytoplasm during symbiosis is an open question. Waters *et al.* (1998) suggest a that ammonium synthesis and subsequent assimilation by alanine dehydrogenase are so tightly coupled in the bacteroid cytoplasm that very little free ammonium is released. Allaway *et al.* (2000), on the other hand, observe a plastic partitioning of ammonium and alanine excretion from the bacteroids and suggest that the ammonium concentration inside the bacteroids is one of the key variables governing the rate of alanine synthesis. To my knowledge, the only published attempt to estimate the free ammonium concentration in bacteroids was made by Streeter (1989) (cf. chapter 2.2). This estimate was based on an extrapolation of time dependent experimental data to time zero as well as on quite a lot of assumptions, so there is a need for a more direct determination of the concentration of free ammonium inside the bacteroid.

The mechanism by which ammonium reaches the plant cytosol remains controversial (Udvardi and Day 1997). It is clear that in soybean and pea the SM contains channels, which have properties that one would expect of a transporter delivering ammonium to the plant (cf. chapter 2.3), but its operation during nitrogen fixation remains to be demonstrated. The gene encoding the putative SM ammonium transporter has not yet been identified, and therefore it has not been possible to prove its function by for instance blocking the expression of the gene using antisense RNA techniques,

A general problem in the study of nodule metabolism is the extrapolation from *in vitro* to *in vivo*. Information on the microenvironment of the different compartments in the nodule is lacking, and the *in vivo* significance of *in vitro* findings is therefore difficult to appreciate. Biochemical reactions in intact living cells can be studied by NMR spectroscopy ideally without interfering with metabolic processes or destroying enzyme complexes. While determinations of enzyme activities in crude extracts may give a first clue as to which ammonium assimilation pathways are active, they cannot be used to predict the *in vivo* flux distribution over competing enzyme systems such as GDH and GS/GOGAT. *In vivo* NMR spectroscopy, especially when used in combination with stable isotope labelling such as ¹⁵N, does allow the characterisation of metabolic activities in the living cell. Other methods for studying metabolic pathways rely on extractions of the tissue and subsequent purification and analysis. These procedures are time consuming and this is a significant disadvantage in time course experiments, where the aim is to follow the label through a pathway.

In vivo ¹⁵N NMR spectroscopy has previously been used successfully in the study of plant, fungal and bacterial nitrogen metabolism. Much of the work has contributed to the understanding of the roles of GDH and the GS/GOGAT cycle in ammonium assimilation in different systems including cell suspensions (Robinson *et al.* 1991; Fox *et al.* 1995; Amancio *et al.* 1997), embryogenic cultures (Joy *et al.* 1996 and 1997), root tissues (Amancio and Santos 1992), Sphagnum fallax (Kahl *et al.* 1997), ectomycorrhizal mycelium (Martin 1985) and Corynebacterium glutamicum (Tesch *et al.* 1999). The elucidation of the nitrogen assimilating pathways has usually been achieved by supplying ¹⁵N-ammonium or ¹⁵N-nitrate to the living biological system and subsequently ¹⁵N NMR analysing the incorporation of the ¹⁵N label into amino acids in the presence and absence of enzyme inhibitors.

Metabolic studies by *in vivo* NMR spectroscopy has the advantage of avoiding artefacts caused by the breakdown of labile compounds during extraction and it also eliminates errors due to incomplete recovery of solutes from the tissue. A crucial factor in studies of bacteroid metabolism and functioning *in vitro* is the degree to which bacteroid preparations are free from contaminating plant enzymes and metabolites. Waters *et al.* (1998) suggested that the presence of plant enzymes in earlier *in vitro* studies of bacteroids had caused significant artefacts in the results concerning metabolic products released from bacteroids (cf. chapter 2.4), but this was subsequently rejected by Li *et al.* (2000). Likewise, major errors might occur in the determination of metabolite levels because of reallocation or degradation of metabolites during extraction, processing of extracts or separation of compartments (Streeter 1987, 1989; Miller *et al.* 1991).

An unfavourable feature of ¹⁵N NMR spectroscopy is that it is a relatively insensitive spectroscopic method. This might have discouraged previous attempts to study nitrogen fixation, because to my knowledge, no reports on ¹⁵N NMR spectroscopy used for studying living nitrogen fixing organisms are available. The only reported ¹⁵N NMR investigation of ¹⁵N₂ fixation is by Belay *et al.* (1988) who incubated methanogenic bacteria with ¹⁵N₂ and subsequently analysed harvested,

dead cells by ¹⁵N NMR. However, the literature contains a few *in vivo* ³¹P NMR spectroscopic studies of symbiotic legume root nodules such as soybean (Mitsumuri *et al.* 1985; Rolin *et al.* 1989a & b) and alfalfa (Nikolaev *et al.* 1994). Results from these investigations demonstrate that it is possible to keep nodules metabolically active, while recording NMR spectra.

In conclusion, accurate estimation of the assimilation and translocation of fixed nitrogen in nodules would benefit from non-invasive and non-destructive measurements of the fate of the fixed nitrogen. The objective of the present study was thus to investigate nitrogen fixation and assimilation in ¹⁵N₂ fixing root nodules root by *in vivo* ¹⁵N NMR spectroscopy.

5 Introduction

The aim of my experimental work has, throughout the project period, been to obtain informative *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing root nodules. However, a lot of equipment and procedures had to be developed and a lot of problems had to be solved first. The process of developing an experimental set-up that maintained the nodules in a physiologically viable state while in the NMR tube will be described in chapter 7. The *in vivo* NMR technique requires in general that the biological system under study is immersed in aqueous medium in order to maximise magnetic field homogeneity to achieve the narrowest signal line widths. The search for the optimal conditions for maximal nitrogen fixation rates of immersed, detached nodules in the NMR tube is described in chapter 8.

When the first *in vivo* ¹⁵N NMR spectra were obtained, the next task was the interpretation of results and the identification of metabolites giving rise to the observed signals. This was by no means straightforward and included several approaches involving both NMR (described in chapter 10) as well as mass spectrometry (described in chapter 11). NMR in general and ¹⁵N NMR especially is a relatively insensitive spectroscopic method (cf. chapter 3). This is not a serious limitation, when large amounts of material are available as in studies of purified organic compounds, but it is one of the main limitations *in vivo*. In order to detect physiological levels of nitrogen metabolites, it was necessary to put an effort into optimising experimental variables influencing the ¹⁵N S/N ratio as described in chapter 10.2 and 10.5.

One of the special and useful features of *in vivo* NMR is that unexpected information, which would escape detection by other analytical methods, might be revealed (reviewed by Ratcliffe 1996). This includes the discovery of novel compounds like the unusual root nodule ³¹P metabolites described in chapter 7.2 as well as information on intracellular environment as illustrated by the unusual annonium chemical shift observed *in vivo* (described in chapter 10.3). These most unusual and unexpected results have been pursued and investigated to some extent as described in the respective chapters, but remain to be fully elucidated.

All presented ³¹P and ¹⁵N NMR spectra have been recorded on a Varian Unity Inova 600 spectrometer using a broadband 10-mm-diameter probehead unless otherwise stated.

6 Biological material

Pea (*Pisum sativum* L.) was the model plant of choice from the beginning and the majority of my work has been done with the pea variety "Solara". The variety "Bodil" was used for some experiments mentioned in chapter 8.1. Seeds were inoculated with suspension cultures of *Rhizobium leguminosarum* by. *viceae* as described in the article manuscript and different

Rhizobium strains were tested as mentioned in chapter 8.1. However, the strain Risø 18a, which was used from the beginning, remained the preferred strain and was used for all presented experiments unless otherwise stressed.

Nodules from soybean (*Glycine max* L. cv. Evans) and *Lotus japonicum* inoculated with *Bradyrhizobium japonicum* strain 110 (Hahn and Hennecke 1984) and *Mesorhizobium loti* strain R7A (Sullivan *et al.* 1995), respectively, were also used for a few experiments described in chapters 7.2 and 10.3. As mentioned in chapter 1.3, *Lotus* nodules are of the indeterminate type like pea nodules, whereas soybean nodules are determinate.

In our laboratory, plants have traditionally been grown in vermiculite-filled pots in an experimental set-up described by Rosendahl and Jakobsen (1987), and I started out by using this growth system. However, I changed to a different, so-called aeroponic growth system in the optimisation process leading to root nodules that retained the highest possible nitrogenase activity under *in vivo* NMR conditions (cf. chapter 8.1). Experiments presented in Figs. 21-22, Fig. 23 B and C, Fig. 25 (partly), Figs. 26-27, Fig. 36 (Exp. 1-6), Figs. 37-39 and Fig. 42 have been performed with nodules grown in vermiculite, and all other results are based on nodules grown in the aeroponic system.

The construction of the aeroponic system was initiated by L. Rosendahl and P. Mouritsen in our laboratory with inspiration from M. J. Harrison, Noble Foundation, Oklahoma, USA where it is used for small *Medicago truncatula* plants. I found it necessary to introduce some minor adaptations for cultivating large amounts of pea plants, and these optimisations will be presented in the following. A detailed description of the aeroponic system construction and handling is given in the article manuscript.

I experienced that exudates from roots caused substantial acidification of the nutrient solution in the aeroponic system, and I therefore included monitoring, buffering and adjusting of pH in the culturing procedures. I tested whether the pH buffer MES had any adverse effects on plant growth and found that 8 mM MES in the nutrient solution provided a reasonable pH buffering without affecting plant growth. Additional pH adjusting by base titration was sometimes necessary depending on the amount and age of plants.

Plants were extremely vulnerable to salt deposits on the stems caused by evaporation of nutrient solution though the holes in the lid of the aeroponic system, and several generations of plants died off in the beginning. I tried different ways of tightening of the lid and managed to minimise salt depositing. It was crucial to immobilise plants in an upright position to avoid tearing of the plastic film covering the lid, and I achieved this by constructing special plant supporting racks.

The acroponic system ended up being a very efficient and convenient system for producing large amounts of nodule material, and nodules were easily harvested with minimal mechanical disturbance.

7 The perfusion system

In order to be able to study nitrogen fixation and assimilation in living pea root nodules by ¹⁵N NMR spectroscopy, specialised equipment had to be constructed and developed, and this will be described in the following chapters. First of all there was a need for an experimental set-up that maintained the root nodules in a physiologically viable and controllable state while in the NMR tube. Secondly it was crucial to construct and handle the system in a way that ensured a high ¹⁵N enrichment throughout incubation experiments.

Maintaining a biological tissue in a functional state within the limited volume of the NMR tube does, in general, imply a continuous supply of oxygen and other nutrients as well as the removal of waste products. A so-called perfusion system where a buffer solution is circulated through the sample volume can meet these demands. Several perfusion system set-ups have been presented in the literature (Lee and Ratcliffe 1983b; Roby *et al.* 1987), and as no expertise was present in our

laboratory in the beginning of my project, I started out by visiting the experienced *in vivo* plant NMR group of C. Roby in Grenoble. Very sophisticated perfusion systems were used in this laboratory for many different purposes, and I acquired plenty of useful practical and technical information.

The first generation perfusion system, which I constructed with inspiration from Grenoble, contained devices for monitoring and adjusting pH as well as O₂ during incubations and in vivo NMR experiments. However, it turned out to be extremely difficult to maintain the ¹⁵N enrichment at a reasonably high level in such a complex system, and many different changes were subsequently tested in order to improve the gas tightness of the perfusion system (see Fig. 20). In brief, this caused the perfu-



Fig. 19: ¹⁵N enrichment (expressed as % of total N₂) in the gas phase of the perfusion system. Different perfusion system designs were tested of which some are given in the table below. Gas samples were taken in the beginning and at the end of incubation experiments, where perfused root nodules were analysed by ¹⁵N NMR spectroscopy. Gas samples were taken from the reservoir with a syringe and transferred to evacuated 3 mL glass containers. The gas composition (H₂, ¹⁶N₂, ¹⁵N₂, O₂, Ar and CO₂) was subsequently analysed by mass spectrometry.

	Perfusion system design										
Symbol in Fig. 19	pH stat	at Number of Filters on return tubes		Peristaltic pump with							
		(from NM reser	IR tube to voir)	silicone tubing	Tygon [●] tubing						
+	•	1			+						
•	-	2	•		+						
II	-	2	-	+							
•	•	2	+	+							
×	+	2	+	+							

sion system for the present study to undergo an evolution in my hands from a very complex system to a rather simple one.

The final perfusion system, which was used for all *in vivo* experiments described in the article manuscript, was a closed system consisting of a reservoir that contained both a gas and a liquid phase, and a system of tubing that allowed the perfusion buffer to be circulated (see Fig. 20). In order to minimise leaks, all trafficking of liquids and gasses in and out of the system was carried out by needle injections through the rubber stopper in the serum bottle that made up the reservoir. The liquid was circulated by the operation of a peristaltic pump, and the gas pressure was regulated by manual expansion/reduction of the closed system volume by syringes. Approximately 1.5 g FW (≈ 0.17 g DW) root nodules could be contained within the NMR tube, and about 1 g FW was within the volume of the NMR detection coil. More details of the perfusion system construction and handling are described in the article manuscript.



7.1 Monitoring of nodule physiological status by ³¹P NMR spectroscopy

It is not trivial to maintain a biological tissue well oxygenated in the NMR tube, and monitoring of the physiological state is therefore required. A common indicator of hypoxic metabolism in biological systems is the relative size of the adenylate pools (reviewed by Roberts and Xia 1996). In hypoxic cells the concentration of ATP is typically lower whereas ADP and AMP pools are higher than those found in aerobic cells. ³¹P NMR spectroscopy offers a convenient non-invasive method for monitoring of adenylate pool sizes and in addition permits determinations of changes in intracellular pH (see chapter 7.3). The ³¹P nucleus is 100% naturally abundant and has a relatively high intrinsic sensitivity i.e. magnetogyric ratio (cf. Table 2, chapter 3), and changes in phosphorus metabolite concentrations can often be measured in a few minutes.

Fig. 21 shows in vivo ³¹P NMR spectra of pea root nodules, which were exposed to various concentrations of O₂ and N₂ dissolved in the perfusion buffer in a time course study. ³¹P NMR signals from adenylates are present in the spectra and clearly change with changing O₂ concentrations. The resonances from α - and γ -ATP are not well resolved from the resonances from α - and β -ADP, respectively, but β -ATP gives rise to a unique, wellresolved signal at -19.0 ppm. A solid quantitative analysis of ATP/ADP levels can be done based on ³¹P NMR spectra, but qualitative results were sufficient for my purpose of ensuring that the root nodules were in a well oxygenated physiological state.



Fig. 21: In vivo ³¹P NMR spectra of pea root nodules perfused at 50 mL min⁻¹ with a buffer in equilibrium with a gas phase containing (A) 50% O_2 + 50% N_2 , (B) 100% N_2 and (C) 100% O_2 . Numbers refer to assignments given in Table 3. MDP (methylene diphosphonic acid) was present as an aqueous solution at pH 8.9 in a capillary and used as a the chemical shift reference at 16.38 ppm, but the resonance is outside the shown spectral window. Buffer composition and NMR acquisition parameters are given in the article manuscript.

In my work I have used the mere presence of the β -ATP signal as an indicator of normoxia, which is the physiological state under which ATP synthesis occurs at a high rate through oxidative phosphorylation (respiration). As seen from the spectra in Fig. 21 the β -ATP signal is present when nodules are perfused with a buffer in equilibrium with 50% O₂, disappears when no oxygen is dissolved in the perfusion buffer and reappears when nodules are perfused with an oxygen saturated buffer.

I recorded ³¹P spectra routinely before and after time course ¹⁵N NMR studies of ¹⁵N₂ fixing root nodules. In general, root nodules could be kept metabolically active in the perfusion system for more that 10 hours, as judged from the presence of β -ATP signals in ³¹P spectra, when sufficient oxygen was provided (see chapter 10.2).

The ³¹P ATP signals from root nodules represent ATP, which is present in both the plant and the bacteroid cytoplasm. I have not been able to find any reported estimates of the relative volumes taken up by the plant and the bacteroid cytoplasm in pea nodules, and it is therefore difficult to ascertain whether the information given by the ATP signals apply to both compartments. Oxygen gradients are present inside the root nodules (cf. chapter 1.4), which may lead to substantially lower oxygen concentrations in infected cells (cf. Fig. 6). There is therefore a risk that the bacteroid respiration might be oxygen limited (Kuzma *et al.* 1999) and that this would not be revealed by a lacking β -ATP signal, if there was still a major contribution to the ATP signal from the plant adenylate pool.

7.2 Usual and unusual phosphorus metabolites in pea, Lotus and soybean nodules

An elaborate analysis of the observed *in vivo* ³¹P NMR resonances from pea root nodules has been beyond the scope of my work. However, tentative assignments of ³¹P resonances have been made (see Table 3) based on comparisons with literature values and acquisition of ³¹P spectra of perchloric acid extracts (see Fig. 22), performed during my stay in the laboratory of C. Roby,



Fig. 22: Fully relaxed and proton decoupled ³¹P NMR spectrum of a neutralised perchloric acid extract from seven-week-old pea root nodules. Numbers refer to assignments given in Table 3. MPA and MDP are the reference compounds methyl phosphoramide and methylene diphosphonic acid, respectively, which had been added during the extraction procedure. The perchloric acid extraction was performed as described by Roby et al. 1987. The spectrum was recorded during my stay in Grenoble on a 200 MHz Bruker spectrometer equipped with a 10 mm broadband probe. Acquisition parameters were: 90° pulse angle, 0.5 s acquisition time, 20 s recycle delay and 8065 transients resulting in a total acquisition time of 45 hours.

Grenoble. All the expected phosphorus metabolites, which are normally observed in plant tissue (e.g. Roby *et al.* 1987, Rolin *et al.* 1989a; Saint-Ges *et al.* 1991), were found to be present in pea root nodules.

		Chemi	Concentration ³	
No*	Phosphorus metabolite	(1	opm)	(µmol/g FW)
		Pea nodules	Pea nodules	Peanodules
1		In vivo	Extract ⁴ pH 7.5	i ca ilociules
}	Glucose-6-phosphate			
1	Mannose-6-phosphate	4,3	4.62, 4.44, 4.33	0.27
	Glycerol-3-phosphate			
	Phosphoethanolamine			
2	3-Phosphoglycerate	3.8	4.03, 3.91	0.41
	Fructose-6-phosphate			
3	Phosphatidyl choline	3.4	3.39	0.60
4	Phasphate	2.1 (cyt.)	2 37	4.03
5	Thosphate	0.3 (vac.)	2.57	4.05
6	Phytate*	1.6, 1.0	1.79, 1.39	
7	Glycerophosphocholine		0.04	0.02
8	Phophoenolpyruvate	-0.7	-0.61	0.03
x	Unknown compound "X"	-1.9	-1.87	0.10
11	ATP a	-10.5	-10.88	
14	β	-19.0	-21.29	0.07
9	Υ	-5.4	-6.01	
11	ADP a			
10	β	-6.2	-6.32	
12	UDPG ^a a	-11.5	-11.11	
13	β	-12.6	-12.46	

Table 3: In vivo and in vitro ³¹P chemical shifts and concentrations of phosphorus metabolites in pea root nodules.

⁵ Numbers refer to annotations in ³¹P NMR spectra in Figs. 21, 22 and 23

^{*} All assignments are based solely on comparisons with literature values. Chemical shifts are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. NMR acquisition conditions are given in the article manuscript and in the legend of Fig. 22

^c The neutralised perchloric acid extract was prepared as described by Roby et al. 1987

⁴ Concentrations were calculated from the fully relaxed, proton-decoupled ³¹P NMR spectrum of a perchloric acid extract shown in Fig. 22 by comparing peak intensities of phosphorus metabolites with the intensity of the internal standard methyl phosphoramide (MPA), which was added to 5.8 g FW root nodules before extraction.

* Meso-inositole hexaphosphate

[©] Uridin-diphospho-glucose

An additional, very unusual ³¹P resonance, not previously reported in plant material, was observed

in pea nodule spectra at around -1.9 ppm (denoted "X" in Table 3 and Figs. 21-23). This signal was observed in several in vivo experiments to be more narrow and persistent during prolonged perfusion than all other ³¹P signals. The same chemical shift was observed in vivo and in vitro in ³¹P NMR spectra of perchloric acid extracts at pH 7.5 and showed no coupling to other ³¹P nuclei (data not shown). The signal appeared as a 1:2:1 triplet with J = 8.9 Hz in proton coupled ³¹P NMR spectra of a root nodule perchloric acid extract (data not shown), which indicates that it is a phosphate group located next to a methylene group. The concentration in the root nodule tissue was estimated to be 0.1 μ mol g⁻¹ FW (cf. Table 3). The yet unidentified peak X was not observed in ³¹P NMR spectra of either pea root tips, a dense suspension of free-living Rhizobium leguminosarum by. vicea, or intact root nodules from Lotus or soybean plants (cf. Fig. 23).

Lotus and soybean nodules were used in the search for experimental conditions that would result in highly informative ¹⁵N NMR spectra (see chapter 10.2). However, another very surprising and unusual ³¹P resonance was observed in two separate experiments with Lotus nodules at 16.3 ppm (cf. Fig. 23 B). This resonance frequency is very close to the frequency of methylene diphosphonic acid (MDP), which is a frequently used reference compound that was <u>not</u> applied in these experiments. The Lotus resonance at 16.3 ppm was never observed in ³¹P NMR



Fig. 23: In vivo ³¹P spectra of (A) pea nodules, (B) Lows nodules, (C) soybean nodules, (D) 3 mm long pea root tips, (E) Rhizobium leguminosarum by, vicea suspension. Nodules and root tips were perfused with buffer (10% D₂O, pH 6) as described in the article manuscript. The buffer was in equilibrium with a gas phase containing 50, 40, 50 and 100% O2 for experiment A, B, C and D, respectively. For experiment E the dense Rhizobium suspension was produced from 2 L of a three-day-old yeast broth culture, which was centrifuged at 4000 rpm and washed with perfusion buffer twice. The bacteria were finally suspended in perfusion buffer (10% D₂O, pH 6), but not perfused during the acquisition. Acquisition parameters for all spectra are as described in the article manuscript, but with different numbers of transients leading to total acquisition times of 30, 30, 30, 60 and 120 min for spectrum A, B, C, D and E, respectively. Numbers refer to assignments given in Table 3. Additional assignments are: PCCG, phospholine-substituted \$-1,3;1,6 cyclic glucan; Ext. Pi, external inorganic phosphate. A capillary containing an aqueous solution of MDP (methylene diphosphonic acid) was present only in experiment E.

spectra of either pea or soybean nodules, and it was not observed in spectra recorded from the buffer after perfusion of *Lotus* nodules. The identity of this unusual compound was not further pursued and remains a mystery.

Soybean nodules have previously been studied by ³¹P NMR and were observed to give rise to an unusual ³¹P resonance at around 0.4 ppm (Rolin *et al.* 1989a and b), which I also observed (cf. Fig. 23 C). This ³¹P metabolite was later identified as a phosphocholine substituted macrocyclic glucan (PCCG) synthesised by the bacterium in both the free-living and the symbiotic state (Rolin *et al.* 1992) and suggested to play a role in osmoregulation (Pfeffer *et al.* 1994).

It is interesting that the three nodule types studied give rise to three different very unusual ³¹P resonances. The identity and function of the unusual ³¹P metabolites in pea and alfalfa nodules have not yet been investigated, but the discovery of these novel compounds illustrate the strong potential of *in vivo* NMR spectroscopy to reveal unexpected information that would escape detection by other analytical methods,

7.3 pH effects on phosphate chemical shifts

Monitoring of the nodule physiological status by ³¹P NMR spectroscopy also includes the detection of possible changes in intracellular pH, because cytoplasmic acidosis is associated with intolerance of low O_2 (reviewed by Xia *et al.* 1995). NMR signals from ionisable metabolites often have pH dependent properties, when the pH of the surrounding medium is close to the pK_s of the ionisable group. Chemical shifts are pH dependent, if there is a shift difference between the acid and the base form, and the proton exchange rate is rapid.

Inorganic phosphate, a ubiquitous ion with a pK_a for the species H_2PO_4 of 6.8, is an excellent probe for the cytoplasmic pH, and although it is less sensitive to pH in the acidic range, it can also be used to put an upper limit on the vacuolar pH. To measure intracellular pH using ³¹P NMR, *in vivo* ³¹P chemical shifts are compared with a titration curve of inorganic phosphate (Moon and Richards 1975).

All the techniques available for measuring intracellular pH are affected by uncertainties in the construction of appropriate calibration curves, and NMR is no exception. Uncertainties about the solute composition of cytoplasm and vacuole, especially total ionic strength and free Mg²⁺ concentration, lead to uncertainties in absolute pH values (Roberts *et al.* 1981). The titration curve of P_i in undiluted maize root tip homogenates resembles that of P_i in 100 mM KCl and 2 mM MgCl₂ (Roberts *et al.* 1981), but to apply the NMR method rigorously to root nodules, one would have to examine the solute composition of this tissue and to know how Mg²⁺ and ionic strength are distributed between the cytoplasm and vacuole. It has been beyond the scope of my work to go deeply into using ³¹P NMR for pH measurements, so I have based my pH estimates on calibration curves of measured chemical shifts for phosphate in two different solutions, whose compositions

were intended to mimic the cytoplasm and vacuole, respectively, as suggested by Spickett *et al.* (1993) (see Fig. 24).

The pH values in the cytoplasm and vacuole of pea root nodules were estimated to be 7.2 and 5.2, respectively, from the P_i chemical shifts at 2.1 ppm and 0.3 ppm, respectively, in the representative ³¹P spectrum presented in Fig. 23 A. The cytoplasmic compartment at pH 7.2 might include both the plant and the bacteroid cytoplasm. The resonance at 1.6 ppm could represent P_i in a different subcellular compartment with a pH of approximately 6.8 or it could be a signal from one of the phosphate groups of phytate. Another possible signal from phytate was ob-



Fig. 24: Calibration curves of phosphate chemical shifts as a function of pH in different solutions, whose ionic compositions were intended to mimic the cytosol and the vacuole as suggested by Spickett *et al.* (1993). The cytosolic calibration solution consisted of 100 mM KCl, 5 mM MgSO₄, 2 mM NaH₂PO₄ and 3 mM Na₂HPO₄. The vacuolar calibration solution contained 20 mM KCl, 50 mM MgCl₂, 10 mM cltric acid, 5 mM nalic acid and 5 mM NaH₂PO₄. pH was regulated with KOH and HCl. ³¹P chemical shifts were measured relative to the signal from methyleoe diphosphonic acid (pH 8.9 in TRIS buffer) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm.

served at 1.0 ppm, but the exact position of the phytate multiplet is highly sensitive to pH and to the chemical environment, making its correct identification difficult (Saint-Ges *et al.* 1991). However, it seems likely that the two small peaks represent phytate, as ³¹P spectra of perchloric acid extracts from root nodules also contained phytate resonances of approximately the same intensity (cf. Fig. 22). I observed that pH in the root nodule cytoplasm as well as in the vacuole remained stable during more than 10 hours in the perfusion system when the oxygen supply was adequate (data not shown).

8 Nitrogen fixation rate

At the beginning of the project it was not known whether detached nodules would be able to take up and fix ${}^{15}N_2$ at a reasonable rate from the water phase under the given conditions of an NMR tube, so this had to be investigated before *in vivo* ${}^{15}N$ NMR experiments could be started out. In the following chapters, the experimental parameters that were tested and varied in order to find the optimal conditions for maximal nitrogen fixation will be described.

Several techniques have previously been developed for measuring nitrogen fixation rates in nodules and the most applied methods are the acetylene reduction assay (ARA), the measurement of H_2 evolution and of ${}^{15}N_2$ -incorporation. Each of these methods has inherent shortcomings and disadvantages, and estimates of nitrogenase activity may be affected by the used assay (reviewed by Hunt and Layzell 1993). In the presence of 10% acetylene virtually all electron flux through nitrogenase is diverted to acetylene reduction, and measurement of the ethylene production rate provides the basis for the ARA (Hardy *et al.* 1968). I started out by using ARA for quantifying nodule nitrogenase activity, but for convenience I changed to using H₂ evolution, which has been used for obtaining most of the results presented in this chapter.

 H_2 production is an obligate part of the N₂ fixation reaction (cf. Eq. 1, chapter 2.2) and thus reflects nitrogenase activity. It is, however, not straightforward to convert measured H₂ evolution to nitrogenase activity, because the relative allocation of electrons by nitrogenase to H⁺ and N₂ reduction is subject to variation (Skøt 1983). The theoretical ratio of H₂ to N₂ given by the nitrogenase equation is 1, but larger ratios are often observed (reviewed by Skøt 1983). Certain legume symbioses possess an uptake hydrogenase enzyme (Hup) that recycles some or all of the H₂ produced by nitrogenase (Truelsen and Wyndaele 1984) and H₂ analysis cannot be used to estimate nitrogenase activity in these Hup⁺ symbioses. The pea symbiont *R. leguminosarum* strain Risø 18a, which I have used for all experiments except when otherwise stressed, is Hup⁻ (Truelsen and Wyndaele 1984), and the measurement of H₂ evolution has been an adequate method for my purpose of comparing how nitrogenase activity was influenced by different treatments and experimental conditions.

8.1 Maximising the nitrogenase activity

The amount of bacteroid-containing nodule tissue in the late symbiotic developmental stage (cf. chapter 1.3) varies with the age of the nodules, and this is one of the major determinants of the extent of N_2 fixation. It is well known that the maximum rate of nodule nitrogenase activity in legumes occurs at flowering or just before the pod-filling stage of seed development (Robertsen and Farnden 1980; Sprent 1982). It is generally assumed that the reason for this change in nitrogenase activity is a change in the source-sink relationships of the plant resulting in a decreased supply to nodules of photosynthates, which support nitrogenase activity (Minchin *et al.* 1981). For the pea variety and the growth conditions that I have mainly employed (see article manuscript) the early pod-filling stage occurs six to seven weeks after sowing and all experiments have been performed with nodules from plants of this age.

Physical disturbance of root nodules, which necessarily occurs at harvesting and nodule excision, causes an inhibition of nitrogenase activity (see Fig. 25) as a result of a decrease in the O_2 permeability (cf. chapter 1.4). The decrease in nitrogenase activity at nodule excision is furthermore attributed to a cessation in the import of phloem-supplied carbohydrate as well as a cessation in the export of the products of N_2 fixation in the xylem. I tried different procedures for gentle washing and excision of root nodules but always ended up with detached nodules, whose nitrogenase activity was at least an order of magnitude smaller than for nodules in an undisturbed

root system (Fig. 25). Introduction of a different plant growth system, the aeroponic system described in chapter 6 and in the article manuscript, made it possible to harvest nodules with minimal physical disturbance, and the nodules retained a higher nitrogenase activity (Fig. 25).

In the search for pea root nodules with the highest possible nitrogenase activity I also tested different biological materials. One different pea variety, "Bodil", and two different *R. leguminosarum* strains, Risø 1A and 128 C53 (Truelsen and Wyndaele 1984), were assayed but did not give rise to root nodules with larger nitrogen fixation rates (data not shown) than the pea variety "Solara" in combination with *R. leguminosarum* Risø 18A, which was used for all other experiments.

