

## Agricultural/biological applications of NMR

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**Abstract** This chapter is designed to introduce the neophyte to a range of nuclear magnetic resonance (NMR) techniques and experiments that have or will have an impact on the solution of agriculture/biological problems. Throughout the various sections I have attempted to use examples from the recent literature as well as work from our own laboratory to illustrate the diversity of applications. The four main topic areas discussed are: (1) multinuclear high field/high resolution methods including 2-D methods for structure determination; (2) *in vivo*, multinuclear high resolution studies of plant material; (3) solid state cross polarization magic angle spinning (CPMAS) NMR which will cover the study of intact polymers, soil matrices, seeds and plant tissue metabolism, and (4) magnetic resonance imaging (MRI) as it is applied to plant and animal tissues and some examples of how it is utilized in conjunction with spatially resolved high resolution surface coil *in vivo* techniques.

### Introduction

The advent of the computer and superconducting magnet technology has immeasurably advanced the state of the art of the modern Nuclear Magnetic Resonance (NMR) spectrometer in terms of its (1) inherent sensitivity, (2) multinuclear capabilities, (3) capability of routinely identifying resonances to solve complex structural problems, and (4) ability to control nuclear spin physics, to evaluate molecular dynamics and elucidate biochemical phenomenon in solution and solid states.

Today's commercial, pulsed superconducting, Fourier transform, (FT) spectrometer operating at magnetic fields as high as 11.8T can obtain spectra of submicrogram quantities of material within reasonable periods of time. In addition the extended frequency range and rapid digitization make it possible to explore a seemingly inexhaustible array of magnetically active nuclei, dipolar and quadrupolar, *in vitro* as well as *in vivo*. With the innovative development of RF controlled multipulse programming to produce nuclear spin "acrobatics" comes the realization that the previously unseen communication between related nuclei could be revealed in a second time dimension, hence the birth of two-dimensional NMR (2-D NMR). Presently, the complete assignment of a  $^{13}\text{C}$  resonance spectrum representing a complex molecule as well as detailed stereochemical, conformational and through space nuclear distances are available with a days instrument time. What's more, the

information is most relevant since unlike the data derived from crystallographic methods, it is descriptive of the chemistry in the native solution state.

Similarly, spin "acrobatics" or nuclear spin manipulation has been exploited to enhance the inherent sensitivity of the NMR experiment in solid state applications. Further refinement of this technique has led to methods for identifying specific molecular entities as well as defining their respective relationships with one another within intact multi component assemblies.

The innovative development of magnetic field gradient technology has given birth to magnetic resonance imaging (MRI) and a new era in medical applications. In addition the combination of MRI and spatially resolved high resolution NMR are just beginning to establish new frontiers in the exploration of a variety of living tissues.

In the previous chapter Dr. Becker has described the many innovations of the modern NMR spectrometer and given us an overview of the new techniques and methods that are reputed to be some of the most potent scientific tools we have today. In this contribution I will attempt to touch on examples from the recent literature and our own work that illustrate the application of the NMR experiment to the solution of Agricultural/Biological problems. The four main topics that will be discussed will include, (a) multinuclear high field/high resolution methods including 2-D methods for structure determination, (b) *in vivo*, multinuclear high resolution studies of plant material, (c) solid state cross polarization magic angle spinning (CPMAS) NMR which will cover the study of intact polymers, soil matrices, seeds and plant tissue metabolism and (d) MRI as it is applied to plant and animal tissues and some examples of how it is utilized in conjunction with spatially resolved high resolution surface coil *in vivo* techniques.

This chapter is designed to introduce the neophyte to a sampling of illustrative examples that demonstrate the Agricultural/Biological applications of NMR spectroscopy. It is by no means to be considered an exhaustive review of the NMR literature. In most instances the material will be confined to the study of agriculturally relevant topics. In some areas where general application of a method is recognized, pertinent review articles will be cited. The reader is referred to the following comprehensive texts which provide excellent in depth coverage of the field of biological NMR (Knowles, Marsh and Rattle, 1976; Shulman 1979; Jardetzky and Roberts, 1981; Gorenstein, 1984).

## High resolution NMR

### *(a) Structure determination (analytical applications)*

To establish the structures of organic biomolecules,  $^1\text{H}$  and  $^{13}\text{C}$ , are, because of their ubiquitous nature, the most widely used nuclei for study by NMR. Although many other nuclei, such as  $^{31}\text{P}$ ,  $^2\text{H}$ ,  $^{15}\text{N}$  and  $^{17}\text{O}$  to name a few, can be examined routinely their usefulness in structural studies is limited and beyond the scope of this chapter.

Early NMR instrumentation was limited in its application to relatively large samples coupled with poor dispersion due to the low fields that were available (1.4–2.5T). With the development of superconducting magnets and higher field sensitivity, resonance shift dispersion for protons was significantly improved. The innovation of pulsed Fourier transform methods and computer averaging made dilute spin nuclei such as  $^{13}\text{C}$ , (1.1% natural abundance) important for structure elucidation. However, the most significant advance that has brought NMR spectroscopy to the fore today is the development of pulse controlled nuclear spin physics and its innovative application to resonance identification, structure determination and molecular dynamics. A comprehensive review of these methods is given by Benn and Günther (1983) and full treatment of exclusively 2-D implementation is found in a book by Bax (1982).

Previously difficult to interpret off resonance or gated decoupling experiments had to be performed in order to establish the identity of a carbon type ( $1^\circ$ ,  $2^\circ$ ,  $3^\circ$  or  $4^\circ$ ) in complex  $^{13}\text{C}$  spectra. In special cases such as carbohydrate molecules, carbon resonance assignments could be made with the aid of deuterium isotope shifts (Pfeffer, Valentine and Parrish, 1979). Today the modern pulse programmed spectrometer can do  $^{13}\text{C}$  resonance multiplicity assignments automatically and more accurately and in a fraction of the time required in the past. Figure 1 illustrates an example of the use of one of these new pulse sequences called Inensitive Nuclei Enhanced by Polarization Transfer (INEPT) for  $^{13}\text{C}$  multiplicity sorting of a  $^{13}\text{C}$  spectrum of ATX-I (I), a mycotoxin produced by *Alternaria* mold growing on wheat or rice. (Stinson, Osman and Pfeffer, 1982). To implement this experiment, one employs different delay times or evolutionary periods in the INEPT pulse sequence based on an average  $^{13}\text{C}$ - $^1\text{H}$  coupling constant for the molecule under study. Varying the evolutionary periods corresponding to different fractions of the average  $1/J$  value produces different dephasing responses in the ( $1^\circ$ ,  $2^\circ$ ,  $3^\circ$ ) proton populations and ultimately the generated  $^{13}\text{C}$  spectra of their bound carbons induced by cross polarization. Quaternary carbon resonance are assigned by difference with this technique since they have

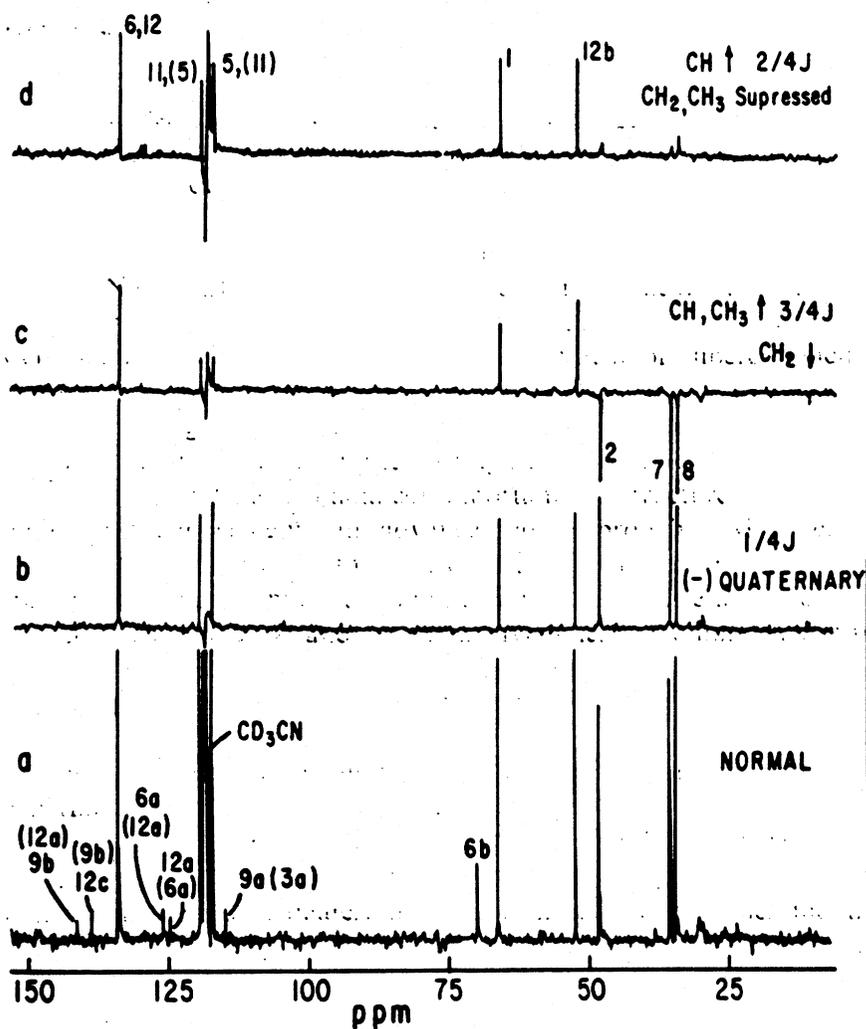


Figure 1. (a) Normal upfield portion of the 67.2 MHz  $^{13}\text{C}$  NMR spectrum of I in  $\text{CD}_3\text{CN}$ , (b) polarization transfer spectrum with delay =  $1/4J$ , (c) delay =  $3/4J$ , (d) delay =  $2/4J$ . Stinson et al., (1982).

no directly bound protons with which to cross polarize and are not seen in the spectra. Other variations on this sequence such as DEPT or APT (Doddrell, Pegg and Bendall, 1982; Patt and Shoolery, 1981) have also been reported and offer certain advantages.

