

## Plant / Microbe Symbioses

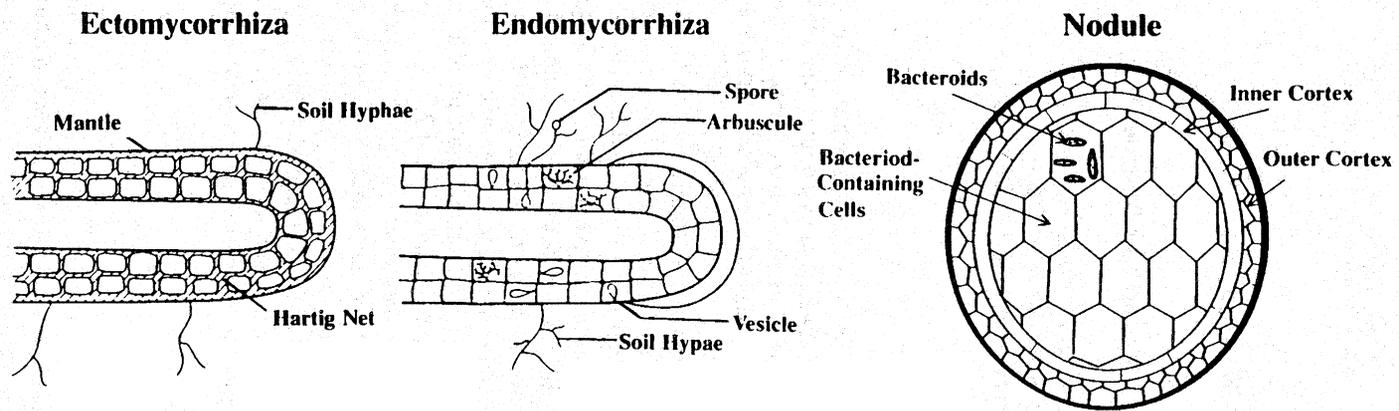
### 3.1 INTRODUCTION

The main subject of this chapter is the contribution of NMR to our understanding of the interactions between plants and their microbial symbionts. The three symbioses to which most attention is devoted: Ectomycorrhizae, Endomycorrhizae (of which only Vesicular Arbuscular Mycorrhizae are considered here), and nitrogen-fixing Nodules, are illustrated in Fig. 1. It is hard to overestimate the ecological and agricultural significance of these systems, and much work has been devoted to their study. Some of the most interesting and practically important questions about mycorrhizae and nitrogen-fixing nodules concern the benefits of the symbiosis to each participant. These benefits hinge on the structural and physiological intimacy between the symbionts. Therefore detailed information on metabolism, transport, and functional anatomy of intact systems is required. The potential to yield such information by non-destructive spatial and chemical discrimination is perhaps the most pronounced advantage of NMR spectroscopy and imaging (see Chapters 1 and 2).

It is for this reason that significant contributions have been made using NMR to such questions as the biochemistry of polyphosphates and the pathways and regulation of carbon and nitrogen metabolism in mycorrhizae as well as utilization and compartmentation of phosphate, barriers to oxygen diffusion, and the structure, role and regulation of cyclic glucans in nitrogen-fixing nodules. While the relevant NMR imaging work is covered in Chapter 2 the present chapter reviews spectroscopic studies.

### 3.2 MYCORRHIZAL SYMBIOSES

The term mycorrhiza which means "fungus root" refers to the mutualistic symbioses formed between plant roots and fungi (Harley and Smith, 1983). In both main classes of mycorrhizae (ectomycorrhizae and vesicular-arbuscular (VA)-



**Figure 1.** Schematic simplified representations of an ectomycorrhizal root, a vesicular arbuscular mycorrhizal root and a nitrogen fixing root nodule. Attention is drawn to the specialized structures and the physical intimacy of the symbionts in each case.

mycorrhizae) fungal structures penetrate the roots and invade the epidermis and cortex, so that host and fungal tissues are in close proximity. For general background material on both ecto- and VA-mycorrhizal systems the reader is referred to the book by Harley and Smith, (1983) and for more recent coverage of the biochemistry, molecular biology and ecophysiology see Varma and Hock (1995).

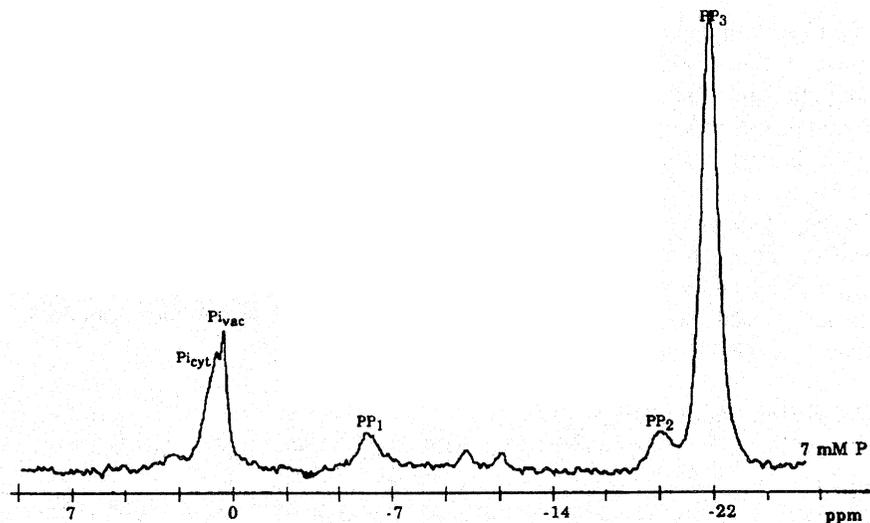
The physiological questions asked are frequently the same for all mycorrhizae, however since there are substantial differences (see Fig. 1) between these classes of mycorrhizae they are discussed separately. The characteristics of these associations and differences in their biochemical profiles as revealed by NMR will be discussed below.

### 3.2.1 Ectomycorrhizae

Ectomycorrhizal fungi form a mutualistic, symbiotic relationship primarily with trees and other woody plants (Harley and Smith, 1983). These fungi colonize host plants by forming a sheath around the root and also extend filamentous hyphae between the root cortical cells to form a branched structure called the Hartig net (see Fig. 1). The fungal symbiont enhances the uptake by the host plant of inorganic phosphate (Pi), among other nutrients, by expanding the absorptive area of the root. In turn, the fungus receives fixed carbon from the host, some of which is returned as nitrogenous compounds (Findlay *et al.* 1988). These fungi can also live as saprophytes in the soil, and consequently it is possible to study them in their free-living as well as their symbiotic states. It is therefore possible to compare transport and metabolic processes in the plant and fungus in their free-living and mycorrhizal forms.

#### 3.2.1.1 Phosphorous Metabolism

Since phosphate is one of the key components involved in the symbiotic exchange of nutrients,  $^{31}\text{P}$  NMR is particularly well suited to the study of ectomycorrhizal physiology. The first  $^{31}\text{P}$  study of axenically cultured ectomycorrhizal fungi (*C. geophilum* and *H. crustuliniforme*) was carried out by Martin and co-workers (1983). This early report demonstrated the presence of polyphosphate (PolyP) whose distinct central phosphate chain resonance was observed at -22ppm in the  $^{31}\text{P}$  spectrum. Fig. 2 shows such a  $^{31}\text{P}$  spectrum of a free-living ectomycorrhizal fungus (*Laccaria bicolor*) taken from a subsequent study (Martin *et al.* 1994). Such spectra show that PolyP (the form in which phosphate is stored by the fungus) represents much of the mobile phosphorous present in the fungal tissue. One should note however that PolyP contains only a fraction (approx. 8%) of the total phosphate in the fungal mycelium (Martin *et al.*, 1983), the rest being in phospholipids, DNA and other forms which do not contribute to these  $^{31}\text{P}$  NMR spectra. Based on the ratio of intensities of the terminal and internal Pi resonances in  $^{31}\text{P}$  spectra such as that of Fig. 2 one can deduce the average chain length of the mobile PolyP. In *H. crustuliniforme* (Martin *et al.*, 1985b) this was measured as 11 residues. Detailed chemical analysis



**Figure 2.** *In vivo*  $^{31}\text{P}$  NMR spectrum of mycelium of *Laccaria bicolor* grown on medium containing 7 mM Pi. The spectrum was obtained in 30 min. Signals marked are from:  $\text{Pi}_{\text{cyt}}$ , cytoplasmic orthophosphate;  $\text{Pi}_{\text{vac}}$ , vacuolar orthophosphate;  $\text{PP}_1$ ,  $\text{PP}_2$ ,  $\text{PP}_3$ , mark the signals from terminal, penultimate and central phosphate residues of Polyphosphate. (Adapted from Martin *et al.*, 1994, with permission).

of this fungus yielded values for the PolyP content of these actively growing cells in agreement with the NMR-derived levels (Rolin *et al.*, 1984). Thus the NMR-observable (mobile, low molecular weight) PolyP corresponds to a minimum of 80% of all the acid-extractable PolyP in the mycelium (Martin *et al.*, 1985b). Further information on the physical state of PolyP in *H. crustuliniforme* was derived from the observation that the spin-lattice relaxation behavior of PolyP is consistent with the formation of "supramolecular aggregates" of these short PolyP chains. *In vivo* spectroscopy showed that under phosphorous starvation, PolyP is degraded to Pi by *H. crustuliniforme* (Martin *et al.*, 1985b). PolyP chain lengths of similar size (15 units/chain) have recently been characterized in *Pisolithus tinctorius* by  $^{31}\text{P}$  NMR (Ashford *et al.*, 1993).