Immersion in water has previously been reported to inhibit nitrogenase activity in soybean nodules and was attributed to a reduction in oxygen supply to the nodules (Sprent 1969). It was reported that increasing the O_2 content to 0.8 atm or more in the gas phase above the immersed no-



Fig. 25: Effect of the treatment of pea root nodules on nitrogenase activity, which was measured as hydrogen evolution rate in atmospheric air. The undisturbed root system was assayed in a closed gas tight root chamber, whereas the washed, detopped root system and the detached nodules were assayed in closed serum bottles. The immersed nodules were covered by perfusion buffer, pH 6 (see article manuscript) and gently shaken throughout the incubation. Nodules harvested from two different plant growth systems (cf. chapter 6) were tested: vermiculite as well as the aeroponic system. Numbers below columns indicate number of replicates. Gas samples were taken at intervals for each replicate, and analysed for H₂ using a gas chromatograph (Hewlett Packard 6890) equipped with a stainless steel column (2 m x 1/8" diam.) containing molecular sieve 5A (80-100 mesh) at 60°C and a thermal conductivity detector. Argon was used as a carrier gas with a flow of 50 mL min⁻¹.

dules and shaking of the incubation vessel, would make the nitrogenase activity return to the rate of dry nodules in a pO_2 of 0.2 atm. Bergersen (1982) reported that detached root nodules with water bathing the nodule surface fixed less N₂ and stated that such results were almost always due to the effects of free water interfering with gas phase diffusion into the porous nodule surface.

I examined the nitrogen fixation capacity of pea nodules immersed in buffer and found substantial inhibition when the gas phase above the solution was atmospheric air (Fig. 25). Nodules grown in the aeroponic system seemed to maintain a higher nitrogenase activity, which may be attributed to the fact that nodules are covered with a water film in the aeroponic system and thus adapted to

taking up oxygen and nitrogen under these circumstances. I found that the degree of shaking of nodules in buffer was crucial for maximising the measured nitrogen fixation rate i.e. the diffusion of gasses through the liquid seems to be rate limiting (data not shown).

The pH of the buffer used for immersion of nodules was tested in the range of pH 5 to pH 7.5 and I found that the nitrogenase activity was inhibited at pH values above 7 (data not shown). Consequently, all further incubations of nodules in the perfusion system were performed with a buffer of pH 6.

The reason for inhibition of nitrogenase at immersion of nodules was assumed to be due to limitation by sub-optimal O_2 concentrations in the infected cells as described above, and I tested whether increasing the O_2 content would lead to increased nitrogen fixation rates. As seen from Fig. 26 it was indeed possible to stimulate the nitrogenase activity by raising pO₂, and a more detailed examination of the optimal oxygen level was undertaken for nodules incubated in the perfusion system.



Fig. 26: Effect of increasing the oxygen content on the nitrogenase activity of pea root nodules. Detached nodules were incubated in closed bottles, which were either filled with gas or with perfusion buffer, pH 6 (see article manuscript) covering the nodules and a gas phase above the solution. Two different oxygen levels were tested. Bottles were shaken gently throughout the incubation period, Gas samples were taken at intervals, and analysed for H₂ as described in legend of Fig. 25.

8.2 Nitrogenase activity in the perfusion system

The actual nitrogenase activity for nodules incubated in the perfusion system (cf. chapter 7), was measured at different O_2 concentrations in the gas phase. It has previously been shown that respiration and nitrogenase activity are O_2 limited in the range of O_2 concentrations measured in infected cells of nodules (Kuzma *et al.* 1993). It was reported that when pO_2 was increased to slightly above the point, where there ceased to be stimulation, nitrogenase activity declined and respiration stabilised or declined. I therefore expected that it would be possible to define an optimal pO_2 for maximal nitrogenase activity.

Results from two different experiments are presented in Fig. 27. Oxygen levels were varied between 30% and 80% and a total gas pressure of 1.5 atm was employed in order to increase the concentrations of gasses dissolved in the perfusion buffer. The first experiment indicated that the nitrogen fixation rate was maximal at around 50% O_2 . Nitrogenase was apparently totally inhibited at 80% O_2 since no hydrogen evolution could be detected. The inhibition seemed to be irreversible as a switch back to 50% O_2 did not result in hydrogen evolution even after 30 min. These results

could, however, not be reproduced in a second experiment, where no variation of the nitrogenase activity was observed with a change in pO_2 .

The experimental reason for these apparently inconsistent results was not pursued, and it was decided to continue incubations in the perfusion system with an O_2/N_2 ratio of 1 and a total gas pressure of 1.5 atm. Further efforts to optimise the experimental conditions for maximal nitrogenase activity in the perfusion system were undertaken for incubations with ¹⁵N₂-fixing nodules as described in chapter 10.2.

In the beginning of the project I purged the perfusion system with pure O_2 , while circulating the buffer through the NMR tube containing the nodules, in order to drive off all ¹⁴N₂ before introducing ¹⁵N₂. I tested the nitrogenase activity of nodules after this treatment and found that nitrogenase was completely inhibited (data not



Fig. 27: The nitrogenase activity for pea root nodules, which were placed in an NMR tube and perfused by the system described in chapter 7 and depicted in Fig. 20. The gas phase in the reservoir was varied to different concentrations of O_2 and N_2 , and nitrogenase activity was measured as the hydrogen evolution rate. The experiment was performed twice with different root nodule samples (Exp. 1 and 2). The oxygen content was increased gradually by 10% in both experiments, and in Exp.1 the gas composition was changed back to 50% O_2 and 50% N_2 at the end of the experiment. Gas samples were analysed for H_2 as described in legend of Fig. 25.

shown). This was surprising because the concentration of oxygen dissolved in the buffer, which is experienced by the nodules, is much smaller than the concentration of oxygen in ambient air. Anyway, I subsequently changed the purging procedure to consist of a period with Ar purge followed by a very short period of O_2 purge, while shunting the NMR tube.

9 Total amino acid pools

Information on the total pool sizes of soluble amino acids in root nodules was needed in order to be able to interpret the data on ¹⁵N labelling that are presented in chapters 10 and 11. However, the equipment for amino acid analysis was not available in our institution, and all amino acid analyses have been performed at the Department of Clinical Genetics, Copenhagen University Hospital by the procedure described in the article manuscript.

The preparation of root nodule extracts for amino acid analysis was performed by me and some optimisation of extraction procedures from the literature was needed to obtain reliable results. I experienced that particularly glutamine (but also asparagine to some extent) was extremely susceptible to deamidation and that it was crucial to keep nodules as well as extracts cold during the entire process of extraction and purification. It was also important to perform the extraction and purification as fast as possible, because deamidation of glutamine would occur even at low temperatures. When extracts were kept at -80 °C glutamine was stable. I tested the improved extraction and purification procedures with standard samples of relevant amino acids and found the same recovery of glutamine and asparagine as of all other tested amino acids (data not shown).

Results from analyses of different root nodule samples are shown in Fig. 28, and it is evident that asparagine was the dominating free amino acid in freshly harvested nodules (172 µmol g⁻¹ DW) as well as in nodules that had been perfused for many hours during an NMR experiment (74-150 µmol g⁻¹ DW). Asparagine is the end product of nitrogen assimilation in indeterminate nodule and the form in which nitrogen is exported from the nodules to the plant (cf. chapter 1.3 and 2.5). The other major α-amino acids glutamine, alanine, aspartate and glutamate were present in much smaller amounts in the range of 5-27 µmol g⁻¹ DW for freshly harvested nodules and 2-14 µmol g⁻¹ DW for perfused nodules. These results indicate that, in general, concentrations of free a-amino acids were slightly lower in perfused nodules. y-Aminobutyric acid (GABA), on the other hand, is not an α amino acid and is not used for protein



Fig. 28: Concentrations of soluble amino acids in freshly harvested pea root nodules and nodules that had been subjected to perfusion for 10 and 8 hours, respectively, in two different experiments. All nodules were from six-week-old plants. Extraction and analysis of amino acids was performed as described in the article manuscript. Values are expressed per DW but may be converted to FW by using a FW/DW ratio of about 8.5 for comparison with Fig. 29. Error bars denote standard errors (n=3).

synthesis, but it constituted a substantial part of the soluble pool of nitrogen-containing metabolites in freshly harvested nodules (12 μ mol g⁻¹ DW) and the pool size apparently increased substantially during perfusion (32-51 μ mol g⁻¹ DW).

The incubation and perfusion of nodules in an NMR tube seemed to have a minor impact on the pool sizes of the free α -amino acids, whereas accumulation of GABA occurred. GABA is synthesised through a decarboxylation of glutamate, and the enzyme glutamate α -decarboxylase

(GAD) is well known in plant tissue and has also been reported to be present in symbiotic rhizobia bacteroids (cf. chapter 2.4 and 2.5). As previously mentioned, the role of GABA is unclear, so at present it is not possible to draw any further conclusions from the observation that GABA accumulated in the root nodules during perfusion in the NMR tube. However, this observation makes it clear that nodule metabolism is affected to some extent by the applied procedures although *in vivo* NMR spectroscopy is usually regarded as a non-invasive method. Whether nodule GABA accumulation also occurs during other forms of incubation in previous investigations of

root nodule metabolism is not known, because the GABA level is usually not measured before and after incubation.

My analyses of total concentrations of amino acids do not allow for a discrimination of amino acids localised in the bacteroid or in the plant cytosol. Literature reports of amino acid levels in root nodules are scarce, but Fig. 29 shows results from a previous study on pea root nodules where bacteroids and plant cytosol were fractionated and analysed separately (Rosendahl *et al.* 1990), and it is obvious that the plant fraction of amino acids was by far the largest.



Fig. 29: Concentrations of soluble amino acids in the plant cytosol and the bacteroid, respectively, as reported in a study of pea root nodules by Rosendahl *et al.* 1990. Values for each of the fractions are expressed per g FW of the entire nodule. All nodules were from five-week-old plants. Error bars denote standard errors (n=3).

10 Nitrogen assimilation studied by ¹⁵N NMR spectroscopy

The development of *in vivo* ¹⁵N NMR spectroscopy for the study of nitrogen fixation and assimilation in pea root nodules turned out to present two major experimental challenges. First of all, a considerable effort was devoted to further optimisation of the experimental conditions for nodules in the perfusion system. The aim was to maximise the ¹⁵N₂ fixation rate in order to make the level of ¹⁵N labelling of metabolites exceed the ¹⁵N NMR detection limit (see chapter 10.2). Secondly, the identification of metabolites giving rise to the observed *in vivo* ¹⁵N signals turned out to be by no means straightforward and led to application of various approaches.

The assignment of ¹⁵N NMR resonances is of course a crucial point and can be approached in several ways. ¹⁵N spectra of living plants, bacteria and fungi, which have been ¹⁵N enriched in some way, can be obtained from the literature (Table 4), and reported chemical shifts may be used

for first tentative assignments. Amino acids and ammonium are the most common NMRobservable ¹⁵N enriched metabolites, but the reported resonance frequencies vary substantially as seen from Table 4. The ¹⁵N chemical shift may be influenced by the nature of the intracellular environment i.e. factors such as ionic composition, pH, paramagnetic compounds, association with other molecules as well as experimental conditions such as temperature and composition of incubation medium. Thus, care should be taken to base assignments purely on literature values from different biological systems. I have therefore used multiple approaches in order to be able to identify *in vivo* resonances unequivocally.

Biological system (Reference)	Gln amide	Asn amide	Ala	Gìn amino	Glu	Asn amino	Asp	GABA	NH₄ ⁺
Brevibacterium lactofermentum (Haran et al. 1983)	90.8	88.1	21.6		19.5		16.8		
Carrot cells (Fox ci al. 1992)	91.2			19	9.8				0
Corynebacterium glutamicum [*] (Tesch et al. 1999)	91		·	2	0				0
Duckweed (Lemnaceae) ¹ (Monselise & Kost 1993)	90.8	90.5	22.0	19),9	19.3		}	0
Ectomycorrhizal mycelium ^t (Martin <i>et al.</i> 1994)	90.4		21.9	19.7	19.6			11.6	0
Green algae (Chlorella fusca) (Kuesel et al. 1989)	92.5		23,3	21.2	20.9				
Maize roots [‡] (Amancio & Santos 1992)	91.7	89.0 "	22.3	20.0	19.8	18	.6	12.0	0
<i>Neurospora crassa</i> mycelium [®] (Kanamori <i>et al.</i> 1982a)	90.6		22.6	20.35	20.16				
Neurospora crassa mycelium (Legenton er al. 1981)	92.2		2 2.3	20).3				0.1
Norway sprace seedlings roots and sterns [*] (Aarnes et al. 1995)	91.1		22.0		19.8			10.6	-0.1

Table 4: Literature values of in vivo ¹⁵N chemical shifts of amino acids and ammonium in plants, bacteria and fungi

All spectra were collected either without D2O or with D2O contained in a capillary

* Chemical shifts were originally reported relative to NO₃^{*} at 0 pmm, but have been converted to a scale that puts area at 55.8 ppm (and NO₃^{*} at 354.6 pmm)

* Chemical shifts are reported relative to intracellular ammonium at 0 ppm

* This value was reported by the authors, but the published in vivo ¹⁵N NMR spectra showed a signal from the asparagine amide group at approximately 91.4 ppm

It was investigated how ¹⁵N chemical shifts are influenced by the following variables: pH, temperature, phosphate and D_2O concentrations by using authentic ¹⁵N amino acid samples (see chapter 10.1). Furthermore, I used different ¹⁵N₂ incubation procedures for obtaining root nodules

with high ¹⁵N enrichment of the amino acid pool, which made it possible to observe a number of resonances during the subsequent recording of ¹⁵N NMR spectra (see chapter 10.3) and to optimise the NMR acquisition parameters for *in vivo* experiments (see chapter 10.5). These nodules were dead and thus not metabolically active when subjected to ¹⁵N NMR spectroscopy, but it was assumed that ¹⁵N amino acids would be in an intracellular environment mimicking the living root nodules. However, it turned out later that the influence of the intracellular environment on some of the NMR properties of ¹⁵N metabolites was substantially different in dead nodules compared to living nodules. ¹⁵N spectra of extracts from ¹⁵N enriched nodules were likewise recorded, and spiking of these extracts with authentic ¹⁵N amino acid standards permitted a very reliable identification of metabolites that were ¹⁵N labelled by assimilation of fixed ¹⁵N₂. Finally, nodule extracts were analysed by different mass spectrometric methods (see chapter 11) in order to provide complementary results on ¹⁵N labelling of amino acids to support the assignment of ¹⁵N NMR resonances.

10.1 Medium effects on ¹⁵N NMR properties of amino acids and ammonium

pH

In aqueous solutions, the protons attached to ammonium and amino group nitrogens undergo basecatalysed exchange with water protons. The rate of exchange, $1/\tau_e$ (where τ_e is the average lifetime between proton transfer), thus increases with pH and furthermore depends on the pK_a of the given amino group. At very low pH the proton exchange rate of ammonium and α -amino groups with a pK_a of 9-10 is slow on the NMR time scale ($1/\tau_e << J$), whereas at neutral pH, exchange is rapid ($1/\tau_e>>J$) (reviewed by Legerton *et al.* 1983). Slow exchange will lead to a multiplet in protoncoupled ¹⁵N (or ¹⁴N) spectra, whereas fast exchange will result in a singlet. This singlet broadens as the pH is reduced, and the line width of proton-coupled ¹⁵N resonances is pH dependent when the proton exchange rate is intermediate. The line widths of α -amino groups of amino acids and ammonium have been used as *in vivo* probes of intracellular and subcellular pH (Legerton *et al.* 1983; Aarnes *et al.* 1995), and the strong pH dependence in the line width of the ammonium signal provided the basis for an *in vivo* investigation of the subcellular distribution of ammonium (Lee and Rateliffe 1993).

In the biological pH range the protons attached to amide groups are in slow exchange and the exchange rate is not influenced by pH.

In the present study, I have generally used full proton decoupling when recording ¹⁵N spectra in order to take full advantage of the NOE (cf. chapter 3.3), and line broadening caused by exchange effects is thus not observed. However, the exchange rate of protons attached to nitrogen has an impact on many of the ¹⁵N NMR observable properties, which will be described in the following chapters.

pH and chemical shift

The pH of the medium may, in addition to the proton exchange rate, also influence the ¹⁵N chemical shifts of ionisable groups, as described in chapter 7.3. pH dependent chemical shifts may be observed for amino groups and ammonium because the proton exchange rate is rapid in the relevant pH range. Deprotonation of the amines with increasing pH shifts the resonance significantly upfield (Berger *et al.* 1997), but under normal physiological conditions the NMR signals will not be affected because the pK_a values of the amino acids that are relevant to my work are too remote from the cytoplasmic and vacuolar pH. Literature values of amino group pK_as are as follows: asparagine 8.84, glutamine 9.00, glutamate 9.69, aspartate 9.72, and GABA 10.56 (Strandgaard Andersen *et al.* 1993).

I measured ¹⁵N chemical shifts of amino acids at different pHs (see Fig. 30 and Table 5) and, as expected, the observed effects clearly reflected the differences in amino group pK, values. The chemical shifts of asparagine and glutamine α -amino groups changed slightly in the upfield direction with pH in the range from 7 to 8 and dramatically from around pH 8 and upwards when increasing deprotonation caused increased shielding of the ¹⁵N nucleus. The chemical shifts of glutamate, aspartate and GABA amino groups only showed minor upfield shifting for pH values above 8.

The amide group shielding of asparagine, but not glutamine, was slightly influenced by the deprotonation of the α -amino group. With increasing pH and increasing deproto-



Fig. 30: Amino acid chemical shifts as a function of pH. 99% ¹⁵N enriched amino acids were dissolved in 0.1 M HCl to a concentration of about 30 mM and titrated to different pH values with KOH and HCl. Chemical shifts are referenced to urea at 55.8 ppm, which was present as an aqueous solution in a capillary. ¹⁵N NMR spectra were acquired as described in the article manuscript.

nation of the amino group, the asparagine amide group becomes deshielded and the resonance is thus shifted downfield. The difference between glutamine and asparagine can be ascribed to the difference in length of the carbon backbone connecting the amino and amide groups. The extra carbon atom in glutamine apparently attenuates the effects on the amide group of the change in electron density with deprotonation of the amino group.

In the physiologically relevant pH range from 7 to 7.5 a variation of maximum 0.1 to 0.2 ppm in the chemical shift of individual amino acids was observed.

Histidine (imidazolium $pK_a = 6.00$) has previously been used as an *in vivo* intracellular pH probe by ¹⁵N NMR spectroscopy of intact *Neurospora crassa* mycelium (Legerton *et al.* 1983), but in my work this has not been possible because root nodules contain only minor amounts of histidine, which is furthermore not ¹⁵N labelled to any great extent (data not shown).

The ¹⁵N chemical shift of ammonium/ammonia depends strongly on pH, because there is a large chemical shift difference between ammonum and ammonia of about 30 ppm. I measured this effect in more detail in the search for an explanation of the very unusual in vivo ammonium chemical shifts that were observed in root nodules (see chapter 10.3). The pK_a value of ammonium at 20 °C is 9.23 (Bates and Pinching 1950), and as seen from Fig. 31, major changes the chemical shift were in observed already between pH 7 and 8. Ammonium was originally dissolved in a solution, whose composition was intended to mimic the cell cytosol (Spickett et al. 1993), but it was only possible



Fig. 31: Ammonium chemical shift as a function of pH. 99% 15 N enriched ammonium chloride was dissolved in different solvents: (1) a "cytosol" solution consisting of 100 mM KCl, 5 mM MgSO₄ and 5mM NaH₂PO₄ (Spickett *et al.* 1993), (2) 0.1 M HCl and (3) water. The ammonium concentrations were about 5, 30 and 5 mM, respectively in the three solvents, and the titrations to different pH values were done with KOH and HCl. Chemical shifts are referenced to urea at 55.8 ppm, which was present as an aqueous solution in a capillary. ¹⁵N NMR spectra were acquired as described in the article manuscript.

to obtain ¹⁵N measurements at pH values lower than 8 because precipitation of MgOH₂ occurred at higher pH. Titration and recording of ¹⁵N spectra from ammonium dissolved in either 0.1 M HCl or pure water demonstrated the expected strong upfield shifting of the ammonium resonance with increasing pH.

pH and NOE

The NOE factors, η (cf. Eq. 7 and 8, chapter 3.3), for standard samples of ¹⁵N labelled ammonium and amino acids were determined as the ratio between signal intensities in ¹⁵N NMR spectra acquired with full decoupling and NOE and spectra with inverse gated decoupling (see Fig. 32). The possible impact of NOE factors on the appearance of *in vivo* ¹⁵N NMR spectra will be discussed in chapter 10.3 and 10.5.

I observed a dramatic change of α -amino group η with pH (Fig. 32 A). At low pH (below 6) NOE factors were near or even below the theoretical minimum value of -5, but with increasing pH the η increased and even passed through -1, which implied a complete loss of the signal at the given pH in spectra with full decoupling. The amino group of asparagine had a η of -1 already at around pH



7.1, whereas the η for amino groups of glutamine, aspartate and glutamate went through -1 at much higher pH values: 8.1, 8.6 and above 9, respectively. The differences between amino acids in the observed pH dependence reflected the amino group pK_x values mentioned above.

Fig. 32: Amino acid and ammonium NOE factors (η) as a function of pH. (A) α -Amino groups and (B) amide groups, ammonium and GABA. 99% ¹⁵N enriched ammonium and amino acids were dissolved in 0.1 M HCl to a concentration of about 30 mM and titrated to different pH values with KOH and HCl. NOE factors were determined as the ratio between signal intensities in spectra with full decoupling and spectra with inverse gated decoupling. NMR acquisition parameters are given in the article manuscript.



An anomalous pH dependence of the NOE factor of ¹⁵N glycine has previously been reported. A reduction in the NOE factor with increasing pH was observed and attributed to scalar and spinrotation relaxation (Cooper *et al.* 1973; Leipert and Noggle 1975). This explanation was later questioned by Irving and Lapidot (1975), who claimed that the pH dependent phenomenon arose purely from contamination by paramagnetic impurities. It was stated that an increasing coordination of amino groups to mainly Cu^{2+} would occur with increasing pH and cause the observed decrease in η . In order to find the explanation for the change of α -amino group η with pH that I observe, further experiments would be needed. In any case it is very difficult to predict what will be happening *in vivo*, because the presence of paramagnetic impurities cannot be controlled.

The η values for ammonium and GABA were, surprisingly, observed to be much smaller than the theoretical minimum of -5 in part of (ammonium) or all of (GABA) the tested pH range (Fig. 32 B). Ammonium NOE factors displayed systematic pH dependence with η values increasing in the range of -10 to -2 for pH values of 6-9. GABA NOE factors were found to vary in a peculiar and unsystematic way between -6 and -10 throughout the tested pH range. I did not pursue these intriguing results further. Amide group η did not change systematically with pH, but were observed to be mainly between -4 and the theoretical minimum of -5 in the tested pH range in accordance with expectations (Fig. 32 B).

Phosphate

From the three titration curves in Fig. 31 it is obvious that the ionic composition of the medium influences the ammonium chemical shift. Phosphate is an ubiquitous anion in the plant cytoplasm with estimated concentrations in the range of 5 to 18 mM (Schachtman et al. 1998; Lee and Ratcliffe 1983a). The phosphate concentration in bacteroids of root nodules has been demonstrated to be even higher by Sa and Israel (1991), who found that phosphorus concentrations in soybean nodules were 4-5 mg g⁻¹ DW plant cell and 10-12 mg g⁻¹ DW bacteroid. I investigated the counter ion effect of phosphate on the ¹⁵N ammonium chemical shift and observed that increasing the phosphate concentrations to much higher levels than encountered in physiological systems only shifted the ammonium resonance slightly towards higher resonance frequencies (see Fig. 33).



Fig. 33: Ammonium chemical shift as a function of the phosphate concentration. 99% 15 N enriched ammonium chloride was dissolved to a final concentration of 0.11 M in 1) water, 2) 0.5 M phosphate buffer pH 7.2 and 3) I M phosphate buffer pH 7.2. No reference compound was present during recording of the spectra, and the chemical shift scale is therefore arbitrary.

D_2O

It is necessary to include some deuterated species in the NMR tube for fieldfrequency locking and when working with biological systems D₂O is an obvious choice. However, deuteration increases the nitrogen shielding by about 0.5-0.7 ppm per hydrogen replaced in aminos or amides (reviewed by Mason 1996). The effects are reported to be smaller in NH4^{*}, about 0.3 ppm per hydrogen. Amide ¹⁵N signals are split by deuteration because of the slow proton exchange rate, and three separate signals are observed from CO¹⁵NH₂, CO¹⁵NHD and CO¹⁵ND₂, respectively. The fast exchange rate of amino and ammonium protons will result in ¹⁵N signals with chemical shift values representing a weighted mean of the deuterated and non-deuterated ¹⁵N nucleus. The ¹⁵N chemical shift thus depends



Fig. 34: Amino acid chemical shifts as a function of the D_2O concentration. 99% ¹⁵N enriched amino acids were dissolved in 0.1 M HCl to a concentration of about 10 mM. The D_2O concentration was regulated to the desired level, and pH was regulated to 5.8. NMR acquisition parameters were as described in the article manuscript. Chemical shifts are referenced to urea at 55.8 ppm, which was present as an aqueous solution in a capillary.

on the D_2O concentration in a predictable way, and the upfield shifting of for instance ammonium in 10% D_2O may be estimated as: 10% * 0.3 ppm /proton * 4 protons = 0.12 ppm.

In order to be able to compare literature values and own ¹⁵N spectra of nodules and extracts with different D₂O concentrations, the D₂O effects on the actual amino acid chemical shifts were quantified (see Fig. 34 and Table 5). As expected, the amide chemical shifts were largely unchanged, whereas the ammonium and amino group resonances were observed to be shifted approximately 0.15 and 0.1 ppm, respectively, upfield with a change in D₂O concentration of 10%. The change in the ammonium chemical shift was close to the expected value, but the amino group resonances were shifted a bit less than predicted from the literature values mentioned above.



Temperature

The amide and amino resonances of amino acids were observed to be shifted in opposite directions with a change in temperature (see Fig. 35 and Table 5). An increase of 10 °C shifted the glutamine amide resonance approximately 0.2 ppm downfield, whereas the ammonium and the amino resonances of glutamine and aspartate were shifted approximately 0.1 ppm upfield. Fig. 35: Amino acid chemical shifts as a function of the temperature. 99% ¹⁵N enriched amino acida were dissolved in 0.1 M HCl (20% D_2O) to a concentration of about 10 mM, and pH was regulated to 5.8. The NMR tube was heated/cooled in the NMR spectrometer to the desired temperatures and left to equilibrate for about five minutes before acquisition of spectra. The chemical shifts at the selected temperatures were measured in two repeated rounds and showed the same values. NMR acquisition parameters were as described in the article manuscript. Chemical shifts are referenced to urea at 55.8 ppm, which was present as an aqueous solution in a capillary

pH	T (°C)	% D ₂ O	Gin amide	Asn amide	Gin amino	Glu	Asn amíno	Asp	GABA	NH.*
6.9 7.0	25	10	90.86	90.51	19.85	19.65	18.90	18.34	11.75	-0.26
8.0 8.1			90.84	90.64	19.18	19.48	17.88		11.70	-0.85
5.8	25	5 15	90.89 90.91	90.48 90.48	19.97 19.86	19.75 19.64	19.01 18.95	18.40 18.29		-0.14 -0.29
5.8	25 50	20	90.99 90.55			19.41 19.77		18.14 18.37		-0.42 -0.17

Table 5: ¹⁵N chemical shifts^{*} of amino acid authentic samples in 0.1 M HCl adjusted to different pH values, D₂O concentrations and temperatures (Figs. 30, 31, 34 and 35)

All chemical shifts are reported relative to urea at 55.8 ppm. Urea was present as an aqueous solution in a capillary

10.2 Optimisation of *in vivo*¹⁵N signal-to-noise ratio by maximising the ¹⁵N incorporation

¹⁵N NMR spectroscopy is not a very sensitive method due to the inherent magnetic properties of the ¹⁵N nucleus, i.e. the low magnetogyric ratio (cf. chapter 3). In order to make the *in vivo* S/N ratio of ¹⁵N labelled metabolites exceed the ¹⁵N detection threshold, it has therefore been crucial to optimise factors influencing the ¹⁵N incorporation.

The ¹⁵N enrichment of the root nodules depends initially on the level of nitrogenase activity, which again depends on inherent properties of the biological material and can mainly be manipulated by changing the oxygen supply (cf. chapters 1.4 and 2.2). I have tested a range of experimental parameters in the search for the ultimate conditions that would result in highly informative ¹⁵N NMR spectra. The first part of the optimisation process was performed by directly measuring nitrogenase activity (cf. chapter 8). Additional experimental variables, which will be described in the present chapter, were tested with metabolically active ¹⁵N₂ fixing root nodules in the perfusion system while recording ¹⁵N NMR spectra. An overview of tested experimental conditions is given in Table 6. The obtained *in vivo* ¹⁵N NMR spectra are presented in Fig. 36 and the assignments of resonances are summarised later in Table 8 (chapter 10.3).

Experiment no.	Plant age (weeks, days)	Incubation time with ¹⁵ N ₄ (b)	O ₂ conc. (%) start → end	Special experimental features
1	6.3	19	50 → 9	:
2	5,4	7	60 → 47	Young nodules
3	4,5	6	62 → 54	Very young nodules
4	5,4	7	63 → 60	Reduced buffer flow rate
5	5,5	6	68 → 58	Gentle harvesting procedure Higher oxygen concentration
6	6,2	4	49 → 44	Different pea variety (Bodil)
7	6,3	5	49 → 41	Aeroponic growth system from here onwards
8	6,2	10	48 → 24	More efficient pump from here onwards
9	6,4	8	48 → 39	Mainly large nodules
10	6,5	9	39 → 30	OBS: Lotus nodules
11	6,6	7	23 → 11	Mainly small nodules Low oxygen concentration
12	6,6	9	45 → 37	Reduced buffer flow rate

Table 6: Experimental conditions for in vivo ¹⁵N NMR spectroscopy of ¹⁵N₂ fixing root nodules

The first varied parameter was the age of the nodules, but no major differences could be observed in the ¹⁵N NMR spectra of nodules from plants aged between four and seven weeks (see Exp. 1-3 in Table 6 and Fig. 36). I thus continued to use nodules from six- to sevenweek-old plants.

The root system of a given pea plant contains nodules of different size and developmental stage (cf. chapter 1.3). The NMR tube puts a limit on the volume of nodules that can be included in an experiment, and as smaller nodules may be packed more densely in the tube they might give rise to a higher total ¹⁵N₂ fixing rate per volume unit, although more mature nodules are known to contain a larger zone of N₂ fixing tissue (cf. chapter 1.3). I tested whether small, young nodules from the lower part of the root system or large, older nodules from the top of the tap root gave better ¹⁵N NMR spectra and found that large nodules were best (see Exp. 9 and 11 in Table 6 and Fig. 36). In all previous experiments I had been using a mixture of different nodule si-



Fig. 36: In vivo ¹⁵N NMR spectra of metabolically active, perfused, $^{15}N_2$ fixing root nodules under the experimental conditions given in Table 6. NMR acquisition parameters were as described in the article manuscript apart from the total acquisition periods. The presented spectra represent total acquisition periods of six hours (Exp. 1-5 and 8-12) or four hours (Exp. 6-7). Chemical shifts are referenced to urea at 55.8 ppm, which was present as an aqueous solution in a capillary except in Exp. 12. Assignments are given in Table 8.

zes, but for subsequent experiments I mainly used large nodules.

The nitrogenase activity is known to vary among pea varieties. Nodules from the pea variety "Solara" were used for the majority of experiments (cf. chapter 6) and the nitrogenase activity measured as hydrogen evolution rate in air was higher for "Solara" nodules than for nodules from the variety "Bodil" (cf. chapter 8.1). It was decided to test "Bodil" nodules also under ¹⁵N₂ fixing

conditions in the perfusion system, but these nodules did, however, not give rise to more informative ¹⁵N NMR spectra than "Solara" nodules (see Exp. 6 in Table 6 and Fig. 36).