To establish the assignments of each of these carbon types, identification of the corresponding proton spectrum and a molecular weight and empirical formula are essential. In spite of present day larger magnetic fields, proton resonances often overlap and are rarely observed in first



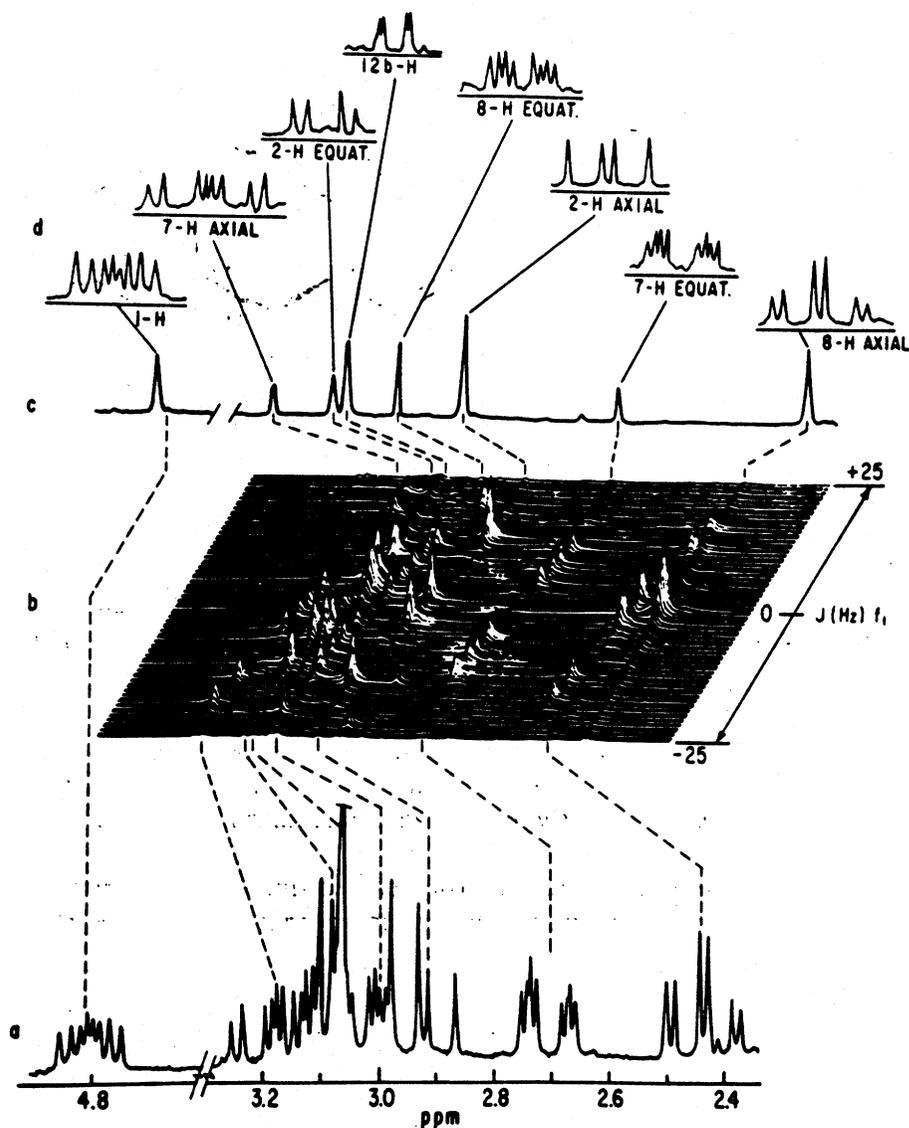


Figure 2. Upfield portion of the  $^1\text{H}$  spectrum of I in  $\text{CDCl}_3$ , measured at 270 MHz. (a) Conventional NMR spectrum, (b) two-dimensional  $J$  spectrum (130 traces are shown; the methine H-1 at  $\delta$  4.77 has been omitted from this plot), (c), projected spectrum of  $b$  onto the horizontal ( $f_2$ ) axis, (d)  $J$  spectra (cross sections of the stacked plot). Stinson et al., (1982).

The solid lines equidistant from the one-dimensional spectrum displayed on the central axis define the through bond scalar  $J$  coupled connectivities between vicinal and germinal protons, (SECSY correlation), and the structure of each carbohydrate residue, i.e. glucose, galac-

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

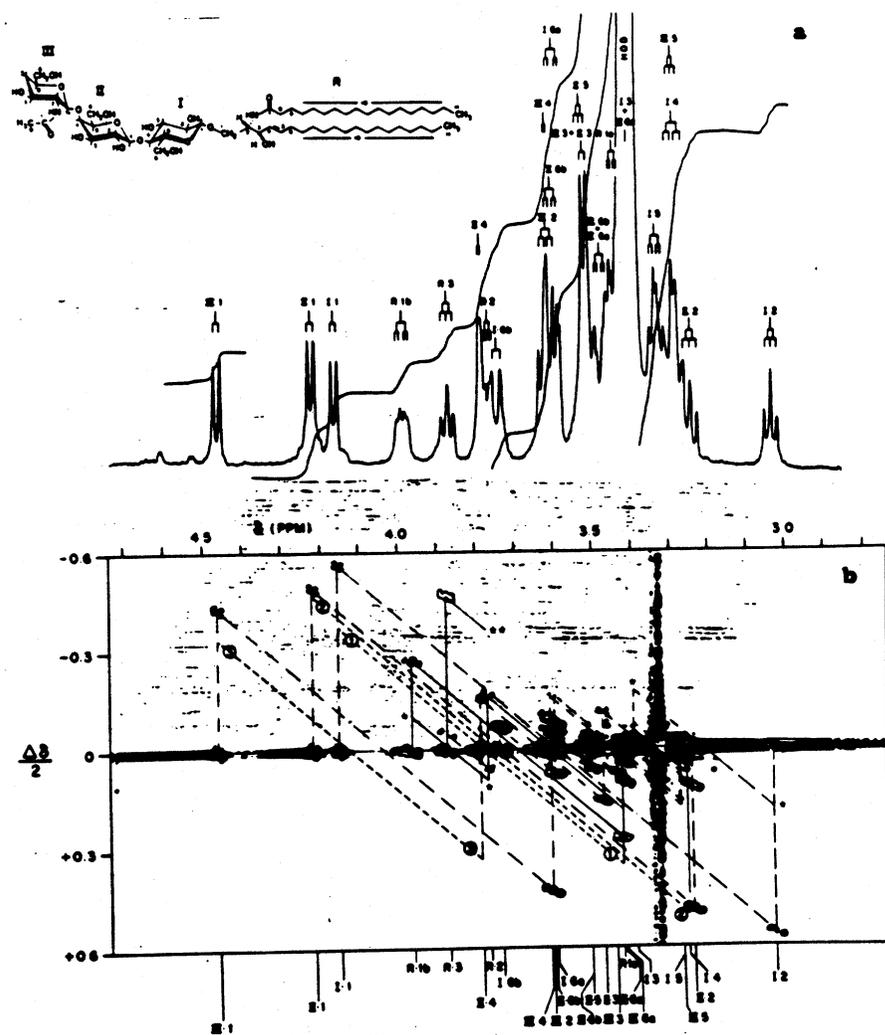


Figure 3. Structure and proton NMR spectra of gangliotriaosylceramide (I): (a) Integrated, one-dimensional spectrum of oligosaccharide ring proton region obtained at 30°C, after 420 pulses in an 8 K data set with a repetition rate of 10 s. Me<sub>4</sub>Si was used as internal reference. (b) Two-dimensional SECSY spectrum obtained at 40°C. A total of 16 pulses in a 256 × 1024 data set were required, taking approximately 2 h. J connectivities are labeled as follows: I,  $\beta$ -galactopyranosyl (---); III, 2-acetamido-2 deoxy- $\beta$ -galactopyranosyl (---); R, ceramide (---). Also shown are the three (numbered) interresidue NOE couplings (---), obtained from the 2-D-NOE spectrum (which used 256 × 88 acquisitions and required 20.5 h). Other notations are for J connectivities observed at slightly lower contour thresholds (\*) and to protons outside the observed window (\*\*). A slight temperature dependence in chemical shift is to be noted, especially for the III-3 and II-3 resonances. Prestegard et al., (1982).

tose, mannose, etc. In addition the dotted line connectivities define the common NOE responses resulting from dipolar through space interactions of protons on adjacent residue carbons attached at glycosidic linkage pairs, e.g. (OCH<sub>2</sub>-I1); (I4-II1); (II4-III1). Also the intensity of the Overhauser effect can be used to determine the proximity of the adjacent residue protons with respect to each other and thereby the conformational relationship of one residue with another. Other workers have exploited the two-dimensional carbon-proton heteronuclear COSY experiment, after establishing the assignments of the proton spectrum, to identify carbon connectivities for the metabolite vomitoxin produced by a fungus growing on cereal crops (Blackwell, Greenhalgh and Bain, 1984). These methods of analysis have also been used for structural studies of oligosaccharides (Morris and Hall, 1981). A complete review of these methods and applications to polysaccharide containing structures is given in a special issue of the journal of carbohydrate chemistry (Pfeffer and Binkley 1984).

NMR methods are clearly challenged to the limit when confronted with the task of evaluating complex protein structure. Before such a study is undertaken one must be well acquainted with the primary structure and often an X-ray crystallographic map. Why then do we need to do an NMR experiment? In essence, the homonuclear proton 2-D COSY experiment can assign residue proton resonances which then can be examined with the 2-D NOESY methodology to establish secondary through space interresidue interactions. This data can then be used to generate three-dimensional computer model structure. Wüthrich (1984), has made major contributions in the 2-D studies of protein. Some of these recent findings suggest that protein conformational preferences as defined by X-ray crystallographic methods in the solid differ from those in solution and membrane bound (MB) state. Figure 4 illustrates the sheer complexity of the proton 2-D COSY spectrum of a relatively simple 29 amino acid MB-polypeptide hormone glucagon (Wüthrich, 1984). While the analysis of this spectrum might take up to a year, new computer methods may shorten this time significantly in the near future (Billeter, Engeli and Wüthrich, 1985).

Analysis of the 2-D NOESY experiment yielded many of the through space interactions leading to the conformational calculations of this structure. Final calculation of the three dimensional structure of the membrane bound molecule based on a detailed analysis of the NMR data revealed that overall the distance from the C-terminal helical region to the N-terminus is appreciably longer than in the glucagon single crystals.