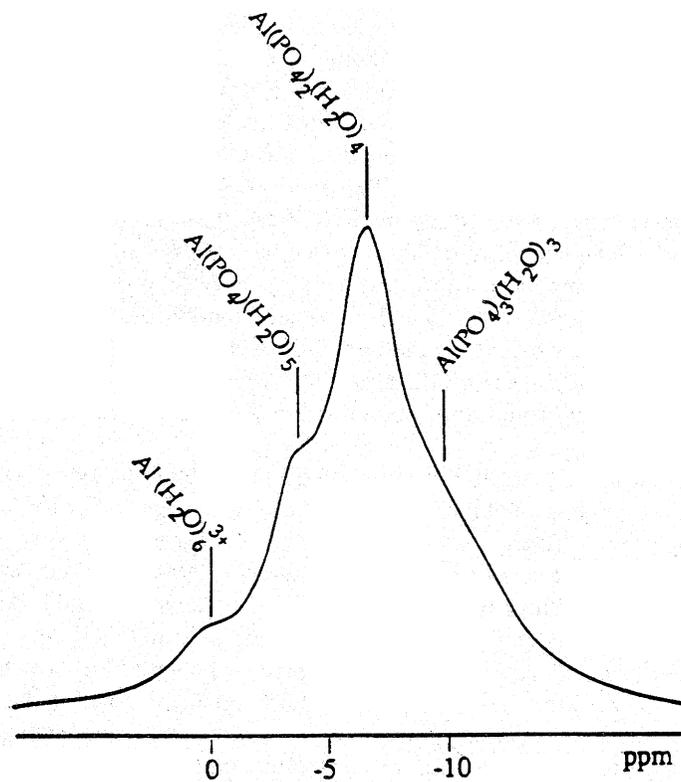
Loughman and Ratcliffe (1984) demonstrated the presence of PolyP and active incorporation of Pi into mobile PolyP in living mycorrhizal beech root tips. *In vivo*  $^{31}\text{P}$  NMR spectra of synthetic mycorrhiza (birch roots colonized with *Paxillus involutus*) (Grellier *et al.*, 1989) showed that levels of PolyP were somewhat higher (10-40% of the total soluble phosphate) than those of free-living mycelium of the fungus. In a study of intact mycorrhizal red pine roots (*Pinus resinosa* Ait colonized with *Hebeloma arenosa*) MacFall and co-workers (1992) showed that the Pi/PolyP ratio remained the same (approximately 1.8) when plants were grown for

19 weeks in either P amended or unamended soil. These findings were interpreted as supporting the idea that the fungus, through controlling the synthesis of PolyP can modulate Pi flow within the mycorrhizal root and thereby regulate the amounts of Pi available for transport to the shoots. In a recent NMR study Gerlitz and Werk (1994) followed levels of Pi and PolyP signals in mycorrhizal pine and beech roots. Their findings suggest that there are small changes in mobile polyphosphates over a period of 20 minutes after exposure to Pi. Signals from mobile PolyP (chain lengths of <100) increased by 5- 10% then returned to their initial levels. The authors speculate that during this short period incoming Pi is incorporated into mobile PolyP which becomes immobilized and thus NMR-invisible. Further work is needed including appropriate chemical and complementary NMR analyses (Rolin *et al.*, 1984; Martin *et al.*, 1985b) to establish if there are significant amounts of immobile PolyP present to account for these observations.

Aluminum in acidic soils is known to cause disruption in root physiology and function including inhibition of nutrient and water uptake and phosphate extrusion by the roots (Haug, 1984; Pfeffer *et al.*, 1987; Kochian and Shaff, 1991). In a unique <sup>27</sup>Al NMR study Martin and coworkers (1994b) have demonstrated that Al is chelated by PolyP in the mycelium of *Laccaria bicolor* and is bound in at least 3 different complexes. Analysis of the spectrum from this study is shown in Fig. 3. The four different components comprising the spectrum show that the Al<sup>3+</sup> ion exists in 4 different states and are almost certainly located in the fungal vacuole. These complexes were found to be very resistant to remobilization, even when the mycelium was transferred to Al-free medium for 9d. These findings suggest that the sequestration of Al in polyphosphate complexes could make a significant contribution to the protection of mycorrhizal plants. This is an example in which NMR is shown to be a powerful and unique tool for quantifying the speciation of a particular metal ion in living cells. Further NMR work will no doubt follow up on the effect of the turnover of these complexes in relation to the nutrient levels, the competition with other cellular ligands, and transfer of Al to the cell roots following PolyP degradation at the fungal/host interface (Martin *et al.*, 1994b).

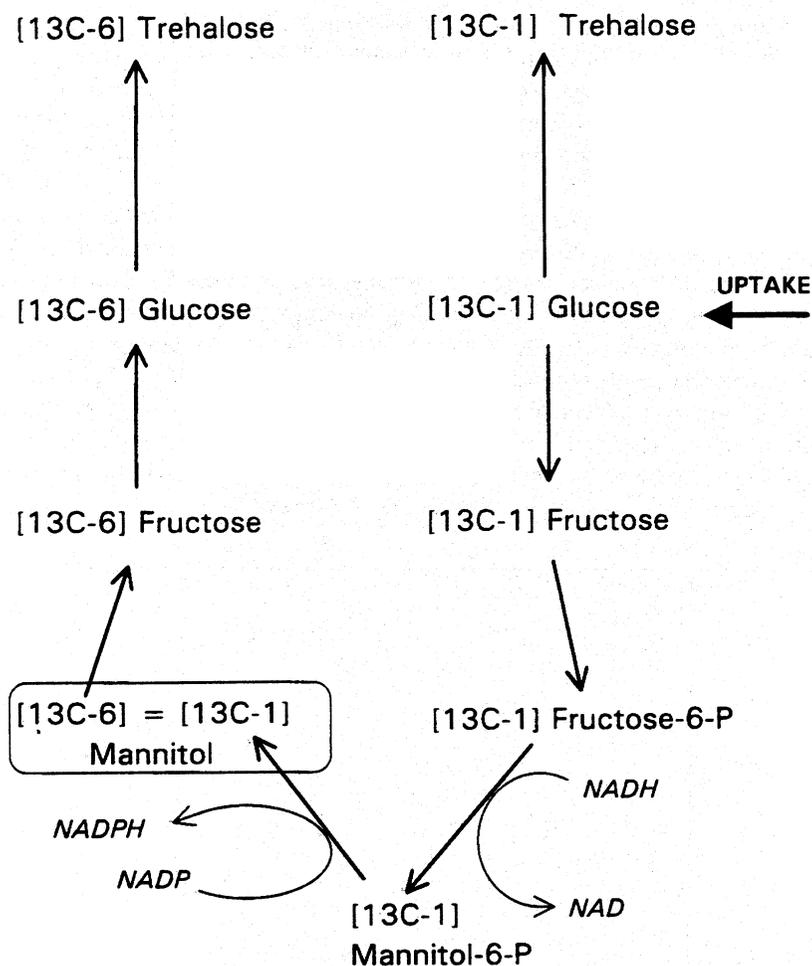
### 3.2.1.2 Carbon Metabolism

One of the strengths of NMR for studying metabolism is the fact that labeling in the different positions of different compounds can be simultaneously measured. Those familiar with the time-consuming and labor-intensive extraction, separation and chemical degradation which is needed to obtain such information using radioactive or mass spectrometric analysis will appreciate the advantages offered by *in vivo* NMR. The body of work on carbon metabolism in ectomycorrhizal fungi by F. Martin and co-workers is the clearest example of this capability as applied to symbiotic systems.



**Figure 3.** Deconvolution of a  $^{27}\text{Al}$  NMR spectrum of *L. bicolor* grown in the presence of 5mM  $\text{AlCl}_3$  for 3d. The spectrum was acquired in 30min and the different components of the signal were assigned as arising from different forms of complexed  $\text{Al}^{3+}$  by comparison with the shifts of established mixed-solvation  $\text{Al}^{3+}$ -PolyP complexes *in vitro*. Peaks are labeled  $\text{Al}(\text{PO}_4)_n(\text{H}_2\text{O})_{6-n}$  where  $n=1, 2$  or  $3$  and refers to Al interacting with one to three P residues of the same PolyP chain or stacked PolyP chains. (Adapted from Martin *et al.*, 1994, with permission.)