Detachment of nodules from the plant root inhibits the nitrogenase activity (cf. chapter 1.4 and 8.1), but using nodules attached to a small root piece can diminish this inhibition. I tested nodules that had been carefully harvested by cutting the root near the nodule, but this implied that much less nodule material could be packed in the NMR tube. For each single NMR spectrum the S/N ratio is proportional to the number of nuclei within the detection coil, but the S/N ratio increases only with the square root of the number of transients (cf. chapter 3.3), and ³¹P spectra showed that the smaller amount of nodules caused an extremely deteriorated S/N ratio (data not shown). I therefore decided not to continue with addition of ¹⁵N₂ because the presumably higher nitrogen fixation rate was estimated to be more than outweighed by the greatly diminished S/N ratio.

Nodules from soybean and *Lotus* plants (cf. chapter 6) were tested, but soybean nodules were considered less suitable for perfusion as no ATP signals were present in ³¹P spectra (cf. Fig. 23 C), and I decided not to continue with addition of ¹⁵N₂ to the perfusion system. *Lotus* nodules, on the other hand, seemed well oxygenated as judged from the presence of β -ATP signals in ³¹P spectra (cf. Fig. 23 B) and ¹⁵N₂ was introduced. The obtained *in vivo* ¹⁵N NMR spectra from *Lotus* nodules displayed less intense signals than spectra from pea nodules (see Exp. 10 in Table 6 and Fig. 36), and *Lotus* nodules were therefore rejected for further experiments. However, the *Lotus* ¹⁵N spectra provided valuable information that aided the assignment of ¹⁵N resonances as described in chapter 10.3.

10.3 Assignment of ¹⁵N resonances in root nodules

Dead nodules

Root nodules were ¹⁵N enriched by different ¹⁵N₂ incubation procedures as described in legends of Figs. 37 and 38 and subjected to ¹⁵N NMR spectroscopy after harvesting and quenching of metabolic activity by freezing in liquid nitrogen. Several ¹⁵N signals from labelled amino acids were observed in the NMR spectra (Figs. 37 and 38). These resonances were tentatively assigned as being GABA, alanine and the amide and amino group of asparagine (Table 7) by comparison with literature values (Table 4) and own measurements of authentic standards (Table 5). Subsequent extraction of the nodules and recording of ¹⁵N spectra from the extract (Fig. 38 and Table 7) showed almost identical chemical shift values compared to dead nodules for metabolites that were ¹⁵N labelled by assimilation of fixed ¹⁵N₂. The assignments were confirmed by spiking the extract with authentic ¹⁵N amino acid standards followed by NMR analysis (Fig. 38 and Table 7). A small signal at -0.14 ppm, presumably from ¹⁵N labelled ammonium, was observed in one of the extracts, but in none of the spectra from dead nodules.

NMR analysis of an extract from nodules that were not investigated by NMR before extraction (extract C in Fig. 39 and Table 7) showed an additional ¹⁵N resonance at 19.84 ppm, which was



Fig. 37: ¹⁵N NMR spectrum from dead pea root nodules. Root nodules were harvested from six-week-old plants and transferred to a 60 mL syringe. The syringe was flushed with Ar, and subsequently O_2 and $^{15}N_2$ was added to a final gas composition of: 6% Ar, 44% O_2 and 50% $^{15}N_2$. After four hours incubation in the ^{15}N enriched atmosphere root nodules were frozen in liquid nitrogen and kept at -80°C until NMR analysis. The dead nodules were immersed in 10 mM MES buffer, pH 6, during NMR analysis. The spectrum was recorded at the University of Oxford on a Bruker CXP300 spectrometer equipped with a 20 mm broadband probe tuned at 30.42 MHz. Acquisition parameters were: 90° pulse angle, 0.25 s acquisition time, recycle delay of 1.75 s, proton decoupling at high power during the acquisition and at low power during the delay. 5.5 kHz sweep width and 28800 transients resulting in a total acquisition time of 16 hours. 8 Hz line broadening was applied. See assignments in Table 7 and GC-MS analyses in Fig. 42 A.



Fig. 38: ¹⁵N NMR spectrum from (A) dead pea root nodules, (B) extract and (C) spiked extract. Pea plants were grown with the entire root system in a gas tight chamber (Rosendalil and Jakobsen 1988). The root chamber of six-week-old plants was flushed with an Ar-O₂ mixture (70%:30%), and ¹⁵N₂ was subsequently added to a final gas composition of: 59% Ar, 24% O₂, 10% ¹⁵N₂ and 7% ¹⁴N₂. After four hours incubation in the ¹⁵N enriched atmosphere root nodules were quickly harvested on ice, immediately frozen in liquid nitrogen and kept at -80°C until NMR analysis. The dead nodules were immersed in perfusion buffer, pH 6, during NMR analysis. After recording of NMR spectra, nodules and the buffer bathing the nodules during NMR analysis were extracted with MeOH/CHCl₃/H₂O (12:5:3, v/v/v). The extract was subsequently purified as described in the article manuscript, redissolved in 0.1 M HCl and pH adjusted to 5.8 before NMR analysis. The extract was spiked with authentic ¹⁵N labelled amino acids. NMR acquisition parameters were as described in the article manuscript apart from the total acquisition periods, which were one hour (A), 16 hours (B) and 20 hours (C). See assignments in Table 7 and GC-MS analyses in Fig. 42 B.

assigned as being the amino group of glutamine based on literature values (Table 4) and own measurements of authentic standards (Table 5).



Fig. 39: ¹⁵N NMR spectrum from a root nodule extract. Root nodules were harvested from six-week-old plants and transferred to a 60 mL syringe that contained 10 mL of the perfusion buffer described in the article manuscript. The syringe was flushed with Ar, and O₂ and ¹⁵N₂ was subsequently added to a final gas composition of: 15% Ar. 45% O₂ and 40% ¹⁵N₂. Root nodules were immersed in the buffer and incubated for four hours under continuous gentle shaking of the syringe to ensure equilibrium of the liquid with the ¹⁵N enriched atmosphere. By the end of the incubation, nodules were immediately frozen in liquid nitrogen and extracted with MeOH/CHCl₂/H₂O (12:5:3, v/v/v). The extract was dried, redissolved in 0.1 M HCl and pH adjusted to 5.8 before NMR analysis. NMR acquisition parameters were as described in the article manuscript but with a total acquisition period of 16 hours. See assignments in Table 7.

	% D ₂ O	Gln amide	Asn amide	Ala	Gln amino	Glu	Asn amino	Asp	GABA	NH4*
Dead nodules A	0		90.4	22.0			19.1		11.8	
Dead nodules B	20		90.6				18.8		11.6	
Extract B1	4		90.48	22.06			19.07		11.79	-0.14
Extract B2	10		90.49	22.04			18.99		11.69	
Spikes to B2	10	90.92	90.49		19.88		18.97	18.35		
Extract C	14		90.70		19.84		19.15		11.91	ref: 0

Table 7: Assignments of amino acid ¹⁵N chemical shifts' in metabolically <u>inactive</u> nodules and extracts thereof⁴

^{*} Chemical shifts for nodules A and B and extracts B are reported relative to urea at 55.8 ppm. Urea was present as an aqueous solution in a capillary in experiments with nodules, whereas urea was added directly to extract B. Ammonium was added to extract C and used as a chemical shift reference at 0 ppm.

Treatments of nodules, NMR acquisition conditions and spectra are given in Fig. 37 (dead nodules A), Fig. 38 (dead nodules B + extract B + spikes) and Fig. 39 (extract C).

Ammonium in metabolically active nodules

Incubation of metabolically active root nodules with ${}^{15}N_2$ in the perfusion system while recording ${}^{15}N$ NMR spectra resulted in the observation of ${}^{15}N$ ammonium in several experiments (see Fig. 36, Table 8 and Fig. 40) and the resonance frequency showed some variability in the range from -4.2 to -3.9. This is an unusually low ammonium frequency compared to *in vitro* measurements (cf.

chapter 10.1) and previous in vivo ¹⁵N NMR studies which all have demonstrated ammonium resonances at around 0 ppm in procaryotic as well as in eucaryotic organisms (cf. Table 4).

The signal at around -4 ppm was assigned to ammonium based on several indications. First, no other well known ¹⁵N metabolite resonates anywhere near -4 ppm. Second, the signal at -4 ppm represents the first detectable ¹⁵N metabolite in ¹⁵N₂ fixing nodules (cf. Fig. 40), which is in accordance with the well-established fact that the product of nitrogenase activity is ammonium (cf. chapter 2.2). Third, the NOE factor is between -1 and 0, which is characteristic for ammonium *in vivo* (Martin 1985; Robinson *et al.* 1991; Fox *et al.* 1992; Joy *et al.* 1997) and gives rise to spectra with oppositely phased signals for amino acids and ammonium, respectively (cf. Figs. 36 and 40). Finally, the signal at -4 ppm is intense in spite of being attenuated by NOE under the applied acquisition parameters, and it must thus originate from a metabolite that is present in large amount, which is what should be expected from ammonium in the bacteroid compartment (cf. chapter 2.2).

The signal from ammonium was not observed in the earliest ¹⁵N NMR spectra (cf. Fig. 36), but only became detectable when perfusion was more efficient because of a new peristaltic pump (cf. Table 6). A possible explanation for this observation could be that the nitrogenase generated ammonium ions were assimilated immediately, when the nitrogen fixation rate was low, and the free ammonium pool therefore remained small and below the detection limit.

The observed enhanced shielding of ammonium could be due to many different factors such as hydrogen bonding to anions or pH effects, and may thus provide valuable information on the intracellular environment of pea root nodules. Some of these possibilities were investigated *in vitro* (cf. chapter 10.1), but I observed no significant effect of phosphate concentrations up to 1 M on the ammonium chemical shift. Base titration of ammonium *in vitro* demonstrated, as expected, large pH effects on the chemical shifts, and an ammonium chemical shift of -4 ppm was observed at pH 8.9. From the present investigation it is not possible to localise the ammonium pool to the bacteroid or plant cytoplasm, but in either case it seems most unlikely that the pH could be so alkaline in a tissue that is still metabolically active.

The unusual ammonium chemical shift could also be a result of different forms of ammonium being present. Fast exchange between free ammonium at one chemical shift and bound ammonium at another, would lead to a weighted chemical shift for the observed signal, but further investigations would be needed to examine this hypothesis.

It is evident that substantial, although not quantifiable under the acquisition mode of the present study, ¹⁵N labelling of ammonium takes place in the metabolically active pea root nodules and that ammonium is the first detectable ¹⁵N labelled compound in the intact symbiosis. This provides new information, since previous estimates of the free ammonium concentration in symbiotic root nodules have been based on extractions and several assumptions (Streeter 1989). The information that is provided by the unusual *in vivo* ¹⁵N ammonium chemical shift remains to be interpreted.

Amino acids in metabolically active nodules

In vivo ¹⁵N NMR spectra from ¹⁵N₂ fixing root nodules showed two amino acid ¹⁵N resonances at 90.7 and 19.6 ppm, which correspond to an amide and an α -amino group, respectively (see Fig. 36, Table 8 and Fig. 40). This was observed in very many experiments. The amide resonance at 90.7 ppm could, by comparison with all previously mentioned analyses, unequivocally be assigned to asparagine.

The assignment of the α -amino resonance at 19.6 ppm posed some problems. The chemical shift value corresponded to all previously observed values for glutamine and glutamate (cf. Table 4 and Table 5), but the analyses of dead nodules and extracts (cf. Table 7) demonstrated that the dominating ¹⁵N labelled α -amino group was the one of asparagine and that it resonated at the expected 18.8-19.1 ppm when inside dead nodules. Analyses of the ¹⁵N labelling by IP-RPLC MS also showed that the asparagine amide and amino groups were the most abundant ¹⁵N labelled species in nodules that were incubated with ¹⁵N₂ in the perfusion system (see Table 10 and Fig. 52). This apparent inconsistency was an intriguing enigma that needed an explanation!

In the search for factors that might cause unexpected changes of ¹⁵N chemical shifts, I came across the pH dependence of NOE factors (cf. chapter 10.1). As described, the amino group of asparagine was observed to have a η of -1 at around pH 7.1 *in vitro*, whereas NOE factors of the glutamine and glutamate amino groups were between -1.5 and -3.5 in the physiologically relevant pH range of 7-7.5. If the NOE factors exhibit the same pH dependency *in vivo* as was demonstrated *in vitro*, it would be expected that the signal from the α -amino group of asparagine would be lost, whereas the signals from the amino group of glutamine and glutamate would be enhanced. The signal at 19.6 ppm that was observed in all *in vivo* ¹⁵N NMR spectra of pea root nodules was therefore assigned to the amino groups of glutamine and glutamate.

Experiment no.	Gin amide	Asn amide	Ala	Gin amino	Glu	Asn amino	Asp	GABA	NH4 ⁺
1-7		90.7		19	.6				
8		90.7		19	.6				-3.9
9		90.7		19	.6				-4.2
10		90.7	19,6		18	.4	11.3	-4.1	
11		90.7		19	.6				-4.0
12	[90,7		19	.6				-3.9

Table 8: Assignments of in vivo ¹⁵N chemical shifts in ¹⁵N₂ fixing root nodules^{*} (Fig. 36)

* All chemical shifts are reported relative to urea at 55.8 ppm, which was present as an aqueous solution in a capillary.
Observations from an experiment with Lotus nodules provided further support for the assignment of the resonance at 19.6 ppm to glutamine/glutamate. Two α -amino resonances were observed at 19.6 and 18.4 ppm, respectively, in an *in vivo* ¹⁵N NMR spectrum from Lotus nodules (Exp. 10 in Fig. 36 and Table 8). The chemical shift difference between asparagine and aspartate had been demonstrated to be around 0.6 ppm (cf. Table 5), and it was therefore unlikely that the resonances at 19.6 and 18.4 ppm originated from asparagine and aspartate. Whether the resonance at 18.4 ppm should be assigned to asparagine or aspartate could not be decided purely based on the chemical shift value, but it scemed likely that it was asparagine, because this amino acid is so abundant in indeterminate nodules (Ta *et al.* 1986). Consequently, the signal at 19.6 ppm in Lotus nodules seemed to be the α -amino group of either glutamine or glutamate. This suggests that the resonance at 19.6 ppm in pea root nodules also originated from glutamine/glutamate, since all other observed ¹⁵N resonances in Lotus nodules occurred at exactly the same frequencies as in pea nodules.

GABA was observed at the expected 11.6, 11.8 and 11.3 ppm in ¹⁵N NMR spectra from dead pea root nodules, an extract from nodules and metabolically active, ¹⁵N₂-fixing *Lotus* nodules, respectively (cf. Table 7 and Exp. 10 in Table 8). The GABA ¹⁵N resonances in spectra from nodules seemed to be broader than resonances from other amino acids. However, GABA was never observed in *in vivo* ¹⁵N NMR spectra from ¹⁵N₂ fixing pea nodules (cf. Figs. 36 and 40), although IP-RPLC MS analyses demonstrated very high ¹⁵N labelling of GABA in these nodules (see Table 10 and Fig. 52). Possible reasons for this apparent inconsistency will be discussed in chapter 12.

The chemical shift of ¹⁴N₂ in aqueous solution has been reported to be 290 ppm (converted to a scale that puts urea at 55.8 ppm) (Joy *et al.* 1997). ¹⁴N and ¹⁵N measurements are interchangeable since primary isotope effects are negligible (Mason 1996), so ¹⁵N₂ would be expected to resonate at the same frequency in ¹⁵N spectra. However, I normally recorded *in vivo* NMR spectra with a spectral window that excluded the observation of signals from ¹⁵N₂, because no relevant information would be provided by a possible signal from ¹⁵N₂. I tested an expanded spectral window in a single experiment and observed no additional signals, so the amount of dissolved ¹⁵N₂ within the volume of the NMR tube was probably too small to exceed the detection limit.

10.4 Time course of ¹⁵N assimilation in ¹⁵N₂ fixing root nodules

Fig. 40 shows a representative series of consecutive *in vivo* ¹⁵N NMR spectra that were started immediately after addition of $^{15}N_2$ at time zero. An ammonium signal at -4.2 ppm was evident during the first hour of incubation and increased in intensity throughout the first five hours, after which steady state seemed to occur. Two more ^{15}N signals emerged in the spectrum representing the period from one to three hours and likewise increased in intensity in the next spectrum from three to five hours. The intensities of these two signals were unchanged in the last spectrum from five to seven hours indicating steady state. The signal at 90.7 ppm was assigned to the amide group of asparagine (cf. chapter 10.3). The amino groups of glutamate and glutamine resonate in a 0.2 ppm interval around 19.7 ppm and are difficult to resolve *in vivo*, so the resonance at 19.6 ppm is



likely to contain contributions from the amino group of glutamine as well as glutamate (cf. chapters 10.1 and 10.3).

Fig. 40: In vivo ¹⁵N NMR spectra showing a time course of nitrogen assimilation in ¹⁵N₂ fixing pea root nodules. Treatment of nodules, perfusion conditions and NMR acquisition parameters were as described in the article manuscript The numbered peaks may be assigned to: 1, asparagine amide-N; 2, glutamine/glutamate amino-N; 3, ammonium. The signal at 55.8 ppm is from the reference compound ¹⁵N-urea, which was present as an aqueous solution in a capillary.

10.5 Acquisition of in vivo ¹⁵N NMR spectra

Optimisation of NMR acquisition conditions was crucial in order to obtain the maximal *in vivo* S/N ratio in the shortest possible time in spectra of ¹⁵N labelled metabolites. Signal intensities in ¹⁵N NMR are affected by a multiplicity of factors, of which field homogeneity, T_1 relaxation times and NOE factors are the most important ones under the experimental conditions of the present study.

Carefully shimming of the magnetic field homogeneity improved the S/N ratio of spectra from perfused root nodules dramatically. The effect of time consuming shimming should of course be weighed against the gradual degradation of the living biological material that occurs with time, but I experienced that time spent on shimming was well invested. I used the line width at half height ($v_{1/2}$, cf. chapter 3.2) of the ¹H signal from H₂O routinely as a measure of the field homogeneity to be able to assess whether additional shimming efforts should be invested, and I usually obtained $v_{1/2}$ (H₂O) of 20-30 Hz for perfused pea root nodules.

The optimal sensitivity per unit time depends on the combination of the recycle delay (T_r) between transients and the pulse rotation angle (θ). The rate at which equilibrium magnetisation of a given nucleus recovers after a perturbation is characterised by the time constant T₁ (cf. chapter 3.2), and the maximum signal is obtained for a rotation angle $\theta_{\rm E}$, known as the Ernst angle (Cavanagh *et al.* 1996), which is given by:

$$\cos \theta_{\rm E} = \exp \left(-T_{\rm r} / T_{\rm l}\right) \tag{Eq. 9}$$

The choice of the appropriate Ernst angle requires that T_1 is known or can be determined, which is not often the case for *in vivo* experiments. *In vivo* T_1 s for ¹⁵N amino acids in different biological systems have been reported (see Table 9), and most T_1 s are around 2-5 s albeit with a lot of variation. Based on this information I have chosen to use a recycle delay of 2 s and a 60° rotation angle, which according to Eq. 9 is the most efficient combination for nuclei with a T_1 of 2 s. I tested different combinations of θ and T_r for obtaining spectra of ¹⁵N enriched dead nodules, and confirmed that the chosen acquisition parameters were the most efficient. Different nuclei have different T_1s and especially ammonium has been shown to exhibit an extremely long T_1 *in vivo* (cf. Table 9). This implies that the choice of a particular set of time optimised acquisition parameters will result in biased spectra, where for instance the intensity of the ammonium signal will be suppressed relative to the other signals.

The proton decoupling efficiency is another important consideration in obtaining optimal sensitivity in *in vivo* ¹⁵N spectra, and I tested different decoupling regimes on ¹⁵N enriched dead nodules (data not shown). I observed that the most intense ¹⁵N signals from amino acids in dead nodules were obtained with full broadband decoupling under both the acquisition period and the delay. When ¹⁵N NMR spectra are acquired with full proton decoupling the individual NOE factors of ¹⁵N containing molecules will strongly influence the size of the observed signals (cf. chapter 3.3). The use of full proton decoupling in my *in vivo* experiments was intended to produce maximal signal enhancement by NOE, but I realised very late that NOE factors for some of the ¹⁵N groups was maybe of an unfavourable size under *in vivo* conditions causing signal intensities to diminish dramatically (cf. chapter 10.1 and 10.3).

¹⁵N NOE factors are special because they may be negative and amount to a value as numerically large as -4.9 resulting in a signal inversion and net enhancement factor of 3.9. However, the NOE factor observed depends on the proportion of the relaxation that takes place by ¹⁵N/¹H dipolar interaction. If this proportion is small the signal intensity is diminished and the signal may even vanish entirely. Several mechanisms have been identified by which ¹⁵N signal can be lost on proton decoupling (reviewed by Mason 1996). The dipolar process diminishes when more efficient modes of relaxation are possible, as in the presence of paramagnetic compounds or averaging by fast chemical exchange. Paramagnetic ions are known to bind much more easily to the α -amino (and the carboxylate) than to an amide group, and thus cause a reduction of the NOE factor for the former *in vivo* (Tesch *et al.* 1999) (see Table 9). The peak area per ¹⁵N concentration in bacterial cells was found to be equal for the α -amino nitrogens of glutamine and glutamate, but was 1.5 times larger for the amide nitrogen of glutamine due to different NOEs (Tesch *et al.* 1999). Likewise, it was observed that NOEs were very different for the different N nuclei in arginine, which is known to be sequestered in vacuoles in *Neurospora crassa* mycelium (see Table 9). Large or highly associated molecules have increased correlation times and this also leads to a decrease in dipole-dipole relaxation and a shrinking NOE factor. This effect is more pronounced at higher fields.

	Biological system	Gln- amide	δ-Arg	ω−Arg	αArg	Pro	Ala	α-amine groups"	GABA*	NH₄⁺	Ref.
	White spruce buds	3.10 (0.11)	5.95 (0.10)	2.74 (0.02)		7.76 (0.87)	6.75 (0.26)	4.24 (0.02)	2.62 (0.10)		1
T ₁ (s) (S.D.)	White spruce tissue culture	3.19 (0.08)					4.44 (0.12)	4.37 (0.12)	1.95 (0.18)		2
	Neurospora crassa mycelium	4.05	1.2	1.1	1.3		5.1			_	3
	Corynebacterium glutamicum					-				>50	4
NOE factor	White spruce buds	-4.31	-4.00	-4.07		0	-3.85	-3.90	-5.13		1
	White spruce tissue culture	-4.37					-3.83	-3.99	-4.94		2
	Neurospora crassa mycelium	-4.9	-2.5	-4.6	-2.7		-4.6			ŀ	3

Table 9: Literature values of in vivo T₁s and NOE factors for amino acids

References: 1) Thorpe et al. 1989. 2) Joy et al. 1997. 3) Kanamori et al. 1982b. 4) Tesch et al. 1999

⁴Mainly α-amino groups of glutamine and glutamate

*Possibly with contributions from other aliphatic amines; ornithine and lysine

I considered whether indirect detection of ¹⁵N labelled metabolites would be potentially advantageous for increasing the sensitivity. The idea was, however, discarded because indirect detection demands that the protons attached to the ¹⁵N nucleus are in slow exchange, which would mean that only amide groups and not amino groups and ammonium would be observed in *in vivo* experiments (cf. chapter 10.1). ¹⁵N detection by the HMQC technique has previously been used with tissue extracts, where indirect detection of amino-N groups is possible over a limited range of acidic pH values (reviewed by Ratcliffe and Shachar-Hill 2001).

In principle, ¹⁵N metabolites may be quantified by integration of NMR spectra, but there are some important considerations in obtaining reasonably reliable integrals (reviewed by Sanders and Hunter 1993):

- All the signals to be integrated must have the same intrinsic intensity. In ¹⁵N spectroscopy this means controlling or suppressing all NOEs very carefully.
- It is absolutely essential that the data be collected under conditions, which allow uniform recovery of all signals that are to be integrated. This usually means, if $\pi/2$ pulses are being used

to read the state of the system, a relaxation delay of at least five times the longest T_1 must be allowed between successive pulses.

 It is particularly dangerous to integrate resolution-enhanced (for instance by exponential multiplication) spectra because their resolution enhancement is derived from the fact that broader lines are suppressed.

As described, I have put an effort into obtaining the maximal ¹⁵N signal intensity in the shortest possible time and this means that none of the above mentioned criteria for possible quantification of ¹⁵N metabolites are met. Relative levels of ¹⁵N metabolites may be estimated tentatively based on the obtained *in vivo* ¹⁵N NMR spectra but should be used with caution.

11 Nitrogen assimilation studied by mass spectrometry

Mass spectrometry is one of the principal methods for analysing stable isotope labelling, and has the advantage of increased sensitivity compared to NMR. The original intention for using mass spectrometric methods in the present project, was to provide complementary information on the ¹³N labelling of amino acids in root nodules in order to be able to assign *in vivo* ¹⁵N NMR resonances unequivocally. However, it turned out to be complicated to obtain information on the positional distribution of the ¹⁵N label in glutamine and asparagine, and new techniques had to be developed (cf. chapter 11.2). Fortunately, advanced spectrometric equipment and expertise was present in our laboratory, which made it possible to design and explore new approaches. The successful application of these new techniques finally provided information that would have been overseen if using ¹⁵N NMR on its own.

11.1 GC-MS analyses of labelling

The separation of amino acids by GC is a standard procedure, and analysis of stable isotopes can be done by mass spectrometry in the form of GC-MS. In general, detection involves post-column derivatisation. I started out by setting up a GC-MS procedure for analysis of ¹⁵N in amino acids (see protocol in appendix), which was a combination of procedures that had previously been described in the literature (Johansen *et al.* 1996; Fortier *et al.* 1986). This procedure was used for all results presented in this chapter, but had several disadvantages:

The derivatisation was based on silvlation, which caused a pronounced increase in intensity of the natural abundance [M+1] and [M+2] ions due to ¹³C and ²⁹Si and made the determination of low levels of ¹⁵N labelling troublesome. Furthermore, the silvlation of glutamine was extremely difficult, and the very low yield of glutamine derivates hindered the quantification of the ¹⁵N labelling in many cases. Finally, there is a need for two derivatisation steps to distinguish amide

and amino labelling in glutamine and asparagine, and this was left undone because of the problems with incomplete derivatisation of glutamine.

A chromatogram of a root nodule extract is shown in Fig. 41, and it is evident that the extract

contained a multitude of compounds, identified as well as unidentified ones. An important result of the GC-MS analyses was that significant ¹⁵N labelling was only found in a few amino acids: asparagine, aspartate, glutamine, glutamate, alanine and GABA (data not shown). This was reproduced in very many analyses, and made it reasonable and convenient to focus on these six amino acids throughout the analytical work with all other applied methods. From the knowledge of nitrogen assimilation pathways in root nodules (cf. chapter 2.4 and 2.5) it would likewise be expected that these were the relevant amino acids, but experimental evidence ensured that it was safe to exclude other amino acids from the analyses presented in chapter 11.2.



Fig. 41: GC-MS chromatogram of a root nodule extract. Amino acids were identified from retention times and mass spectra by comparison with authentic standards. Extraction and derivatisation procedures as well as GC-MS parameters are given in the protocol in appendix.

Nodules that had been incubated with ${}^{15}N_2$, and studied by ${}^{15}N$ NMR spectroscopy after harvesting and quenching of metabolic activity by freezing in liquid nitrogen. (Figs. 37 and 38) were also analysed by GC-MS (see Fig. 42). The amount of labelling varied a lot among amino acids and between experiments. At the time when these experiments were performed, the analysis of total amino acid pool sizes was not yet possible. A comparison between the NMR spectra and the ${}^{15}N$ atom% enrichments is thus difficult, because the pool sizes will determine the total amount of ${}^{15}N$ label to be found in each amino acid. However, the results encouraged further work because they demonstrated that a significant proportion of the amino acid pools were accessible for ${}^{15}N$ labelling and could be detected by NMR.





11.2 Ion-pair reverse-phase liquid chromatography mass spectrometry (IP-RPLC MS)

Only the more hydrophobic amino acids can be separated using classic reverse-phase chromatography. However, ion-pair reverse-phase liquid chromatography (IP-RPLC) has been reported to constitute a promising method for analysis of the most polar amino acids, like aspartate, asparagine, glutamate and glutamine. Perfluorinated carboxylic acids have been reported to be excellent ion-pairing reagents for this purpose (Petritis *et al.* 1999; Kwon and Moini 2001). In addition, electro-spray ionisation mass spectrometry can be used for studying the individual amino acids using those systems (Petritis *et al.* 2000).

We started up the IP-RPLC separation technique of amino acids in our laboratory and extended this method to include an on-line determination of the amount of ¹⁵N labelling based on the MS ion pattern of the individual amino acid. Analysis of small amounts of labelling could be done with enhanced sensitivity and accuracy compared to the analyses described in chapter 11.1, because the natural abundance background constitutes a smaller proportion of mass intensities in spectra of underivatised ¹⁵N enriched amino acids.

Furthermore, we have developed a new technique, whereby also the <u>position</u> of the ¹⁵N labelling of glutamine (Gln) and asparagine (Asn) in either the amide or the amino group could be analysed in the same experiment based on MS/MS and MS/MS/MS, respectively. The fragmentation paths were elucidated by studies of standard samples of ¹⁵N mono labelled Gln and Asn with a specific

position of the label in either the amide or the amino group (isotopomers). The theoretical background and our experimental experiences with the positional labelling analysis will be presented in the following. Extracts from ¹⁵N enriched root nodules were analysed by this new method and the obtained results will be presented. The details of the IP-RPLC MS materials and methods are given in the article manuscript.

Position of ¹⁵N label in glutamine

The position of the ¹³N labelling of Gln in either the amide or the amino group was determined by MS/MS experiments. The mass of the parent molecular Gln ion (MH⁺) is 147, and with one ¹⁵N in the amino or amide group the mass becomes 148. This mono labelled precursor ion is trapped and subjected to collision activation whereby NH₃ is lost (Fig. 43) in agreement with previous studies



Fig. 43: Sketch of the fragmentation pattern of mono ¹⁵N labelled glutamine when subjected to collision activation.

of the fragmentation of protonated Gln (Dookeran *et al.* 1996). The lost NH_3 may or may not contain the ¹⁵N label (i.e. mass 18 or 17 is lost), and the resulting MS/MS spectrum (Fig. 44) will therefore contain the daughter ions 130 and 131 in a proportion that reflects the position of the ¹⁵N label.



Fig. 44; Reference MS/MS spectra of 100% mono ¹⁵N labelled glutamine isotopomers.

NH₃ is lost from the amide as well as from the amino group but in <u>unequal</u> amounts. Inherent chemical properties of the molecule determine the tendency to loose either of the groups, irrespectively of the position of the ¹⁵N label, because isotope effects of heavy nuclei like ¹⁵N are negligible (Levsen 1978). By inspection of the MS/MS spectra from samples of 100% ¹⁵N enriched Gln isotopomers (Fig. 44), it can be calculated that 84% of the NH₃ loss comes from the amide group and the remaining 16% of course from the amino group. This is in excellent agreement with recent theoretical calculations on the fragmentation mechanisms of protonated α -amino acids, which predict the elimination of NH₃ to be an energetically strongly favoured reaction of protonated Gln (Rogalewics *et al.* 2000). The estimated activation energies for NH₃ loss from the amino and amide groups were 164 and 144 kJ/mol, respectively.