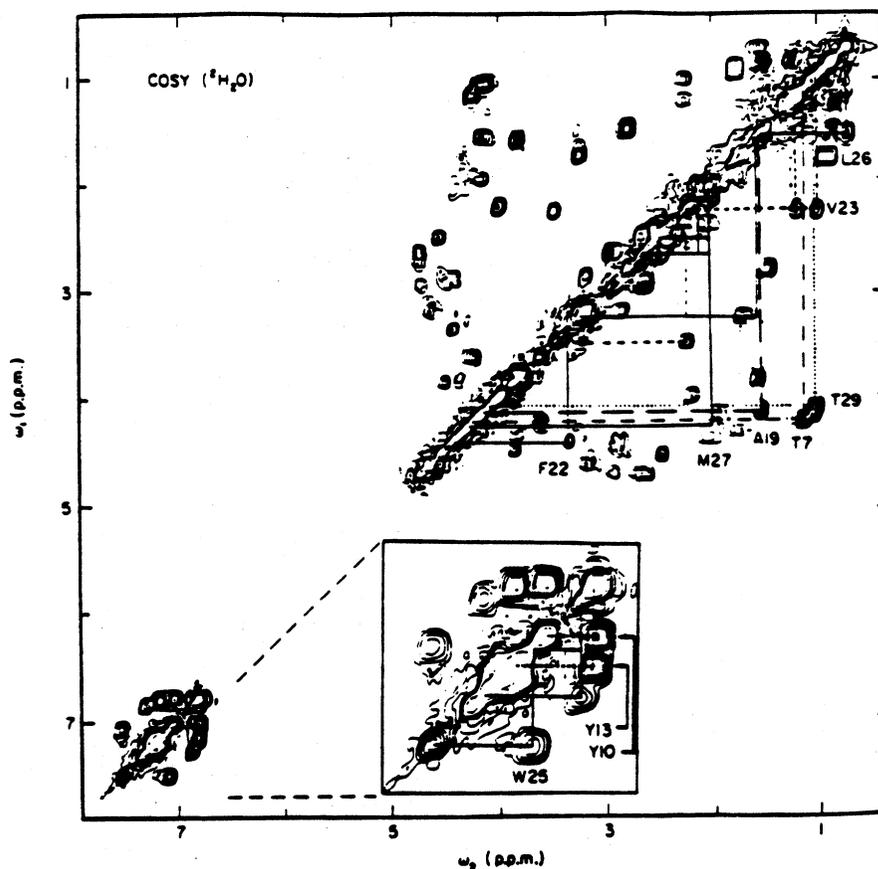


Figure 4. Contour plot of a 360 MHz  $^1\text{H}$  COSY spectrum of glucagon bound to perdeuterated dodecylphosphocholine micelles in  $^2\text{H}_2\text{O}$ . The sample contained 0.015M glucagon,  $0.7\text{M} [^2\text{H}_3]_n$ -dodecylphosphocholine, 0.05M phosphate buffer,  $p^2\text{H}$  6.0,  $T = 37^\circ\text{C}$ . Under these conditions the predominant species in the solution are mixed micelles of 1 glucagon molecule and  $\sim 40$  detergent molecules with a molecular weight of about 17 000. The spectrum was recorded in 24 h, the digital resolution is 5.88 Hz/point. The symmetrized absolute value spectrum is shown. The aromatic region is also presented on an expanded scale. Proton-proton J-connectivities are indicated for the following residues: Thr 7 (---), Ala 19 (—), Phe 22 (—), Val 23 (---), Leu 26 (—), Met 26 (—), Thr 29 (····) and the aromatic rings of Tyr 10 (····), Tyr 13 (---) and Trp 25 (—). In order not to overcrowd the figure, only the  $\text{C}^{\alpha}\text{H}$  connectivity with the lower field  $\text{C}^{\beta}\text{H}$  line is shown. Crosspeaks originating from residual protons in the perdeuterated dodecylphosphocholine are marked (x). Wüthrich, (1984).

#### (b) Biosynthetic pathways and molecular interactions

Multinuclear NMR methods have revolutionized the study of biosynthetic pathways and molecular interaction as they relate to enzyme active sites and biochemical processes. Because of the voluminous literature:

available in this area, I will confine this discussion to some key review articles, references and a single example, in hopes that the interested reader will be stimulated to pursue these topics in further detail. In general the great majority of the studies involving macromolecular structure and interactions require the enrichment of a dilute spin nucleus such as  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ , or the use of a particular metal ion as a probe. A recent study in our laboratory has utilized the incorporation of  $^{13}\text{C}$  acetate label to elucidate the biosynthetic pathway for the production of the metabolite alternariol from *Alternaria*, a fungus that grows on wheat or rice (Stinson et al., 1986). After we established the complete and unambiguous  $^{13}\text{C}$  resonance assignments for this molecule, the fungus

was fed  $^{13}\text{CH}_3\text{-}^{13}\text{C}\text{-ONa}$  to produce alternariol containing intact incorporated acetate units. To elucidate the pathway for its production the enriched compound was subjected to a 2-dimensional multiple quantum coherence analysis-INADEQUATE (Incredible Natural Abundance Double Quantum Transfer Experiment) (Bax et al., 1981). The pulse sequence in this experiment generates a  $^{13}\text{C}$  spectrum (Fig. 5) of those  $^{13}\text{C}$  nuclei coupled directly to adjacent  $^{13}\text{C}$  nuclei. In the natural abundance compound the probability of having a  $^{13}\text{C}$  nucleus next to another  $^{13}\text{C}$  is 1/10 000. Essentially the double quantum filtering experiment suppresses the signals from any  $^{13}\text{C}$  nuclei that would be positioned next to a  $^{12}\text{C}$  and so the results are diagnostic for detecting a synthetic pathway requiring the splitting of dilabeled acetate. The  $^{13}\text{C}$ - $^{13}\text{C}$  connectivities given in the contour plot shown in Fig. 5 defines the sequential labelling pattern and biosynthetic polyketide pathway for alternariol given in the structure in Fig. 5. A number of examples that use  $^{13}\text{C}$  and  $^2\text{H}$  enrichment techniques for elucidating biosynthetic pathways is given by (Steyn, 1980).

$^{15}\text{N}$  enrichment has been used to study enzymic binding sites in carboxypeptidase (Bachovehin et al., 1982) the  $pK_a$  values of specific amino acid residues in serine proteases (Kanamori and Roberts, 1983) and base pairing and motion in DNA (James, James, and Lapidot, 1981) and tRNA (Gonnella et al., 1982). Often because of the difficulties of incorporating an  $^{15}\text{N}$  isotope into a large biomolecule, the label is put into a small substrate and its dynamic properties can be examined in the macromolecule's active binding site (Morishima and Inubushi, 1978). High resolution  $^{15}\text{N}$  and  $^{13}\text{C}$  NMR have also been effectively used to characterize the structure of wheat proteins as a function of concentration and heating (Baianu, Johnson and Waddell, 1982). Because of the low sensitivity and negative NOE,  $^{15}\text{N}$  NMR has not seen the wide

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

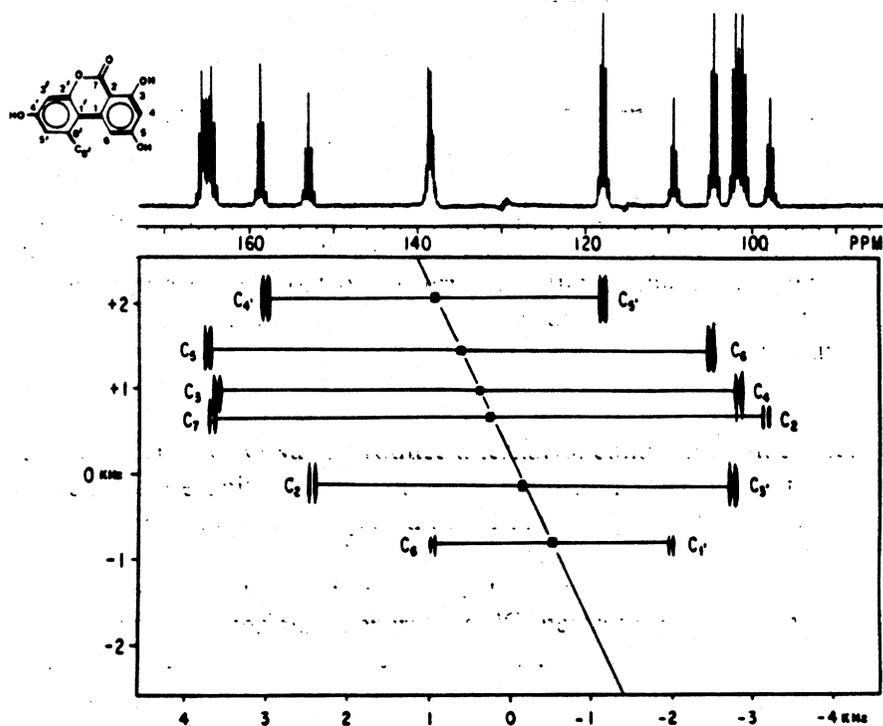


Figure 5. 100 MHz  $^{13}\text{C}$  2-D INADEQUATE contour plot for a 0.47 M solution of alternariol (biosynthetically prepared from  $[1,2\text{-}^{13}\text{C}]$  acetate). Only the 5 KHz region in the vicinity of  $F_1 = 0$  is shown. Total acquisition time for this spectrum was 38 h. Stinson et al., (1986).

application in *in vitro* biological systems. However, its use in *in vivo* studies because of its unusual correlation time ( $T_c$ ) dependent NOE have made it a good probe for monitoring cellular viscosity, (this will be described in the next section).

Although  $^2\text{H}$  has low inherent NMR sensitivity its ease of incorporation and usefulness of its quadrupolar relaxation for probing motion are responsible for its most popular application in studies of protein mobility (Wooten and Cohen 1979; Brown, Kumosinski and Pfeffer, 1983) and membrane lipid and lipid protein interactions (Mantsch, Saito and Smith 1976; Jacobs and Oldfield, 1981). In order to obtain motional information for highly oriented, relatively rigid structures as found in membranes, large quadrupolar splittings must be measured with specialized instrumentation capable of rapid digitization of spectral widths in the MHz range. In addition high resolution  $^2\text{H}$  NMR has been used to

examine the molecular interactions of dietary fiber components with digestive bile acids as a means of evaluating a proposed mechanism suggested for the lowering of serum cholesterol (Pfeffer et al., 1981).

Metal ion NMR spectroscopy has become an important tool of the biologically inclined spectroscopist since the late 70's. In general both high natural abundance metals such as  $^{23}\text{Na}$ ,  $^{113}\text{Cd}$  and the less sensitive quadrupolar nuclei  $^{25}\text{Mg}$ ,  $^{39}\text{K}$  and  $^{43}\text{Ca}$  have found important applications to studies of macromolecular equilibria, exchange rates and the structure and dynamics of metal ion-ligand interactions. The breath of these studies runs the gamut from proteins and enzymes to membranes and charged polysaccharides. A most comprehensive review of the up-to-date literature in this area is given by Braunlin, Drakenberg and Forsen (1985).

### High resolution *in vivo* NMR of plant materials

#### (a) $^{31}\text{P}$ studies

High resolution NMR has become the most widely utilized method for examining functioning isolated cells, organelles from plant and animal tissues and microorganisms, primarily because it is non-invasive i.e., it does not require ionizing radiation, dehydration of cell contents or isolation and or fractionation of cellular components. NMR applications to the study of mammalian tissues and tissue disorders has been thoroughly reviewed (Roberts and Jardetzky, 1981; Gadian, 1982; Barany and Glonek, 1984).

The two most often used nuclei for whole tissue and cell studies are  $^{31}\text{P}$  and  $^{13}\text{C}$ , however more interests in  $^{14}\text{N}$ ,  $^{15}\text{N}$  and  $^{23}\text{Na}$  has recently been sparked (Martin, 1985). Although the  $^1\text{H}$  nucleus is the most sensitive, it has seen only limited use in *in vivo* applications because of the dominance of the large tissue  $\text{H}_2\text{O}$  resonance in spectra, the relatively narrow chemical shift range ( $\sim 10$  ppm) and broad overlapping lines of a multitude of components. Brown and Campbell (1980) have dealt with these problems by employing an elegant spin echo technique for examining the red blood cell.