**Carbohydrates.** Free-living Ectomycorrhizal fungi produce substantial quantities of mannitol and to a lesser extent trehalose (Harley and Smith, 1983). There is evidence indicating that significant rates of metabolic cycling occur in a range of fungal species due to simultaneous synthesis and degradation of mannitol. Postulated roles for this cycling include: regulation of glucose metabolism, the production of NADPH and regulation of carbohydrate storage (Ramstedt *et al.*, 1986). A simplified metabolic scheme for this cycle is shown in Fig. 4 which is drawn from an NMR spectroscopic perspective -*i.e.* omitting some of the intermediates (such as glucose phosphates) which are not observed in the C NMR spectrum and emphasizing the movement of label supplied as  $^{13}\text{C}_1$  glucose through this pathway. According to this scheme, glucose is converted to mannitol via

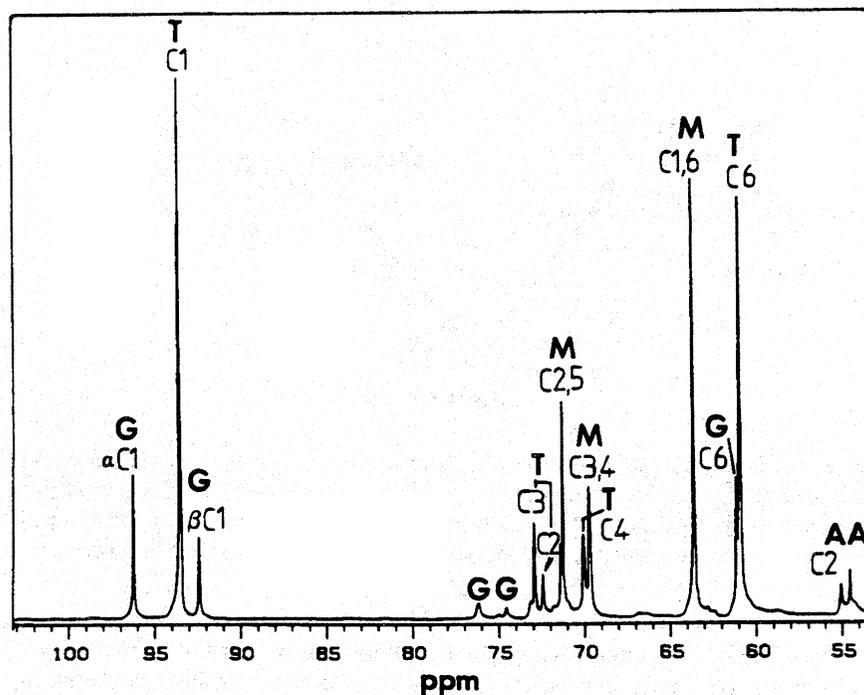


**Figure 4.** Simplified metabolic chart to explain the labeling patterns observed in  $^{13}\text{C}$  spectra of extracts of ectomycorrhizal fungi incubated with  $^{13}\text{C}_1$  glucose. Metabolic cycling from glucose to mannitol and back is responsible for scrambling of label from  $\text{C}_1$  to  $\text{C}_6$  in glucose and the subsequent labeling of  $\text{C}_6$  of trehalose.

fructose 6-P. Subsequently, mannitol is oxidized to fructose from which glucose is formed. Because mannitol is a symmetrical molecule, cycling of carbohydrates from glucose to mannitol and back results in the "scrambling" of any label between different positions of glucose. Metabolic cycling through mannitol was demonstrated in  $^{13}\text{C}$  NMR studies of *S. Brunea* and *C. Geophilum*, in which the timecourse of labeling of metabolites was followed after incubation with  $^{13}\text{C}_1$  glucose (Martin, 1985, Martin *et al.*, 1988). Labeling in the  $\text{C}_1$  of mannitol (which is equivalent to the  $\text{C}_6$  in this symmetrical molecule) was observed and

subsequently glucose and trehalose became labeled in their C<sub>6</sub> as well as C<sub>1</sub> positions. This is consistent with flux of label back from mannitol into sugars.

Fig. 5 is a <sup>13</sup>C spectrum of an extract of *C. graniforme* after incubation with <sup>13</sup>C<sub>1</sub> glucose (Martin *et al.*, 1985a). Signals from the different carbons of glucose, trehalose and mannitol show by their intensities the extent of <sup>13</sup>C enrichment in the different positions of these compounds. It can be seen that the C<sub>6</sub> positions of glucose trehalose and mannitol have become significantly labeled through scrambling of the label supplied as C<sub>1</sub>. Comparison of this spectrum with those obtained either without labeling or after labeling with <sup>13</sup>C<sub>6</sub> labeled glucose allowed the authors to determine that cycling through mannitol accounted for most of the observed labeling in C<sub>6</sub> of glucose and trehalose. Enzyme assays demonstrated the presence of the key enzymes of mannitol formation and breakdown. To illustrate the high information content of such spectra one may see for example that trehalose is labeled almost as much in its C<sub>6</sub> carbons as in C<sub>1</sub>, thus most of the trehalose was formed from glucose molecules which had cycled through mannitol rather than having been incorporated directly after uptake. Closer examination of this spectrum



**Figure 5.** <sup>13</sup>C NMR spectra of extracts of *C. graniforme* mycelia prepared after the labeling with <sup>13</sup>C<sub>1</sub> glucose. Sampling time was 60 h after <sup>13</sup>C glucose addition. Peaks are labeled: (AA), amino acids; (G), glucose; (M), mannitol; (T), trehalose. (Adapted from Martin *et al.*, 1985 with permission.)

shows that the glucose C<sub>6</sub> signal is significantly less than the C<sub>1</sub> signals so that the trehalose must have been synthesized from a sub-pool of glucose. Also the mannitol has a lower fractional enrichment in the C<sub>1</sub>=C<sub>6</sub> positions than does trehalose, therefore the cycling of material from glucose to mannitol and back must have involved a sub-pool of the mannitol also. This example serves to make clear the wealth of quantitative metabolic information obtained directly from the use of <sup>13</sup>C labeled substrates. Also illustrated in this case is the potential both to test metabolic hypotheses (in this case about the role of mannitol in metabolic cycling) and to give evidence of other unlooked-for features of metabolism (here the existence of different metabolic pools).

*Amino Acid Metabolism.* Glutamate and glutamine accumulate in ectomycorrhizal fungi when NH<sub>4</sub><sup>+</sup> is supplied (Martin and Botton, 1993), and the pattern of labeling in these amino acids reflects the labeling of Krebs cycle intermediates. Therefore, labeling with <sup>13</sup>C<sub>1</sub> glucose and analysing the isotopic distribution in glu and gln allowed Martin and co-workers (1988), to assess which pathways of glucose breakdown contributed to amino acid synthesis. The relative enrichments at C<sub>2</sub>, C<sub>3</sub> and C<sub>4</sub> positions in glu/gln showed that the flow of carbon is via pyruvate dehydrogenase into the Krebs cycle and that there is substantial dark fixation of CO<sub>2</sub>. The carboxylation of pyruvate (or Phosphoenol pyruvate) in dark fixation to generate 4 carbon units serves to replenish Krebs cycle intermediates used for biosynthesis. The extent of this dark fixation was determined, relative to the flux through pyruvate dehydrogenase, by analysis of the single and multiple labeling in glu/gln of *S. brunnea* (Martin *et al.*, 1988).

In studies of *C. geophilum* incubated with <sup>13</sup>C<sub>2</sub> acetate, the <sup>13</sup>C NMR spectra become extremely complex due to the multiple <sup>13</sup>C labeling of the amino acids and citrate resulting from flux of label through multiple turns of the TCA cycle. Because of the complexity caused by the resonance overlap and multiple couplings of the signals of various isotopomers these labeling patterns could not be analyzed from conventional <sup>13</sup>C spectra. Boudot *et al.*, 1988 adapted a spectral editing methodology which uses the <sup>13</sup>C-<sup>13</sup>C couplings to separate signals from different isotopomers. Thus analysis of labeled glutamate from <sup>13</sup>C<sub>2</sub> acetate labeling of *C. geophilum*, into its isotopomers was possible. This can give direct information on the number of turns of the Krebs cycle through which label from glucose had flowed before being diverted for biosynthesis (Martin *et al.*, 1988).

The studies described above have focused on axenically cultured mycelium; the application of these methods to quantifying the contributions of different metabolic pathways for carbon substrates in colonized mycorrhizal roots is an exciting prospect.

### 3.2.1.3 Nitrogen Metabolism

Axenically cultured ectomycorrhizal fungi have a preference for the assimilation of ammonium over nitrate (Marschner and Dell, 1994), some of the species can reduce nitrate efficiently (Scheromm *et al.*, 1990). These nitrate-

reducing fungi could be useful in agricultural soils in which nitrate dominates in early years of tree establishment (Marschner and Dell, 1994) or for tree species which are better able to metabolize ammonium than nitrate. In seeking to determine the pathways and regulation of nitrogen assimilation  $^{15}\text{N}$  NMR has proven very useful. This is because nitrate, ammonium, amine and amide nitrogens give distinct signals in the  $^{15}\text{N}$  spectrum (see *e.g.* Chapter 4). Thus NMR time course experiments make it relatively straightforward to follow the kinetics of the labeling in amine and amide of glutamine and other amino acids, with the drawback of rather low sensitivity (see Chapter 1).