The ratio of the intensities of the ions m/z 130 and 131 in MS/MS spectra for samples containing different amounts of ¹⁵N label in the amino or amide group, respectively, is given by the following equation:

$$\left(\frac{130}{131}\right)_{\text{meas.}} = \frac{(148)_{\text{n.e.}} \cdot F(130)_{\text{n.e.}} + (148)_{15N} \left(x \cdot F(130)_{\text{amino}} + (1-x) \cdot F(130)_{\text{amide}}\right)}{(148)_{\text{n.e.}} \cdot F(131)_{\text{n.e.}} + (148)_{15N} \left(x \cdot F(131)_{\text{amino}} + (1-x) \cdot F(131)_{\text{amide}}\right)}$$
(Eq. 10)

where experimentally determined constants are:

 $(148)_{n.s.}$ = intensity of m/z 148 natural abundance (normalised against m/z 147) = 7.44

 $F(130)_{n.a.}$ / $F(131)_{n.a.}$ = fractions of intensities of m/z 130 and 131, respectively, in unlabelled sample = 0.06 / 0.94

 $F(130)_{amino} / F(131)_{amino} \approx$ fractions of intensities of m/z 130 and 131, respectively, in 100% ¹⁵N amino labelled sample = 0.16 / 0.84

 $F(130)_{amide} / F(131)_{amide} =$ fractions of intensities of m/z 130 and 131, respectively, in 100% ¹⁵N amide labelled sample = 0.84 / 0.16

and variables are:

(130/131)_{meas.} = measured ratio of intensities of m/z 130 and 131

 $(148)_{15N}$ = measured intensity of m/z 148 (normalised against m/z 147) minus $(148)_{na}$

x = motar ratio of ¹⁵N amino labelled Gln molecules

 $1 \cdot x = molar ratio of ¹⁵N amide labelled Gln molecules$

Simulated standard curves based on Eq. 10 are shown in Fig. 45. Dilution series of isotopomeric ¹⁵N Gln standards were subjected to the analysis in order to test the procedure, and the experimental data are plotted in Fig. 45. It is obvious that the ratio of intensities 130/131 is not very sensitive to changes in the amount of amino ¹⁵N label, because only a minor part of the NH₃ lost from the 148 ion comes from the amino group. The ¹⁴NH₃ loss from the amide group, in molecules with ¹⁵N-amino labelling, can be considered as a sort of a large background value for the 131 ion, upon which the ¹⁴NH₃ from the amino group is superimposed. On the other hand, changes in the amount



Fig. 45: The ratio of the ions m/z 130 and 131 in MS/MS spectra at a given ¹⁵N enrichment of the mono ¹⁵N labelled glutamine molecule (atom% excess) depends on the position of the ¹⁵N label in either the amino or the amide group. Simulated standard curves based on Eq. 10 are shown as well as experimental data from analysis of dilution series of isotopomeric ¹⁵N glutamine standards. IP-RPLC MS procedures and parameters are given in the article manuscript.

of ¹⁵N labelling in the amide group will result in large changes in the 130/131 ratio.

Position of ¹⁵N label in asparagine

The position of the ¹⁵N labelling of Asn in either the amide or the amino group could be determined by MS/MS/MS experiments. The mass of the parent molecular Asn ion (MH⁺) is 133 and with one ¹⁵N in the amino or amide group the mass becomes 134. When this mono labelled precursor ion is trapped and subjected to collision activation it does <u>not</u> loose NH₃ like Gln, but instead the carboxylic acid group (CO + H₂O, M=46) is eliminated (Fig. 46). Thus, the fragmentation of protonated Asn resembles that of simple aliphatic amino acids, whereas the fragmentation of



Fig. 46: Sketch of the fragmentation pattern of mono ¹³N labelled asparagine when subjected to two tounds of collision activation.

protonated Gln seems to be driven by the formation of protonated pyroglutamic acid by loss of NH₃. In addition, the shorter carbon backbone of Asn opens up for stabilisation of the amide group by hydrogen bonding between the amide oxygen and the protonated amino group. This difference between the two amino acids Gln and Asn, which seem very alike when judged from the chemical structure, was very surprising!

The daughter ion resulting from the carboxylic acid loss has a mass of 88 and when trapped and subjected to another round of collision activation, HNCO is lost from the amide group. If the amide group contains ¹⁵N, the loss will be 44, and the remaining "grand-daughter" ion will weigh 44. If the ¹⁵N label is in the amino group, only mass 43 is lost with the amide group, and a "grand-daughter" ion of mass 45 is created. Thus, the ratio between the ions 44 and 45 in the MS/MS/MS spectrum (Fig. 47) contains information on the position of the ¹⁵N label.



Fig. 47: Reference MS/MS/MS spectra of 100% mono ¹⁵N labelled asparagine isotopomers.

The ratio of the intensities of the ions m/z 44 and 45 in MS/MS/MS spectra for samples containing different amounts of ¹⁵N label in the amino or amide group, respectively, is given by the following equation:

$$\left(\frac{44}{45}\right)_{\text{meas.}} = \frac{(134)_{n.a.} \cdot F(44)_{n.a.} + (134)_{15N} \left(x \cdot F(44)_{\text{amino}} + (1-x) \cdot F(44)_{\text{amide}}\right)}{(134)_{n.a.} \cdot F(45)_{n.a.} + (134)_{15N} \left(x \cdot F(45)_{\text{amino}} + (1-x) \cdot F(45)_{\text{amide}}\right)}$$
(Eq. 11)

where experimentally determined constants are:

 $(134)_{n,k}$ = intensity of m/z 134 natura) abundance (normalised against m/z 133) = 5.25

 $F(44)_{n.s.}$ / $F(45)_{n.s.} =$ fractions of intensities of m/z 44 and 45, respectively, in unlabelled sample = 0.28 / 0.72

 $F(44)_{amino}$ / $F(45)_{amino}$ = fractions of intensities of m/z 44 and 45, respectively, in 100% ¹⁵N amino labelled sample = 0.20 / 0.80

 $F(44)_{smide}$ / $F(45)_{smide}$ = fractions of intensities of m/z 44 and 45, respectively, in 100% ¹⁵N amide labelled sample = 0.84/0.16

and variables are:

 $(45/45)_{mean}$ = measured ratio of intensities of m/z 44 and 45

 $(134)_{15N}$ = measured intensity of m/z 134 (normalised against m/z 133) minus $(134)_{n_A}$

x = molar ratio of ¹⁵N amino labelled Asn molecules

 $1 - x = molar ratio of {}^{15}N$ amide labelled Asn molecules

Simulated standard curves based on Eq. 11 are shown in Fig. 48. Mono labelled isotopomeric ¹⁵N Asn standard samples were analysed and the obtained data are shown in Fig. 48. The measured 44/45ratios for ¹⁵N amino labelled Asn standards agreed well with the simulated curve, whereas the experimental data for ¹⁵N amide labelled standards displayed more variation.



Fig. 48: The ratio of the ions m/z 44 and 45 in MS/MS/MS spectra at a given ¹⁵N enrichment of the mono ¹⁵N labelled asparagine molecule (atom% excess) depends on the position of the ¹⁵N label in either the amino or the amide group. Simulated standard curves based on Eq. 11 are shown as well as experimental data from analysis of dilution series of isotopometic ¹⁵N asparagine standards. IP-RPLC MS procedures and parameters are given in the article manuscript.

Analysis of root nodule extracts

The developed IP-RPLC MS technique was used successfully for determining the amount and position of ¹⁵N labelling of root nodule amino acids. The technique has been used for several analyses, but all the results presented in the following chapter are obtained from a single representative sample of root nodules. These nodules were incubated for eight hours with ¹⁵N₂ in

the perfusion system while the *in vivo* ¹⁵N NMR spectra shown in Fig. 40 were recorded. Nodules were subsequently extracted and the extract could be analysed directly after a minor purification process (see article manuscript). The ¹⁵N enrichment (= atom% excess) was calculated by Eq. 12 from the intensities of the molecular ions MH⁺ and MH⁺+1 in MS spectra for each of the relevant amino acids (see Table 10). Parallel analyses of the ¹⁵N enrichment by GC-MS and IP-RPLC MS showed good agreement as seen from Fig. 49.

Atom% excess ¹⁵N, APE =
$$\frac{R_s - R_{n.a.}}{1 + R_s - R_{n.a.}} \cdot 100\%$$
 (Eq. 12)

where R_s and $R_{n.a.}$ are ratios of intensities of the ions [MH⁺+1]/[MH⁺] in the sample under study and an unlabelled sample, respectively.



Fig. 49: ¹⁵N enrichment (atom% excess) of amino acids in pea root nodules determined by GC-MS and IP-RPLC MS. Nodules were extracted (see article manuscript) after eight hours incubation with $^{15}N_2$ in the perfusion system and acquisition of the *in vivo* ¹⁵N NMR spectra shown in Fig. 40. Subsamples of extract were analysed by GC-MS (see protocol in appendix) and IP-RPLC MS (see article manuscript). GC-MS results are given as the mean of two replicates, whereas the number of replicates varied a lot for IP-RPLC MS results. From left to right n = 11, 12, 2, 5, 4, 7, 10, 5. Error bars denote standard errors.

The intensities of the Gln ions m/z 130 and 131 and the Asn ions m/z 44 and 45 in MS/MS and MS/MS/MS spectra, respectively, were measured in single ion scan mode in order to obtain the highest possible sensitivity (see Fig. 50). The measured ion ratios are plotted as a function of the amount of mono ¹⁵N labelling in Fig. 51. By comparison with the simulated standard curves it is seen that the mono ¹⁵N labelling in Gln seems to be located almost exclusively in the amino position, whereas the ¹⁵N in mono labelled Asn is found in both positions, although mainly in the amino position. The exact values of the distribution between amino and amide mono ¹⁵N labelled molecules were calculated by Eq. 10 (Gln) and 11 (Asn) and are presented in Table 10.



Fig. 50: Chromatograms showing single ion mass scans of (A) m/z 131 and 130 after collision activation of mono ¹⁵N labelled glutamine (m/z 148) and (B) m/z 45 and 44 after two rounds of collision activation of mono ¹⁵N labelled asparagine (m/z 134) and the daughter ion (m/z 88), respectively. The analyses were performed on a sample of pea root nodule extract as described in the article manuscript.



Fig. 51: Determination of the position of ¹⁵N labelling in (A) glutamine and (B) asparagine in pea root nodule extracts from ratios of ions m/z 130 and 131 in MS/MS spectra and ions m/z 44 and 45 in MS/MS/MS spectra, respectively. The ¹⁵N enrichment of the mono ¹⁵N labelled amino acids (atom% excess) was calculated by Eq. 12. IP-RPLC MS procedures and parameters are given in the article manuscript. Error bars denote standard error (numbers of replicates are given in

When the position of the ${}^{15}N$ mono label had been determined, all the data were available for calculating the much-coveted total amount of ${}^{15}N$ in each of the amino acid groups (see Table 10 and Fig. 52).

GABA, glutamate and alanine were the most highly ¹³N enriched compounds after the eight-hour

incubation period with ¹⁵N excess of 33.4, 27.9 and 25.0 atom%, respectively, but all examined amino acids were found to be significantly ¹⁵N labelled. When the pool sizes were taken into account, asparagine and GABA were found to contain most of the ¹⁵N label of soluble amino acids in the nodule tissue, namely 2.03 and 1.91 µmol ¹⁵N g⁻¹ FW, respectively. Alanine and glutamate contained a minor part of the total ¹⁵N label (0.31 and 0.10 µmol ¹⁵N g⁻¹ FW, respectively) despite the high atom% excess, because of their smaller pool sizes. Asparagine was ¹⁵N labelled in both the amino group (1.33 µmol ¹⁵N g⁻¹ FW) and the amide group (0.70 µmol ¹⁵N g⁻¹ FW). Glutamine was also more labelled in the amino group (0.34 µmol ¹⁵N g⁻¹ FW) than in the amide group (0.11 µmol 15N g-1 FW).



Fig. 52: Concentrations of unlabelled, ¹⁵N-amino-labelled and ¹⁵N-amide-labelled soluble amino acids in pea root nodules after an eight-hour incubation period with ¹⁵N₂ in the perfusion system and acquisition of the *in vivo* ¹⁵N NMR spectra shown in Fig. 40.

Total aminu acid conc. (µmol/g FW)		Atom% excess ¹⁵ N (APE)				Position of mono ¹⁵ N label (%)				Total ¹⁵ N concentration (µmol/g FW)	
16.91	1.65	mono ¹³ N	8,17	0.27	11	amino	73	6	6	amino	1.33
					1	amide	27	6	6		
		double ¹⁵ N	1.91	0.12	12					amide	0.70
5.72	0.67		33.39		2						1.91
1.55	0.20	mono ¹⁵ N	15.74	2.08	5	amino	98	2	3	amino	0.34
1					Í	amide	2	2	3		
		double ¹⁵ N	6.59	1.10	4					amide	0.11
1.24	0.12		25.02	0.80	7						0.31
0.76	0.05		14.22	1.27	10						0,11
0.35	0.04		27.86	0.98	5	<u> </u>				_ 	0.10
	Total ar acid co (μmol/g Mean ^r 16.91 5.72 1.55 1.24 0.76 0.35	Total amino acid conc. (μmol/g FW) Mean ^μ 5.72 0.67 1.55 0.20 1.24 0.76 0.35	Total amino Atom9 acid cone. (μmol/g FW) (# Mean ^µ S.E. (# 16.91 1.65 mono ¹⁵ N double ¹⁵ N	Total aminu Atom% excess acid conc. (μrnol/g FW) (APE) Mean ^µ S.E. Mean 16.91 1.65 mono ¹⁵ N 8.17 double ¹⁵ N 1.91 5.72 0.67 33.39 1.55 9.20 mono ¹⁵ N 15.74 double ¹⁵ N 15.74 4000000000000000000000000000000000000	Total aminu Atom % excess ^{15}N acid conc. (μ rnol/g FW) (APE) Mean ^a s.e. Mean s.e. 16.91 1.65 mono ^{15}N 8.17 0.27 double ^{15}N 1.91 0.12 5.72 0.67 33.39 1.55 0.20 mono ^{15}N 15.74 2.08 double ^{15}N 6.59 1.10 1.24 0.12 25.02 0.80 0.76 0.05 14.22 1.27 0.35 0.04 27.86 0.98	$\begin{array}{ c c c c c c c c c c c c c c c c c c c$	Total aminu Atom % excess ^{15}N Posit acid conc. (APE) (APE) Mean ^a S.E. Mean S.E. n 16.91 1.65 mono ^{15}N 8.17 0.27 11 amino acid conc. double ^{15}N 1.91 0.12 12 12 5.72 0.67 33.39 2 1.55 2.08 5 atmino 1.55 0.20 mono ^{15}N 15.74 2.08 5 atmino 1.24 0.12 25.02 0.80 7 0.76 0.05 14.22 1.27 10 0.35 0.04 27.86 0.98 5 5 5	Total aminu Atom % excess ${}^{15}N$ Position of π acid conc. (μ mol/g FW) (APE) (%) Mean ^a S.E. Mean S.E. Nean 16.91 1.65 mono ${}^{15}N$ 8.17 0.27 11 arnino 73 double 15N 1.91 0.12 12 12 11 arnino 98 5.72 0.67 33.39 2 11.55 arnino 98 arnide 2 1.55 0.20 mono ${}^{15}N$ 15.74 2.08 5 atmino 98 amide 2 2 25.02 0.80 7 110 4 1.24 0.12 25.02 0.80 7 12 12 127 10 0.35 0.04 27.86 0.98 5 5 5 5	Total aminu Atom % excess ^{15}N Position of mono acid conc. (μ rnol/g FW) (APE) (%) Mean ^a S.E. Mean S.E. Nean S.E. (%) 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 15N 1.91 0.12 12 12 12 12 12 12 12 12 12 12 12 133.39 2 133.39 2 14.22 12 12 12 13 13 14 12 14 12 14 12 14 14 12 14 12 12 12 10 14 12 12 12 12 12 12 12 12 12 12 12 12 12 12 13 <	Total aminu acid conc. Atom % excess ¹⁵ N Position of mono 1 ⁵ N label $(\mu mol/g FW)$ (APE) (%) Mean ^a S.E. Mean S.E. Mean S.E. Mean S.E. N 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 double 15N 1.91 0.12 12 12 12 12 12 5.72 0.67 33.39 2 1 15N 191 0.12 12 12 12 1.55 0.20 mono 15N 15.74 2.08 5 amino 98 2 3 double 15N 6.59 1.10 4 1 2 2 3 1.24 0.12 25.02 0.80 7 1 <td< td=""><td>Total aminu Atom % excess ¹⁵N Position of mono Total aminu acid conc. (μmol/g FW) (APE) (%) (μmol/g FW) Mean^r S.E. Mean S.E. N Mean S.E. n 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 11.55 0.20 mono 15N 1.91 0.12 12 </td></td<>	Total aminu Atom % excess ¹⁵ N Position of mono Total aminu acid conc. (μ mol/g FW) (APE) (%) (μ mol/g FW) Mean ^r S.E. Mean S.E. N Mean S.E. n 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 16.91 1.65 mono 15N 8.17 0.27 11 amino 73 6 6 amino 11.55 0.20 mono 15N 1.91 0.12 12

n = 3

"The values given for amino and amide group total ¹⁵N concentration include both mono and double labelled molecules

12 Discussions and conclusions

Development of new methodologies

A considerable part of my project time has been devoted to developing and optimising new methodologies. It turned out to be more challenging and complicated to investigate introgen fixation and assimilation in ¹⁵N₂ fixing root nodules by *in vivo* ¹⁵N NMR spectroscopy, than anticipated from the beginning. However, at the end of the project period a whole range of costummade equipment and analytical techniques, which supplemented each other well, were available.

The developed perfusion system (cf. chapter 7) fulfilled its purpose well of maintaining root nodules in a physiologically functional and nitrogen fixing state for long incubation periods of up to 10 hours. In vivo ³¹P NMR spectroscopy provided a convenient and excellent means for monitoring the physiological state. Furthermore, the perfusion system made it possible to supply nodules with highly ¹⁵N enriched N₂ gas dissolved in the circulating buffer during the incubation period.

The experimental set-up was optimised to ensure that the oxygen supply experienced by the root nodules was not inhibiting the oxygen labile nitrogenase enzyme complex but still adequate for maintaining the high oxidative phosphorylation level needed to support nitrogen fixation (cf. chapters 8 and 10.2). 50% oxygen was found to be optimal. The maximal nitrogen fixation rate of nodules in the perfusion system was substantially reduced as compared to nodules on the intact plant root system, but still adequate for sufficient incorporation of ${}^{15}N_2$ to allow detection of ${}^{15}N$ labelling by ${}^{15}N$ NMR.

A time course of *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing pea root nodules has been recorded for the first time. The interpretation of information provided by the ¹⁵N spectra proved difficult, and will be discussed further below, and revealed some of the limitations in the use of *in vivo* ¹⁵N NMR spectroscopy. Nevertheless, it was demonstrated that it was indeed possible to apply *in vivo* ¹⁵N NMR spectroscopy to the study of nitrogen fixation and assimilation in root nodules, which has never been reported before.

We developed new mass spectrometric techniques (cf. chapter 11) involving separation of amino acids by IP-RPLC, whereby it was possible to analyse the position of ¹⁵N in mono labelled glutarnine and asparagine by using MS/MS and MS/MS/MS, respectively. The analysis could be done in a single experiment without any previous derivatisation procedures, which is a major simplification and improvement compared to GC-MS procedures, which are usually used for analysis of positional labelling.

In vivo ¹⁵N-ammonium NMR signal in root nodules

The ¹³N NMR ammonium signal in living pea and *Lotus* root nodules was in several experiments observed at around -4 ppm, which is an unusually low ammonium frequency. Previous *in vivo* ¹⁵N

NMR studies have all demonstrated ammonium resonances at around 0 ppm in biological systems as diverse as maize root tips (Amancio and Santos 1992), carrot cells (Fox *et al.* 1992), *Corynebacterium glutamicum* (Tesch *et al.* 1999) and ectomycorrhizal mycelium (Martin *et al.* 1994). The observed enhanced shielding of ammonium can be due to many different factors such as hydrogen bonding to anions or pH effects, and may thus provide valuable information on the intracellular environment of root nodules.

I investigated some of these possibilities *in vitro*, but observed no significant effect of phosphate concentrations up to 1 M on the ammonium chemical shift. Base titration of ammonium *in vitro* demonstrated, as expected, large pH effects on the chemical shifts, and an ammonium chemical shift of -4 ppm was observed at pH 8.9. From the present investigation it was not possible to localise the ammonium pool to the bacteroid or plant cytoplasm, but in either case it seems most unlikely that the pH could be that alkaline in a tissue that is metabolically active. Streeter (1989) estimated that free ammonium in soybean nodules was primarily confined to bacteroids, and it has been anticipated that the bacteroid cytoplasm is slightly alkaline (Day *et al.* 2001), because of the proton pumping activity of the electron transport chain in the bacteroid inner membrane as well as the proton consuming nitrogenase activity. It is thus possible that a minor contribution to the shielding of the observed ¹⁵N NMR ammonium signal was due to a higher pH in the bacteroids. However, the major cause for the shielding remains unidentified.

The free ammonium concentration in symbiotic root nodules has never been solidly quantified. Based on extractions and several assumptions Streeter (1989) estimated that soybean bacteroids contained 1.6 µmol ammonium g⁻¹ FW nodule, whereas the concentration of ammonium in the plant cytosol was essentially nil. The ¹⁵N NMR acquisition mode of the present study only allowed for a rough estimate of the minimum nodule ammonium concentration. The intensity of the ^{15}N ammonium NMR signal was of a comparable size to the signal from the asparagine amide group after an 8-hour incubation with ${}^{15}N_2$ (Fig. 40). NMR signal intensities are influenced by both T₁ and NOE, which have not been quantified under in vivo conditions in the present study, but from other studies it can be ascertained that both processes would cause the ammonium signal to diminish relative to the asparagine amide signal. In vivo NOE factors for amino acid amide groups have previously been shown to be of the order of -4.5 (cf. Table 9), whereas the ammonium NOE factor is between 0 and -1 in the present study. Amino acid amide groups T₁s of 3-4 s in plant tissue have been reported (cf. Table 9), whereas very long ammonium T₁s of up to 50 s are commonly observed in vivo (Tesch et al. 1999). Thus, the concentration of ¹⁵N ammonium must be at least as large as that of ¹⁵N labelled asparagine amide, which was estimated to constitute 0.7 µmol g⁻¹ FW nodule based on IP-RPLC MS measurements.

In summary, it is evident that substantial, although not quantifiable, ¹⁵N labelling of ammonium took place in the metabolically active pea root nodules in the present study and that ammonium was the first detectable ¹⁵N labelled compound in the intact symbiosis. This is in contrast to recent results by Waters *et al.* (1998), where no ¹⁵N labelling of ammonium could be detected in ¹⁵N₂-fixing soybean bacteroids, whereas alanine was demonstrated to contain 98 atom% excess ¹⁵N, and

to be the primary product excreted by isolated bacteroids, when these were incubated at high bacteroid densities under microaerobic conditions.

Nitrogen assimilation in pea nodules studied by in vivo ¹⁵N NMR and IP-RPLC MS

The IP-RPLC MS analyses were originally intended to provide additional information for the assignment of *in vivo* ¹⁵N NMR resonances, but the obtained results by the two analytical methods turned out to be apparently contradictory regarding some of the ¹⁵N labelled metabolites. It is not easy to ascertain, which of the methods is providing the most complete and correct picture of the ¹⁵N labelling pattern, because both methods have inherent shortcomings and disadvantages. *In vivo* ¹⁵N NMR spectra have the advantage of representing non-invasive measurements, but the levels of ¹⁵N metabolites relate to the signal intensities in an unpredictable way under the acquisition mode that has been applied in the present study. IP-RPLC MS analyses give accurate and quantitative results on the level of ¹⁵N labelling, but the analyses are performed on extracts and thus comprise all the possibilities of artefacts and errors mentioned in chapter 4.

The most conspicuous discrepancy between results from the two methods was that the most abundant amino acid, asparagine, was found to contain about twice as much ¹⁵N labelling in the amino group than in the amide group when analysed by IP-RPLC MS (cf. Table 10). A large asparagine amide resonance was observed in *in vivo* ¹⁵N NMR spectra from pea root nodules in many experiments, but an ¹⁵N amino group NMR signal was never observed *in vivo* (cf. Fig. 36 and 40). However, the asparagine ¹⁵N amino as well as the amide resonance was seen in NMR spectra of nodule extracts.

These apparent inconsistencies were indeed disturbing, until I came across the pH dependence of NOE factors (cf. chapter 10.1). If the NOE factors exhibit the same pH dependency *in vivo* as was demonstrated *in vitro*, it would be expected that the signal from the amino group of asparagine would be lost in *in vivo* ¹⁵N NMR spectra, whereas the signal from the asparagine amide group would be enhanced. This mechanism may thus provide the explanation to make ends meet, and conclude that asparagine is ¹⁵N labelled *in* both the amino and the amide groups, although the amino resonance is invisible in ¹⁵N NMR spectra with full decoupling.

If it is correct that the *in vivo* NOE factor of the asparagine amino group is of an unfavourable size, which actually causes elimination of the signal in spectra recorded with full decoupling, it is surprising that this has not been reported previously, since the majority of *in vivo* ¹⁵N NMR studies applies full decoupling. On the other hand, very few *in vivo* ¹⁵N NMR studies have actually observed a signal from the asparagine amino group (cf. Table 4). To my knowledge, only a single ¹⁵N NMR study, namely the investigation of ¹⁵N ammonium metabolism in duckweeds by Monselise and Kost (1993), demonstrated the presence of ¹⁵N labelling in the amino group of asparagine. Amancio and Santos (1992) assigned an ¹⁵N signal at 18.6 ppm to the amino groups of both asparagine and aspartate, but in my opinion only aspartate would resonate at this low frequency. The absence of reported asparagine ¹⁵N amino signals in the *in vivo* NMR literature may of course reflect that no labelling occurs or that the asparagine pool of the studied organisms was

below the detection limit, but it may also be caused by NOE signal elimination. Very few investigations have included parallel analyses by *in vivo* ¹⁵N NMR and other analytical methods that would reveal a possible NMR invisible asparagine amino signal.

In root nodules the amide group for the biosynthesis of asparagine from aspartate may either come from direct incorporation of ammonium or from the glutamine amide group, and previous results have indicated that both pathways occur in alfalfa nodules (Snapp and Vance 1986; Ta *et al.* 1986). My data indicate that some asparagine synthesis from highly ¹⁵N enriched ammonium is taking place in pea root nodules, since the low level of ¹⁵N amide labelling of glutamine was not sufficient to account for the observed asparagine amide enrichment. The high total ¹⁵N labelling of asparagine is consistent with its generally accepted role as the end product of the primary nitrogen assimilation processes taking place in indeterminate root nodules.

Another obvious difference between results obtained by the different analytical methods was that GABA contained a very large amount of the total ¹⁵N labelling, when analysed by IP-RPLC MS (cf. Table 10), but did not appear in pea nodule *in vivo* ¹⁵N NMR spectra at all (cf. Fig. 36 and 40).

I investigated the *in vitro* pH dependence of the GABA NOE factor, and found no indications that NOE could cause elimination of the ¹⁵N NMR signal from GABA. ¹⁵N GABA has previously been observed by *in vivo* ¹⁵N NMR spectroscopy (cf. Table 4), although some investigators have noted that the signal possibly also contained contributions from ¹⁵N labelled ornithine and lysine. *In vivo* T₁s of GABA have been reported shorter than of other amino acids, and *in vivo* NOE factors for GABA were found to be numerically larger than for other amino acids (cf. Table 9). These two variables affect signal intensities, but under the experimental conditions of the present study, both of the reported values would imply an enhancement and not an attenuation of the ¹⁵N GABA signals relative to signals of other amino acids.

GABA is synthesised from glutamate in a reaction catalysed by the enzyme glutamate α -decarboxylase, which is well known in plant tissue (reviewed by Bown and Shelp 1997) and has also been reported to be present in symbiotic rhizobia bacteroids (Miller *et al.* 1991; Fitzmaurice and O'Gara 1991). The ¹⁵N-labelling of GABA found by IP-RPLC MS could reflect a decarboxylation of glutamate occurring after termination of the *in vivo* ¹⁵N NMR acquisition. However this seems unlikely when considering the high ¹⁵N-enrichment of GABA together with the short time period between acquisition of the last NMR spectrum and the quenching of metabolic activity by transfer of root nodules to liquid nitrogen.

A substantial amount of GABA (labelled as well as unlabelled) was shown to accumulate in root nodules during incubation in the NMR tube (cf. Fig. 28). This may reflect an increase in synthesis and/or a decrease in catabolism. The results concerning the size of the GABA pool in the nodule tissue do not allow for a discrimination of GABA localised in the bacteroid or in the plant cytoplasm. It is, however, evident from the high GABA ¹⁵N labelling that the GABA is formed from a newly synthesised pool of glutamate. The GABA shunt pathway (cf. Fig. 13) has previously

been suggested to play a prominent role in *R. meliloti* bacteroids (Fitzmaurice and O'Gara 1988; Miller *et al.* 1991), although the function of GABA remains unclear.

Some experimental evidence indicates that the locations of GABA production and accumulation are not identical, and that accumulated GABA is sequestered within organelles, possibly in the vacuoles (reviewed by Shelp *et al.* 1999). It has also been demonstrated that nodules of many legume species accumulate bound forms of GABA amounting to as much as 20% of the total N content of the nodule (Larher *et al.* 1983).). The apparent NMR invisibility of ¹⁵N-labelled GABA in pea root nodules in *in vivo* experiments may result from GABA being immobilised in some way *in vivo*. Immobilised GABA may be released from the tissue by the applied extraction procedures and/or enzymatic reactions and therefore detected by IP-RPLC MS. A support for such a suggestion is the observation of broad resonances of GABA in dead nodules indicating that GABA is less tightly bound under such conditions but still not free in e.g. the cytoplasm.

Results from both NMR and IP-RPLC MS analyses agreed that glutamine was predominantly ¹⁵N labelled in the amino group and not in the amide group. I never observed any resonances from glutamine ¹⁵N amide in ¹⁵N NMR spectra despite the fact that amide groups seem to possess favourable NOE factors for the detection *in vivo*. Glutamine ¹⁵N amide groups did not give rise to any signals in NMR spectra of extracts, not even when signals from glutamine ¹⁵N amino groups were present. IP-RPLC MS results demonstrated ¹⁵N in mono labelled glutamine was almost exclusively positioned in the amino group, and when the amount of double labelled glutamine molecules was taken into account, the distribution between amino and amide ¹⁵N labelling was 3:1.

The concurrent findings of glutamine being predominantly ¹⁵N labelled in the amino position are very controversial as the prevailing scheme for the assimilation of fixed nitrogen states that ammonium is first incorporated into the amide group of glutamine, catalysed by glutamine synthetase.

Glutamate and alanine were shown by IP-RPLC MS to be among the most highly ¹⁵N enriched metabolites, but the total pool sizes of glutamate and alanine were rather small compared to the other studied amino acids, which justifies that they were not observed by *in vivo* ¹⁵N NMR. The high ¹⁵N enrichment of these two amino acids and of GABA might indicate that these metabolites were synthesised directly from highly ¹⁵N enriched ammonium via glutamate dehydrogenase (GDH) and the GABA shunt pathway: $NH_4^+ \rightarrow glutamate \rightarrow GABA \rightarrow alanine$.