This section will deal with the use of  $^{31}\text{P}$  NMR, drawing on some examples from our ongoing work on metal ion transport in maize root tissue. Work dealing with other nuclei such as  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{14}\text{N}$  and  $^{23}\text{Na}$  will also be mentioned. For additional references concerning plant metabolism and general NMR application to plant materials the reader is referred to reviews by Roberts (1984), and Loughman and Ratcliffe (1984), respectively.

$^{31}\text{P}$  combines the advantages of 100% natural abundance, relatively

#### AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

high sensitivity, and a chemical shift range for metabolic compounds of 30 + ppm which make it most attractive for the examination of plant tissue and cells. In addition because of the occurrence of the relatively few key phosphorus containing metabolites such as sugar phosphates, various nucleotides, (NTP, NDP, NAD and UDPG) and intracellular orthophosphate ( $P_i$ ), the important energetic status of the cell can be monitored from multistacked experiments in relatively short periods of time. Also, since the position of the  $P_i$  resonance is strongly pH dependent in the range of 5.0–8.0, one can easily measure ongoing changes in intracellular pH directly from the spectra (Roberts et al., 1980). While spectral analysis is often relatively straightforward one must keep in mind that we can only examine mobile, low molecular weight compounds with standard high resolution instrumentation. This means that a large fraction of the phosphorus signal is NMR invisible, e.g. signals from membrane phospholipids and relatively immobile nucleic acids and phosphoproteins cannot be observed. This can be a serious problem e.g., if one wants to follow the pathway of the production of polymeric materials in which fluxes are rapid through low steady state concentrations of metabolites (Jacob et al., 1985). Also, if metabolites are immobilized by precipitation or by tight binding to cell walls or membranes, they will be undetectable. In terms of establishing quantitative relationships between resonance responses, careful attention must be paid to the relaxation properties of each resonance in the spectrum in order to account for ongoing changes and molecular interconversions within the cells (Schleich, Willis and Matson, 1984; Pfeffer et al., 1986).

To keep plant tissue viable it must be continually perfused with a solution containing sufficient oxygen to maintain adequate metabolic activity. Recent designs (Lee and Ratcliffe, 1983) with additional modifications (Pfeffer et al., 1986) allow experiments to proceed up to 72 hours with little change in viability of the tissue. Figure 6 shows spectra of excised maize root tips including the chemical shift assignments. Spectrum *A* demonstrates the loss of viability due to inadequate replenishing of  $O_2$  while *B* shows full viability after 23 hrs provided by a recycling perfusate (45–50 ml/min) saturated with oxygen. Under optimum aerobic conditions two  $P_i$  resonances are detectable corresponding to the vacuolar compartment pH  $\sim$  5.5 (high field  $P_i$  resonance) and the cytoplasmic compartment pH  $\sim$  7.6 (Roberts et al., 1980). pH values were obtained from a calibration curve of  $P_i$  pH vs. chemical shift (Pfeffer et al., 1986). Under anaerobic conditions cytoplasmic acidification occurs leading to a shift in the  $P_i$  (cyt) peak corresponding to a pH of 6.2 and a loss of viability due to local  $CO_2$  buildup around the tissue. A similar

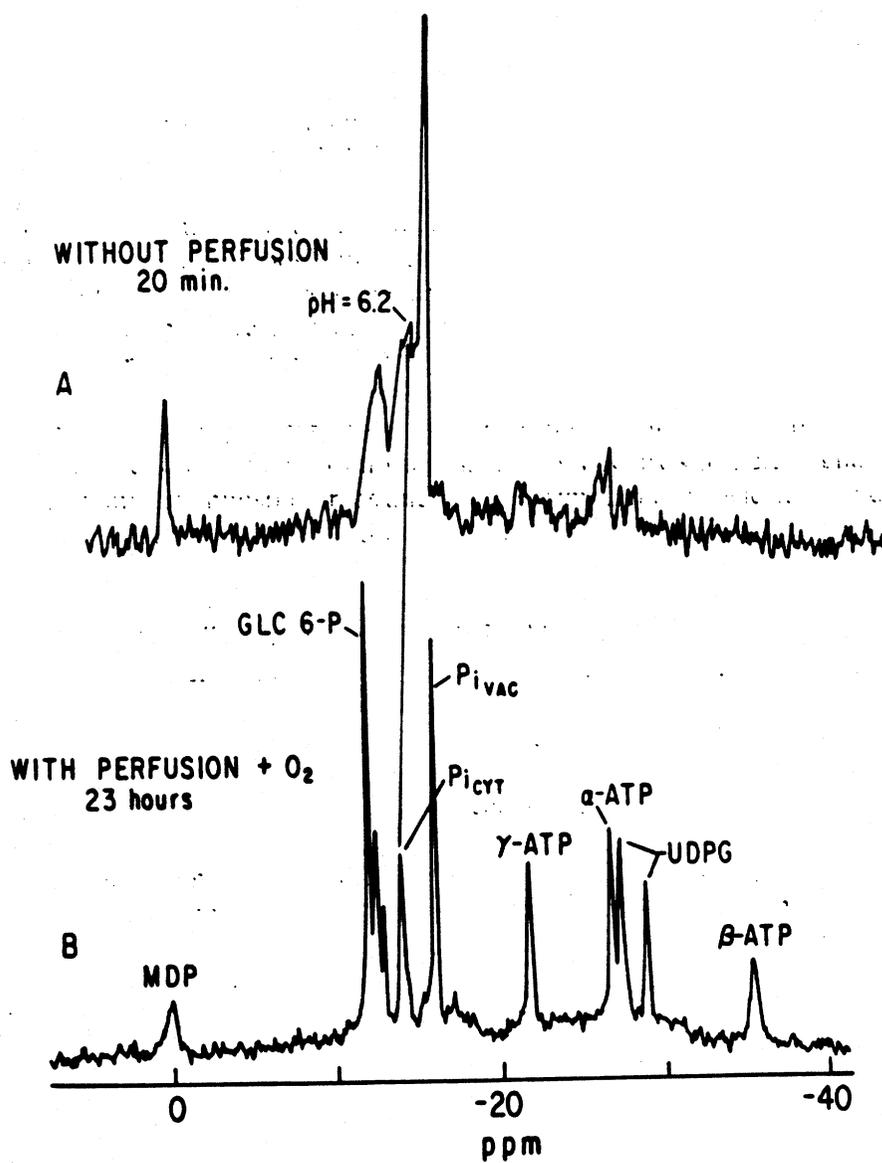


Figure 6. 161.7 MHz  $^{31}\text{P}$  spectrum of approximately 900 excised root tips (5–7 mm) taken under fast-acquisition conditions: (a) 5000 scans, after remaining in the NMR tube with no perfusion for 20 minutes; (b) 10000 scans after 23 hours of perfusion with oxygen saturated perfusate.

#### AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

cytoplasmic acidification will take place, however, without loss of viability if hypoxic conditions are maintained, i.e., if rapid circulation is continued with nitrogen saturated perfusate (Roberts et al., 1984; Roberts, Andrade and Anderson, 1985). This condition is rapidly reversed to the normal state with reoxygenation of the perfusate even after periods of 20 hrs, except for certain genetically deficient varieties that cannot control their internal pH (Roberts, et al., 1984).

The pH gradient between the cytoplasm and vacuolar (across the tonoplast) is maintained through proton pumping driven by ATPase. When this tissue is exposed to a protonophore such as 2,4-dinitrophenol (DNP) or the protonophore, FCCP the loss of intracellular pH gradients occurs (Kime, Loughman and Ratcliffe, 1982; Pfeffer unpublished results) signifying a lack of mitochondrial control and membrane integrity, respectively. Kime *et al.* (1982) and we (1986) have observed vacuole trapping of paramagnetic  $Mn^{2+}$  in the vacuole of maize root tips. The phenomenon is shown in Fig. 7 in which the vacuolar  $P_i$  resonance fails to regenerate after extended washing of the tissue. In contrast, only trace amounts of  $Mn^{2+}$  are transported into the tissue when it is exposed in a hypoxic state (Pfeffer et al., 1986) and full restoration of the  $P_i$  vacuolar resonance is seen on reoxygenation. Similar observations are made when this tissue is energetically stressed with 2-deoxyglucose or denied an exogenous carbohydrate source (Pfeffer et al., 1986a). Clearly, movement of such divalent metals as  $Mn^{2+}$  is a facilitated process dependent on the level of ATP available in the cell.

The  $P_i$  (nutrition) uptake in various plant tissues has been extensively studied (Lee Ratcliffe, 1983a). Over the range of phosphorus nutrition investigated, the quantity of vacuolar  $P_i$  per unit fresh weight of root increased considerably, whereas cytoplasmic  $P_i$  remained constant. Roberts (1984a) has demonstrated that salt stress can cause stimulation of  $P_i$  uptake and the loss of regulation of cytoplasmic  $P_i$  levels. We have observed that in the absence of exogenous  $P_i$  maize root tips generate up to 125% additional mobile  $P_i$ , (primarily found in the vacuole) over a period of 34 hours (Pfeffer et al., 1986a). The source of this phosphate is not clear, however suppression of this additional generated signal area with aluminum ion at low pH suggests that  $P_i$  may be migrating from locations in the cell wall. Aluminum has been shown to have an inhibitory effect on the acid phosphatase generation of extracellular  $P_i$  in plant cell cultures of *Agrostis capillaris* (McCain and Davies, 1984). Figures 8 and 9 illustrate the effect of aluminum ion on  $P_i$  generation in maize tips over a period of 20 hrs.