It is known that ectomycorrhizae can enhance the uptake of nitrogen into roots (Hogberg, 1989) especially ammonium (Findley *et al.*, 1988). However, it is not known if the uptake mechanisms of the mycorrhizal hyphae absorb nitrate and ammonium more efficiently than those of colonized roots. Martin (1985) and Martin and co-workers (1994a) used *in vivo*  $^{15}\text{N}$  NMR to follow the metabolism of ammonium in mycelia of *C. graniforme* and *L. laccaria* and demonstrated that ammonium is predominantly incorporated via glutamine synthetase/glutamate synthase (GS/GOGAT) with a smaller proportion metabolized via the GDH pathway to glutamate and alanine. However, under N-limited conditions or when MSX was used to inhibit GS in these species,  $^{15}\text{N}$  labeling patterns showed that the flux through GDH increased several fold (Martin, 1985, Martin *et al.*, 1994a). Thus it was demonstrated that the relative contribution of the GDH pathway to N assimilation increases with decreasing extracellular  $\text{NH}_4^+$  concentrations. This is not the case for higher plants which exclusively use the GS/GOGAT pathway for  $\text{NH}_4^+$  assimilation (Robinson *et al.*, 1991). The author (Martin, 1993) concludes that there appears to be a parallel activity of both GDH and GS in the assimilation of ammonium by these free living fungi and GOGAT and the coupled glutamine transaminase  $\omega$ -amidase pathway makes little if any contribution to glutamate biosynthesis.

Arginine was substantially labeled in *C. geophilum* supplied with  $^{15}\text{NH}_4^+$  which is consistent with the operation of the ornithine cycle (Martin, 1991). Since the  $^{15}\text{N}$  signal of arginine was not observed *in vivo* but was observed in the extract spectrum it was concluded that arginine is located in the vacuole where it can form a large, relatively immobile, NMR-invisible complex with polyphosphate. The authors speculate that movement of polyphosphate granules within hyphae could be responsible for a joint translocation of nitrogen and phosphorus to the root.

In contrast to the free-living ectomycorrhizal fungi, Beech (*Fagus sylvatica* L.) roots colonized with *Lactarius* sp. and *Russula* sp. gave somewhat different metabolic responses to  $\text{NH}_4^+$  feeding (Martin *et al.*, 1986). Using  $^{15}\text{N}$  NMR to monitor the progress of specific labeling of amino acid metabolites Martin (1985) demonstrated that like free-living mycelia the amide-N of glutamine was most enriched, consistent with the operation of the GS/GOGAT pathway. Also the treatment of the tissue with MSX and albazine inhibitors of GS/GOGAT blocked incorporation of  $^{15}\text{N}$  into amino acids and caused an accumulation of  $\text{NH}_4^+$ . From

these results it appears that in contrast to the free-living fungi, the ectomycorrhizas do not utilize the GDH pathway in  $\text{NH}_4^+$  assimilation. These results suggest that the fungal GDH is suppressed in the intact ectomycorrhizas, however the authors caution that it was not possible to obtain a good sampling of the  $^{15}\text{N}$  amino acids from the fungal mantle (see Fig. 1) and so these amino acids may be under represented in the overall analyses.

### 3.2.2 Vesicular-Arbuscular Mycorrhizae

Vesicular-Arbuscular mycorrhizae (VAM) are more ubiquitous than their ectomycorrhizal counterparts and occur in more than 80% of all land plants (Harley and Smith 1983). In these associations fungal hyphae grow both in the apoplastic space and also inside host cells. Inside host cells the hyphae terminate in swellings called vesicles or highly branched structures known as arbuscules (see Fig. 1), hence the name vesicular-arbuscular mycorrhizae. These structures do not disrupt the host cell's plasmalemma but penetrate the cell wall and invaginate into the cells so that fungal and host membranes are in close contact. The arbuscules are thought to be involved in phosphorus (Smith, 1980) and perhaps carbon (Cox *et al.*, 1975) transport between the fungus and plant host, while vesicles appear to function as storage compartments for lipids and polyphosphates (Cox, 1975). Vesicles may not always be present in these fungi and arbuscules may only appear and turn over rapidly during certain periods of the symbiotic life cycle (Harley and Smith, 1983). As with the ectomycorrhizae external hyphae extend out into the surrounding soil to help the root absorb nutrients such as phosphate and other minerals.

In contrast to ectomycorrhizal fungi that can develop and complete their life-cycle in a free-living state, VAM fungi are obligate symbionts and therefore their metabolism can only be fully evaluated in association with the host plant. This, together with the fact that the fungus only represents a small fraction of the tissue volume in VA mycorrhizal roots, has been a serious hindrance to studying this system. As a result, much less is known about the metabolism of VA mycorrhizae than about ectomycorrhizae. Recent applications of molecular methods have begun to separate the gene expression of the symbionts and their interregulation. We believe that the potential to spectroscopically differentiate host from fungal metabolites holds a similar promise for studying biochemistry in this intimate symbiosis.

#### 3.2.2.1 Identification of Storage Compounds

Based on studies of ectomycorrhizas and other symbiotic systems, it has long been thought that in all mycorrhizal associations the fungus converts carbohydrates received from the host into forms that cannot be metabolized by the plant (Smith *et al.*, 1969). This conversion would then act as a one-way biochemical valve preventing backflow of carbon to the host (Harley and Smith, 1983). Efforts to find in VA mycorrhizae, fungal-specific carbohydrates that are known to exist in ectomycorrhizae (trehalose, mannitol and glycogen), were unsuccessful because

the colonizing fungus represents in most cases, as little as 1% of the root tissue. An HPLC-based study (Amijee and Stribley, 1987) reported the presence of trace quantities of trehalose and another unknown carbohydrate in the extraradical mycelia of *Glomus mosseae* and *Glomus caledonium*. Confirmatory evidence for the presence of trehalose was lacking until the detection and positive identification of trehalose by *in vivo*  $^{13}\text{C}$  NMR of *Glomus etunicatum* and *Gigaspora margarita* chlamydospores (Becard *et al.*, 1991). These spectra showed the presence of trehalose (1.1-1.6% dry weight) and also gave a direct assessment of the lipid composition (triglycerides, free fatty acids and glycerol) in the intact spores. No trehalose was seen in spectra of *Glomus interatices* spores though low levels (0.06% dry weight) were detected by HPLC. Subsequently, gas chromatography was used to detect the presence of trehalose in roots colonized with *Glomus versiforme* and *Glomus Mosseae* and in their corresponding sporocarps (Schubert *et al.*, 1992).

In contrast to reports of large quantities of mannitol present in ectomycorrhizal mycelia (reviewed in Martin, 1991), no evidence of this compound was observed in any of the three species of spores described above (Becard *et al.*, 1991). Furthermore, glycogen was only detectable by  $^{13}\text{C}$  NMR in these spores 5 days after the onset of germination and then only in trace amounts (Shachar-Hill and Pfeffer, unpublished results). This glycogen may be present prior to germination in larger immobile granules whose NMR signal is too broad for detection. Upon germination, depolymerization of the large glycogen molecules into smaller, more mobile, oligosaccharide chains would then explain the appearance of a  $^{13}\text{C}$  signal.

$^{31}\text{P}$  NMR spectra of *Glomus etunicatum* spores (Shachar-Hill *et al.*, 1995) showed a large Pi resonance at 2.0 ppm corresponding to an internal compartment at pH 6.9 as well as PolyP resonances (see above) at -22 ppm, -19 ppm and -6.5 ppm corresponding to central, penultimate and terminal phosphate residues. The ratio of these three polyphosphate resonances (1:2:2 respectively) corresponds to an average chain length of 5 phosphate units.

The  $^{31}\text{P}$  spectrum of excised leek (*Allium porrum*) roots colonized with *Glomus etunicatum* shows a polyphosphate resonance at -22 ppm, whereas the uncolonized does not (Shachar-Hill *et al.*, 1995). The absence of PolyP peaks from terminal and penultimate residues suggests that PolyP are markedly larger in mycorrhizal root than in the spores of the same fungus. Vacuolar and cytoplasmic pH's and nucleotide levels in colonized and control roots were indistinguishable. The PolyP content of the colonized root was estimated as equivalent to approximately 10mM phosphate in the fungus, based on a comparison with nucleotide resonances and the assumption that the fungus occupies one-tenth of the volume of the host cytoplasm. This is the first example in which the "chemical presence" of VAM in root tissue was detected non-destructively.

Turning to the natural abundance  $^{13}\text{C}$  spectrum of the same colonized roots (Fig. 6) the presence of fatty acid resonances is noted with a group of peaks centered around 30 ppm. These correspond to the fungal lipids, located in the

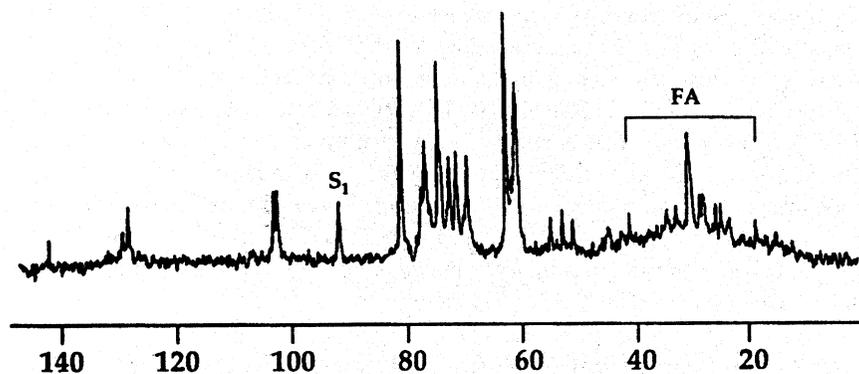
vesicles and are the only distinguishing features in the natural abundance  $^{13}\text{C}$  spectrum that differentiates this tissue from uncolonized roots.