The direct incorporation of ammonium into glutamate catalysed by GDH is normally not considered an important reaction in the plant cytoplasm of root nodules (cf. chapter 2.5). Substantial bacteroid GDH activity has been reported in many different host plants (cf. chapter 2.4; Brown and Dilworth, 1975; Miller et al., 1991), but the *in planta* significance of GDH activity remains uncertain. My results indicate that substantial GDH catalysed glutamate synthesis may occur under the given experimental conditions, although it is not possible to localise the reaction to a specific compartment.

If synthesis of glutamate and alanine via the GDH and GABA shunt pathway was taking place in the bacteroid compartment, the excretion product from bacteroid to plant cytoplasm could be a highly ¹⁵N-labelled amino acid. All of the necessary enzymes have been reported to be present in bacteroids (cf. chapter 2.4; Brown and Dilworth, 1975; Miller et al., 1991; Fitzmaurice and O'Gara, 1991). If amino acids rather than ammonium were the excretion products under certain conditions, as has been suggested previously (Waters et al., 1998; Allaway et al., 2000), the ammonium pool of the plant cytoplasm is expected to contain very little ¹⁵N-label. In that way glutamine would be synthesised in the plant cytoplasm from highly labelled glutamate and poorly labelled ammonium resulting in glutamine molecules being higher labelled in the amino group (originating from glutamate) that in the amide group (originating from ammonium). It has previously been estimated that the plant cytoplasm contains only a minor free ammonium pool (Streeter, 1989), so the suggested way of synthesis of glutamine would depend on ammonium being provided from other sources. This speculative hypothesis would of course need further investigation, but at present it could serve as a possible explanation of my controversial observation of glutamine being predominantly ¹⁵N-labelled in the amino position.

Discovery of a novel phosphorus compound in pea nodule ³¹P NMR spectra

I observed a new very narrow and well resolved ³¹P signal at -1.91 ppm from an unknown compound in pea root nodules, which was absent in root nodules from both *Lotus* and soybean. It has not been reported in previous ³¹P NMR studies of soybean nodules (Rolin *et al.* 1989a and b) or alfalfa nodules (Nikolaev *et al.* 1994). I have only performed preliminary analyses to elucidate the structure and origin of this compound. However, it seemed that the phosphate group neighboured a methylene group and that it was a symbiosis specific compound since it was neither found in uninfected plant root tissue nor in free-living *Rhizobium* bacteria.

The chemical shift of the unknown compound was identical when observed in living root nodules and in a perchloric acid extract at pH 7.5, which makes it unlikely that the compound is localised in the vacuole since the acidic conditions in this compartment would be expected to change the chemical shift. The signal was less line broadened than other ³¹P metabolites in *in vivo* spectra, and this may be because the compound was confined to a specific compartment in the nodule tissue, possibly the bacteroid, and thus experienced less field inhomogeneity because of the limited volume. The fact that the unidentified ³¹P signal persisted during prolonged perfusion of root nodules, when other ³¹P signals became weaker, suggested that the compound was not metabolised and not washed out, which may indicate that it is bound in some way, though still highly mobile.

Concluding remarks

The present work presents the first report of *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing pea root nodules. The results demonstrate that it is indeed possible to apply *in vivo* ¹⁵N NMR spectroscopy to the study of nitrogen fixation and assimilation in root nodules. By using NMR it was possible to observe directly the incorporation of ¹⁵N into living nodules, but the detection was limited to ammonium and some of the more abundant amino acids.

The fully decoupled *in vivo* ¹⁵N NMR spectra did not include a signal from the amino group of asparagine, which may reflect that the *in vivo* NOE factor of the asparagine amino group is of an unfavourable size, since LC-MS results revealed that asparagine was indeed ¹⁵N-labelled at the amide as well as the amino nitrogen. LC-MS analyses showed that GABA was ¹⁵N-labelled in considerable amounts, but ¹⁵N GABA was NMR invisible in living pea root nodules. This may be due to immobilisation of GABA in root nodules.

A substantial pool of free ammonium was observed in the metabolically active, intact symbiosis by in vivo ¹⁵N NMR. The in vivo ¹⁵N NMR spectra further envisaged that glutamine was more highly ¹⁵N-labelled at the amino nitrogen than at the amide nitrogen. Both of these findings represent information that has not been made available by other techniques used to study nitrogen assimilation in legume nodules. The intracellular environment led to very unusual *in vivo* ¹⁵N ammonium chemical shifts, whereas no changes in the expected *in vivo* chemical shifts of amino acids were observed. This may suggest that ammonium and amino acids reside in different compartments: ammonium residing in the bacteroids, and ¹⁵N-labelled glutamine/glutamate and asparagine located in the plant cytoplasm.

References

- Aarnes, H., Eriksen, B., and Southon, T.E. (1995) Metabolism of nitrate and ammonium in seedlings of Norway spruce (*Picen abies*) measured by *in vivo* N-14 and N-15 NMR spectroscopy. *Physiol.Plant.* 94, 384-390.
- Allaway, D., Lodwig, E.M., Crompton, L.A., Wood, M., Parsons, R., Wheeler, T.R., and Poole, P.S. (2000) Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Mol.Microbiol.* 36, 508-515.
- Amancio, S. and Santos, H. (1992) Nitrate and ammonium assimilation by roots of maize (Zea mays L.) seedlings as investigated by *in vivo* ¹⁵N-NMR. J.Exp.Bot. 43, 633-639.
- Amancio, S., Clarkson, D.T., Diogo, E., Lewis, M., and Santos, H. (1997) Assimilation of nitrate and ammonium by sulphur deficient Zea mays cells. Plant Physiol. Biochem. 35, 41-48.
- Appels, M.A. and Haaker, H. (1988) Identification of cytoplasmic nodule-associated forms of malate dehydrogenase involved in the symbiosis between *Rhizobium leguminosarum* and *Pisum sativum*. *Eur.J.Biochem.* 171, 515-522.
- Appels, M.A. and Haaker, H. (1991) Gluramate oxaloacetate transaminase in pca root nodules. Participation in a malate/aspartate shuttle between plant and bacteroid. *Plant Physiol.* 95, 740-747.
- Appleby, C.A. (1984) Leghemoglobin and Rhizobium respiration. Ann. Rev. Plant Physiol. 35, 443-478.
- Appleby, C.A. (1992) The origin and functions of hemoglobin in plants. Sci. Progr. 76, 365-398.
- Atkins, C.A. (1991) Ammonia assimilation and export of nitrogen from the legume nodule. In: Biology and Biochemistry of Nitrogen Fixation, 293-319. Edited by Dilworth, M. and Glenn, A., Amsterdam, Elsevier.
- Bates, R.G. and Pinching, G.D. (1950) J.Am. Chem. Soc. 72, 1393
- Belay, N., Sparling, R., Choi, B.S., Roberts, M., Roberts, J.E., and Daniels, L. (1988) Physiological and ¹⁵N-NMR analysis of molecular nitrogen fixation by Methanococcus thermolithotrophicus, Methanobacterium bryantii and Methanospirillum hungatei. Biochim.Biophys.Acta 971, 233-245.
- Bengtsson, G. and Odham, G. (1979) A micromethod for the analysis of free amino acids by gas chromatography and its application to biological systems. Anal.Biochem. 92, 426-443.
- Berger, S., Braun, S., and Kalinowski, H.O. (1997) NMR spectroscopy of the non-metallic elements, Chichester, John Wiley & Sons Ltd.
- Bergersen, F.J. and Turner, G.L. (1967) Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. *Biochim.Biophys.Acta* 141, 507-515.
- Bergersen, F.J. (1982) Root nodules of legumes: Structure and functions, 1-164, Research Studies Press.
- Bergersen, F.J. (1997) Regulation of nitrogen fixation in infected cells of leguminous root nodules in relation to O₂ supply. *Plant Soil* 191, 189-203.
- Bligny, R. and Douce, R. (2001) NMR and plant metabolism. Curr. Opin. Plant Biol. 4, 191-196.
- Boland, M.J., Farnden, K.J.F., and Robertson, J.G. (1980) Ammonia assimilation in nitrogen-fixing legume nodules. In: Nitrogen Fixation, Volume II, 33-51. Edited by Newton, W.E. and Orme-Johnson, W.H., Baltimore, University Park Press.

- Bown, A.W. and Shelp, B.J. (1997) The metabolism and functions of gamma-aminobutyric acid. *Plant Physiol.* 115, 1-5.
- Brewin, N.J. (1991) Development of the legume root nodule. Ann. Rev. Cell Biol. 7, 191-226.
- Brewin, N.J. (1998) Tissue and cell invasion by *Rhizobium*: The structure and development of infection threads and symbioses. In: *The Rhizobiaceae*, 417-429. Edited by Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J., Dordrecht, Kluwer Academic Publishers.
- Brown, C.M. and Dilworth, M.J. (1975) Ammonia assimilation by *Rhizobium* cultures and bacteroids. *J.Gen.Microbiol.* 86, 39-48.
- Carroll, A.D., Fox, G.G., Laurie, S., Phillips, R., Ratcliffe, R.G., and Stewart, G.R. (1994) Ammonium assimilation and the role of gamma-aminobutyric acid in pH homeostasis in carrot cell suspensions. *Plant Physiol.* 106, 513-520.
- Cavanagh, J., Fairbrother, W.J., Palmer III, A.G., and Skelton, N.J. (1996) Protein NMR spectroscopy. Principles and practice, San Diego, Academic Press Inc.
- Cohn, J., Day, R.B., and Stacey, G. (1998) Legume nodule organogenesis. Trends Plant Sci. 3, 105-110.
- Cooper, R.A., Lichter, R.L., and Roberts, J.D. (1973) pH-dependent nuclear magnetic resonance spectra of ¹⁵N-enriched glycine. Line shape and relaxation studies. J.Am. Chem. Soc. 95, 3724-3729.
- Cullimore, J.V., Lara, M., Lea, P.J., and Miflin, B.J. (1983) Purification and properties of two forms of glutamine synthetase from the plant fraction of *Phaseolus* root nodules. *Planta* 157, 245-253.
- Cullimore, J.V. and Bennett, M.J. (1992) Nitrogen assimilation in the logume root nodule: Current status of the molecular biology of the plant enzymes. *Can.J.Microbiol.* 38, 461-466.
- Day, D.A., Price, G.D., and Udvardi, M.K. (1989) Membrane interface of the Bradyrhizobium japonicum-Glycine max symbiosis: Peribacteroid units from soybean nodules. Aust.J.Plant Physiol. 16, 69-84.
- Day, D.A. and Copeland, L. (1991) Carbon metabolism and compartmentation in nitrogen-fixing legume nodules. *Plant Physiol Biochem.* 29, 185-201.
- Day, D.A. and Udvardi, M.K. (1992) Metabolite exchange across symbiosome membranes. Symbiosis 14, 175-189.
- Day, D.A., Poole, P., Tyerman, S.D., and Rosendahl, L. (2001) Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. *Cell.Mol.Life Sci.* 58, 61-71.
- Denarie, J. and Cullimore, J.V. (1993) Lipo-oligosaccharide nodulation factors A minireview new class of signaling molecules mediating recognition and morphogenesis. *Cell* 74, 951-954.
- Dixon, R.O.D. and Wheeler, C.T. (1986) Nitrogen fixation in plants, New York, Chapman and Hall.
- Donald, R.G.K. and Ludwig, R.A. (1984) Rhizobium sp. strain ORS571 ammonium assimilation and nitrogen fixation. J.Bacteriol. 158, 1144-1151.
- Dookeran, N.N., Yalcin, T., and Harrison, A.G. (1996) Fragmentation reactions of protonated α-amino acids. J.Mass Spectrom. 31, 500-508.
- Emerich, D.W., Waters, J.K., Hughes, B.L., Purcell, L.C., Gerhardt, K.O., and Mahwhinney, T.P. (2000) Alanine excretion from Bradyrhizobium japonicum bacteroids. In: Nitrogen Fixation: From Molecules to Crop Productivity, 355-358. Edited by Pedrosa, F.O., Hungria, M., Yates, M.G., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.

- Fitzmaurice, A.M. and O'Gara, F. (1988) Involvement of glutamate as a carbon source in supporting nitrogen fixation activity in R. meliloti. In: Nitrogen Fixation: Hundred Years after, 558. Edited by Bothe, G., de Bruijn, F.J., and Newton, W.E., Stuttgart, New York, Gustav Fischer.
- Fitzmaurice, A.M. and O'Gara, F. (1991) Glutamate catabolism in Rhizobium meliloti. Arch. Microbiol. 155, 422-427.
- Forde, B.G., Day, H.M., Turton, J.F., Shen, W., Cullimore, J.V., and Oliver, J.E. (1989) Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. *Plant Cell* 1, 391-401.
- Fortier, G., Tenaschuk, D., and MacKenzie, S.L. (1986) Capillary gas chromatography micro-assay for pyroglutamic, glutamic and aspartic acids, and glutamine and asparagine. J.Chromatograph. 361, 253-261.
- Fox, G.G., Ratcliffe, R.G., Robinson, S.A., Slade, A.P., and Stewart, G.R. (1992) Detection of ¹⁵N-labeled ammonium. *In vivo* ¹⁵N NMR versus mass spectrometry. *J.Magn.Reson.* 96, 146-153.
- Fox, G.G., Ratcliffe, R.G., Robinson, S.A., and Stewart, G.R. (1995) Evidence for deamination by glutamate dehydrogenase in higher plants: Commentary. Can.J.Bot. 73, 1112-1115.
- Galibert, F., Finan, T.M., Long, S.R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M.J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R.W., Dreano, S., Federspiel, N.A., Fisher, R.F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R.W., Jones, T., Kahn, D., Kahn, M.L., Kalman, S., Keating, D.H., Kiss, E., Komp, C., Lalaure, V., Masuy, D., Palm, C., Peck, M.C., Pohl, T.M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F.J., Weidner, S., Wells, D.H., Wong, K., Yeh, K.C., and Batut, J. (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti. Science* 293, 668-672.
- Glenn, A.R. and Dilworth, M.J. (1981) The uptake and hydrolysis of disaccharides by fast- and slow-growing species of *Rhizobium*. Arch. Microbiol. 129, 233-239.
- Glenn, A.R. and Dilworth, M.J. (1984) Methylamine and ammonium transport systems in *Rhizobium leguminosarum* MNF3841. *J.Gen.Microbiol.* 130, 1961-1968.
- Gordon, A.J., Ryle, G.J.A., Mitchell, D.F., and Powell, C.E. (1985) The flux of ¹⁴C-labelled photosynthate through soybean root nodules during N₂ fixation. *J.Exp.Bot.* **36** (166), 756-769.
- Gordon, A.J. (1992) Carbon metabolism in the legume nodule. Chapter 7, pp. 133-162.
- Gordon, A.J. (1995) Sucrose metabolism to support N₂ fixation in legume root nodules. In: Nitrogen Fixation: Fundamentals and Applications, 533-538. Edited by Tikhonovich, I.A., Provorov, N.A., Romanov, V.I., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.
- Hadfield, K.L. and Bulen, W.A. (1969) Adenosine triphosphate requirement of nitrogenase from Azobacter vinelandli. Biochem. 8, 5103-5108.
- Hadri, A.-E., Spaink, H.P., Bisseling, T., and Brewin, N.J. (1998) Diversity of root nodulation and rhizobial infection processes. In: *The Rhizobiaceue*, 347-361. Edited by Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J., Dordrecht, Kluwer Academic Publishers.
- Halm, M. and Hennecke, H. (1984) Localized mutagenesis in Rhizobium japonicum. Mol.Gen.Genet. 193, 46-52.
- Hansen, A.P. (1994) Symbiotic N₂ Fixation of Crop Legumes: Achievements and Perspectives, Stuttgart, Centre for Agriculture in the Tropics and Subtropics.

- Haran, N., Kahana, Z.E., and Lapidot, A. (1983) In vivo ¹⁵N NMR studies of regulation of nitrogen assimilation and amino acid production by Brevibacterium lactofermentum, J.Biol.Chem. 258, 12929-12933.
- Hardy, R.W.F., Holsten, R.D., Jackson, E.K., and Burns, R.C. (1968) The acetylene-ethylene assay for N₂ fixation: Laboratory and field evaluation. *Plant Physiol.* 43, 1185-1207.
- Herrada, G., Puppo, A., and Rigaud, J. (1989) Uptake of metabolites by bacteroid-containing vesicles and by free bacteroids from french bean nodules. J.General Microbiol. 135, 3165-3171.
- Hoult, D.I. and Richards, R.E. (1976) The signal-to-noise ratio of the nuclear magnetic resonance experiment. J.Magn.Reson. 24, 71-85.
- Howard, J. and Rees, D.C. (2000) Structure of the nitrogenase protein components. In: Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process, 43-54. Edited by Triplett, E.W., Norfolk, Horizon Scientific Press.
- Howitt, S.M., Udvardi, M.K., Day, D.A., and Gresshoff, P.M. (1986) Ammonia transport in free-living and symbiotic Rhizobium sp. ANU289. J.Gen.Microbiol. 132, 257-261.
- Hunt, S. and Layzell, D.B. (1993) Gas exchange of legume nodules and the regulation of nitrogenase activity. Ann. Rev. Plant Physiol. 44, 483-511.
- Hynes, M.F. and Finau, T.M. (1998) General genetic knowledge. In: The Rhizobiaceae, 25-43. Edited by Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J., Dordrecht, Kluwer Academic Publishers.
- Irving, C.S. and Lapidot, A. (1975) Origin of the anomalous pH dependence of the 15-[1H] Nuclear Overhauser effect of amino and amide nitrogens. J.Am. Chem. Soc. 97, 5945-5946.
- Jin, H.N., Dilworth, M.J., and Glenn, A.R. (1990) 4-Aminobutyrate is not available to bacteroids of cowpea Rhizobium Mnf2030 in snake bean nodules. Arch. Microbiol. 153, 455-462.
- Johansen, A., Finlay, R.D., and Olsson, P.A. (1996) Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol. 133, 705-712.
- Jording, D., Uhde, C., Schmidt, R., and Puhler, A. (1994) The C-4-dicarboxylate transport system of *Rhizobium meliloti* and its role in nitrogen fixation during symbiosis with alfalfa (*Medicago sativa*). *Experientia* 50, 874-883.
- Joy, R.W., Mcintyre, D.D., Vogel, H.J., and Thorpe, T.A. (1996) Stage-specific nitrogen metabolism in developing carrot somatic embryos. *Physiol.Plant.* 97, 149-159.
- Joy, R.W., Vogel, H.J., and Thorpe, T.A. (1997) Inorganic nitrogen metabolism in embryogenic white spruce cultures: A nitrogen 14/15 NMR study. J. Plant Physiol. 151, 306-315.
- Kahl, S., Gerendas, J., Heeschen, V., Ratcliffe, R.G., and Rudolph, H. (1997) Ammonium assimilation in bryophytes. L-glutamine synthetase from Sphagnum fallax. Physiol. Plant. 101, 86-92.
- Kahn, M.L., Kraus, J., and Sommerville, J.E. (1985) A model of nutrient exchange in the *Rhizobium*-legume symbiosis. In: *Nitrogen Fixation Research Progress*, 193-199. Edited by Evans, H.J., Bottomley, P.J., and Newton, W.E., Dordrecht, Martinus Nijhoff.
- Kahn, M.L., McDermott, T.R., and Udvardi, M.K. (1998) Carbon and nitrogen metabolism in rhizobia. In: *The Rhizobiaceae*, 461-485. Edited by Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J., Dordrecht, Kluwer Academic Publishers.
- Kaiser, B.N., Finnegan, P.M., Tyerman, S.D., Whitehead, L.F., Bergersen, F.J., Day, D.A., and Udvardi, M.K. (1998) Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. *Science* 281, 1202-1206.

- Kaminski, P.A., Batut, J., and Boistard, P. (1998) A survey of symbiotic nitrogen fixation by rhizobia. In: *The Rhizobiaceae*, 431-460. Edited by Spaink, H.P., Kondorosi, A., and Hooykaas, P.J.J., Doordrecht, Kluwer Academic Publishers.
- Kanamori, K., Legerton, T.L., Weiss, R.L., and Roberts, J.D. (1982b) Nitrogen-15 spin-lattice relaxation times of amino acids in *Neurospora crassa* as a probe of intracellular environment. *Biochem.* 21, 4916-4920.
- Kanamori, K., Legerton, T.L., Weiss, R.L., and Roberts, J.D. (1982a) Effect of the nitrogen source on glutamine and alanine biosynthesis in *Neurospora crassa*. An *in vivo* ¹⁵N nuclear magnetic resonance study. J.Biol.Chem. 257, 14168-14172.
- Kennedy, I.R. (1966) Primary products of symbiotic nitrogen fixation. II. Pulse labelling of Seradella nodules with ¹⁵N₂. Biochim.Biophys.Acta 130, 295-303.
- Köckenberger, W. (2001) Nuclear magnetic resonance micro-imaging in the investigation of plant cell metabolism. J.Exp. Bot. 52, 641-652.
- Kondorosi, A., Svab, Z., Kiss, G.B., and Dixon, R.A. (1977) Ammonia assimilation and nitrogen fixation in *Rhizobium meliloii. Mol.Gen.Genet.* 151, 221-226.
- Kouchi, H., Fukai, K., and Kihara, A. (1991) Metabolism of glutamate and aspartate in bacteroids isolated from soybean root nodules. J.Gen.Microbiol. 137, 2901-2910.
- Kuesel, A.C., Kuhn, W., Sianoudis, J., Grimme, L.H., Leibfritz, D., and Mayer, A. (1989) N-15 in vivo NMR spectroscopic investigation of nitrogen deprived cell suspensions of the green alga Chlorella fusca. Arch.Microbiol. 151, 434-438.
- Kurz, W.G.W., Rokosh, D.A., and LaRue, T.A. (1975) Enzymes of ammonia assimilation in *Rhizobium* leguminosarum bacteroids. Can.J.Microbiol. 21, 1009-1012.
- Kuzma, M.M., Hunt, S., and Layzell, D.B. (1993) Role of oxygen in the limitation and inhibition of nitrogenase activity and respiration rate in individual soybean nodules. *Plant Physiol.* 101, 161-169.
- Kuzma, M.M., Winter, H., Storer, P., Oresnik, I., Atkins, C.A., and Layzell, D.B. (1999) The site of oxygen limitation in soybean nodules. *Plant Physiol.* 119, 399-407.
- Kwon, J.Y. and Moini, M. (2001) Analysis of underivatized amino acid mixtures using high performance liquid chromatography/ dual oscillating nebulizer atmospheric pressure microwave induced plasma ionisation-mass spectrometry. J.Am.Soc.Mass Spectrom. 12, 117-122.
- Larher, F., Goas, G., le Rudulier, D., Gerard, J., and Hamelin, J. (1983) Bound 4-aminobutyric acid in root nodules of *Medicago sativa* and other nitrogen fixing plants. *Plant Science Lett.* 29, 315-326.
- Layzell, D.B. (1998) Oxygen and the control of nodule metabolism and N₂ fixation. In: *Biological Nitrogen Fixation for the 21st Century*, 435-440. Edited by Elmerich, C., Kondorosi, A., and Newton, W.E., Netherlands, Kluwer Academic Publishers.
- Layzell, D.B. (2000) Oxygen regulation and adenylates in legume nodules. In: Nitrogen Fixation: From Molecules to Crop Productivity, 367-368. Edited by Pedrosa, F.O., Hungria, M., Yates, M.G., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.
- Lee, R.B. and Ratcliffe, R.G. (1983b) Development of an aeration system for use in plant tissue NMR experiments. J.Exp.Bot. 34, 1213-1221.
- Lee, R.B. and Ratcliffe, R.G. (1983a) Phosphorus nutrition and the intracellular distribution of inorganic phosphate in pea root tips: A quantitative study using ³¹P-NMR. *J.Exp. Bot.* 34, 1222-1244.
- Lee, R.B. and Rateliffe, R.G. (1993) Observations on the subcellular distribution of the ammonium ion in maize root tissue using *in vivo* N-14-Nuclear-Magnetic-Resonance spectroscopy. *Planta* 183, 359

- Legerton, T.L., Kanamori, K., Weiss, R.L., and Roberts, J.D. (1981) ¹⁵N NMR studies of nitrogen metabolism in intact mycelia of *Neurospora crassa*. *Proc.Natl.Acad.Sci. USA* 78, 1495-1498.
- Legerton, T.L., Kanamori, K., Weiss, R.L., and Roberts, J.D. (1983) Measurements of cytoplasmic and vacuolar pH in *Neurospora* using nitrogen-15 nuclear magnetic resonance spectroscopy. *Biochem*, 22, 899-903.
- Leipert, T.K. and Noggle, H.N. (1975) The nitrogen-15 magnetic resonance of glycine. J.Am. Chem. Soc. 97, 269-272.
- Levsen, K. (1978) Fundamental aspects of organic mass spectrometry (Progress in mass spectrometry; Vol. 4), Weinheim, New York, Verlag Chemie.
- Li, Y.Z., Green, L.S., Day, D.A., and Bergersen, F.J. (2000) Ammonia and alanine efflux from nitrogenfixing soybean bacteroids. In: Nitrogen Fixation: From Molecules to Crop Productivity, 391-391. Edited by Pedrosa, F.O., Hungtia, M., Yates, M.G., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.
- Marini, A.M., Springael, J.Y., Frommer, W.B., and Andre, B. (2000) Cross-talk between ammonium transporters in yeast and interference by the soybean SAT1 protein. *Mol.Microbiol.* 35, 378-385.
- Martin, F. (1985) ¹⁵N-NMR studies of nitrogen assimilation and amino acid biosynthesis in the ectomycorrhizal fungus *Cenococcum graniforme*. FEBS Lett. **182**, 350-354.
- Martin, F., Cote, R., and Canet, D. (1994) NH₄⁺ assimilation in the ectomycorrhizal basidiomycete Laccaria bicolor (Maire) Orton, a ¹⁵N-NMR study. New Phytol. 128, 479-485.
- Mason, J. (1996) Nitrogen NMR. In: Encyclopedia of Nuclear Magnetic Resonance. Vol. 5, 3222-3251. Edited by Grant, D.M. and Harris, R.K., John Wiley & Sons.
- McCormack, D.K., Farnden, K.J.F., and Boland, M.J. (1982) Purification and properties of glutamine synthetase from the plant cytosol fraction of lupin nodules. Arch. Biochem. Biophys. 218, 561-571.
- McDermott, T.R., Griffith, S.M., Vance, C.P., and Graham, P.H. (1989) Carbon metabolism in Bradyrhizobium bacteroids. FEMS Microbiol. Rev. 63, 327-340.
- McParland, R.H., Guevara, J.G., Becker, R.R., and Evans, H.J. (1976) The purification and properties of the glutamine synthetase from the cytosol of soya-bean root nodules. *Biochem.J.* 153, 597-606.
- Meijer, A.J. and van Dam, K. (1974) The metabolic significance of anion transport in mitochondria. Biochim.Biophys.Acta 346, 213-244.
- Miller, R.W., McRae, D.G., and Joy, K.W. (1991) Glutamate and gamma-aminobutyrate metabolism in isolated Rhizobium meliloti bacteroids. Mol. Plant-Micr. Interact. 4 (1), 37-45.
- Minchin, F.R., Summerfield, R.J., Hadley, P., Roberts, E.H., and Rawsthorne, S. (1981) Carbon and nitrogen nutrition of nodulated roots of grain legumes. *Plant Cell Envir.* 4, 5-26.
- Mitsch, M., Cowie, A., Voegele, R., and Finan, T.M. (2000) Carbon metabolism and symbiotic needs of root nodule bacteria. In: Nitrogen Fixation: From Molecules to Crop Productivity, 359-364. Edited by Pedrosa, F.O., Hungria, M., Yates, M.G., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.
- Mitsumori, F., Yoneyama, T., and Ito, O. (1985) Phosphorus-31 nuclear magnetic resonance studies on higher plant tissue. Plant Sci. 38, 87-92.
- Monselise, E.B.-I. and Kost, D. (1993) Different ammonium-ion uptake, metabolism and detoxification efficiencies in two *Lemnaceae*. A ¹⁵N-nuclear magnetic resonance study. *Planta* 189, 167-173.

- Moon, R.B. and Richards, J.H. (1973) Determination of intracellular pH by ³¹P magnetic resonance. J.Biol.Chem. 248, 7276-7278.
- Moreno, S., Meza, R., Guzman, J., Carabez, A., and Espin, G. (1991) The glnA gene of Rhizobium leguminosarum by. phaseoli and its role in symbiosis. Mol.Plant-Micr.Interact. 4 (6), 619-622.
- Mouritzen, P. and Rosendahl, L. (1997) Identification of a transport mechanism for NH₄⁺ in the symbiosome membrane of pea root nodules. *Plant Physiol.* 115, 519-526.
- Nagatani, H., Shimizu, M., and Valentine, R.C. (1971) The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch.Mikrobiol. 79, 164-175.
- Newcomb, W. (1976) A correlated light and electron microscope study of symbiotic growth and differentiation in *Pisum sativum* root nodules. *Can.J.Bot.* 54, 2163-2186.
- Nikolaev, B.P., Provorov, N.A., Simarov, B.V., and Shlyakov, A.M. (1994) Transformation of phosphates by free-living and symbiotic root-nodule bacteria *Rhizobium meliloti* followed by ³¹P NMR spectroscopy, *Microbiol.* 63, 16-20.
- O'Brian, M.R. and Maier, R.J. (1989) Molecular aspects of the energetics of nitrogen-fixation in *Rhizobium*legume symbioses. *Biochim.Biophys.Acta* 974, 229-246.

Orme-Johnson, W.H. (1992) Nitrogenase structure - Where to now? Science 257, 1639-1640.

- Ou Yang, L.J., Udvardi, M.K., and Day, D.A. (1990) Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. *Planta* 182, 437-444.
- Ou Yang, LJ. and Day, D.A. (1992) Transport properties of symbiosomes isolated from sizatro nodules. Plant Physiol.Biochem. 30, 613-623.
- Parsons, R. and Day, D.A. (1990) Mechanism of soybean nodule adaptation to different oxygen pressures. Plant Cell Envir. 13, 501-512.
- Petritis, K.N., Chaimbault, P., Elfakir, C., and Dreux, M. (1999) Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent. J. Chromatograph.A 833, 147-155.
- Petritis, K., Chaimbault, P., Elfakir, C., and Dreux, M. (2000) Parameter optimization for the analysis of underivatized protein amino acids by liquid chromatography and ionspray tandem mass spectrometry. J. Chromatograph.A 896, 253-263.
- Pfeffer, P. and Shachar-Hill, Y. (1996) Plant/microbe symbioses. In: Current Topics in Plant Physiology. An American Society of Plant Physiologists Series. Vol. 16. Nuclear magnetic resonance in plant biology, 77-108. Edited by Shachar-Hill, Y. and Pfeffer, P., Rockville, American Society of Plant Physiologists.
- Pfeffer, P.E., Becard, G., Rolin, D.B., Uknalis, J., Cooke, P., and Tu, S.I. (1994) In vivo nuclear magnetic resonance study of the osmoregulation of phosphocholine-substituted beta-1,3;1,6 cyclic glucan and its associated carbon metabolism in Bradyrhizobium japonicum USDA 110. Appl.Envir.Microbiol. 60, 2137-2146.
- Pfeffer, P.E., Bago, B., and Shachar-Hill, Y. (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol. 150, 543-553.
- Poole, P. and Allaway, D. (2000) Carbon and nitrogen metabolism in *Rhizobium. Adv. Microb. Physiol.* 43, 117-163.
- Poole, P.S., Franklin, M., Glenn, A., and Dilworth, M. (1985) The transport of L-glutamate by *Rhizobium* leguminosarum involves a common amino acid carrier, J.Gen.Microbiol. 131, 1441-1448.