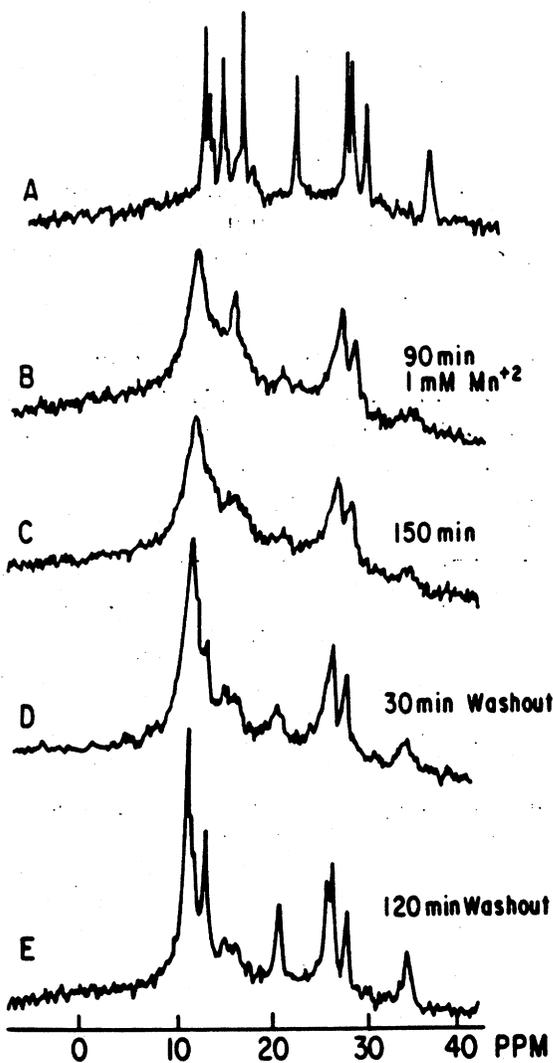
Mn<sup>2+</sup> UPTAKE IN VACUOLE

Figure 7. <sup>31</sup>P spectrum (10000 transients) taken as above after 3 h of perfusion with a solution of 0.1 mM CaSO<sub>4</sub>, 50 mM glucose, 10 mM Mes buffer (pH 6.0), O<sub>2</sub>. B, same as above except for the addition of 1.0 mM MnCl<sub>2</sub> and exposure for 54 to 81 min. C, Same as (B), exposure 108 to 135 min. D, Sample perfused with buffer as in (A) for 0 to 27 min. E, Same as (D) for 81 to 108 min. Pfeffer et al., (1986).

The presence of 0.1 mM Cd<sup>2+</sup> in the perfusion medium decreased the cytoplasmic levels of ATP in corn roots by 50% in 1½ hours and the cytoplasmic pH dropped from 7.6 to 6.8 (Pfeffer et al., 1986a). Addition of 1.0 mM Ca<sup>2+</sup> prevents this acute toxicity by competing favorably with

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

GENERATION OF MOBILE PHOSPHATE pH 4.0

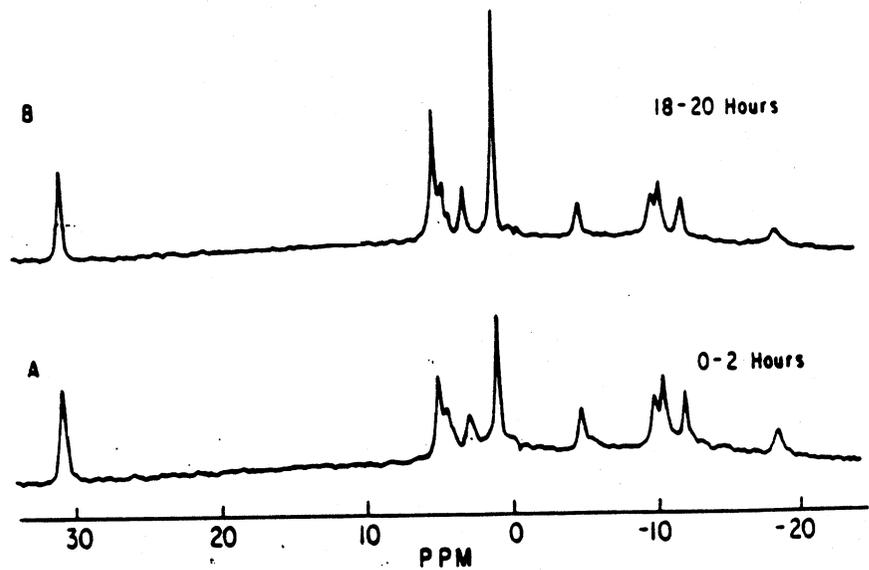


Figure 8. Below, 161.7MHz,  $^{31}\text{P}$  spectrum of approximately 900 excised root tips (5-7 mm) taken under the fast acquisition conditions, 30° pulse. (12  $\mu\text{sec}$ ) 2000 data points zero filled to 16000, recycling time 0.162s. 20000 transients, frequency of 16 KHz, 15 Hz linebroadening. Perfusate containing 0.1 mM  $\text{CaSO}_4$  and 50 mM glucose pH 4.0. (a) 0-2 hours; (b) 18-20 hours. Internal standard of 120 mM HMPA in a capillary referenced as 30.74 ppm relative to 85%  $\text{H}_3\text{PO}_4$  taken as 0.0 ppm.

$\text{Cd}^{2+}$  for entry through the cell membrane. A similar inhibition of  $\text{Mn}^{2+}$  entry is also observed in the presence of excess  $\text{Ca}^{2+}$  (Pfeffer et al., 1986a).

To obtain quantitative information on the state of metabolism as it pertains to ATP synthesis rates and oxygen consumption we must be able to measure *in vivo* exchange processes. Saturation transfer is an NMR technique whereby low-power rf field saturation of a spin on a nucleus, will transmit that saturation to any other nucleus with which it is exchanging (Shulman, 1979). In order to measure unidirectional reaction rates the systems must be in a steady state; the exchanging partners, e.g.  $\gamma\text{-ATP}$  and  $P_i$  must have separate and detectable NMR signals and the rate constants for both forward and reverse reactions must be of the magnitude of the spin lattice relaxation rate of each of the exchanging nuclei (Roberts, 1984). Using oxygen tension measurements Roberts & Wemmer and Jardetzky, (1984) have demonstrated that the P/O ratio which is 3 in normal metabolizing root tips drops to 2 when succinate is substituted for glucose. This observation indicates that a secondary

## ALUMINUM SUPPRESSION OF MOBILE PHOSPHATE pH 4.0

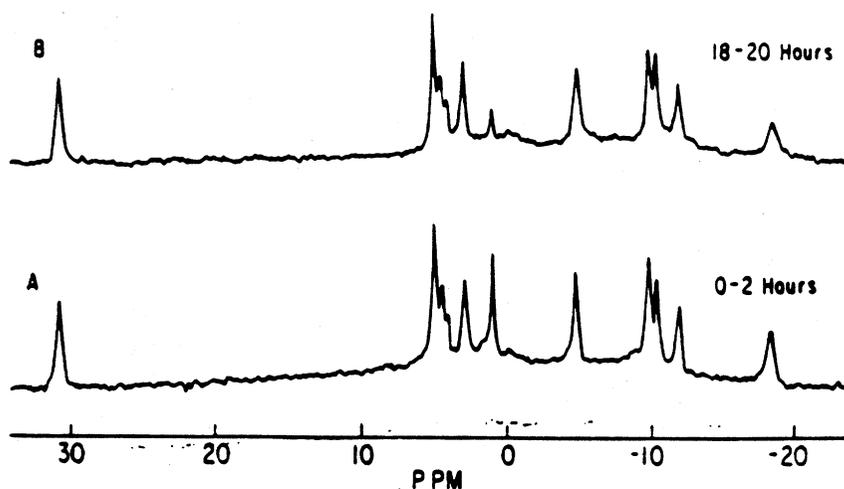


Figure 9. Above. Same as in Fig. 8 except perfusate contained, in addition to 0.1 mM  $\text{CaSO}_4$  and 50 mM glucose, 2.5 mM  $\text{Al}_2(\text{SO}_4)_3$ , at pH 4.0.

pathway in the electron transport system has been activated in which  $\text{FADH}_2$  is the principal electron donor. Addition of cyanide with succinate, suppresses the rate of ATP synthesis completely while cyanide and glucose still give a P/O ratio of 1. It is hypothesized from these results that this plant tissue contains a cyanide resistant respiration path, in which electrons travel to a terminal oxidase from a point between the first and second coupling sites of the mitochondrial electron transfer chain and, therefore only the first coupling site of the electron transfer chain is used. Figure 10 illustrates the saturation transfer experiment in which the  $\gamma$ -ATP resonance is saturated and the transfer is seen at the cytoplasmic  $P_i$  resonance.

A study by Kallas and Dahlquist, (1981) demonstrated that  $^{31}\text{P}$  NMR could be used to follow the alkalization of the cytoplasm of the photosynthetic cyanobacterium *Synechococcus* under light irradiation. This was followed by an extension of this method to the investigation of spinach protoplasts and chloroplasts (Foyer et al., 1982). Unlike the previously examined intact bacteria (Kallas and Dahlquest, 1981), these workers observed that the intracellular pH is not tightly controlled but determined by the pH of the suspending medium. Most recently, Waterton, Bridges and Irving (1983) have been able to lessen the effects of bulk magnetic susceptibility and obtain  $^{31}\text{P}$  spectra of intact photosynthetic

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

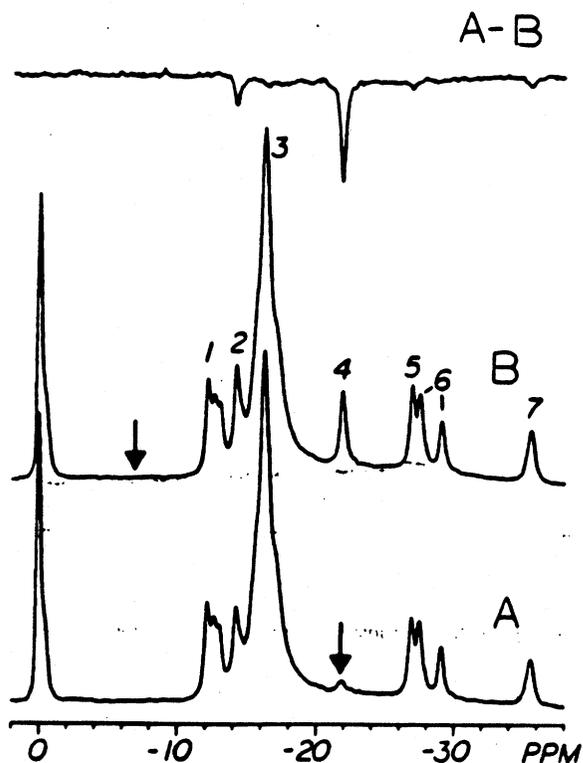


Figure 10. Saturation transfer from  $\gamma$ -ATP to cytoplasmic  $P_i$  in aerobic maize root tips. Saturation transfer spectra obtained with root tips perfused with  $O_2$ -saturated 50 mM Glic + 0.1 mM  $CaSO_4$ , 40 ml/min. Spectrum A obtained with selective presaturation of the  $\gamma$ -ATP resonance; spectrum B obtained with selective irradiation of a point equidistant from the cytoplasmic  $P_i$  line (peak 2) on the low field side of the spectrum (as indicated by arrows). Spectrum A-B is the difference between spectrum A and spectrum B, showing the transfer of saturation from the  $\gamma$ -ATP line to the cytoplasmic  $P_i$  line. Roberts et al. (1984).

wheat-leaf tissue using (1) magic-angle spinning, (2) saturation recovery and (3) infiltrating of water into the tissue air spaces. These spectra also demonstrate the extrusion of protons from the chloroplast stroma and alkalization as reported by the extravascular  $P_i$  signal.