### 3.2.2.2 Observations on Spore Germination

$^{31}\text{P}$  can be used to monitor the germination process in VAM spores (Pfeffer, Becard, Rolin and Douds, unpublished results): *Gigaspora margarita* spores were incubated at  $30^\circ\text{C}$  for 5d and  $^{31}\text{P}$  spectra taken each day. Initially only polyphosphate signals were detected indicating an average chain length of 11 units. As the spores began to germinate, narrow signals associated with newly formed Pi, UDPG, NAD(P)(H) and phosphomonesters were seen as well as a broad resonance (extending from 10 to  $-20$  ppm) from relatively immobile, phospholipid membranes.

That stored trehalose is utilized in the early stages of germination was revealed by *in vivo*  $^{13}\text{C}$  NMR time course experiments which demonstrated a 50-58% breakdown over a period of 5 days after germtube emergence. No significant reduction of lipid signals from triacylglycerols and free fatty acids was detected during this time, indicating that lipid stores are probably metabolized to sustain hyphal growth only after the carbohydrate has been exhausted. This observation has recently been supported in a study of lipid composition in *Glomus versiforme* spores following germination (Gaspare *et al.*, 1994). This study found that although the total amount of mobile lipids was little changed in the first 5 days of germination its composition changes with the ratio of saturated:unsaturated fatty acid chains decreasing.

To examine the metabolic events associated with carbohydrate utilization during germination, *Glomus etunicatum* spores were incubated with  $50\text{mM }^{13}\text{C}_1$  glucose for 70h (Shachar-Hill *et al.*, 1995). Spectra taken of the extracts of the



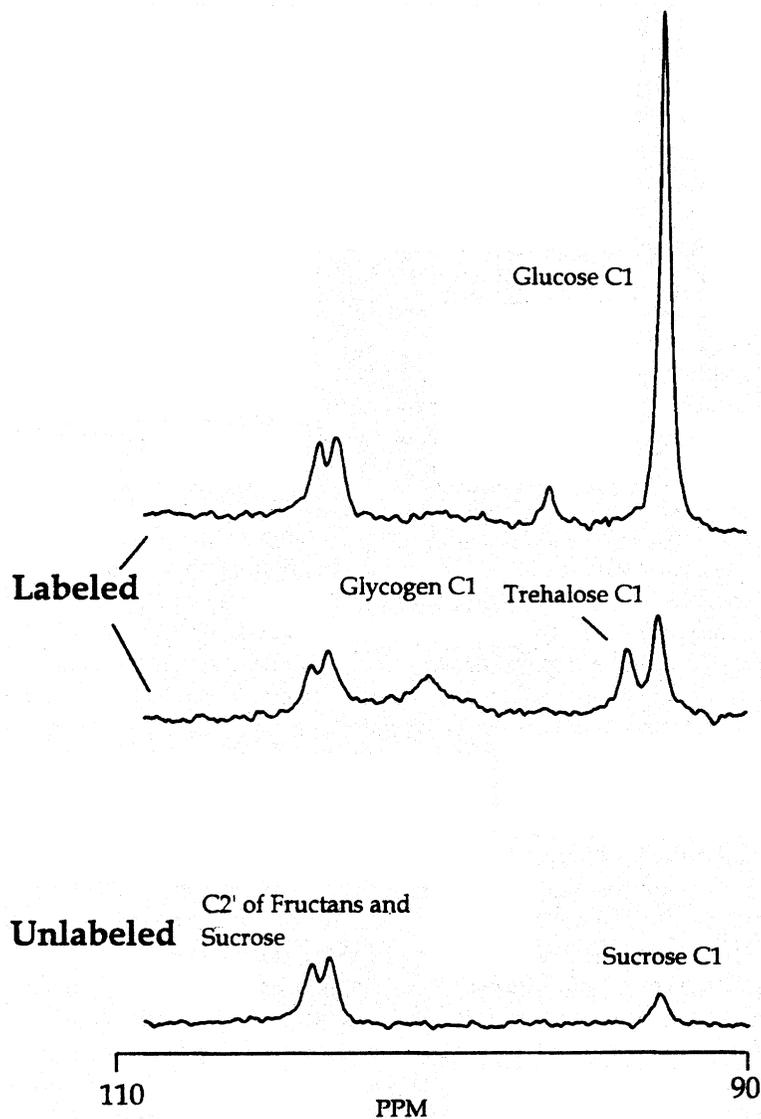
**Figure 6.** *In vivo*  $^{13}\text{C}$  NMR spectrum of excised roots of leek, colonized by *Glomus etunicatum*. The spectrum was obtained during perfusion of the tissue with oxygenated buffer and took 3h to acquire. Natural abundance signals of host carbohydrates and fungal lipids dominate the spectrum of this unlabeled sample.

germinating spores and hyphae indicated that relatively little labeling had taken place overall. Mannitol became labeled in the C<sub>1</sub>=C<sub>6</sub> position, trehalose in the C<sub>1</sub> position and a resonance attributed to the C<sub>3</sub> of alanine was also observed. These were the only compounds labeled from glucose. From the intensity of the natural abundance resonances, mannitol and trehalose were found to be in approximately equal concentrations while mannitol had some 15 times as much % labeling. This is consistent with the observation (Becard *et al.*, 1991) that the spores contain high levels of trehalose but not mannitol before germination and that trehalose is utilized during germination. The absence of scrambling of <sup>13</sup>C label between C<sub>1</sub> and C<sub>6</sub> in trehalose demonstrated that there is no cycling through mannitol during germination. This contrasts markedly with observations in free-living ectomycorrhizal (see 3.2.1.2) and other fungal species (Dutsch and Rast, 1972; Martin *et al.* 1985a; Ramstedt *et al.*, 1986). It is tempting to speculate that the function of mannitol synthesis is different in ectomycorrhizal fungi than in VAM fungi.

### 3.2.2.3 Carbon Metabolism in the Symbiotic State

VAM fungi in their symbiotic state occupy a very small volume fraction within colonized host roots and neither dissection nor axenic culturing has yielded sufficient quantities of the organism for detailed metabolic study (Becard and Fortin, 1988). Therefore little is known about the pathways by which host photosynthate is utilized by the fungus. Even less is known about the effects of the host on fungal metabolism. There has been a tacit assumption by many that the carbon metabolism of VA mycorrhizae is similar to that in ectomycorrhizae. Our recent work indicates that this is not so. Fig. 7 shows the results of labeling mycorrhizal and non-mycorrhizal leek root tissue by incubation of the roots of intact plants with <sup>13</sup>C<sub>1</sub> glucose for 20h. These partial spectra show the production of C<sub>1</sub> labeled trehalose and glycogen in mycorrhizal roots, whereas in uncolonized roots only labeling of sucrose is seen (Shachar-Hill *et al.*, 1995). The labeling of sucrose by the host cells is suppressed in the mycorrhizal roots despite the high levels of glucose supplied (50mM) and the very low fractional volume occupied by the fungus. It has been postulated that Pi mimics the metabolic effect of colonization on the host tissue (Koide, 1991). To examine this effect on carbohydrate metabolism, <sup>13</sup>C<sub>1</sub> glucose labeling was examined in uncolonized tissue which was supplied with elevated Pi for 25d. The presence of Pi did not suppress the labeling of host sucrose and thus does not appear to mediate the reduced labeling in sucrose by the host tissue.

The fact that neither trehalose nor glycogen showed evidence of isotopic scrambling between C<sub>1</sub> and C<sub>6</sub> positions in hexose pools in symbiotic VA-mycorrhizae (see Fig. 4 above) is in marked contrast with observations in free living ectomycorrhizal and VA-mycorrhizal fungal spores (Martin, 1991; Shachar-Hill *et al.*, 1995). Another difference is that very little labeling of mannitol was seen in VA mycorrhizal leek, this also distinguishes metabolism in the mycorrhizal