- Rastogi, V.K. and Watson, R.J. (1991) Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. J.Bacteriol. 173 (9), 2879-2887.
- Ratcliffe, R.G. (1996) In vivo NMR spectroscopy: Biochemical and physiological applications to plants. In: Current Topics in Plant Physiology. An American Society of Plant Physiologists Series. Vol. 16. Nuclear magnetic resonance in plant biology, 1-32. Edited by Shachar-Hill, Y. and Pfeffer, P., Rockville, American Society of Plant Physiologists.
- Ratcliffe, R.G. and Shachar-Hill, Y. (2001) Probing plant metabolism with NMR. Ann. Rev. Plant Physiol. Plant Mol. Biol. 52, 499-526.
- Reibach, P.H. and Streeter, J.G. (1983) Metabolism of ¹⁴C-labeled photosynthate and distribution of enzymes of glucose metabolism in soybean nodules. *Plant Physiol.* **72**, 634-640.
- Reid, C.J., Walshaw, D.L., and Poole, P.S. (1996) Aspartate transport by the Dct system in Rhizobium leguminosarum negatively affects nitrogen-regulated operons. Microbiol.- UK 142, 2603-2612.
- Reid, R.J., Loughman, B.C., and Ratcliffe, R.G. (1985) ³¹P NMR measurements of cytoplasmic pH changes in maize root tips. J.Exp.Bot. 36 (167), 889-897.
- Reitzer, L.J. and Magasanik, B. (1986) Transcription of GlnA in E.coli is stimulated by activator bound to sites far from the promotor. Cell 45, 785-792.
- Rhijn, P. and Vanderleyden, J. (1995) The Rhizobium-plant symbiosis. Microbiol. Rev. 59, 124-142.
- Rivers, R.L., Dean, R.M., Chandy, G., Hall, J.E., Roberts, D.M., and Zeidel, M.L. (1997) Functional analysis of nodulin 26, an aquaporin in soybean root nodule symbiosomes. J.Biol. Chem. 272, 16256-16261.
- Roberts, J.K.M., Wade-Jardetzky, N., and Jardetzky, O. (1981) Intracellular pH measurements by ³¹P nuclear magnetic resonance. Influence of factors other than pH on ³¹P chemical shifts. *Biochem.* 20, 5389-5394.
- Roberts, J.K.M. and Xia, J.H. (1996) NMR contributions to understanding plant responses to low-oxygen stress in plants. In: Current Topics in Plant Physiology. An American Society of Plant Physiologists Series. Vol. 16. Nuclear magnetic resonance in plant biology, 155-180. Edited by Shachar-Hill, Y. and Pfeffer, P., Rockville, American Society of Plant Physiologists.
- Robertson, J.G. and Farnden, K.J.F. (1980) Ultrastructure and metabolism of the developing legume root nodule. Biochem. Plants 5, 65-113.
- Robinson, D.L., Kahn, M.L., and Vance, C.P. (1994) Cellular localization of nodule enhanced aspartate aminotransferase in *Medicago sativa* L. *Planta* 192, 202-210.
- Robinson, S.A., Slade, A.P., Fox, G.G., Phillips, R., Ratcliffe, R.G., and Stewart, G.R. (1991) The role of glutamate dehydogenase in plant nitrogen metabolism. *Plant Physiol.* 95, 509-516.
- Roby, C., Martin, J.B., Bligny, R., and Douce, R. (1987) Biochemical changes during sucrose deprivation in higher plant cells. Phosphorus-31 nuclear magnetic resonance studies. J.Biol. Chem. 262, 5000-5007.
- Rogalewicz, F., Hoppilliard, Y., and Ohanessian, G. (2000) Fragmentation mechanisms of α-amino acids protonated under electrospray ionization: a collision activation and ab initio theoretical study. *Int.J.Mass Spectrom.* 195/196, 565-590.
- Rolin, D.B., Pfeffer, P.E., Boswell, R.T., Schmidt, J.H., and Tu, S.I. (1989b) In vivo ³¹P NMR spectroscopic studies of soybean Bradyrhizobium symbiosis. Compartmentation and distribution of P metabolites. FEBS Lett. 254, 203-206.
- Rolin, D.B., Boswell, R.T., Sloger, C., Tu, S.I., and Pfeffer, P.E. (1989a) In vivo³¹P NMR spectroscopic studies of soybean Bradyrhizobium symbiosis. I. Optimization of parametres. Plant Physiol. 89, 1238-1246.

- Rolin, D.B., Pfeffer, P.E., Osman, S.F., Szwergold, B.S., Kappler, F., and Benesi, A.J. (1992) Structural studies of a phosphocholine substituted B-(1,3);(1,6) macrocyclic glucan from Bradyrhizobium japonicum USDA 110. Biochim.Biophys.Acta 1116, 215-225.
- Rosendahl, L. and Jakobsen, I. (1987) Rhizobium strain effects on pea: The relation between nitrogen accumulation, phosphoenolpyrovate carboxylase activity in nodules and asparagine in root bleeding sap. Physiol.Plant. 71, 281-286.
- Rosendahl, L. and Jakobsen, I. (1988) Effects of age, supra-ambient oxygen and repeated assays on acetylene reduction and root respiration in pea. *Physiol.Plant.* 74, 77-82.
- Rosendahl, L., Vance, C.P., and Pedersen, W.B. (1990) Products of dark CO₂ fixation in pea root nodules support bacteroid metabolism. *Plant Physiol.* 93, 12-19.
- Rosendahl, L., Dilworth, M.J., and Glenn, A.R. (1992) Exchange of metabolites across the peribacteroid membrane in pea root nodules. J.Plant Physiol. 139, 635-638.
- Rosendahl, L., Mouritzen, P., and Rudbeck, A. (2001) Nitrogen transfer in the interface between the symbionts in pea root nodules. *Plant Soil* 230, 31-37.
- Roth, E., Jeon, K., and Stacey, G. (1988) Homology in endosymbiotic systems: The term "symbiosome". In: Molecular Genetics of Plant-Microbe Interactions, 220-225. Edited by Palacios, R. and Verma, D.P.S., American Phytopathology Society.
- Rudbeck, A., Mouritzen, P., and Rosendahl, L. (1999) Characterization of aspartate transport across the symbiosome membrane in pea root nodules. J. Plant Physiol. 155, 576-583.
- Sa, T.M. and Israel, D.W. (1991) Energy status and functioning of phosphorus-deficient soybean nodules. Plant Physiol. 97, 928-935.
- Saint-Ges, V., Roby, C., Bligny, R., Pradet, A., and Douce, R. (1991) Kinetic studies of the variations of cytoplasmic pH, nucleotide triphosphates (³¹P-NMR) and lactate during normoxic and anoxic transitions in maize root tips. *Eur.J.Biochem.* 200, 477-482.
- Salminen, S.O. and Streeter, J.G. (1987) Involvement of glutamate in the respiratory metabolism of Bradyrhizobium japonicum bacteroids. J.Bacteriol. 169, 495-499.
- Salminen, S.O. and Streeter, J.G. (1990) Factors contributing to the accumulation of glutamate in Bradyrhizobium japonicum bacteroids under microaerobic conditions. J.Gen.Microbiol. 136, 2119-2126.
- Salminen, S.O. and Streeter, I.G. (1992) Labeling of carbon pools in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* by viciae bacteroids following incubation of intact nodules with ¹⁴CO₂. *Plant Physiol.* 100, 597-604.
- Sanders, J.K.M. and Hunter, B.K. (1993) Modern NMR Spectroscopy. A Guide for Chemists, 2nd Ed., Oxford, Oxford University Press.
- Schachtmann, D.P., Reid, R.J., and Ayling, S.M. (1998) Phosphorus uptake by plants; From soil to cell. Plant Physiol. 116, 447-453.
- Schultze, M. and Kondorosi, A. (1998) Regulation of symbiotic root nodule development. Ann. Rev. Gen. 32, 33-57.
- Shelp, B.J. and Atkins, C.A. (1984) Subcellular location of enzymes of ammonia assimilation and asparagine synthesis in root nodules of Lupinus albus L. Plant Science Lett. 36, 225-230.
- Shelp, B.J., Bown, A.W., and Mclean, M.D. (1999) Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

- Simonsen, A.C.W. (1998) Origin of de novo synthesized proteins in the interface between pea-Rhizobium in root nodules. Ph.D. thesis,
- Skøt, L. (1983) Relationship between C₂H₂ reduction, H₂ evolution and ¹⁵N₂ fixation in root nodules of pea (*Pisum sativum*). *Physiol.Plant.* 59, 581-584.
- Smith, B.E. (2000) Chemistry and biochemistry of nitrogenase. In: Nitrogen Fixation: From Molecules to Crop Productivity, 11-12. Edited by Pedrosa, F.O., Hungria, M., Yates, M.G., and Newton, W.E., Dordrecht, Kluwer Academic Publishers.
- Snapp, S.S. and Vance, C.P. (1986) Asparagine biosynthesis in alfalfa (Medicago sativa L.) root nodules. Plant Physiol. 82, 390-395.
- Spickett, C.M., Smirnoff, N., and Ratcliffe, R.G. (1993) An *in vivo* nuclear magnetic resonance investigation of ion transport in maize (Zea mays) and Spartina anglica roots during exposure to high salt concentrations. *Plant Physiol.* 102, 629-638.
- Sprent, J.I. (1969) Prolonged reduction of acetylene by detached soybean nodules. Planta 88, 372-375.
- Sprent, J.I. (1982) Nitrogen fixation by grain legumes in the UK. Philos. Trans. R. Soc. London B269, 387-395.
- Strandgaard Andersen, E., Jespersgaard, P., and Grønbæk Østergaard, O. (1993) Databog fysik kemi, 7 Ed., Copenhagen, F & K Forlaget.
- Streeter, J.G. (1987) Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol.* 85, 768-773.
- Streeter, J.G. (1989) Estimation of ammonium concentration in the cytosol of soybean nodules. *Plant Physiol.* 90, 779-782.
- Streeter, J.G. and Salminen, S.O. (1990) Periplasmic metabolism of glutamate and aspartate by intact Bradyrhizobium japonicum bacteroids. Biochim.Biophys.Acta 1035, 257-265.
- Streeter, J.G. (1991) Transport and metabolism of carbon and nitrogen in legume nodules. Adv. Bot. Res. 18, 129-185.
- Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., and Ronson, C.W. (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc.Natl.Acad.Sci.USA* 92, 8985-8989.
- Szafran, M.M. and Haaker, H. (1995) Properties of the peribacteroid membrane ATPase of pea root nodules and its effect on the nitrogenase activity. *Plant Physiol.* 108, 1227-1232.
- Ta, T.C., Faris, M.A., and Macdowall, F.D.H. (1986) Pathways of nitrogen metabolism in nodules of alfalfa (Medicago sativa L.). Plant Physiol. 80, 1002-1005.
- Taiz, L. and Zeiger, E. (1998) *Plant Physiology*, 2nd Ed., Sunderland Massachusetts, Sinauer Associates, Inc. Publishers.
- Temple, S.J., Vance, C.P., and Gantt, J.S. (1998) Glutamate synthase and nitrogen assimilation. *Trends Plant* Sci. 3, 51-56.
- Tesch, M., de Graaf, A.A., and Sahm, H. (1999) In vivo fluxes in the ammonium-assimilatory pathways in Corynebacterium glutamicum studied by N-15 magnetic nuclear magnetic resonance. Appl.Envir.Microbiol. 65, 1099-1109.
- Thorpe, T.A., Bagh, K., Cutler, A.J., Dunstan, D.I., Mcintyre, D.D., and Vogel, H.J. (1989) A ¹⁴N and ¹⁵N nuclear magnetic resonance study of nitrogen metabolism in shoot-forming cultures of white spruce (*Picea glauca*) buds. *Plant Physiol.* **91**, 193-202.

- Thumfort, P.P., Layzell, D.B., and Atkins, C.A. (1999) Diffusion and reaction of oxygen in the central tissue of ureide-producing legume nodules. *Plant Cell Envir.* 22, 1351-1363.
- Truelsen, T.A. and Wyndaele, R. (1984) Recycling efficiency in hydrogenase uptake positive strains of Rhizobium leguminosarum. Physiol. Plant. 62, 45-50.
- Tyerman, S.D., Whitehead, L.F., and Day, D.A. (1995) A channel-like transporter for NH₄⁺ on the symbiotic interface of N₂-fixing plants. *Nature* 378, 629-632.
- Udvardi, M.K., Salom, C.L., and Day, D.A. (1988b) Transport of L-glutamate across the bacteroid membrane but not the peribacteroid membrane from soybean root nodules. *Mol.Plant-Micr.Interact.* 1(6), 250-254.
- Udvardi, M.K., Price, G.D., Gresshoff, P.M., and Day, D.A. (1988a) A dicarboxylate transporter on the perihacteroid membrane of soybean nodules. FEBS 231 (1), 36-40.
- Udvardi, M.K. and Day, D.A. (1990) Ammonia (¹⁴C-methylamine) transport across the bacteroid and peribacteroid membranes of soybean root nodules. *Plant Physiol.* 94, 71-76.
- Udvardi, M.K., Lister, D., and Day, D.A. (1991) ATPase activity and anion transport across the peribacteroid membrane of isolated soybean symbiosomes. Arch.Microbiol. 156, 362-366.
- Udvardi, M.K. and Kahn, M.L. (1992) Evolution of the (*Brady*)Rhizobium-legume symbiosis: Why do bacteroids fix nitrogen? Symbiosis 14, 87-101.
- Udvardi, M.K. and Day, D.A. (1997) Metabolite transport across symbiotic membranes of legume nodules. Ann.Rev.Plant Physiol.Plant Mol.Biol. 48, 493-523.
- Vance, C.P., Stade, S., and Maxwell, C.A. (1983) Alfalfa root nodule carbon dioxide fixation. I. Association with nitrogen fixation and incorporation into amino acids. *Plant Physiol.* **72**, 469-473.
- Vance, C.P. and Heichel, G.H. (1991) Carbon in N₂ fixation: Limitation or exquisite adaptation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42, 373-392.
- Vance, C.P., Gregerson, R.G., Robinson, D.L., Miller, S.S., and Gantt, J.S. (1994) Primary assimilation of nitrogen in alfalfa nodules: Molecular features of the enzymes involved. *Plant Sci.* 101, 51-64.
- Vance, C.P. (2000) Amide biosynthesis in root nodules of temperate legumes. In: Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process, 589-607. Edited by Triplett, E.W., Norfolk, Horizon Scientific Press.
- Vasse, J., Debilly, F., Camut, S., and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen-fixation in alfalfa nodules. J.Bacteriol. 172, 4295-4306.
- Walshaw, D.L. and Poole, P.S. (1996) The general L-amino acid permease of *Rhizobium leguminosarum* is an ABC uptake system that also influences efflux of solutes. *Mol.Microbiol.* 21, 1239-1252.
- Walshaw, D.L., Reid, C.J., and Poole, P.S. (1997b) The general amino acid permease of *Rhizobium* leguminosarum strain 3841 is negatively regulated by the Ntr system. FEMS Microbiol.Lett. 152, 57-64.
- Walshaw, D.L., Wilkinson, A., Mundy, M., Smith, M., and Poole, P.S. (1997a) Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. *Microbiol.* - UK 143, 2209-2221.
- Waters, J.K., Hughes, B.L., Purcell, L.C., Gerhardt, K.O., Mawhinney, T.P., and Emerich, D.W. (1998) Alanine, not ammonia, is excreted from N-2-fixing soybean nodule bacteroids. *Proc.Natl.Acad.Sci.USA* 95, 12038-12042.

- Waters, J.K. and Emerich, D.W. (2000) Transport of metabolites to and from symbiosomes and bacteroids. In: Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process, 549-558. Edited by Triplett, E.W., Norfolk, Horizon Scientific Press.
- Watson, R.J., Rastogi, V.K., and Chan, Y.K. (1993) Aspartate transport in *Rhizobium meliloti*. J.Gen.Microbiol. 139, 1315-1323.
- Werner, D., Mörschel, E., and Stripf, R.A. (1980) Development of nodules of Glycine max infected with an ineffective strain of Rhizobium japonicum. Planta 147, 320-329.
- Werner, D. (1992) The Rhizobium/Bradyrhizobium-Fabales symbiosis. In: Symbiosis of Plants and Microbes, 49-167. Edited by Werner, D., London, Chapman & Hall.
- Whitehead, L.F., Tyerman, S.D., Salom, C.L., and Day, D.A. (1995) Transport of fixed nitrogen across symbiotic membranes of legume nodules. *Symbiosis* 19, 141-154.
- Whitehead, L.F. and Day, D.A. (1997) The peribacteroid membrane. Physiol. Plant. 100, 30-44.
- Whitehead, L.F., Young, S., and Day, D.A. (1998) Aspartate and alanine movement across symbiotic membranes of soybean nodules. Soil Biol. Biochem. 30, 1583-1589.
- Witty, J.F., Skøt, L., and Revsbech, N.P. (1987) Direct evidence for changes in the resistance of legume root nodules to O₂ diffusion. J.Exp. Bot. 38 (192), 1129-1140.
- Witty, J.F. and Minchin, F.R. (1990) Oxygen diffusion in the legume root nodule. In: Nitrogen Fixation: Achievements and Objectives, 285-292. Edited by Gresshoff, P.M., Roth, E., Stacey, G., and Newton, W.E., New York. London, Chapmann and Hall.
- Wycoff, K.L., Hunt, S., Gonzales, M.B., Vandenbosch, K.A., Layzell, D.B., and Hirsch, A.M. (1998) Effects of oxygen on nodule physiology and expression of nodulins in alfalfa. *Plant Physiol.* 117, 385-395.
- Xia, J.H., Saglio, P., and Roberts, J.K.M. (1995) Nucleotide levels do not critically determine survival of maize root tips acclimated to a low-oxygen environment. *Plant Physiol.* 108, 589-595.
- Yoshioka, H., Gregerson, R.G., Samac, D.A., Hoevens, K.C.M., Trepp, G., Gantt, J.S., and Vance, C.P. (1999) Aspartate aminotransferase in alfalfa nodules: Localization of mRNA during effective and ineffective nodule development and promoter analysis. *Mol.Plant-Micr.Interact.* 12, 263-274.

Exploring Symbiotic Nitrogen Fixation and Assimilation in Pea Root Nodules by *in vivo* ¹⁵N NMR Spectroscopy and LC-Mass Spectrometry

Anne Marie Scharff^{*}, Helge Egsgaard^{*}, Poul Erik Hansen[#] and Lis Rosendahl^{*}

* Risoe National Laboratory, Plant Research Department * Roskilde University Centre, Department of Life Sciences and Chemistry Roskilde, Denmark

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N Assimilation in Pea Nodules Studied by in vivo 15N NMR

Corresponding author:

Anne Marie Scharff Plant Research Department Risoe National Laboratory PRD-301, P.O. Box 49 DK-4000 Roskilde Denmark

Tel: +45 4677 4159 E-mail: <u>anne.marie.scharff@risoe.dk</u> Fax: +45 4677 4122

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Abbreviations:

GABA, γ-aminobutyric acid GDH, glutamate dehydrogenase GOGAT, glutamate synthase GS, glutamine synthetase IP-RPLC, ion-pair reverse-phase liquid chromatography NMR, nuclear magnetic resonance NOE, nuclear Overhauser effect LC-MS, liquid chromatography mass spectrometry
Abstract

Nitrogen fixation and assimilation in pea (*Pisum sativum* L.) root nodules was studied by exposing detached nodules to ${}^{15}N_2$ via a perfusion medium, while recording a time course of *in vivo* ${}^{15}N$ NMR spectra. *In vivo* ${}^{31}P$ NMR spectroscopy was used to monitor the physiological state of the metabolically active nodules. Root nodules were extracted after NMR studies and analysed for total soluble amino acid pools and ${}^{15}N$ -labelling of individual amino acids by LC-MS.

Asparagine was observed to be ¹⁵N-labelled at the amide as well as the amino nitrogen by LC-MS. The *in vivo* ¹⁵N NMR signal from the asparagine amino group was lacking in fully decoupled spectra, which is probably caused by an unfavourable size of the NOE factor. Likewise, γ -aminobutyric acid (GABA) was observed by LC-MS to be highly ¹⁵N-labelled, but ¹⁵N-GABA was NMR invisible in living pea nodules. This may be due to immobilisation of GABA in nodules. Glutamine was more ¹⁵N-labelled in the amino than the amide nitrogen as evident from LC-MS as well as *in vivo* NMR.

A substantial pool of free ammonium was recorded in the metabolically active, intact symbiosis by *in vivo* ¹⁵N NMR. The intracellular environment resulted in unusual *in vivo* ¹⁵N ammonium chemical shifts, whereas no changes in *in vivo* chemical shifts of amino acids were observed. This may suggest that ammonium and amino acids reside in different compartments.

Introduction

Symbiotic nitrogen fixation, the process whereby nitrogen fixing bacteria enter into associations with plants, provides the major source of nitrogen for the biosphere. Nitrogenase, a bacterial enzyme, catalyses the reduction of atmospheric dinitrogen to ammonium. In rhizobia-leguminous plant symbioses, a widely accepted and simple model of nitrogen transfer from the symbiotic form of the bacterium, called a bacteroid, to the plant implies that nitrogenase-generated ammonia diffuses across the bacteroid membrane and is assimilated into amino acids in the plant compartment of the nodule tissue. However, the transport of symbiotically fixed nitrogen across the membranes surrounding the bacteroid and the form in which this occurs has been a matter of controversy.

The size of the free ammonium pool in the bacteroid and plant cytoplasm during symbiosis is an open question. It has been suggested that ammonium synthesis and subsequent assimilation by alanine dehydrogenase are so tightly coupled in the bacteroid cytoplasm that very little free ammonium is released (Waters et al., 1998). On the other hand, Allaway et al. (2000), observed a plastic partitioning of ammonium and alanine excretion from isolated bacteroids and suggested that the ammonium concentration inside the bacteroids was one of the key variables governing the rate of alanine synthesis. The free ammonium concentration in bacteroids was estimated by Streeter (1989). This estimate was based on an extrapolation of time dependent experimental data to time

zero and many other assumptions, and consequently there is a need for a more direct determination of the concentration of free ammonium inside the bacteroid.

Until recently it has been generally accepted that *Rhizobium* bacteroids do not assimilate ammonium to a great extent during symbiosis. However, more recent results challenge this view and suggest a possible involvement of amino acids as the form of fixed nitrogen delivered from the bacteroid to the plant (reviewed by Poole and Allaway, 2000; Day et al., 2001). At present, consensus has not been reached as to whether substantial nitrogen assimilation takes place in the nodule bacteroid compartment, and new investigations of nodule nitrogen metabolism are required.

An overall problem in the study of nodule metabolism is the extrapolation from *in vitro* to *in vivo*. Information on the microenvironment of the different compartments in the nodule is lacking, and the *in vivo* significance of *in vitro* findings is therefore difficult to predict. Biochemical reactions in intact living cells can be studied by NMR spectroscopy ideally without interfering with metabolic processes or destroying enzyme complexes. While determinations of enzyme activities in crude extracts may give a first clue as to which ammonium assimilation pathways are active, they cannot be used to predict the *in vivo* flux distribution over competing enzyme systems such as GDH and GS/GOGAT. *In vivo* NMR spectroscopy, especially when used in combination with stable isotope labelling such as ¹⁵N, does allow the characterisation of metabolic activities in the living cell. Other methods for studying metabolic pathways rely on extractions of the tissue and subsequent purification and analysis. These procedures are time consuming and this is a significant disadvantage in time course experiments, where the aim is to follow the label through a pathway.

In vivo ¹⁵N NMR spectroscopy has previously been used successfully in the study of plant, fungal and bacterial nitrogen metabolism. Much of the work has contributed to the understanding of the roles of GDH and the GS/GOGAT cycle in ammonium assimilation in different systems including cell suspensions (Robinson et al., 1991; Fox et al., 1995; Amancio et al., 1997), embryogenic cultures (Joy et al., 1996 and 1997), root tissues (Amancio and Santos, 1992), Sphagnum fallax (Kahl et al., 1997), ectomycorrhizal mycelium (Martin, 1985) and Corynebacterium glutamicum (Tesch et al., 1999). The elucidation of the nitrogen assimilating pathways has generally been achieved by supplying ¹⁵N-ammonium or ¹⁵N-nitrate to the living biological system and subsequently ¹⁵N NMR analysing the incorporation of the ¹⁵N-label into amino acids in the presence and absence of enzyme inhibitors.

Metabolic studies by *in vivo* NMR spectroscopy has the advantage of avoiding artefacts caused by the breakdown of labile compounds during extraction and it also eliminates errors due to incomplete recovery of solutes from the tissue. A crucial factor in studies of bacteroid metabolism and functioning *in vitro* is the degree to which bacteroid preparations are free from contaminating plant organelles, enzymes and metabolites. Waters et al. (1998) suggested that the presence of plant enzymes in earlier *in vitro* studies of bacteroids had caused significant artefacts in the results concerning metabolic products released from bacteroids, but this was subsequently contradicted by Li et al. (2000). Likewise, major errors might occur in the determination of metabolite levels because of reallocation or degradation of metabolites during extraction, processing of extracts or separation of compartments (Streeter, 1987, 1989; Miller et al., 1991).

An unfavourable feature of ¹⁵N NMR spectroscopy is that it is a relatively insensitive spectroscopic method. This might have discouraged previous attempts to study nitrogen fixation by this method, because to our knowledge, no reports on ¹⁵N NMR spectroscopy used for studying living, nitrogen fixing organisms are available. The only reported ¹⁵N NMR investigation of ¹⁵N₂ fixation is by Belay et al. (1988), who incubated methanogenic bacteria with ¹⁵N₂ and subsequently analysed harvested, dead cells by ¹⁵N NMR. However, the literature contains a few *in vivo* ³¹P NMR spectroscopic studies of symbiotic soybean (Mitsumuri et al., 1985; Rolin et al., 1989a & b) and alfalfa root nodules (Nikolaev et al., 1994). Results from these investigations demonstrate that it is possible to maintain nodules in a metabolically active state, while recording NMR spectra.

In conclusion, accurate estimation of the assimilation and translocation of fixed nitrogen in nodules would benefit from non-invasive and non-destructive measurements of the fate of the fixed nitrogen. The objective of the present study was thus to investigate nitrogen fixation and assimilation in ¹⁵N₂ fixing root nodules root by *in vivo* ¹⁵N NMR spectroscopy. This included optimisation of experimental conditions for maximal ¹⁵N incorporation. The investigation presents *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing root nodules, which in combination with LC-MS results demonstrate the intracellular build-up of ¹⁵N-ammonium and ¹⁵N-amino acids.

Materials and methods

Plant material

Surface sterilised seeds of pea (Pisum sativum L. cv. Solara) were germinated in humid vermiculite inoculated with a three-day-old yeast broth suspension culture of Rhizobium leguminosarum by. viceae strain Risø 18a. After six days the seedlings were transferred to an aeroponic system consisting of a large plastic caisson tank equipped with a mist generator circulating 12 L of nutrient solution from the bottom of the tank. The nutrient solution was prepared as described previously (Rosendahl and Jakobsen, 1987) with the addition of 8 mM MES to provide some pH buffering, pH was kept at 6-6.5 by adjusting with 5 M KOH when necessary. The nutrient solution was inoculated once a week with 50 mL of the Rhizobium suspension culture. The roots of seedlings were pushed through holes in the lid of the tank and thus exposed to the mist. The lid was carefully tightened around the stems of the plants in order to avoid salt deposition on the stems, which may result in plant death. Pea nodules were harvested from this system with minimal physical disturbance and were observed to retain a higher nitrogenase activity than pea nodules harvested from the below mentioned vermiculite growth system (data not shown). Nodules grown in the aeroponic system maintained a higher nitrogenase activity when immersed in water than vermiculite grown nodules (data not shown). This may be attributed to the fact that nodules are covered with a water film in the aeroponic system and thus adapted to taking up oxygen and nitrogen under these circumstances.

Nodules from *Lotus japonicum* inoculated with *Mesorhizobium loti* strain R7A (Sullivan et al., 1995) were used for a few experiments. Plants were grown in vermiculite-filled pots in an experimental set-up described by Rosendahl and Jakobsen (1987).

All plants were cultured in a growth chamber under a 16/8 h light/dark cycle at 20/16°C and a photosynthetically active photon flux density of 600 μ mol m⁻² s⁻¹. Nodules were excised from the roots immediately before NMR experiments when plants were six to seven weeks old (early pod-filling stage) and nitrogenase activity is maximal.

Experimental design of in vivo studies

In vivo NMR spectra were recorded from detached root nodules that were incubated with ${}^{15}N_2$ in a perfusion system (Fig. 1). After perfusion the nodules were quickly removed from the NMR tube, gently rinsed with water, and immediately frozen in liquid nitrogen. The nodules were kept at -80°C until further analysis. Subsamples were later taken for determination of FW/DW ratios, total ${}^{15}N$ incorporation, soluble amino acid pools, and ${}^{15}N$ -labelling of individual amino acids. Spectra and matching analyses from a single experiment are presented in this paper, but similar results were obtained in several experiments.

Perfusion and ¹⁵N₂ incubation of nodules for in vivo NMR

Approximately 1.5 g FW root nodules were placed in a 10 mm NMR tube, which was connected to a perfusion system (Fig. 1). Nodules were maintained in a physiologically viable and controllable state by perfusion with an oxygenated nutrient buffer consisting of 25 mM glucose, 25 mM malate, 0.1 mM CaSO₄, 10 mM MES (pH 6.0), 10% D₂O at a temperature of 21-22°C. A teflon inlet tube delivered the buffer to the bottom of the NMR tube below the nodules and the buffer left through two output tubes above the nodules. The tubes and nodules were kept in place by a plastic rod insert fixed to the screw cap top of the threaded NMR tube. All tubing outside the NMR tube was made of Tygon (R-3603, 1.6 mm wall. Cole-Parmer, USA), which is only slightly permeable to N2 and O2. A peristaltic pump attached to the two output tubes circulated the 700 mL nutrient buffer at 45 mL min⁻¹ through the nodule-filled volume, and recycled it via a closed gas-tight reservoir. The reservoir consisted of a 500 mL rubber stopped serum bottle that contained a liquid phase and an 80 mL gas phase. Equilibrium between the phases was ensured by continuous stirring of the liquid phase and by spraying the perfusate returning from the NMR tube through the gas phase. To achieve a high enrichment of ${}^{15}N_2$ in the perfusion system, the reservoir was first flushed for 30 min with an O₂/Ar mixture (1:1) while circulating the nutrient buffer in order to drive off all ¹⁴N₂. Then the reservoir was filled with O2, and ¹⁵N2 (99.95 atom% ¹⁵N. Isotec Inc., Ohio, USA) was introduced in the system by injecting it into the reservoir. The composition and ¹⁵N-enrichment of the final gas mixture in the perfusion system was 49% N₂ (92 atom%¹⁵N), 48% O₂ and 3% Ar as determined by mass spectrometry of a subsample. The gas mixture in the perfusion system was compressed to a total pressure of 1.6 atm during the entire experiment in order to dissolve more gas in the circulating liquid phase and to reduce intrusion of atmospheric air. At the end of the experiment after eight hours of perfusion of the nodules the composition of the gas phase was 56% N2 (73 atom% ¹⁵N), 39% O2, 4% Ar and 0.4% CO2. The pH value of the perfusate was measured after the experiment to ensure that pH was unchanged. The perfusate was also examined by ¹⁵N NMR spectroscopy to ensure that all observed ¹⁵N resonances originated from intracellular ¹⁵N metabolites.

Total ¹⁵N incorporation

Nodules were dried and homogenised, and the ¹⁵N-enrichment was determined on an isotope ratio mass spectrometer (Finnigan MAT Delta E) coupled in continuous flow mode to an EA 1110 elemental analyser as described previously (Egsgaard et al., 1989).