Although the investigation of less complex plant cell cultures offer certain advantages over the relatively heterogeneous tissues, maintaining viability, good S/N and good spectral resolution has been problematic (Lee and Ratcliffe 1983). Rébeillé et al. (1985) have obtained excellent  $^{31}P$  spectra of suspended sycamore cells with simple air bubbling. Their experiments demonstrated when cells are deprived of sucrose, starch hydrolysis cannot maintain the high phosphate esters levels and respiration rates synonymous with high cellular metabolism.

(b) *Plant material studies with other nuclei*

$^{13}\text{C}$  in natural abundance (1.1%) has been used almost exclusively for examining the oil composition of soybeans (Schaefer and Stejskal, 1975) and seeds (Rutar et al., 1977), however recently, Colnago and Seidler (1983) have observed raffinose and stachyose in germinating soybeans. Use of  $^{13}\text{C}$  enrichment has dominated the voluminous numbers of reports describing carbon fluxes in microorganisms (Den Hollander and Shulman 1983; Dickinson et al., 1983; Baxter, 1985). This methodology has not yet attained widespread applications in the area of *in vivo* plant studies although a number of reports on algae and fungi have recently appeared and have been reviewed (Martin, 1985). Stidham et al., 1983 have successfully used the  $^{13}\text{C}$  signal from malate in intact *Kalanchoe tubiflora* leaves (introduced by exposure to  $^{13}\text{CO}_2$ ) to follow the decarboxylation of malate of this CAM plant in the light. The position of the  $^{13}\text{C}$  resonance was also useful for determining the pH of the vacuole during light and dark periods. Also, a substantial level of carbonic anhydrase activity has been detected in the CAM plant using double labeled  $^{13}\text{C}$   $^{18}\text{O}_2$ . Since isolated malate from the CAM plant leaves contained only a small amount of  $^{18}\text{O}$  this experiment demonstrated that the intervention of carbonic anhydrase facilitated the exchange of  $^{18}\text{O}$  with cellular water (Holtum et al., 1984). Ashworth and Mettler (1984) have done metabolic studies in both cultured tobacco and corn cells to establish intracellular glycine and serine pool sizes. These workers utilized 2- $^{13}\text{C}$  glycine and observed signals from 2- $^{13}\text{C}$  glycine, 2- $^{13}\text{C}$  serine and 3- $^{13}\text{C}$  serine and 2,3- $^{13}\text{C}$  serine *in vivo*. Assignments were verified by  $^{13}\text{C}$  homonuclear COSY experiments. The results of these studies suggest that serine formation from glycine is the result of a tight coupling between glycine decarboxylase and serine hydroxymethyl transferase. The metabolism of 1- $^{13}\text{C}$  glucose has also been followed in the elaboration of cell wall polysaccharide biosynthesis in protoplasts from cell suspensions of millet (Heyser and Sillerud, 1984).

Natural abundance  $^{14}\text{N}$  NMR has gotten very little attention in *in vivo* studies because of an extremely rapid relaxation and broad lines due to its quadruple moment. However, if certain symmetrical probe molecules such as  $\text{NH}_4^+$  or  $\text{NO}_3^-$  are used, relatively narrow line spectra are attainable (Richards & Thomas, 1974). With this in mind Belton et al., (1985) examined the intracellular uptake of nitrate and ammonium in maize, barley and pea root tissue. Nitrate was found to accumulate in the vacuole of all these tissues while ammonium-grown barley roots showed a biphasic buildup of the ammonium resonance, consistent with possible intracellular compartmentation.

Despite the need for isotopic enrichment  $^{15}\text{N}$  is generally the nucleus

## AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

of choice for most NMR applications. Its spin of  $\frac{1}{2}$  gives it narrow lines and its dominant dipolar relaxation make it a useful ~~problem~~ for examining microviscosities. Kanamori et al., (1982) capitalized on the dipolar relaxation of intracellular  $^{15}\text{N}$  labelled glutamine, alanine and arginine to establish that the viscosity of the cytoplasm of *N. crassa* is substantially less than its vacuole due to polyanion interactions in the latter.  $^{15}\text{N}$  has also been used as an intracellular pH probe for *N. crassa* mycelia. Both linewidths as well as chemical shifts are diagnostic for the pH environment of  $^{15}\text{N}$  labelled amino acids, histidine, arginine, alanine and proline used in the study (Legerton et al., 1983). The chemical shift pH dependence of  $N_c$  of histidine was found to be most sensitive in the pH range of 5-7, (characteristic of the vacuole) whereas,  $P_i$  whose  $pK$  is closer to 6.8 is relatively insensitive for reporting the relatively acidic vacuole pH.

Movement of metal ions in and out of plant root tissue is an important phenomenon to monitor. Gupta and Gupta (1982) demonstrated that dysprosium tripolyphosphate shift reagents could be used to shift the resonance of external sodium ions relative to the internal sodium ions of intact cells and tissues. Sillerud and Heyser (1984) took advantage of this method to measure the  $^{23}\text{Na}$  efflux kinetics of adapted (adapted for rapid growth at 130 mM NaCl) and non-adapted Proso millet cell suspensions. The non-adapted cells showed little measurable Na efflux after preloading, while the adapted cells demonstrated biphasic efflux kinetics characteristic of vacuole and cytoplasmic emptying. Similarly, excised corn root tips have been investigated by  $^{23}\text{Na}$  NMR for their uptake of sodium ions. At the same time the energy status and intracellular pH associated with the sodium ion influx was concurrently monitored with  $^{31}\text{P}$  NMR (Gerasimowicz, Tu and Pfeffer, 1986). Figures 11(a) and (b) show the concurrently obtained  $^{23}\text{Na}$  and  $^{31}\text{P}$  influx spectra of corn root tips taken under normal aerobic conditions. The rate of sodium ion influx decreased from the normal aerobic state under the following conditions cyanide > hypoxia > FCCP. Although treatment of maize roots tips with cyanide and succinate has been demonstrated by saturation transfer to completely inhibit oxidative phosphorylation (Roberts, Wemmer and Jardetzsky, 1984), sodium influx was only slightly inhibited (Gerasimowicz et al., 1986). These findings suggest that an alternate, non-ATPase dependent pump i.e., perhaps an NADH linked proton pump is responsible for the sustained facilitated sodium influx.

### Solid state NMR

With the innovations of cross polarization, magic angle spinning and high power decoupling, solid state NMR spectra of dilute spin nuclei are

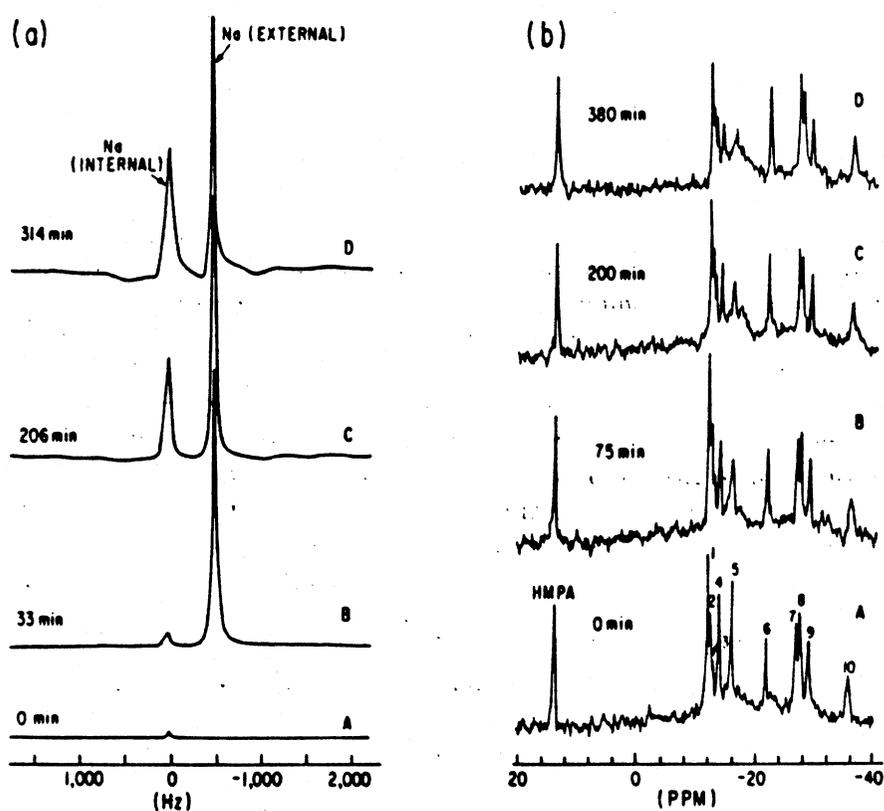


Figure 11a. (left) Sodium Influx into Aerobic Root Tissue. 3.0 mM of  $Dy(PPP)_2^{-7}$  was added along with the appropriate amount of NaCl to keep the concentration of  $Na^+$  at 160 mM. Spectrum (A) represents the endogenous  $Na^+$  concentration. Spectra (B), (C), and (D) represent the distribution of  $Na^+$  between cellular and external spaces at the indicated time after the addition of shift reagent and NaCl. Each spectrum was time averaged for 10.83 minutes. Other experimental conditions used were: frequency range 8 KHz; 16 K data points zero filled to 16 K; 548 transients per spectrum; repetition time 1.124 sec; 28.5  $\mu$ sec ( $90^\circ$  pulse); zero broadening factor. 11b. (right) Effects of  $Na^+$  Stress on Aerobic Roots. The roots were perfused for 2 hours (A). Then, 160 mM NaCl was added to the perfusion medium. (B), (C), and (D) represent the status of roots at the indicated time after the addition of NaCl. The  $^{31}P$  spectra were obtained sequentially on disk, and each spectrum was normalized in terms of S/N so that the relative concentrations of the components could be compared to the initial spectrum of each series. Each spectrum was signal averaged for 27 minutes. Other parameters used were: frequency range 16 KHz; 2 K data points zero filled to 16 K; 10 K transients per spectrum; repetition time 162 msec; 12  $\mu$ sec ( $30^\circ$  pulse); broadening factor 15 Hz. Gerasimowitz et al. (1986).

now attainable with a resolution that can approach that observed in solution studies. A detailed description of this methodology is beyond the scope of this chapter, however those interested in the basic concepts and aspects of quantitation are directed to works by Yannoni, (1982) and Fyfe, (1983). Biological applications are covered in a review by

## AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

Ganish (1984), the carbohydrate and polysaccharide literature has been covered by Pfeffer (1984), Fyfe et al., (1983) and Pfeffer (1985), and proteins by Hatfield and Maciel (in press).

### *(a) Composition and structural studies*

As an analytical tool solid state NMR made some early contributions to studies of the composition of intact seeds. In 1974 Schaefer and Stejskal utilized dipolar-decoupling to generate an oil seed  $^{13}\text{C}$  spectrum showing the oil, starch and protein resonances. Later with the application of the cross polarization technique, the signals from the oil were suppressed to afford a clear  $^{13}\text{C}$  spectrum of the immobile protein and carbohydrate (Schaefer and Stejskal, 1975; Rutar and Blinc, 1980).