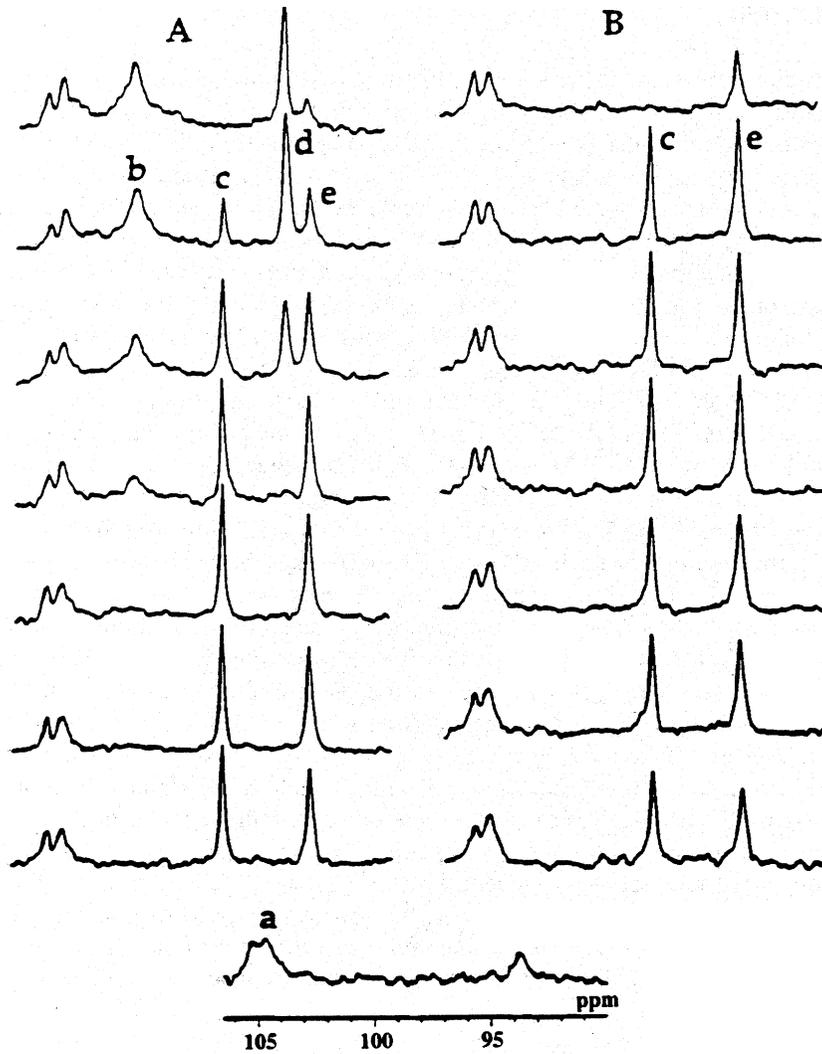
**Figure 7.** Part of the *in vivo*  $^{13}\text{C}$  NMR spectrum of excised leek roots colonized by *Glomus etunicatum* with no labeling (A); colonized with *Glomus etunicatum* and labeled for 20h with 50mM [ $^{13}\text{C}_1$ ] Glc (B); and uncolonized and labeled for 20 h with 50mM [ $^{13}\text{C}_1$ ] Glc (C). This region shows no differences between colonized and control roots before labeling. After labeling, incorporation of label by the host is much higher in the control than in the mycorrhizal roots. Fungal metabolites are only detected after labeling. (Adapted from Shachar-Hill *et al.*, 1995.)

state from that of germinating spores. Ectomycorrhizae have yet to be investigated to determine if they produce C<sub>1</sub>, C<sub>6</sub> scrambling through mannitol cycling.

Fig. 8 shows two time-courses of *in vivo* <sup>13</sup>C NMR spectra taken of mycorrhizal and non-mycorrhizal leek root segments perfused with 0.5mM <sup>13</sup>C<sub>1</sub> glucose. Such experiments demonstrated a significant lag (12-15h) in the production of <sup>13</sup>C<sub>1</sub> trehalose and glycogen. This lag may be shortened by manipulation of the host's metabolism and may be related to the availability of host photosynthate. The labeling of fungal metabolites after exposure to <sup>13</sup>C<sub>1</sub> glucose was measured in extracts by NMR and mass spectrometry and found to be at high enrichment. This and the above observations of mutual influence on glucose metabolism in the intact mycorrhizae, all point to a central role for glucose metabolism and suggest that hexoses may serve as common substrates for fungus and host root cells (Shachar-Hill *et al.*, 1995). We have also observed (Pfeffer, Shachar-Hill, and Douds, unpublished results) that trehalose and glycogen labeling did not occur when *Glomus etunicatum* colonized leek roots did not contain arbuscules. However, earlier in the year of colonization when arbuscules were present, labeled trehalose and glycogen were produced. These findings suggest that the arbuscules may be a storage site (Cox *et al.*, 1975) for fungal carbohydrate storage compounds that will be used by the daughter spores for germination (Becard *et al.*, 1991) (see above discussion of VA-mycorrhizal spore germination, 3.2.2.2).

Labeling studies of carbon metabolism in mycorrhizae (whether using NMR or other techniques) in which substrates other than CO<sub>2</sub> are used, are severely restricted in duration because of interference from other microorganisms. It is therefore desirable to have a sterile system if longer term storage and mobilization is to be examined. For such long term labeling experiments we have therefore begun to use a dual culture system (Becard and Fortin, 1988) utilizing t-RNA transformed carrot roots colonized with *Glomus intraradices* growing on solidified medium in petri plates. In the sterile plates, external hyphae but not the colonized transformed roots themselves spread into a second compartment; this allows separate labeling of the colonized roots and of the external hyphae over extended periods (3-4 weeks). Such experiments (unpublished data) show that external hyphae do not take up glucose from their surroundings, whereas internal fungal structures do. Most of the long term labeling observed was in lipids, of which a C16 ω5 unsaturated fatty acid predominates. From considerations of fractional enrichment in host versus fungal metabolites it appears that glucose can be used directly by the fungus without equilibration with the plant metabolite pools.

This difference between transport by internal and external fungal structures mirrors a recent finding of differences in the levels of expression of a phosphate transporter between internal and external hyphae of *Glomus versiforme* in VA mycorrhizal alfalfa roots (Harrison and van Buuren., 1996).



**Figure 8.** Time courses of *in vivo*  $^{13}\text{C}$  NMR spectra of colonized (A) and uncolonized (B) leek roots excised and perfused with 0.6 mM  $^{13}\text{C}_1$  glucose in Hoagland's solution. Each successive spectrum was acquired in 3 h. The first spectrum (at the bottom of the figure) was acquired before the addition of the  $^{13}\text{C}_1$  glucose to the perfusion medium and was essentially identical for both mycorrhizal and control roots. Labeling of peaks: a,  $\text{C}_2'$  of sucrose and  $\text{C}_2$  of fructans; b,  $\text{C}_1$  of glycogen; c,  $\text{C}_1$  of  $\beta$ -glucose; d,  $\text{C}_1$  of trehalose; e,  $\text{C}_1$  of  $\alpha$ -glucose and  $\text{C}_1$  of sucrose. Last spectrum in each timecourse was acquire after washout with glucose-free solution after 24 h of exposure to glucose. (Adapted from Shachar-Hill, 1995.)

### 3.3 LICHEN

Lichens are symbioses between an alga and a fungus in which photosynthetically fixed carbon is provided to the fungus by the alga. The form of carbon transferred seems to be predominantly as polyols (Smith, 1980). On the basis of observations of metabolism in free living fungi (Lewis and Smith, 1967) it has been proposed that ribitol is the transferred substrate and that this algal product is metabolized by the fungus to mannitol via xylulose-5-phosphate and the pentose phosphate pathway. This proposal is difficult to test using  $^{14}\text{C}$  labeling because of the necessity of separating compounds (MacFarlane and Kershaw, 1985) and more problematically of identifying the structure of  $^{14}\text{C}$  labeled isotopomers.  $^{13}\text{C}$  NMR spectroscopy of extracts of lichen (*Xanthia calcicola*) obtained after labeling with  $^{13}\text{CO}_2$  followed by a variable chase period were used to circumvent these problems (Lines *et al.*, 1989). Labeling of ribitol was seen within 2h of exposure to  $^{13}\text{CO}_2$  in the light. In the subsequent 22h chase, mannitol was the predominant species labeled. These observations are consistent with the majority of label being fixed into ribitol and the flux of a substantial fraction of fixed carbon to the fungus. In addition to these findings this study also explored the effects of light and temperature on polyol production. For example it was observed that there was no change in ribitol and mannitol levels during a dark chase period. This finding suggests that there was no fungal pool of ribitol in lichen since one does not expect the pentose phosphate pathway to be light-dependent. Multiple labeling in algal and fungal metabolites was evident in the spectra because  $^{13}\text{C}$ - $^{13}\text{C}$  J-coupling (see 1.2.4 and Glossary) results in splittings of the  $^{13}\text{C}$  resonances. The clear differences in the extent of such splittings in the three signals of mannitol was interpreted as resulting from the action of the pentose phosphate pathway in the fungus. Limitations due to overlap of some of the multiply labeled ribitol peaks prevented quantification of the isotopomers - this should now be possible through the application of the spectroscopic method described by Boudot *et al.* (1988) preferably at a higher field strength. This would allow a more definitive determination of the pathways and intermediates involved.