Preparation of ¹⁵N-enriched biological material and solutions

In order to aid the initial assignments of ¹⁵N metabolites, highly ¹⁵N-enriched pea nodules were prepared in a separate experiment and studied by ¹⁵N NMR spectroscopy after quenching of all metabolic activity. These pea plants were grown with the entire root system in a gas tight chamber (Rosendahl and Jakobsen, 1988). The root chamber of six-week-old plants was flushed with an Ar-O₂ mixture (70%:30%), and ¹³N₂ was subsequently added to a final gas composition of: 59% Ar, 24% O₂, 10% ¹⁵N₂ and 7% ¹⁴N₂. After four hours incubation in the ¹⁵N-enriched atmosphere root nodules were quickly harvested on ice, immediately frozen in liquid nitrogen and kept at -80°C until NMR analysis. After recording of NMR spectra, nodules and the medium bathing the nodules during NMR analysis were extracted with MeOH/CHCl₃/H₂O (12:5:3, v/v/v). The extract was subsequently purified as described below, redissolved in 0.1 M HCl and pH adjusted to 5.8 before NMR analysis. Identification of ¹⁵N-labelled metabolites was obtained by spiking the extract with authentic ¹⁵N-labelled amino acids.

Standard solutions of ¹⁵N-enriched ammonium and amino acids were prepared by dissolving authentic 99% ¹⁵N-enriched compounds, purchased from Icon Services Inc., Summit, USA, in 0.1 M HCI to a concentration of about 30 mM. The solutions were titrated to different pH values with KOH and HCl for the study of pH dependence of NOE factors.

Extraction of amino acids from nodules

Frozen nodules (approx. 0.4 g FW) were homogenised in a mortar on liquid nitrogen, and α aminobutyric acid was added as an internal standard. The homogenate was transferred to a tube and kept on ice, and amino acids were extracted as described by Johansen et al. (1996) by three subsequent additions of 4 mL MeOH/CHCl₃/H₂O (12:5:3, v/v/v), vortex mixing for 1 min and centrifugation (2000 g, 5 min, 5°C). All subsequent purification of extracts was performed on ice. The supernatants were pooled and CHCl₃ (14 mL) and water (3 mL) was added. The tube was vortexed and centrifuged (2000 g, 2 min, 5°C) to facilitate phase separation. The methanol-water phase containing the amino acids was evaporated in a Speed-Vac concentrator, and the amino acids were finally taken up in 1 mL of 0.1 M HCl. An amino acid standard solution was subjected to the extraction procedure and analysed in parallel with nodule extracts to ensure that in particular glutamine and asparagine were not degraded during the applied procedures.

Determination of soluble amino acid pools

Nodule extracts were deproteinised by addition of sulphosalicylic acid to a final concentration of 0.1 M, and norleucine was added as an internal standard. The samples were left for 15 min at room temperature before they were centrifuged (4000 rpm, 30 min, 5°C), and the supernatant was applied to a Waters HPLC system modified as an amino acid analyser with cooled autosampler and fluorometer. The instrument used cation exchange chromatography for separation of the amino acids and post column reaction with ortho-phthaldialdehyde for quantification. Millenium software from Waters was used for control of the instrument and for integration of the amino acid peaks.

Analysis of ¹⁵N-labelling of amino acids by LC-MS

Underivatised amino acids were analysed using the ion-pair reverse-phase chromatographic strategy as pioneered by Petritis et al. (2000). Asp, Asn, Gln, Glu and Ala were baseline separated using a Purospher RP-18e (125 x 4 mm, 5 μ m) column with 0.5 mM pentadecafluoroctanoic acid as mobile phase (0.5 mL/min). The rather difficult separation of GABA and α -aminobutyric acid was achieved using 10 mM nonafluorpentanoic acid as mobile phase. In all cases a sample size of 50 uL was used. The isotope analyses were carried out on-line using a LCQ (Classic) MSⁿ system bundled with an ESI (electrospray ionisation) source and a complete TSP HPLC system. Specific scan events were designed to meet the analysis of the individual amino acids. The ¹⁵N content was determined using the single ion traces of the MH⁺ and {M+1}H⁺ ions. The ¹⁵N content was calculated directly from the measured ratios and corrected for natural abundances.

The positional labelling of Gln was quantified by monitoring the daughter ions formed by the loss NH₃ from the $[M+1]H^+$ ions using MS/MS experiments. Thus, the daughter ions $([[M+1] - NH_3]H^+$ and $[[M+1] - {}^{15}NH_3]H^+)$ were monitored using two different MS/MS experiments. The positional labelling of Asn was quantified using the granddaughter ions arising from the consecutive loss of $[CO + H_2O]$ and HNCO from the $[M+1]H^+$ ions by MS/MS/MS experiments. Thus, the granddaughter ions ($[[M+1] - [CO + H_2O] - HNCO]H^+$ and $[[M+1] - [CO + H_2O] - {}^{15}HNCO]H^+$) were quantified using two different MS experiments. The isolation widths in the MS⁶ experiments were carefully optimised with respect to sensitivity and accuracy. The positional labelling was calculated on the principles of isotope dilution.

NMR spectroscopy

³¹P and ¹³N spectra were recorded at 242.8 and 60.79 MHz, respectively, on a Varian Unity Inova 600 spectrometer using a broadband 10-mm-diameter probehead.

In vivo ³¹P spectra were acquired with a 30° pulse angle (12 μ s), 0.125 s acquisition time, proton decoupling by WALTZ-16 composite pulse sequence, 11.2 kHz sweep width, 13824 transients and 20 Hz line broadening. ³¹P chemical shifts were measured relative to the signal from methylene diphosphonic acid (pH 8.9 in TRIS buffer) contained in a capillary included in the NMR tube and are quoted relative to the resonance of 85% phosphoric acid at 0 ppm. Assignments of ³¹P signals were based on literature reports (Saint-Ges et al., 1991) and own ³¹P NMR analyses (data not shown) of neutralised perchloric acid extracts performed as described by Roby et al. (1987).

Estimates of intracellular pH were based on cytoplasmic and vacuolar calibration curves, respectively, that were made as suggested by Spickett et al. (1993).

¹⁵N spectra of metabolically active, ¹⁵N₂ fixing nodules as well as of dead ¹⁵N-enriched nodules were acquired with a 60° pulse angle, 0.25 s acquisition time, recycle delay of 1.75 s, proton decoupling at high power during the acquisition and at low power during the delay by WALTZ-16 composite pulse sequence, 9.3 kHz sweep width, 1792 or 3584 transients leading to total acquisition times of one or two hours, respectively. 8 Hz line broadening was applied.

¹⁵N spectra of nodule extracts were acquired with a 90° pulse angle, 2 s acquisition time, 3 s delay 1, 5 s delay 2, proton decoupling at high power during the acquisition and at low, power during delay 2 by WALTZ-16 composite pulse sequence, 18 kHz sweep width and 5800 transients leading to a total acquisition time of 16 hours. 5 Hz line broadening was applied.

¹⁵N NMR spectra of standard solutions of ¹⁵N-enriched ammonium and amino acids were acquired with a 90° pulse angle, 2 s acquisition time, 10 s delay 1, 10 s delay 2 and 9.3 kHz sweep width. No line broadening was applied. NOE factors were determined as the ratio between signal intensities in 1) spectra where NOE was applied by WALTZ-16 modulated proton decoupling during delay 2 as well as during the acquisition and 2) spectra with inverse gated decoupling. Line widths were similar in the two types of spectra.

¹⁵N chemical shifts were measured relative to the signal at 55.8 ppm from 0.25 M aqueous ¹⁵Nurea. For ¹⁵N NMR analyses of nodules, urea was contained in a capillary included in the NMR tube, whereas urea was added directly to extracts and ¹⁵N standard solutions.

Results

Optimisation of experimental conditions for maximal ¹⁵N incorporation

¹⁵N NMR spectroscopy is not a very sensitive method due to the inherent magnetic properties of the ¹⁵N nucleus, i.e. the low magnetogyric ratio. In order to make the *in vivo* S/N ratio of ¹⁵Nlabelled metabolites exceed the ¹⁵N detection threshold, it was therefore crucial to optimise factors influencing the ¹⁵N incorporation.

The ¹⁵N-enrichment of the root nodules depends initially on the level of nitrogenase activity, which again depends on inherent properties of the biological material. The proportion of nitrogen fixing nodule tissue varies with age, and nodules from plants of different ages were tested. However, no major differences could be observed in *in vivo* ¹⁵N NMR spectra of nodules from plants aged between four and seven weeks, and six- to seven-week-old plants were used throughout the study. The root system of a given plant contains nodules of different sizes and developmental stages. The size of the NMR tube limits the amount of nodules that can be included in an experiment, and as smaller nodules may be packed more densely in the tube they might give rise to a higher total ¹⁵N₂

fixing rate per volume unit, although more mature nodules are known to contain a larger zone of N_2 fixing tissue. We tested whether small, young nodules from the lower part of the root system or large, older nodules from the top of the tap root gave better ¹⁵N NMR spectra and it was found that large nodules were best. Different pea varieties in combination with different bacterial strains were likewise tested and the most efficient combination (cf. materials and methods) was used for all described experiments.

It was tested whether nodules from *Lotus* plants would give rise to more informative *in vivo* ¹⁵N NMR spectra, but the obtained spectra (Fig. 3 C) displayed less intense signals than spectra from pea nodules, and *Lotus* nodules were therefore rejected for further experiments. However, the *Lotus* ¹⁵N spectra provided valuable information that aided the assignment of ¹⁵N resonances as described later.

Root nodules in the perfusion system were immersed in liquid, and this is well known to cause a decrease in nitrogenase activity, which, however, may be partially recovered by increasing the oxygen supply to the nodules (Sprent, 1969). The O₂ level experienced by the nodules in the NMR tube depends on a number of factors such as the composition and pressure of the gas phase, the equilibrium between the perfusion buffer and the gas phase, the perfusion buffer flow rate and the packing of nodules in the NMR tube. An adequate flow rate must be maintained to prevent significant gradients of oxygen tension across the sample, due to both depletion of oxygen by cells nearest the medium inlet, and non-uniform flow over individual pieces of tissue. The most intense *in vivo* ¹⁵N signals occurred when nodules were perfused at a flow rate of 40-50 mL min⁻¹ with a buffer in equilibrium with a gas phase containing about 50% O₂ and a total gas pressure of 1.6 atm. When the O₂ concentration was raised to about 70%, less intense ¹⁵N NMR signals were observed, which indicated an inhibition of nitrogenase activity.

The total amount of ¹⁵N incorporated by pea nodules during an eight-hour incubation period in the perfusion system was determined by mass spectrometry to be 1.97 μ mol ¹⁵N g⁻¹ FW. The mean nitrogenase activity could thus be estimated to be 0.12 μ mol ¹⁵N₂ g⁻¹ FW h⁻¹. The nitrogenase activity was presumably higher in the beginning of the incubation and decreased throughout the incubation period.

Monitoring of physiological state of root nodules

During NMR experiments the energy status of root nodules and possible changes in intracellular pH, which might have indicated hypoxic conditions, were monitored by acquiring ³¹P NMR spectra. Fig. 2 shows representative in vivo ³¹P spectra obtained at the beginning (0 h) and at the end (8 h) of the ¹⁵N experiment shown in Fig. 5. ATP concentrations did not change significantly throughout the experiment as seen from the unchanged intensity of the well resolved signals from the β - and γ -phosphate groups of ATP at -19.0 and -5.5 ppm, respectively. The intracellular pH can be estimated from the chemical shift of the P_i signal based on calibration curves (see materials and methods), and we observed that pH in the cytoplasm as well as in the vacuole remained stable during the course of the experiment. The pH values in the cytoplasm and vacuole were estimated to

be 7.2 and 5.2, respectively, from the P_i chemical shifts at 2.1 ppm and 0.3 ppm, respectively. The cytoplasmic compartment at pH 7.2 might include both the plant and the bacteroid cytoplasm. The resonance at 1.6 ppm could represent P_i in a different subcellular compartment with a pH of approximately 6.8 or it could be a signal from one of the phosphate groups of phytate. Another possible signal from phytate was observed at 1.0 ppm, but the exact position of the phytate multiplet is highly sensitive to pH and to the chemical environment, making its correct identification difficult (Saint-Ges et al., 1991). However, it seems likely that the two small peaks represent phytate as ³⁴P spectra of perchloric acid extracts from root nodules also contained phytate resonances of approximately the same intensity (data not shown).

The ³¹P spectra from pea root nodules showed a large, unexpected signal (X) at -1.9 ppm (Fig. 2), which was observed in several experiments to be more narrow and persistent during prolonged perfusion than all other ³¹P signals. The same chemical shift was observed in ³¹P NMR spectra of root nodule perchloric acid extracts at pH 7.5 and showed no coupling to other ³¹P nuclei (data not shown). The signal appeared as a 1:2:1 triplet with J = 8.9 Hz in proton coupled ³¹P NMR spectra of perchloric acid extracts (data not shown), which indicated that it was a phosphate group located next to a methylene group. The concentration in the root nodule tissue was estimated to be 0.1 μ mol g⁻¹ FW from a ³¹P NMR spectra of an extract. The yet unidentified peak X was not observed in ³¹P NMR spectra of either pea root tips, a dense suspension of free-living *Rhizobium leguminosarum* bv. vicea, or intact root nodules from *Lotus* or soybean (data not shown). On the other hand, *in vivo* ³¹P NMR spectra of *Lotus* nodules showed a very distinct, intense and unusual resonance at 16.3 ppm (data not shown), which was not observed in pea or soybean nodules.

Assignment of ¹⁵N resonances

Rather different ¹⁵N NMR chemical shifts of amino acids have previously been published (see Table 1) and make it problematic to base assignments purely on literature values. The variability could mean that chemical shifts are strongly influenced by the matrix, and amino acids may be erroneously assigned, as the chemical shifts of the amino resonances of glutamate and glutamine on one side and asparagine and aspartate on the other side are very close. One reason for the variation in the reported chemical shifts could be pH differences between the biological systems. However, we performed pH titration studies of the relevant amino acids and observed rather modest changes in the chemical shifts at pH values below 8. Another cause of uncertainty in the literature reports is the use of different reference compounds. ¹⁵N urea was used in the present study either as an internal standard or in a capillary and resonated at the same frequency under both circumstances. Some studies use intracellular ammonium as a reference compound, but as the ammonium chemical shift may be strongly influenced by the nature of the intracellular environment it seems a rather unsuitable reference. We studied whether temperature effects on chemical shifts could explain some of the differences in the literature reports. This seems not to be the case as an increase of 10°C shifted the glutamine amide resonance only approximately 0.2 ppm downfield, whereas the ammonium and the amino resonances of glutamine and aspartate were shifted approximately 0.1 ppm upfield.

Assignment of ¹⁵N resonances in dead nodules

Several ¹⁵N signals from amino acids that were labelled by assimilation of fixed ¹⁵N₂ were observed in an NMR spectrum from dead root nodules (Fig. 3 A). These resonances were tentatively assigned as being GABA, alanine and the amide and amino group of asparagine (Table 2) by comparison with literature values and own measurements of authentic standards. Subsequent extraction of the nodules and recording of an ¹⁵N spectrum from the extract (Fig. 3 B) showed almost identical chemical shift values compared to dead nodules. The assignments were confirmed by spiking extracts with authentic ¹⁵N amino acid standards followed by NMR analysis. The observed resonance at 20.2 ppm could not be assigned as spiking with ¹⁵N-labelled glutamate and glutamine demonstrated that both amino resonances were downfield of 20.2 ppm. A small signal at -0.14 ppm, presumably from ¹⁵N-labelled ammonium, was observed in the extract, but not in the spectrum from dead nodules.

Assignment of ammonium in metabolically active nodules

Incubation of metabolically active root nodules with ${}^{15}N_2$ in the perfusion system while recording ${}^{15}N$ NMR spectra resulted in the observation of intracellular ${}^{15}N$ ammonium in experiments with both pea and *Lotus* (see Fig. 3 C and Fig. 5). The resonance frequency showed some variability in the range from -4.2 to -3.9 ppm. This is an unusually low ammonium frequency compared to own *in vitro* measurements and previous *in vivo* ${}^{15}N$ NMR studies, which all have demonstrated ammonium resonances at around 0 ppm in procaryotic as well as in eucaryotic organisms (cf. Table 1).

The signal at around -4 ppm was assigned to ammonium based on several indications. First, no other well known ¹⁵N metabolite resonates anywhere near -4 ppm. Second, the signal at -4 ppm represents the first detectable ¹⁵N metabolite in ¹⁵N₂ fixing nodules (cf. Fig. 5), which is in accordance with the well-established fact that the product of nitrogenase activity is ammonium. Third, the NOE factor is between -1 and 0, which is characteristic for ammonium *in vivo* (Martin, 1985; Robinson et al., 1991; Fox et al., 1992; Joy et al., 1997) and gives rise to spectra with oppositely phased signals for amino acids and ammonium, respectively. Finally, the signal at -4 ppm is intense in spite of being attenuated by NOE under the applied acquisition parameters, and it must thus originate from a metabolite that is present in large amount, which is what should be expected from ammonium in the bacteroid compartment.

Acquisition of ¹⁵N NMR spectra from the perfusion buffer after the incubation was ended demonstrated that no extracellular ¹⁵N-labelled ammonium was present (data not shown).

Assignment of amino acids in metabolically active nodules

Considering the very unusual chemical shift of the ammonium ions in root nodules, the assignments of the resonances of amino acid ¹⁵N amino groups required some attention. The amino acid amide resonances are much less likely to be severely perturbed. *In vivo* ¹⁵N NMR spectra from ¹⁵N₂ fixing pea root nodules showed two amino acid ¹⁵N resonances at 90.7 and 19.6 ppm (Fig. 5), which correspond to an amide and an α -amino group, respectively. This was observed in several

experiments. The amide resonance at 90.7 ppm could, by comparison with own *in vitro* measurements and previously observed values (Table 1), unequivocally be assigned to the amide resonance of asparagine.

The assignment of the α -amino resonance at 19.6 ppm posed some problems. The chemical shift value corresponded to glutamine and glutamate according to own *in vitro* measurements as well as previously observed values (Table 1), but the analyses of dead nodules and extracts (cf. Fig. 3 A and B) demonstrated that the dominating ¹⁵N-labelled α -amino group was the one of asparagine and that it resonated at the expected 18.8-19.1 ppm when inside dead nodules. Analyses of the ¹⁵N-labelling by LC-MS also showed that the asparagine amide and amino groups were the most abundant ¹⁵N-labelled species in nodules that were incubated with ¹⁵N₂ in the perfusion system (Table 3 and Fig. 7). This apparent inconsistency needed an explanation, and further investigations indicated that the explanation seemed to relate to NOE factors (see later).

Observations from an experiment with *Lotus* nodules indicated that the resonance at 19.6 ppm could be assigned to glutamine/glutamate. Two α -amino resonances were observed at 19.6 and 18.4 ppm, respectively, in an *in vivo* ¹⁵N NMR spectrum from *Lotus* nodules (Fig. 3 C). The chemical shift difference between asparagine and aspartate was demonstrated *in vitro* to be around 0.6 ppm (cf. Table 2), and it was therefore unlikely that the resonances at 19.6 and 18.4 ppm originated from asparagine and aspartate. Whether the resonance at 18.4 ppm should be assigned to asparagine or aspartate could not be decided purely based on the chemical shift value, but it seemed likely that it was asparagine, because this amino acid is so abundant in indeterminate nodules (Ta et al., 1986). Consequently, the signal at 19.6 ppm in *Lotus* nodules seemed to be the α -amino group of either glutamine or glutamate. This suggests that the resonance at 19.6 ppm in pea root nodules also originated from glutamine/glutamate, since all other observed ¹⁵N resonances in *Lotus* nodules occurred at exactly the same frequencies as in pea nodules.

GABA was observed at the expected 11.6, 11.8 and 11.3 ppm in ^{15}N NMR spectra from dead pea root nodules, an extract from nodules and metabolically active, $^{15}N_2$ fixing *Lotus* nodules, respectively (cf. Fig. 3). However, GABA was never observed in *in vivo* ^{15}N NMR spectra from $^{15}N_2$ fixing pea nodules (Fig. 5), although LC-MS analyses demonstrated very high ^{15}N -labelling of GABA in these nodules (see Table 3 and Fig. 7). Possible reasons for this apparent inconsistency will be discussed later. The GABA ^{15}N resonances in spectra from nodules were in several experiments observed to be broader than resonances from other amino acids.

NOE effects

In order to observe quantitative results, *in vivo* ¹⁵N spectra should ideally be recorded without NOE effects. This turned out not to be feasible in the present study. However, the NOE effects lead to variation in intensities for the various amino acids resonances. This was investigated as a function of pH in standard solutions as shown in Fig. 4. The effects are clearly related to the pK, values of the compounds, except for GABA and ammonium where NOE factors smaller than the theoretical minimum of -4.9 were observed. The amino group of asparagine was observed to have a NOE

factor of -1 at around pH 7.1 *in vitro*, whereas NOE factors of the glutamine and glutamate amino groups were between -1.5 and -3.5 in the physiologically relevant pH range of 7-7.5. If the NOE factors exhibit the same pH dependency *in vivo* as was demonstrated *in vitro*, it would be expected that the signal from the α -amino group of asparagine would be lost, whereas the signals from the amino groups of glutamine and glutamate would be enhanced.

In vivo ¹⁵N NMR time course of ¹⁵N fixation and assimilation

Fig. 5 shows a representative series of consecutive *in vivo* ¹⁵N NMR spectra that were started immediately after addition of $^{15}N_2$ at time zero. An ammonium signal at -4.2 ppm was evident during the first hour of incubation and increased in intensity throughout the first five hours, after which steady state seemed to occur. Two more ¹⁵N signals emerged in the spectrum representing the period from one to three hours and likewise increased in intensity in the next spectrum from three to five hours. The intensities of these two signals were unchanged in the last spectrum from five to seven hours indicating steady state. The signal at 90.7 ppm was assigned to the amide group of asparagine. The amino groups of glutamate and glutamine resonate in a 0.2 ppm interval around 19.7 ppm and are difficult to resolve *in vivo*, so the resonance at 19.6 ppm is likely to contain contributions from the amino group of glutamine as well as glutamate.

Total amino acid pools and ¹⁵N-labelling

Pea root nodules contained asparagine as the dominating free amino acid, and its concentration amounted to as much as 19.9 μ mol g⁻¹ FW in freshly harvested nodules and 16.9 μ mol g⁻¹ FW in nodules that had been perfused for eight hours during the NMR experiment (Fig. 6). This was more than an order of magnitude more than each of the other major amino acids glutamine, alanine, aspartate and glutamate. In general, the concentrations of free α -amino acids were slightly lower in perfused nodules than in freshly harvested nodules. γ -aminobutyric acid (GABA) is not an α -amino acid and is not used for protein synthesis, but it constituted a substantial part of the soluble pool of nitrogen-containing metabolites in freshly harvested nodules (1.4 μ mol g⁻¹ FW) and the pool size apparently increased substantially during perfusion (5.7 μ mol g⁻¹ FW).

A new mass spectrometric technique involving separation of amino acids by IP-RPLC, made it possible to analyse the position of ¹⁵N in mono labelled glutamine and asparagine by using MS/MS and MS/MS/MS, respectively. The analysis could be done in a single experiment without any previous derivatisation procedures, which is a major simplification and improvement compared to GC-MS procedures, which are usually used for analysis of positional labelling. GABA, glutamate and alanine were the most highly ¹⁵N-enriched compounds after the eight-hour incubation period with ¹⁵N excess of 33.4, 27.9 and 25.0 atom%, respectively, but all examined compounds were found to be significantly ¹⁵N-labelled (Table 3). When the pool sizes were taken into account, asparagine and GABA were found to contain most of the ¹⁵N-label in soluble amino acids in the nodule tissue, namely 2.03 and 1.91 μ mol ¹⁵N g⁻¹ FW, respectively (Table 3 and Fig. 7). Alanine and glutamate contained a minor part of the total ¹⁵N-label (0.31 and 0.10 μ mol ¹⁵N g⁻¹ FW, respectively), because of their smaller pool sizes. Asparagine contained more ¹⁵N-label in the amino group (1.33 μ mol ¹⁵N g⁻¹ FW) than in the amide group (0.70 μ mol ¹⁵N g⁻¹ FW). Glutamine

was also more labelled in the amino group (0.34 μ mol¹⁵N g⁻¹ FW) than in the amide group (0.11 μ mol¹⁵N g⁻¹ FW).

Discussion

In vivo ¹⁵N-ammonium NMR signal in root nodules

The ¹⁵N NMR ammonium signal in living pea and *Lotus* root nodules was in several experiments observed at around -4 ppm, which is an unusually low ammonium frequency. Previous *in vivo* ¹⁵N NMR studies have all demonstrated ammonium resonances at around 0 ppm in biological systems as diverse as maize root tips (Amancio and Santos, 1992), carrot cells (Fox et al., 1992), *Corynebacterium glutamicum* (Tesch et al., 1999) and ectomycorrhizal mycelium (Martin et al., 1994). The observed enhanced shielding of ammonium can be due to many different factors such as pH effects, bonding to anions, macromolecules or paramagnetic ions and may thus provide valuable information on the intracellular environment.

Counter ions in large excess are known to perturb the ¹⁵N ammonium chemical shift (Stefaniak et al. 1986). We investigated, whether the observed unusual chemical shift could be explained by the presence of one of the more common anions, phosphate, and found no significant effects of physiologically relevant phosphate concentrations on the ammonium chemical shift (data not shown). Base titration of ammonium in vitro demonstrated, as expected, large pH effects on the chemical shifts, and an ammonium chemical shift of -4 ppm was observed at pH 8.9. From the present investigation it was not possible to localise the ammonium pool to the bacteroid or plant cytoplasm, but in either case it seems most unlikely that the pH could be that alkaline in a tissue that is metabolically active. Streeter (1989) estimated that free ammonium in soybean nodules was primarily confined to bacteroids, and it has been anticipated that the bacteroid cytoplasm is slightly alkaline (Day et al., 2001), because of the proton pumping activity of the electron transport chain in the bacteroid inner membrane as well as the proton consuming nitrogenase activity. It is thus possible that a minor contribution to the shielding of the observed ¹⁵N NMR ammonium signal was due to a higher pH in the bacteroids. However, it should be noted that as pH is raised, more and more of the ammonium will be converted to ammonia. This would cause a change in the ¹⁵N chemical shift but would also imply that the ammonium-ammonia couple, due to the latter, would complex metal ions much better. This in turn may lead to a shift in the chemical shift. If the large chemical shift is caused by pH and binding to paramagnetic sites, this would be likely to have an impact on both ammonium ions and the amino acids. However, no changes in the expected in vivo chemical shifts of amino acids were observed, and it may therefore be suggested that the ammonium ions and the amino acids are in different compartments. This could point to the ammonium ions being in the bacteroid, whereas the labelled glutamine/glutamate and asparagine were located in the plant cytoplasm.

The free ammonium concentration in symbiotic root nodules has never been solidly quantified. Based on extractions and several assumptions Streeter (1989) estimated that soybean bacteroids contained 1.6 μ mol ammonium g⁻¹ FW nodule, whereas the concentration of ammonium in the plant cytoplasm was essentially nil. The ¹⁵N NMR acquisition mode of the present study only allowed for a rough estimate of the minimum nodule ammonium concentration. The intensity of the ¹⁵N ammonium NMR signal was of a comparable size to the signal from the asparagine amide group after an eight-hour incubation with ¹⁵N₂ (cf. Fig. 5). NMR signal intensities are influenced by both T₁ and NOE, which were not quantified under *in vivo* conditions in the present study, but from other studies it can be ascertained that both processes would cause the ammonium signal to diminish relative to the asparagine amide signal. *In vivo* NOE factors for amino acid amide groups have previously been shown to be of the order of -4.5 (Kanamori et al., 1982b; Thorpe et al., 1989; Joy et al., 1997), whereas the ammonium NOE factor is between 0 and -1 in the present study. Amino acid amide groups T₁s of 3-4 s in plant tissue have been reported (Kanamori et al., 1982b; Thorpe et al., 1989; Joy et al., 1997), whereas very long ammonium T₁s of up to 50 s are commonly observed *in vivo* (Tesch et al., 1999). Thus, the concentration of ¹⁵N ammonium must be at least as large as that of ¹⁵N-labelled asparagine amide, which was estimated to constitute 0.7 μ mol g⁻¹ FW nodule based on LC-MS measurements

In summary, it is evident that substantial, although not quantifiable, ¹⁵N-labelling of ammonium took place in the metabolically active pea root nodules in the present study and that ammonium was the first detectable ¹⁵N-labelled compound in the intact symbiosis. This is in accordance with many previous reports (reviewed by Day et al. 2001) but in contrast to recent results by Waters et al. (1998), where no ¹⁵N-labelling of ammonium could be detected in isolated ¹⁵N₂ fixing soybean bacteroids, when these were incubated at high densities under microaerobic conditions. In the experimental set-up used by Waters et al. (1998) alanine was demonstrated to contain 98 atom% excess ¹⁵N, and to be the primary product excreted by the bacteroids.

An inversion of the ammonium resonance during decoupling (the NOE factor being between -1 and 0) has previously been observed for ammonium ions in solutions with added cell debris, whereas no effect was seen for glutamate (Fox et al., 1992). A similar finding concerning ammonium ions was made by Altenburger et al. (1991), who explained the effect as being due to the ammonium ions being bound to ion-exchange sites in the cell wall. It is interesting to notice that neither a change in the chemical shifts nor any apparent line broadening occurred.

Nitrogen assimilation in pea nodules studied by in vivo ¹⁵N NMR and LC-MS

Some of the results from *in vivo* ¹⁵N NMR and LC-MS seemed at first to be contradictory. The most abundant amino acid, asparagine, was found to contain about twice as much ¹⁵N-labelling in the amino group than in the amide group when analysed by LC-MS (cf. Table 3). A large asparagine amide resonance was observed in *in vivo* ¹⁵N NMR spectra from pea root nodules in many experiments, but an ¹⁵N amino group NMR signal was never observed *in vivo* (cf. Fig. 5). However, the asparagine ¹⁵N amino as well as the amide resonance was seen in NMR spectra of nodule extracts. These apparent inconsistencies could, however, be explained if the NOE factors exhibit the same pH dependency *in vivo* as was demonstrated *in vitro*. It would then be expected that the signal from the amino group of asparagine would be lost in *in vivo* ¹⁵N NMR spectra,

whereas the signal from the asparagine amide group would be enhanced. This mechanism may thus provide the explanation to make ends meet, and conclude that asparagine is ¹⁵N-labelled in both the amino and the amide groups, although the amino resonance is invisible in ¹⁵N NMR spectra with full decoupling.

The variation in the NOE effect with pH has been debated intensely over the years (Cooper et al., 1973; Leipert and Noggle, 1975; Irving and Lapidot, 1975). Exchange modulated scalar relaxation, although suggested by Cooper et al. (1973), has been ruled out (Leipert and Noggle, 1975; Irving and Lapidot, 1975). The latter advocated relaxation due to paramagnetic impurities, but this was contradicted by Leipert and Noggle (1975), as they claimed to prevent this by addition of EDTA. No agreement has so far been reached. From our *in vitro* experiments (cf. Fig. 4) it seems obvious that the NOE effect is related to the pK, values. Our results were to a large extent similar to those published by Leipert and Noggle (1975).