A greater refinement in resolution was subsequently attained by the addition of magic angle spinning. This combination of techniques has greatly refined  $^{13}\text{C}$  solid state methodology, now called cross polarization magic angle spinning (CPMAS), to the point where the spectra of seeds can be directly used for the evaluation of protein relative to starch content (O'Donnell, Ackerman and Maciel, 1981; Rutar, 1982). Careful attention to cross polarization parameters can also allow one to examine the liquid-like triglyceride fraction as well as the immobile components in intact soy beans (Haw and Maciel 1983).

The relative signal intensity from mobile liquid-like and immobile components is very sensitive to the cross polarization contact time used in the CPMAS experiment. At short contact times the rigid components are favored for cross polarization while longer contact times cause an attenuation of the signals from rigid components through spin lattice relaxation in the rotating frame and favor the signals from the mobile components. Figures 12(a) and (b) illustrate the phenomenon in which the rigid components in the seed (carbohydrates and proteins) are observed (12a) with a short 100 microsecond contact time while only the lipid components are preferentially observed with a 20 millisecond contact time (Fig. 12(b)).

With careful attention to the parameters used to generate spectra,  $^{13}\text{C}$  CPMAS has become an important analytical technique for studying the structures, composition and degradation of plant tissues *in vitro*. The use of dipolar dephasing, a method by which preferential loss of  $^{13}\text{C}$  signals attributed to protonated carbons, (except for methyl carbons) simplifies the spectra of multi component matrices is illustrated. Figure 13 shows the use of dipolar dephasing which reveals the spectrum of the non-protonated carbons and  $\text{OCH}_3$  groups associated with the minor lignin component in intact wood cellulose (Gerasimowicz, Hicks and Pfeffer, 1985). Note the striking similarity of the dipolar dephased spectrum with

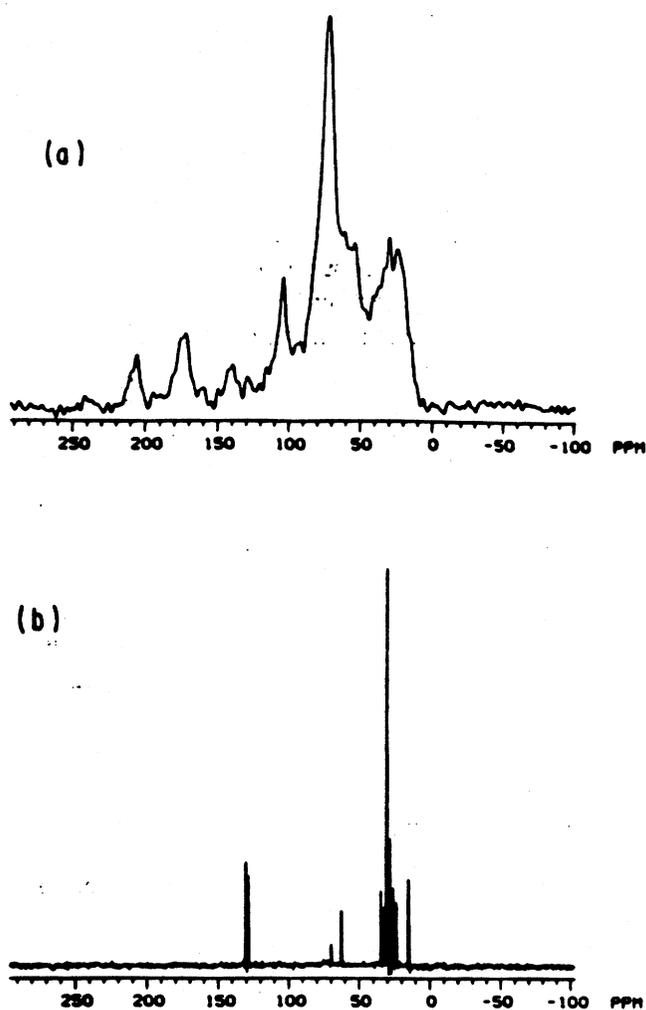


Figure 12(a). A 50.3-MHz  $^{13}\text{C}$  CPMAS spectrum of two intact soybeans: 100- $\mu\text{s}$  contact time, 8000: 2-s repetitions. The Hartmann-Hahn match was carefully adjusted. 12(b). A 50.3-MHz  $^{13}\text{C}$  CPMAS spectrum of two intact soybeans; 20-ms contact time, 2080 3-s repetitions. The Hartmann-Hahn match was carefully adjusted. Haw and Maciel, (1983).

that of the wood cellulose sample, exhaustively treated with cellulase. Relaxation methods were also utilized in this study to evaluate the level of phase homogeneity present in this lignin-carbohydrate matrix. Haw et al., (1984) have thoroughly addressed the question of quantitation for evaluating the levels of lignin in wool pulps which have undergone different types of processing. Compositional changes brought on by microbial modification of lignin or degradation of wood has been qualitatively explored by Schaefer et al., (1981) and Hedges et al., (1985)

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

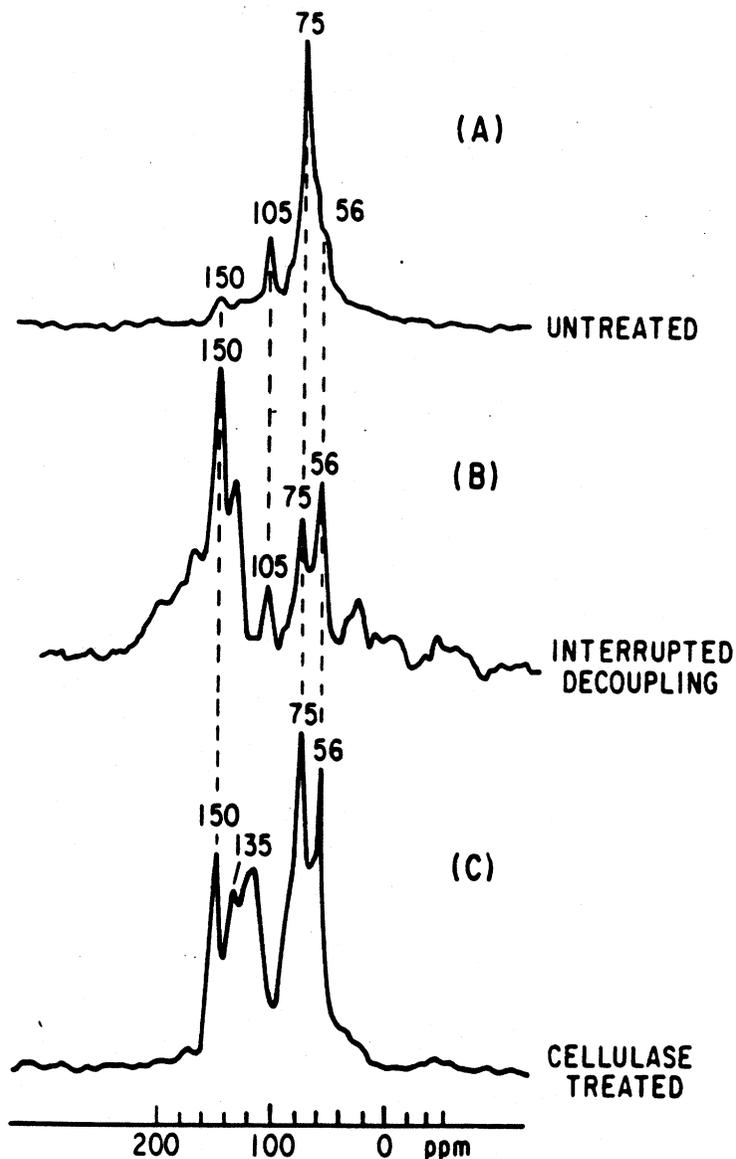


Figure 13. *Piceae glauca* pulp  $^{13}\text{C}$  CPMAS spectra. Spectra A, B and C were obtained with 0.5 msec contact times and decoupling was interrupted in spectrum B for 40  $\mu\text{sec}$ . Gerasimowicz et al., (1984).

respectively. Eflonson et al., (1984) have examined numerous forages by  $^{13}\text{C}$  CPMAS and dipolar dephasing methods to distinguish between signals attributed to protein, lignin and possibly fructose resonances emanating from easily digestible fructoside polymers. A similar study aimed at determining the ratio of carbohydrate, lignin and protein in

grass species has also been reported (Himmelsback, Barton and Windham, 1983).

Schaefer (1985) has recently used  $^{13}\text{P}$  solid state NMR to determine in part the composition of the phosphorous-containing compounds of intact lyophilized alfalfa tissue. In addition, when the tissue was grown in culture under both nonregenerating and regenerating conditions, the  $^{31}\text{P}$  spectra showed that a low average intracellular pH accompanied the latter.

Polysaccharide breakdown or disordering in ripening apple cell wall tissue was monitored as a function of change in molecular dynamics of the polymer matrix (Irwin et al., 1984). Spin lattice relaxation times were used to estimate changes in polymer mobilities as a function of ripening. The polyuranide carbonyl resonances were of particular interest since they showed a 63% drop in their proton  $T_1$  over a period of 21 days. These data correlate well with a decrease in fruit tissue firmness over this period indicative of the pectin matrix breakdown and overall disordering.

Two-dimensional methods are beginning to be exploited in solid state NMR to obtain more detailed information on protein structure by monitoring through space dipolar interactions between dilute spins (Cross, Frey and Opella, 1983). Two-dimensional spin-exchange experiments conducted on uniformly labeled and selectively  $^{15}\text{N}$  labelled coat protein (containing 50 amino acids) showed several cross peaks or sites of interaction consistent with the proposed  $\alpha$ -helical structure.