### 3.4 NITROGEN FIXATION SYMBIOSES

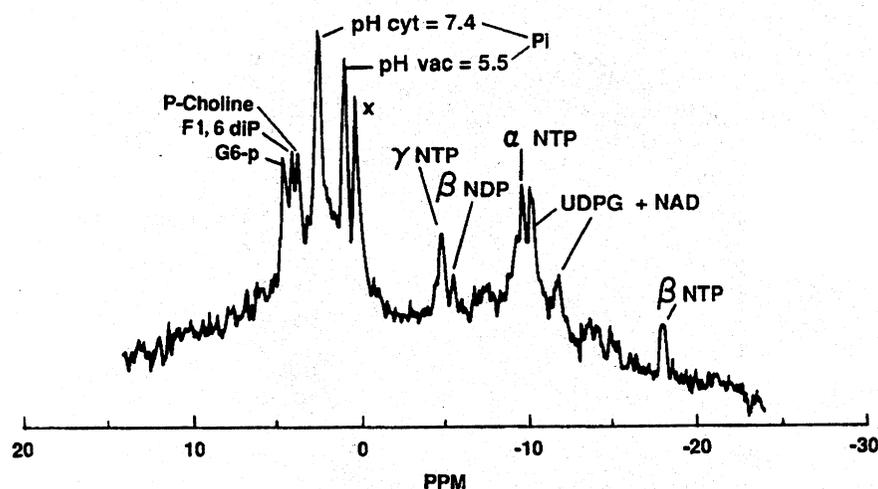
#### 3.4.1 Nod Factors

In nitrogen fixation symbioses, soil-borne bacteria of the Rhizobiaceae colonize the roots of leguminous plants and induce them to form specialized root nodules (see Fig. 1). In these root nodules a differentiated form of the rhizobium, bacteroids, are able to reduce nitrogen to ammonia, which is used by the plant (Long, 1989). Successful symbioses are formed only between particular combinations of bacterial species and leguminous hosts (Spaink, 1992). However before nodule formation can occur several interactions between the bacteria and host plant are required (Fisher *et al.*, 1992; Spaink, 1992). Flavonoids released from the root induce the

expression of *nod* genes in the bacteria resulting in production of different nod factors or nod hormones. These factors elicit from the host a numbers of responses including root hair curling, formation of pre-infection threads and cortical cell division (Lerouge *et al.*, 1990). Nod factors are oligosaccharides of N-acetyl glucosamine (n=4 or 5) with various substituents at the reducing end and an N-acyl fatty acid chain linked to the terminal non-reducing sugar. The identity of the substituents at the reducing end appears to be associated with host specificity (Sanjuan *et al.*, 1992; Spaink, 1994; Ehrhardt *et al.*, 1995), whereas the nature of the fatty acid chain (chain length and degree and positions of unsaturation) is associated with the specific response elicited in the host root (Spaink, 1992). The structures of the various nod factors were determined largely by modern 2D NMR methods (Lerouge *et al.*, 1990; Carlson *et al.*, 1993; Ehrhardt, 1995). However a detailed review of the applications of NMR to this and other chemical structural analyses (notwithstanding their importance for understanding the physiology of the symbiosis) is beyond the scope of this chapter. (See Chapter 6 for an introduction to 2D NMR spectroscopy as applied to metabolite profiling.)

### 3.4.2 Cyclic Glucans

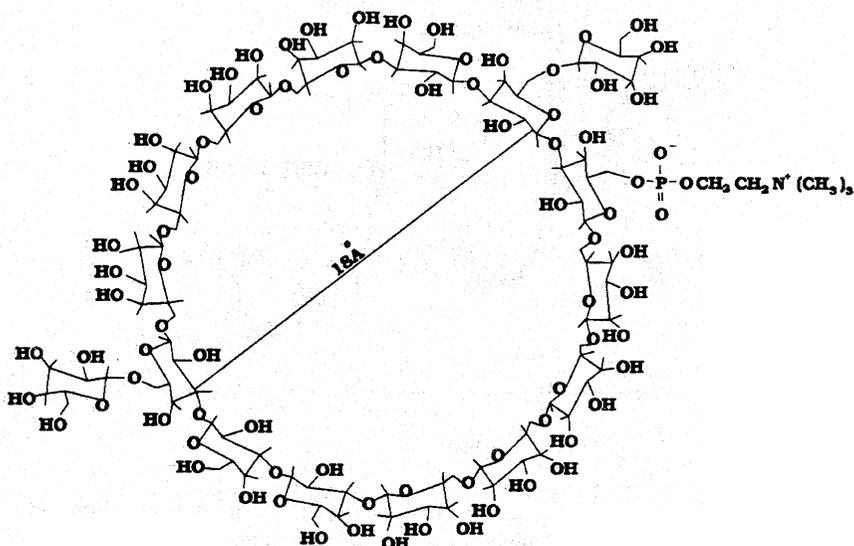
In studying the *in vivo*  $^{31}\text{P}$  spectra of nodules of *Glycine max* with *Bradyrhizobium japonicum* (see 3.4.3), an unusual resonance, not previously



**Figure 9.** *In vivo*  $^{31}\text{P}$  NMR spectrum of nitrogen fixing nodules taken from *B. japonicum* USDA110 colonized soybean (glycine max) roots then split and perfused with  $\text{O}_2$ -saturated buffer solution at pH7.5 containing 50mM glucose. The spectrum was obtained in 53 minutes. Resonances are identified in the figure. Signal "X" turned out to be from a novel cyclic glucan whose structure is shown in Fig. 10. (Adapted from Rolin *et al.* 1992.)

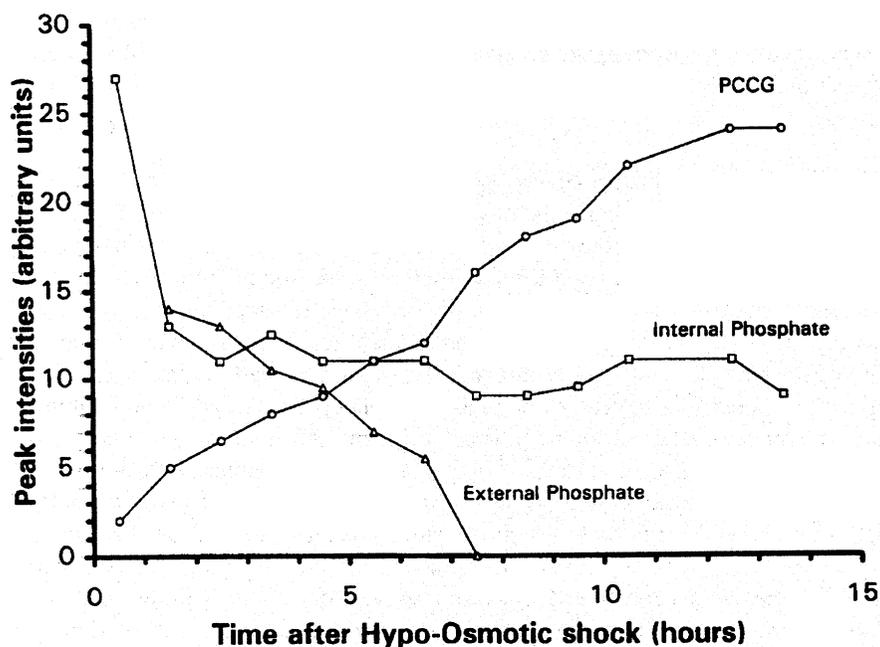
observed in plant material was noted at 0.44 ppm (marked X in Fig. 9) (Rolin *et al.*, 1989a). Spectra of bacteroids isolated from these nodules verified that this compound was contained within the bacteria and not the host plant cells. This resonance was also seen in  $^{31}\text{P}$  spectra of extracts of *Bradyrhizobium japonicum* and of two other slow-growing *B. japonicum* species (Rolin *et al.*, 1992). Following isolation and a detailed characterization using 2D NMR, the structure of the compound was determined to be a phosphocholine-substituted  $\beta$ -1,3;1,6 branched cyclic glucan (PCCG) as shown in Fig. 10 (Rolin *et al.*, 1992). The unsubstituted version of this cyclic glucan (CG) which occurs in four different ring sizes (Miller *et al.*, 1990), was also detected.

The various cyclic  $\beta$ -glucans form a class of compounds found in the *Rhizobiaceae*, they are found in the periplasmic space of the bacteria and most of them contain glucose units linked by  $\beta$ -1,2 bonds. The glucose units are either unsubstituted or substituted at the  $\text{C}_6$  position with primarily phosphoglycerol joined through a phosphodiester linkage. Glucan rings can range in size from 17 to 40 (Breedveld and Miller, 1994) but have also been observed with 12 glucose units (rolin *et al.*, 1992). The synthesis of these compounds is osmotically regulated: they are produced in large quantities under hypoosmotic conditions but not at high osmotic pressure. It has been suggested that the osmo-regulation of glucan production serves to control the relative volumes of the periplasmic and cytoplasmic compartments (Miller *et al.*, 1986). However an exception to these



**Figure 10.** Structure of the novel  $\beta$  1,3; 1,6-phosphocholine-substituted macrocyclic glucan isolated from *Bradyrhizobium japonicum* and nitrogen fixing soybean nodules. (Adapted from Rolin *et al.*, 1992.)

observations has been found using *in vivo*  $^{13}\text{C}$  NMR (Breedveld *et al.*, 1993). *Rhizobium leguminosarum* was shown to produce and excrete large amounts of unsubstituted cyclic  $\beta$ -glucans under both low and high osmotic conditions. For a comprehensive review of this topic see Breedveld and Miller (1994). Given the important function(s) of cyclic glucans and the sensitivity of  $^{31}\text{P}$  NMR for detecting the presence of PCCG, experiments were initiated to explore the conditions under which PCCG is synthesized in *Bradyrhizobium japonicum* (Pfeffer *et al.*, 1994). An airlift system (Fox *et al.*, 1989) see also Chapter (4) was used to maintain the cells during  $^{31}\text{P}$  NMR experiments. Fig. 11 shows the timecourse of PCCG production and concomitant changes in intracellular and extracellular phosphate following transfer of cells grown under high osmotic conditions to hypotonic medium. This experiment demonstrated that these cells first release intracellular Pi in response to the drop in osmotic pressure and subsequently reabsorb this Pi and produce PCCG/CG. These changes are interpreted as responses by the cells to minimize volume changes in their intracellular and periplasmic compartments (Czonka, 1989).