If it is correct that the *in vivo* NOE factor of the asparagine amino group is of an unfavourable size, which causes elimination of the signal in spectra recorded with full decoupling, it is surprising that this has not been reported previously, since the majority of *in vivo* ¹⁵N NMR studies applies full decoupling. On the other hand, very few *in vivo* ¹⁵N NMR studies have observed a signal from the asparagine amino group (cf. Table 1). To our knowledge, only a single ¹⁵N NMR study, namely the investigation of ¹⁵N ammonium metabolism in duckweeds by Monselise and Kost (1993), demonstrated the presence of ¹⁵N-labelling in the amino group of asparagine. Amancio and Santos (1992) assigned an ¹⁵N signal at 18.6 ppm to the amino groups of both asparagine and aspartate, but in our opinion only aspartate would resonate at this low frequency. The absence of reported asparagine ¹⁵N amino signals in the *in vivo* NMR literature may reflect that no labelling occurs or that the asparagine pool of the studied organisms was below the detection limit, but it may also be caused by NOE signal elimination. Very few investigations have included parallel analyses by *in vivo* ¹⁵N NMR and other analytical methods that would reveal a possible NMR invisible asparagine amino signal.

In root nodules the amide group for the biosynthesis of asparagine from aspartate may either come from direct incorporation of ammonium or from the glutamine amide group, and previous results have indicated that both pathways occur in alfalfa nodules (Snapp and Vance, 1986; Ta et al., 1986). Our data indicate that some asparagine synthesis from highly ¹⁵N-enriched ammonium is taking place in pea root nodules, since the low level of ¹⁵N amide-labelling of glutamine makes it unlikely to account for all of the observed asparagine amide enrichment. The high total ¹⁵N-labelling of asparagine is consistent with its generally accepted role as the end product of the primary nitrogen assimilation processes taking place in indeterminate root nodules.

Another obvious difference between results obtained by the different analytical methods was that GABA contained a large amount of the total ¹⁵N-labelling, when analysed by LC-MS (cf. Table 3), but did not appear in pea nodule *in vivo* ¹⁵N NMR spectra at all (cf. Fig. 5). The *in vitro* pH dependence of the GABA NOE factor gave no indications that NOE could cause elimination of the

¹⁵N NMR signal from GABA. ¹⁵N GABA has previously been observed by *in vivo* ¹⁵N NMR spectroscopy (cf. Table 1), although some investigators have noted that the signal possibly also contained contributions from ¹⁵N-labelled ornithine and lysine. *In vivo* T₁s of GABA have been reported shorter than of other amino acids, and *in vivo* NOE factors for GABA were found to be numerically larger than for other amino acids (Thorpe et al., 1989; Joy et al., 1997). These two variables affect signal intensities, but under the experimental conditions of the present study, both of the reported values would imply an enhancement and not an attenuation of the ¹⁵N GABA signals relative to signals of other amino acids.

GABA is synthesised from glutamate in a reaction catalysed by the enzyme glutamate α decarboxylase, which is well known in plant tissue (reviewed by Bown and Shelp, 1997) and has also been reported to be present in symbiotic rhizobia bacteroids (Miller et al., 1991; Fitzmaurice and O'Gara, 1991). The ¹⁵N-labelling of GABA found by LC-MS could reflect a decarboxylation of glutamate occurring after termination of the *in vivo* ¹⁵N NMR acquisition. However this seems unlikely when considering the high ¹⁵N-enrichment of GABA together with the short time period between acquisition of the last NMR spectrum and the quenching of metabolic activity by transfer of root nodules to liquid nitrogen.

A substantial amount of GABA (labelled as well as unlabelled) was shown to accumulate in root nodules during incubation in the NMR tube (cf. Fig. 6). This may reflect an increase in synthesis and/or a decrease in catabolism. The results concerning the size of the GABA pool in the nodule tissue do not allow for a discrimination of GABA localised in the bacteroid or in the plant cytoplasm. It is, however, evident from the high GABA ¹⁵N-labelling that the GABA is formed from a newly synthesised pool of glutamate. The GABA shunt pathway has previously been suggested to play a prominent role in *R. meliloti* bacteroids (Fitzmaurice and O'Gara, 1988; Miller et al., 1991), although the function of GABA remains unclear.

Some experimental evidence indicates that the locations of GABA production and accumulation are not identical, and that accumulated GABA is sequestered within organelles, possibly in the vacuoles (reviewed by Shelp et al., 1999). It has also been demonstrated that nodules from many legume species accumulate bound forms of GABA amounting to as much as 20% of the total N content of the nodule (Larher et al., 1983). The apparent NMR invisibility of ¹⁵N-labelled GABA in pea root nodules in *in vivo* experiments may result from GABA being immobilised in some way *in vivo*. Immobilised GABA may be released from the tissue by the applied extraction procedures and/or enzymatic reactions and therefore detected by LC-MS. A support for such a suggestion is the observation of broad resonances of GABA in dead nodules indicating that GABA is less tightly bound under such conditions but still not free in e.g. the cytoplasm.

Results from both NMR and LC-MS analyses agreed that glutamine was predominantly ¹⁵Nlabelled in the amino group and not in the amide group. No resonances from glutamine ¹⁵N amide were observed in ¹⁵N NMR spectra despite the fact that amide groups seemed to possess favourable NOE factors for the detection *in vivo*. Glutamine ¹⁵N amide groups did not give rise to any signals in NMR spectra of extracts, not even when signals from glutamine ¹⁵N amino groups were present. LC-MS results demonstrated that ¹⁵N in mono labelled glutamine was almost exclusively positioned in the amino group, and when the amount of double labelled glutamine molecules was taken into account, the distribution between amino and amide ¹⁵N-labelling was 3:1. The concurrent findings of glutamine being predominantly ¹⁵N-labelled in the amino position are very controversial as the prevailing scheme for the assimilation of fixed nitrogen states that ammonium is first incorporated into the amide group of glutamine, catalysed by glutamine synthetase.

Glutamate and alanine were shown by LC-MS to be among the most highly ¹⁵N-enriched metabolites, but the total pool sizes of glutamate and alanine were rather small compared to the other studied amino acids, which justifies that they were not observed by *in vivo* ¹⁵N NMR. The high ¹⁵N-enrichment of these two amino acids and of GABA might indicate that these metabolites were synthesised directly from highly ¹⁵N-enriched ammonium via glutamate dehydrogenase (GDH) and the GABA shunt pathway: $NH_4^+ \rightarrow glutamate \rightarrow GABA \rightarrow alanine.$

The direct incorporation of ammonium into glutamate catalysed by GDH is normally not considered an important reaction in the plant cytoplasm of root nodules. Substantial bacteroid GDH activity has been reported in many different host plants (Brown and Dilworth, 1975; Miller et al., 1991), but the *in planta* significance of GDH activity remains uncertain. Our results indicate that substantial GDH catalysed glutamate synthesis may occur under the given experimental conditions, although it is not possible to localise the reaction to a specific compartment.

If synthesis of glutamate and alanine via the GDH and GABA shunt pathway was taking place in the bacteroid compartment, the excretion product from bacteroid to plant cytoplasm could be a highly ¹⁵N-labelled amino acid. All of the necessary enzymes have been reported to be present in bacteroids (Brown and Dilworth, 1975; Miller et al., 1991; Fitzmaurice and O'Gara, 1991). If amino acids rather than ammonium were the excretion products under certain conditions, as has been suggested previously (Waters et al., 1998; Allaway et al., 2000), the ammonium pool of the plant cytoplasm is expected to contain very little ¹⁵N-label. In that way glutamine would be synthesised in the plant cytoplasm from highly labelled glutamate and poorly labelled ammonium resulting in glutamine molecules being higher labelled in the amino group (originating from glutamate) that in the amide group (originating from ammonium). It has previously been estimated that the plant cytoplasm contains only a minor free ammonium pool (Streeter, 1989), so the suggested way of synthesis of glutamine would depend on ammonium being provided from other sources. This speculative hypothesis would of course need further investigation, but at present it could serve as a possible explanation of the controversial observation of glutamine being predominantly ¹⁵N-labelled in the amino position.

Discovery of a novel phosphorus compound in pea nodule ³¹P NMR spectra

A new, very narrow and well resolved ³¹P signal at -1.9 ppm from an unknown compound in pca root nodules was observed. This resonance was absent in spectra from both *Lotus* and soybean root nodules. It has not been reported in previous ³¹P NMR studies of soybean nodules (Rolin et al.,

1989a) or alfalfa nodules (Nikolaev et al., 1994). Based on the observed coupling constants and the chemical shifts the phosphate group must be neighbouring a CH_2 group. It seemed to be a symbiosis specific compound since it was neither found in uninfected plant root tissue nor in free-living *Rhizoblum* bacteria.

The chemical shift of the unknown compound was identical when observed in living root nodules and in a perchloric acid extract at pH 7.5, which makes it unlikely that the compound is localised in the vacuole since the acidic conditions in this compartment would be expected to change the chemical shift. The signal was less line broadened than other ³¹P metabolites in *in vivo* spectra, and this may be because the compound was confined to a specific compartment in the nodule tissue, which might be the bacteroid, and thus experienced less field inhomogeneity because of the limited volume. The fact that the unidentified ³¹P signal persisted during prolonged perfusion of root nodules, when other ³³P signals became weaker, suggested that the compound was not metabolised and not washed out, which may indicate that it is bound in some way, though still highly mobile.

Concluding remarks

The present work presents the first report of *in vivo* ¹⁵N NMR spectra of ¹⁵N₂ fixing pea root nodules. The results demonstrate that it is indeed possible to apply *in vivo* ¹⁵N NMR spectroscopy to the study of nitrogen fixation and assimilation in root nodules. By using NMR it was possible to observe directly the incorporation of ¹⁵N into living nodules, but the detection was limited to ammonium and some of the more abundant amino acids.

The fully decoupled in vivo ¹⁵N NMR spectra did not include a signal from the amino group of asparagine, which may reflect that the *in vivo* NOE factor of the asparagine amino group is of an unfavourable size, since LC-MS results revealed that asparagine was indeed ¹⁵N-labelled at the amide as well as the amino nitrogen. LC-MS analyses showed that GABA was ¹⁵N-labelled in considerable amounts, but ¹⁵N GABA was NMR invisible in living pea root nodules. This may be due to immobilisation of GABA in root nodules.

A substantial pool of free ammonium was observed in the metabolically active, intact symbiosis by *in vivo* ¹⁵N NMR. The *in vivo* ¹⁵N NMR spectra further envisaged that glutamine was more highly ¹⁵N-labelled at the amino nitrogen than at the amide nitrogen. Both of these findings represent information that has not been made available by other techniques used to study nitrogen assimilation in legume nodules. The intracellular environment led to very unusual *in vivo* ¹⁵N ammonium chemical shifts, whereas no changes in the expected *in vivo* chemical shifts of amino acids were observed. This may suggest that ammonium and amino acids reside in different compartments: ammonium residing in the bacteroids, and ¹⁵N-labelled glutamine/glutamate and asparagine located in the plant cytoplasm.

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Literature cited

- Aarnes H, Eriksen B, Southon TE (1995) Metabolism of nitrate and ammonium in seedlings of Norway spruce (*Picea abies*) measured by *in vivo* N-14 and N-15 NMR spectroscopy. Physiol.Plant. 94:384-390
- Allaway D, Lodwig EM, Crompton LA, Wood M, Parsons R, Wheeler TR, Poole PS (2000) Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Mol.Microbiol. 36:508-515
- Altenburger R, Abarzua S, Callies R, Grimme LH, Mayer A, Leibfritz D (1991) Ammonia rhythm in Microcystis firma studied by in vivo ¹⁵N and ³¹P NMR spectroscopy. Arch.Microbiol. 156:471-476
- Amancio S, Santos H (1992) Nitrate and ammonium assimilation by roots of maize (Zea mays L.) seedlings as investigated by in vivo ¹⁵N-NMR. J.Exp.Bot. 43:633-639
- Amancio S, Clarkson DT, Diogo E, Lewis M, Santos H (1997) Assimilation of nitrate and ammonium by sulphur deficient Zea mays cells. Plant Physiol.Biochem. 35:41-48
- Belay N, Sparling R, Choi BS, Roberts M, Roberts JE, Daniels L (1988) Physiological and ¹⁵N-NMR analysis of molecular nitrogen fixation by Methanococcus thermolithotrophicus, Methanobacterium bryantii and Methanospirillum hungatei. Biochim.Biophys.Acta 971:233-245
- Bown AW, Shelp BJ (1997) The metabolism and functions of gamma-aminobutyric acid. Plant Physiol. 115:1-5
- Brown CM, Dilworth MJ (1975) Ammonia assimilation by *Rhizobium* cultures and bacteroids. J.Gen.Microbiol. 86:39-48
- Cooper RA, Lichter RL, Roberts JD (1973) pH-dependent nuclear magnetic resonance spectra of ¹⁵Nenriched glycine. Line shape and relaxation studies. J.Am.Chem.Soc. 95:3724-3729
- Day DA, Poole P, Tyerman SD, Rosendahl L (2001) Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell.Mol.Life Sci. 58:61-71
- Egsgaard H, Larsen E, Jensen ES (1989) Evaluation of automated analysis of ¹⁵N using on-line Dumas combustion. Anal.Chem.Acta 226:345-349
- Fitzmaurice AM, O'Gara F (1988) Involvement of glutamate as a carbon source in supporting nitrogen fixation activity in *R. meliloti.* In: Bothe G, de Bruijn FJ, Newton WE (eds)Nitrogen Fixation: Hundred years after. Gustav Fischer, Stuttgart, New York, pp 558-558
- Fitzmaurice AM, O'Gara F (1991) Glutamate catabolism in Rhizobium meliloti. Arch.Microbiol. 155:422-427
- Fox GG, Ratcliffe RG, Robinson SA, Slade AP, Stewart GR (1992) Detection of ¹⁵N-labeled ammonium. In vivo ¹⁵N NMR versus mass spectrometry. J.Magn.Reson, 96:146-153

- Fox GG, Ratcliffe RG, Robinson SA, Stewart GR (1995) Evidence for deamination by glutamate dehydrogenase in higher plants: Commentary. Can.J.Bot, 73:1112-1115
- Haran N, Kahana ZE, Lapidot A (1983) In vivo ¹⁵N NMR studies of regulation of nitrogen assimilation and amino acid production by Brevibacterium lactofermentum. J.Biol.Chem. 258:12929-12933
- Irving CS, Lapidot A (1975) Origin of the anomalous pH dependence of the 15-[1H] Nuclear Overhauser effect of amino and amide nitrogens. J.Am.Chem.Soc. 97:5945-5946
- Johansen A, Finlay RD, Olsson PA (1996) Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol. 133:705-712
- Joy RW, Mcintyre DD, Vogel HJ, Thorpe TA (1996) Stage-specific nitrogen metabolism in developing carrot somatic embryos. Physiol.Plant, 97:149-159
- Joy RW, Vogel HJ, Thorpe TA (1997) Inorganic nitrogen metabolism in embryogenic white spruce cultures: A nitrogen 14/15 NMR study. J.Plant Physiol. 151:306-315
- Kahl S, Gerendas J, Heeschen V, Ratcliffe RG, Rudolph H (1997) Ammonium assimilation in bryophytes. Lglutamine synthetase from Sphagnum fallox. Physiol.Plant. 101:86-92
- Kanamori K, Legerton TL, Weiss RL, Roberts JD (1982a) Effect of the nitrogen source on glutamine and alanine biosynthesis in *Neurospora crassa*. An *in vivo* ¹⁵N nuclear magnetic resonance study. J.Biol.Chem. 257:14168-14172
- Kanamori K, Legerton TL, Weiss RL, Roberts JD (1982b) Nitrogen-15 spin-lattice relaxation times of amino acids in *Neurospora crassa* as a probe of intracellular environment. Biochem. 21:4916-4920
- Kuesel AC, Kuhn W, Sianoudis J, Grimme LH, Leibfritz D, Mayer A (1989) N-15 in vivo NMR spectroscopic investigation of nitrogen deprived cell suspensions of the green alga Chlorella fusca. Arch.Microbiol. 151:434-438
- Larher F, Goas G, le Rudulier D, Gerard J, Hamelin J (1983) Bound 4-aminobutyric acid in root nodules of Medicago sativa and other nitrogen fixing plants. Plant Science Lett. 29:315-326
- Legerton TL, Kanamori K, Weiss RL, Roberts JD (1981) ¹⁵N NMR studies of nitrogen metabolism in intact mycelia of *Neurospora crassa*. Proc.Natl.Acad.Sci.USA 78:1495-1498
- Leipert TK, Noggle HN (1975) The nitrogen-15 magnetic resonance of glycine. J.Am.Chem.Soc. 97:269-272
- Li YZ, Green LS, Day DA, et al (2000) Ammonia and alanine efflux from nitrogen-fixing soybean bacteroids. In: Pedrosa FO, Hungria M, Yates MG, et al (eds)Nitrogen Fixation: From molecules to crop productivity. Kluwer Academic Publishers, Dordrecht, pp 391-391
- Martin F (1985) ¹⁵N-NMR studies of nitrogen assimilation and amino acid biosynthesis in the ectomycorrhizal fungus *Cenococcum graniforme*. FEBS Lett. 182:350-354
- Martin F, Cote R, Canet D (1994) NH₄⁺ assimilation in the ectomycorrhizal basidiomycete Laccaria bicolor (Maire) Orton, a ¹⁵N-NMR study. New Phytol. 128:479-485
- Miller RW, McRae DG, Joy KW (1991) Glutamate and gamma-aminobutyrate metabolism in isolated Rhizobium meliloti bacteroids. Mol.Plant-Micr.Interact. 4 (1):37-45
- Mitsumori F, Yoneyama T, Ito O (1985) Phosphorus-31 nuclear magnetic resonance studies on higher plant tissue. Plant Sci. 38:87-92
- Monselise EB-I, Kost D (1993) Different ammonium-ion uptake, metabolism and detoxification efficiencies in two Lemnaceae. A ¹⁵N-nuclear magnetic resonance study. Planta 189:167-173

- Nikolaev BP, Provorov NA, Simarov BV, Shlyakov AM (1994) Transformation of phosphates by free-living and symbiotic root-nodule bacteria *Rhizobium meliloti* followed by ³¹P NMR spectroscopy. Microbiol. 63:16-20
- Petritis K, Chaimbault P, Elfakir C, Dreux M (2000) Parameter optimization for the analysis of underivatized protein amino acids by liquid chromatography and ionspray tandem mass spectrometry. J.Chromatograph.A 896:253-263
- Poole P, Allaway D (2000) Carbon and nitrogen metabolism in Rhizobium. Adv. Microb. Physiol. 43:117-163
- Robinson SA, Slade AP, Fox GG, Phillips R, Ratcliffe RG, Stewart GR (1991) The role of glutamate dehydogenase in plant nitrogen metabolism. Plant Physiol. 95:509-516
- Roby C, Martin JB, Bligny R, Douce R (1987) Biochemical changes during sucrose deprivation in higher plant cells. Phosphorus-31 nuclear magnetic resonance studies. J.Biol.Chem. 262:5000-5007
- Rolin DB, Boswell RT, Sloger C, Tu SI, Pfeffer PE (1989a) In vivo ³¹P NMR spectroscopic studies of soybean Bradyrhizobium symbiosis. I. Optimization of parametres. Plant Physiol, 89:1238-1246
- Rolin DB, Pfeffer PE, Boswell RT, Schmidt JH, Tu SI (1989b) In vivo ³¹P NMR spectroscopic studies of soybean Bradyrhizobium symbiosis. Compartmentation and distribution of P metabolites. FEBS Letters 254:203-206
- Rosendahl L, Jakobsen I (1987) Rhizobium strain effects on pea: The relation between nitrogen accumulation, phosphoenolpyrovate carboxylase activity in nodules and asparagine in root bleeding sap. Physiol.Plant. 71:281-286
- Rosendahi L, Jakobsen I (1988) Effects of age, supra-ambient oxygen and repeated assays on acetylene reduction and root respiration in pea. Physiol.Plant, 74:77-82
- Saint-Ges V, Roby C, Bligny R, Pradet A, Douce R (1991) Kinetic studies of the variations of cytoplasmic pH, nucleotide triphosphates (³¹P-NMR) and lactate during normoxic and anoxic transitions in maize root tips. Eur.J.Biochem. 200:477-482
- Shelp BJ, Bown AW, Mclean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4:446-452
- Snapp SS, Vance CP (1986) Asparagine biosynthesis in alfalfa (Medicago sativa L.) root nodules. Plant Physiol. 82:390-395
- Spickett CM, Smirnoff N, Ratcliffe RG (1993) An in vivo nuclear magnetic resonance investigation of ion transport in maize (Zea mays) and Spartina anglica roots during exposure to high salt concentrations, Plant Physiol. 102:629-638
- Sprent JI (1969) Prolonged reduction of acetylene by detached soybean nodules. Planta 88:372-375
- Stefaniak L, Webb GA, Witanowski M (1986) Ann.Report NMR Spectrosc. 18:90
- Streeter JG (1987) Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. Plant Physiol 85:768-773
- Streeter JG (1989) Estimation of ammonium concentration in the cytosol of soybean nodules. Plant Physiol. 90:779-782
- Sullivan JT, Patrick HN, Lowther WL, Scott DB, Ronson CW (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc.Natl.Acad.Sci.USA 92:8985-8989
- Ta TC, Faris MA, Macdowall FDH (1986) Pathways of nitrogen metabolism in nodules of alfalfa (Medicago sativa L.). Plant Physiol. 80:1002-1005

- Tesch M, de Graaf AA, Sahm H (1999) In vivo fluxes in the ammonium-assimilatory pathways in Corynebacterium glutamicum studied by N-15 magnetic nuclear magnetic resonance. Appl.Envir.Microbiol. 65:1099-1109
- Thorpe TA, Bagh K, Cutler AJ, Dunstan DI, Mcintyre DD, Vogel HJ (1989) A ¹⁴N and ¹⁵N nuclear magnetic resonance study of nitrogen metabolism in shoot-forming cultures of white spruce (*Picea glauca*) buds. Plant Physiol. 91:193-202
- Waters JK, Hughes BL, Purcell LC, Gerhardt KO, Mawhinney TP, Emerich DW (1998) Alanine, not ammonia, is excreted from N-2-fixing soybean nodule bacteroids. Proc.Natl.Acad.Sci.USA 95:12038-12042

Legends

Fig. 1. Schematic drawing of the perfusion system used for studying nitrogen fixation and assimilation in living pea root nodules by ¹⁵N NMR spectroscopy. Approximately 1.5 g FW root nodules could be contained within the NMR tube, and about 1 g FW was within the volume of the NMR detection coil. The symbol \otimes indicates a three-way stop-cock.

Fig. 2. In vivo ³¹P NMR spectra of pea root nodules showing the unchanged metabolic status during an eight-hour incubation period. The 30 min spectra were recorded (A) before the addition of ¹⁵N₂ and (B) at the end of the treatment period after acquisition of the *in vivo* ¹⁵N spectra shown in Fig. 5. The numbered peaks may be assigned to: 1, several phosphomonoesters including glucose 6phosphate (1a) and phosphocholine (1c); 2, cytoplasmic P_i; 3, vacuolar P_i; 4, unidentified compound; 5, 6 and 9, the γ -, α - and β - phosphates of nucleoside triphosphate; 7, UDP-glucose and NAD(P)(H); and 8) UDP-glucose. Chemical shifts are quoted relative to 85% H₃PO₄ at 0 ppm

Fig. 3. ¹⁵N NMR spectra from (A) dead ¹⁵N-enriched pea root nodules immersed in buffer (pH 6), (B) extract thereof (pH 5.8) and (C) metabolically active, ¹⁵N₂ fixing *Lotus* nodules. The numbered peaks may be assigned to: 1, asparagine amide-N; 2, alanine; 3, glutamine/glutamate amino-N; 4, asparagine amino-N; 5, aspartate; 6, GABA; 7, ammonium. The signal at 55.8 ppm is from the reference compound ¹⁵N-urea

Fig. 4. Amino acid and ammonium NOE factors at different pH values. (A) α -Amino groups and (B) amide groups, ammonium and GABA. NOE factors were determined as the ratio between signal intensities in spectra with full decoupling and spectra with inverse gated decoupling

Fig. 5. In vivo ¹⁵N NMR spectra showing a time course of nitrogen assimilation in ¹⁵N₂ fixing pea root nodules. The numbered peaks may be assigned to: 1, asparagine amide-N; 3, glutamine/glutamate amino-N; 7, ammonium. The signal at 55.8 ppm is from the reference compound ¹⁵N-urea

Fig. 6. Concentrations of soluble amino acids in freshly harvested pea root nodules and nodules that have been subjected to perfusion for eight hours. Error bars denote standard errors (n=3)

Fig. 7. Concentrations of unlabelled, ¹⁵N-amino-labelled and ¹⁵N-amide-labelled soluble amino acids in pea root nodules after an eight-hour incubation period with ¹⁵N₂ in the perfusion system and acquisition of the *in vivo* ¹⁵N NMR spectra shown in Fig. 5.

Fig. 1





























Biological system (Reference)	Gin amide	Asn amide	Ala	Gln amino	Glu	Asn amino	Asp	GABA	NH4*
Brevibacterium lacsofermensum (Haran et al., 1983)	90.8	88.1	21.6		19.5		16.8		
Carrot cells* (Fox et al., 1992)	91.2			19	9.8				0
Corynebacterium glutamicum [*] (Tesch et al., 1999)	91			2	:0		•		0
Duckweed (<i>Lemnaceae</i>) [†] (Monselise & Kost, 1993)	90.8	90.5	22.0	19).9	19.3			0
Ectomycorthizal mycelium ⁶ (Martin et al., 1994)	90.4		21.9	19.7	19.6			11.6	0
Green algae (<i>Chlorella fusca</i>) [*] (Kuesel et al., 1989)	92.5		23.3	21.2	20.9				
Maize roots ¹ (Amancío & Santos, 1992)	91.7	89.0*	22.3	20.0	19.8	18.6		12.0	0
Neurospora crassa mycelium [®] (Kanamori et al., 1982a)	90,6		22.6	20.35	20.16				
Neurospora crassa mycelium [°] (Legerton et al., 1981)	92.2		22.3	20.3					0.1
Norway spruce seedlings roots and stems [*] (Aarnes et al., 1995)	91.1		22.0		19.8			10.6	-0.1

Table 1: Literature values of *in vivo* ¹⁵N chemical shifts of amino acids and ammonium in plants, fungi and bacteria

All spectra were collected either without D_2O or with D_2O contained in a capillary

* Chemical shifts were originally reported relative to NO_3 at 0 pmm, but have been converted to a scale that puts urea at 55.8 ppm (and NO_3 at 354.6 pmm)

* Chemical shifts were reported relative to intracellular ammonium at 0 ppm

"This value was reported by the authors, but the published in vivo ¹⁵N NMR spectra showed a signal from the asparagine amide group at approximately 91.4 ppm

	% D ₂ O	Gin amide	Asn amide	Ala	GIn amino	Glu	Asn amino	Asp	GABA	NH₄*
Authentic ¹⁵ N standards (pH 7)	10	90.86	90.51		19.85	19.65	18.90	18.34	11.75	-0.26
Dead ¹⁵ N-enriched pea nodules inumersed in buffer (pH 6)	20		90.6				18.8		11.6	
Extract from dead nodules (pH 5.8)	4		90.48	22.06			19.07		11.79	-0.14
Metabolically active, ¹⁵ N ₂ fixing pea nodules	10		90.7		19.6				•	-4.2
Metabolically active, ¹⁵ N ₂ fixing Lotus nodules	10		90.7		19.6		18	18.4		-4.1

Table 2. Assignments of amino acid and ammonium ¹⁵N chemical shifts

· · · · · ·	Total amino acid conc. (µmol/g FW)		Atom%	excess	¹⁵ N		Position of mono				Total ¹⁵ N	
					(W-1806)	concentration						
			(,	(APE)				(%)	(µmovgrw)			
	Mean [#]	S.E.		Mean	\$.E.			Mean	S.E.	R		Mean
Asn	16.91	1.65	mono ¹⁵ N	8.17	0,27	П	amino	73	6	6	amino	1.33
	ļ	I					amide	27	6	6		
	1		double ¹⁵ N	1.91	0.12	12					amide	0.70
Gaba	5.72	0.67		33.39		2						1.91
Gln	1.55	0.20	mono ¹³ N	15.74	2.08	5	amino	98	2	3	amino	0.34
	Ì						amide	2	2	3		
-			double ¹⁵ N	6.59	1.10	4					amide	0.11
Ala	1.24	0.12		25.02	0.80	٦						0.31
Asp	0.76	0.05		14.22	1.27	10						0.11
Glu	0.35	0.04		27.86	0.98	5						0.10

Table 3. ¹⁵N-labelling of soluble amino acids in pea root nodules after eight hours incubation with $^{15}N_2$ in the perfusion system

* n = 3

* The values given for amino and amide group total ¹⁵N concentration include both mono and double labelled molecules

Extraction and GC-MS analysis of amino acids from root nodules

All procedures were performed as cold as possible - i.e. on liquid nitrogen or ice

Extraction (modified after Johansen et al. 1996)

- Frozen nodules (approx. 0.4 g FW) were homogenised in a mortar on liquid nitrogen, and 40 μL 10 mM sarcosin were added as an internal standard
- The homogenate was transferred to a tube and kept on ice, and amino acids were extracted by three subsequent additions of 4 mL MeOH/CHCl₃/H₂O (12:5:3, v/v/v), vortex mixing for 1 min and centrifugation (2000 g, 5 min, 5 °C)

Purification (modified after Johansen et al. 1996)

- The supernatants from the three extractions were pooled and CHCl₃ (14 mL) and water (3 mL) was added. The tube was vortexed and centrifuged (2000 g, 2 min, 5 °C) to facilitate phase separation
- The chloroform phase was discarded
- The methanol-water phase containing the amino acids was evaporated in a Speed-Vac concentrator, and the amino acids were finally taken up in 1 mL of 0.1 M HCi and kept at -80 °C until further analysis

Derivatisation for GC-MS (modified after Fortier et al. 1986)

- 100 µl of the purified extract was transferred to a microvial with conical shaped bottom and evaporated again in a SpeedVac
- Immediately afterwards derivatisation was performed by addition of 15 μl MTBSTFA (N-methyl-N-(*tert.*butyldimethylsilyl)trifluoroacetamid), 15 μl pyridin and 1 μl triethylamin, and incubating the vials for 30 min at 75 °C

GC-MS analysis

- The GC-MS analysis were performed on a Varian Saturn2 gas chromatograph ion trap mass spectrometer equipped with a capillary column (DB-5, length 25 m, inner diameter 0.32 mm, 0.25 µm film), Helium was used as the carrier gas (50 mL min⁻¹)
- 1 μl of sample was injected and split in a ratio of 1:50. The injector, transfer line and manifold temperatures were 250, 280 and 200 °C, respectively. The oven temperature was initially held at 60 °C for 5 min and then linearly increased at 10 °C min⁻¹ to 290 °C, where it remained for 5 min
- Separated amino acid derivatives were ionised by electron impact. Mass spectra were recorded in the interval 50-550 m/z. Amino acids were identified from retention times and mass spectra by comparison with authentic standards
- ¹⁵N-enrichment was calculated from the distribution of the M-57 and M-57+1 ion with a correction for the natural abundance of these ions:

¹⁵N atom% excess = 100% * (Obs_{M-57+1} - NA_{M-57+1}) / (NA_{M-57} + Obs_{M-57+1} - NA_{M-57+1}),

where Obs denotes the observed intensity of the given ion in the mass spectrum and NA denotes the natural abundance

^{*} In the beginning of the project I included a purification of the extracts on a cation exchange column according to Bengtson and Odham (1979), but this was later left out, because I experienced that too much material was lost during this step.