**Figure 11.** A plot showing the time course of movement of phosphate and synthesis of PCCG in a suspension of *B. japonicum* cells that were transferred from a high osmotic growth medium (650mosmol) to a low osmotic medium (17 mosmol) at time 0. Each point represents the  $^{31}\text{P}$  resonance intensity of each signal obtained from 1h NMR spectra. (Adapted from Pfeffer *et al.*, 1994.)

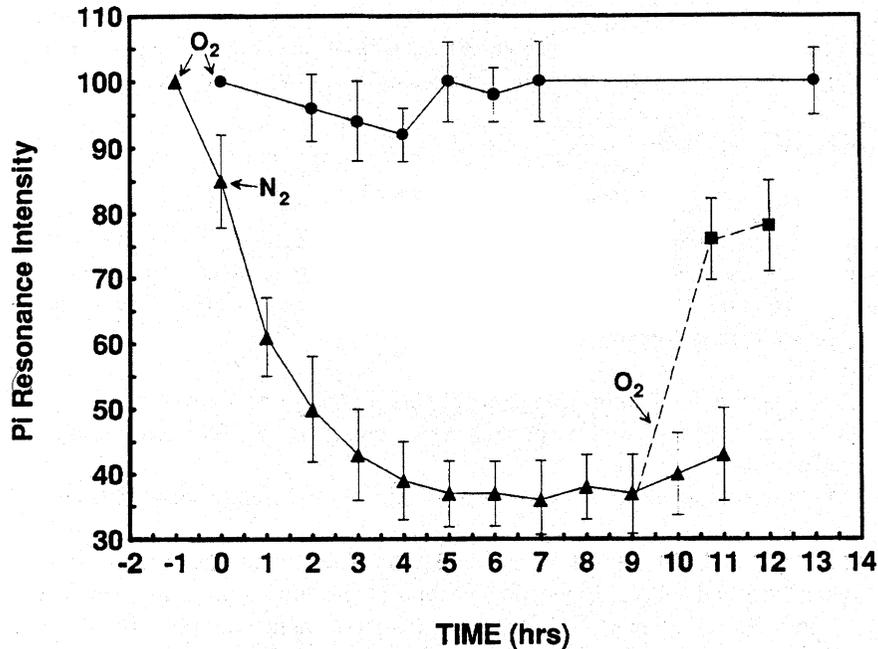
Natural abundance  $^{13}\text{C}$  spectra of cells grown under high osmotic conditions in the presence of glucose, showed that glycogen and trehalose accumulated, whereas those not given glucose did not make glycogen. Nor did such cells produce PCCG/CG when hypoosmotically shocked (Pfeffer *et al.*, 1994) but only released trehalose. Thus a glucose storage polymer such as glycogen is required as a precursor for PCCG/CG production (Pfeffer *et al.*, 1994).

In general, mutants that cannot produce these glucans are impaired in their ability to grow under hypoosmotic conditions and are also defective in their ability to infect plants (Dylan *et al.*, 1990). Adaptation to different osmotic conditions may be an important factor in the ability to infect plants since osmotic pressures may vary between rhizosphere, infection thread and peribacteroid unit within the host cell (Nagpal *et al.*, 1992).

Recent results on *Rhizobium meliloti* further support a link between the function of cyclic glucans in osmoregulation and infectivity (unpublished results): a mutant of *Rhizobium meliloti* which does not make cyclic glucans, was found to be limited both in its ability to adapt to hypoosmotic conditions and in its infectivity. Both of these functions were restored by introducing a gene locus from *Bradyrhizobium japonicum* (Bhagwat *et al.*, 1992) and this complementation resulted in the formation of a novel ten membered ring  $\beta$ -1,3 branched cyclic glucan (cyclolaminarinose) different from cyclic glucans found in either wild type *Rhizobium meliloti* or *Bradyrhizobium japonicum* (Pfeffer *et al.*, unpublished results).

### 3.4.3 Compartmentation of Phosphate in Soybean Nodules

The first effort to optimize the methodology for obtaining *in vivo*  $^{31}\text{P}$  spectra of soybean nodules was relatively successful in defining many of the parameters that influence the energetics of this plant/microbe association (Rolin *et al.* 1989b). Perfused, detached and split nodules at pH 7.5 gave resolved spectra for up to 24h (see Fig. 9). However, no resolution of plant and bacterial cytoplasmic Pi could be achieved. The cytoplasmic and vacuolar pH's were maintained at 7.4 and 5.5, respectively. Spectra of nodules from plants grown under temperature- and-drought stressed conditions showed significant differences from controls in their Pi content. Nodules of stressed plants had a high vacuolar Pi (Rolin *et al.*, 1989b) which suggested that Pi may have remained in the nodule plant cell vacuoles instead of being transported to the shoots since transpiration rates are low under these stress conditions. Perfusion with glucose and 2-deoxyglucose established that glucose was readily metabolized to glucose-6-phosphate, uridine diphosphoglucose and their deoxy derivatives, however, it was not clear whether glucose was utilized by both host cells and bacteroids.  $^{31}\text{P}$  spectra of intact nodules, nodules with cortex tissue (exterior host cells), excised cortex tissue and isolated bacteroids were taken at intervals from 4 to 12 weeks after inoculation (Rolin *et al.*, 1989b). The data show that in the early stages of growth 61% of the Pi was in the cytoplasm of the central matrix. After 7 weeks the majority of the Pi (77%) was in the vacuoles (43% in cortical tissue, 34% in uninfected central matrix



**Figure 12.** Plot of the changes in the integrated intensity of the  $^{31}\text{P}$  NMR signal of phosphate in a low pH compartment of tissue from the cortex of root nodules of soybean. Spectra were taken before during and after exposure to  $\text{N}_2$ -saturated perfusate. The intensity of the Pi signal is inversely related to the relaxation time T1 in the compartment from which the signal arises. At zero time, gassing of the perfusion medium was switched from  $\text{O}_2$  to  $\text{N}_2$ . Each point represents a replicate of four independent experiments: ▲,  $\text{O}_2/\text{N}_2$  timecourse; ●, control timecourse with  $\text{O}_2$  alone; ■, experiment in which  $\text{O}_2$  was reintroduced after 9h of  $\text{N}_2$  treatment. (Adapted from Pfeffer *et al.*, 1992.)

mechanism the oxygen tension affected T1, standard solutions and soybean root segments were exposed to oxygen or nitrogen. No changes in T1 were observed. However, solutions of Pi containing various amounts of ethylene glycol showed a reduction in T1 with increased viscosities. From this model system it was estimated that to duplicate the T1 shortening observed in the cortical signal under oxygen perfusion would require the relative viscosity within the cells to increase by a factor of five. A magnetic resonance imaging (MRI) study performed on attached nodules produced T1-weighted images in which the T1 values for water in the cortex were found to be much shorter in the presence of high oxygen as opposed to nitrogen (MacFall *et al.*, 1992). See 2.7 for details of MRI methods and a discussion of this study. These observations correspond to those above and indicate that the mechanism of enhanced T1 relaxation under oxygen affects both water and Pi. If the proposed change in viscosity is due to the synthesis of glycoprotein

(whose levels have been reported to rise in the intercellular spaces of the nodule cortex, James *et al.*, 1991) then one may predict that the timecourse of glycoprotein synthesis should mirror the dynamic changes in T1 shown in Fig.12.

### 3.5 CONCLUSIONS

It is interesting to consider how the contributions made by NMR to understanding symbiotic systems are related to its strengths and limitations. In particular three distinguishing characteristics of NMR spectroscopy seem to us to have played significant roles in generating information that would probably not have been otherwise obtained.

These are:

- 1) The potential to generate biochemical data in a non-destructive way.
- 2) The sensitivity of *in vivo* spectroscopic signals to physical and chemical environment.
- 3) The discrimination *in vivo* and in crude extracts of different metabolites and of labeling in different positions within metabolites.

Thus the information obtained on compartmentation of Pi and other metabolites in mycorrhizas and nodules depended on 1) and 2). So did the information yielded on the physical state, levels, chelation, and dynamic changes in polyphosphates and the data on local changes in nodules with changes in oxygen levels.

Studies to define which metabolic pathways are active (eg. the mannitol cycle in ectomycorrhizal fungi) are made much easier by the ability to discriminate labeling patterns and fractional enrichments in different positions. This discrimination (*i.e.* 3) removes the need for separation and chemical degradation of metabolites.

The discovery and subsequent structural determination of PCCG was derived from two aspects of 3): (a) that all mobile metabolites present in relatively high abundance give signals in the NMR spectrum, whether their presence was expected or not and (b) that within a molecule, different atoms and their connection to one another can be analyzed. Application of 1) and 3) has made possible the timecourse studies of metabolism and transport in intact symbioses, relying on spectroscopic rather than physical separation between the partners. This has revealed information for example on the storage compounds and their carbon metabolism in VA mycorrhizae and nitrogen metabolism in ectomycorrhizae and demonstrated differences between the free-living and symbiotic states.

A major limitation on information from NMR as applied to symbiotic systems has also to do with (2) since the NMR invisibility of immobile compounds limits the information on large or immobilized compounds and creates uncertainty about potentially invisible pools of, for example, high molecular weight polyphosphates and glycogen in mycorrhizae. In addition, limitations on sensitivity allow one to exploit (3) only after isotopic labeling with substrate, since the level of detection of the fungal metabolites in VA-mycorrhizae for example, are well below the NMR  $^{13}\text{C}$  natural abundance limits of detection.

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