Perhaps the most complex and intractable agricultural material to be examined by solid state NMR is soil, amended soils and soil components. Of particular importance in these studies is the composition and disposition of the organic matter as the soil undergoes different degradative processes. Wilson et al., (1983) have characterized the degradation of the organic matter in whole soils with  $^{13}\text{C}$  CPMAS methods. With the aid of dipolar dephasing techniques, estimates of non-protonated carbons and the degree of condensation of the aromatic rings or alkyl ring substitution and demethylation were made for different periods of humification. Figure 14 shows the emphasis of different parts of the spectrum of aged pine leaves with and without dipolar dephasing. As with studies of wood (Gerasimowicz et al., 1985), the non-protonated resonances associated with the carbonyl and condensed and substituted aromatic carbons dominate the dipolar dephased spectrum 14b. As time proceeds preferential microbial degradation of the carbohydrate components enrich this material in lignin. A comprehensive review of the CPMAS  $^{13}\text{C}$  spectra, composition and diagnosis of humic substances, fulvic acids and humins derived from different soil samples is given by

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

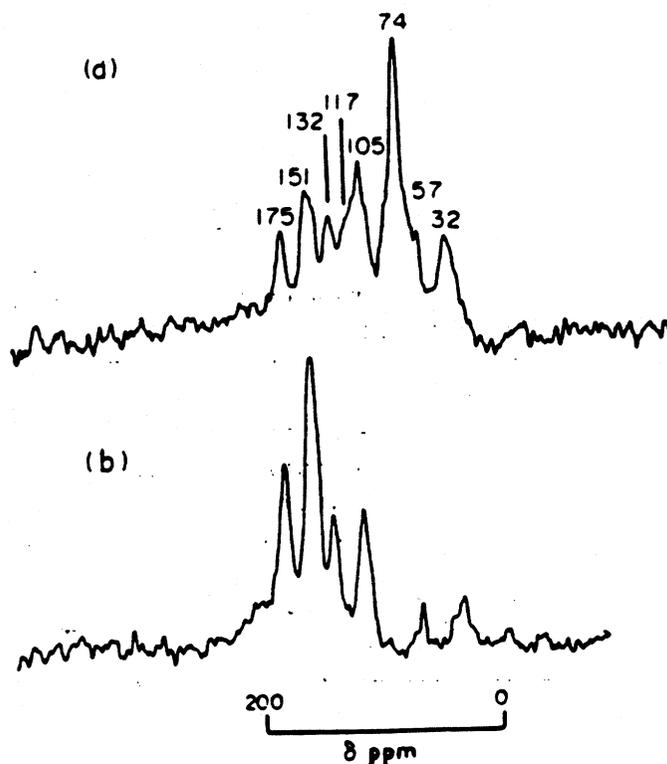


Figure 14. Dipolar dephased spectra of aged pine leaves. (a)  $T_d = 1 \mu\text{sec}$ ; (b) summation of 15 spectra with  $T_d$  incremented by  $5 \mu\text{sec}$  from 40–190  $\mu\text{sec}$ . Wilson et al., (1983).

Hatcher et al., (1983). The accompanying table summarizes the  $^{13}\text{C}$  resonance absorption positions for the different functional carbons found in spectra of soils (Hatcher et al., 1983). Mixtures of representative components present in amended soils and composts were used to simulate the spectra of these complex matrices (Piotrowski, Valentine and Pfeffer, 1984). Additionally, a careful examination of the relaxation parameters associated with the relative magnetization responses of these various components uncovered inherent spectral distortions in the presence of paramagnetic  $\text{Fe}^{3+}$  (Pfeffer, Gerasimowicz and Piotrowski, 1984). Figure 15 illustrates the loss of signal intensity of the carbon resonances representing the hydrophilic cellulosic components. This phenomenon results from a significant shortening of  $T_{\rho\rho}$  (spin lattice  $T_{\rho\rho}$  relaxative time in the rotating frame) due to preferential  $\text{Fe}^{3+}$  interactions. Clearly, to obtain reliable spectra one must be fully aware of the presence of paramagnetic metals in such complex materials. Also the complete relaxation profile of the components should be evaluated before a quantitative assessment of these spectra is undertaken. Later

Table 1. <sup>13</sup>C-NMR chemical-shift regions for various types of carbons typically present in humic substances and soils\*

Region I (0 to 50 ppm)	Region II (50 to 110 ppm)	Region III (110 to 150 ppm)	Region IV (160 to 190 ppm)	Region V (190 to 220 ppm)
paraffinic $\text{C}-\text{C}-\text{C}^*-\text{C}$	alcohols $\text{C}-\text{OH}$	olefinic-C	carboxyl $\text{C}-\text{OH}$	aldehyde $\text{C}-\text{H}$
$\text{C}-\text{C}^*-\text{C}$	amines ( $\text{C}-\text{NH}_2$ , $\text{C}-\text{NHR}$ , $\text{C}-\text{NR}_2$ )	aromatic-C	ester $\text{C}-\text{OR}$	ketone $\text{C}-\text{O}$
$\text{C}-\text{C}^*-\text{C}$	carbohydrates		amide $\text{C}-\text{N}$	
$\text{C}-\text{C}^*-\text{C}$	ethers ( $\text{C}-\text{O}-\text{C}$ )			
$\text{C}-\text{CH}_3$	methoxyl ( $\text{OCH}_3$ )			
	acetals $\text{C}-\text{O}$			

\* Hatcher et al., (1983).

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

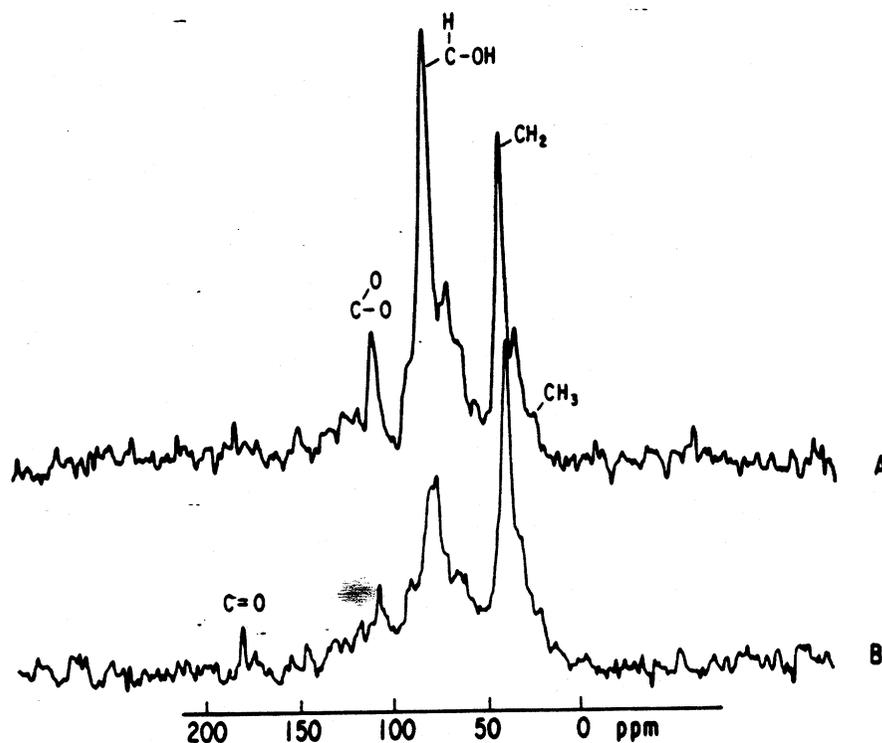


Figure 15. Comparison of  $^{13}\text{C}$  CPMAS NMR spectra of model sludge mixtures (a) with no  $\text{Fe}^{3+}$  and (b) containing 1.9%  $\text{Fe}^{3+}$ . Each spectrum was obtained under the optimized conditions, 14 000 scans, 16-s pulse delays, a 0.5-ms contact time, and 20-Hz line broadening. Pfeffer et al., (1984).

reports of the spectra of organic soil samples which contain large natural amounts of copper (Copper mucks) verified the preferential paramagnetic effects on quantification resulting from the loss in intensity of the cellulosic resonances (Preston et al., 1984). Complementary  $^{13}\text{C}$  solid state NMR and FTIR spectroscopic studies of humic acids derived from different stages of sludge treatment and composting gave more refined information on the nature of the carbonyl components (amides and carbonyl groups) than could CPMAS alone (Gerasimowicz and Byler, 1985).

*(b) Metabolic studies*

Over the past five years Schaefer and co-workers have effectively used  $^{13}\text{C}$  and  $^{15}\text{N}$  labeling techniques in solid state CPMAS NMR to complement high resolution  $^{13}\text{C}$  and  $^{15}\text{N}$  NMR which measures the flux of stable isotopes through soluble pools of cellular systems *in vivo*.

Jacob et al., (1980) demonstrated the efficacy of  $^{15}\text{N}$  CPMAS methodology for examining  $^{15}\text{N}$  incorporation into the amino acids resulting

via nitrogen reductase activity on  $^{15}\text{N}$  nitrate in lyophilized mycelia from *Neurospora Crassa*. Other applications of this method led to the investigation of the amount of amide and  $\alpha$ -amino nitrogens produced in the pod and seeds of a soybean plant at various stages of development (Schaefer et al., 1979). Soybean cotyledons grown on asparagine showed preferential incorporation of amino nitrogen of asparagine into storage proteins over amide nitrogen (Skokut et al., 1982).

Subsequent development of an elegant technique called double cross polarization  $^{13}\text{C}$  and  $^{15}\text{N}$  NMR has expanded the scope of metabolic studies in whole plant tissue. In these experiments, the magnetization is transferred first from  $^1\text{H}$  to  $^{13}\text{C}$  or  $^{15}\text{N}$  and subsequently from  $^{13}\text{C}$  to  $^{15}\text{N}$  or  $^{15}\text{N}$  to  $^{13}\text{C}$ . The double cross polarization mechanism, in fact, labels all  $^{13}\text{C}$  signals or all  $^{15}\text{N}$  signals coming from a  $^{13}\text{C}$ - $^{15}\text{N}$  bond. By subtracting the signals from  $^{13}\text{C}$  or  $^{15}\text{N}$  derived through non-mutual cross polarization from the mutually cross polarized signal a resonance area is obtained which gives a direct quantitative measure of the uptake and metabolism of intact double labels (Schaefer et al., 1984). An example of this method is given in Fig. 16. Here we see the single  $^1\text{H} \rightarrow ^{15}\text{N}$  cross polarization  $^{15}\text{N}$  spectra given for plants grown on  $^{15}\text{N}$  enriched fertilizer with and without  $^{13}\text{CO}_2$ . The difference spectra resulting from the double cross polarization spectra minus the single cross polarization experiment show almost complete cancelling of the resonance line in the single  $^{15}\text{N}$  labelled plant due to the almost complete absence of  $^{13}\text{C}$ - $^{15}\text{N}$  species in the protein, whereas a significant amount of double labelled species is observed in the others grown with  $^{15}\text{N}$  and  $^{13}\text{CO}_2$ . Exploitation of this methodology was demonstrated in the evaluation of protein turnover in soybean leaves (Schaefer et al., 1981). These experiments involved exposing soybean plants massively labeled with  $^{15}\text{N}$  (from fertilizer) to a pulse of  $^{13}\text{CO}_2$ . The isolated and lyophilized leaf tissue was then subjected to the double cross  $^{13}\text{C}$ - $^{15}\text{N}$  NMR experiment as a function of time. The findings indicate that the proteins in the mature leaf, as measured by incorporation of double label are very stable before senescence, and have a turnover constant of about 30 days. The double label method was also utilized to assess the fate of (4- $^{13}\text{C}$ , amide- $^{15}\text{N}$ ) asparagine in developing cotyledons (Schaefer et al., 1981a). Here 50% of all the asparagine residues were incorporated intact within the soybean protein. In contrast no intact double label was observed from the metabolism of  $^{13}\text{C}$ - $^{15}\text{N}$  labeled allantoin in soybean cotyledons (Coker and Schaefer, 1985). These data are consistent with allantoin's degradation to two molecules of urea and one two-carbon fragment.

In general all of the solid state methods, while more technically de-

## AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

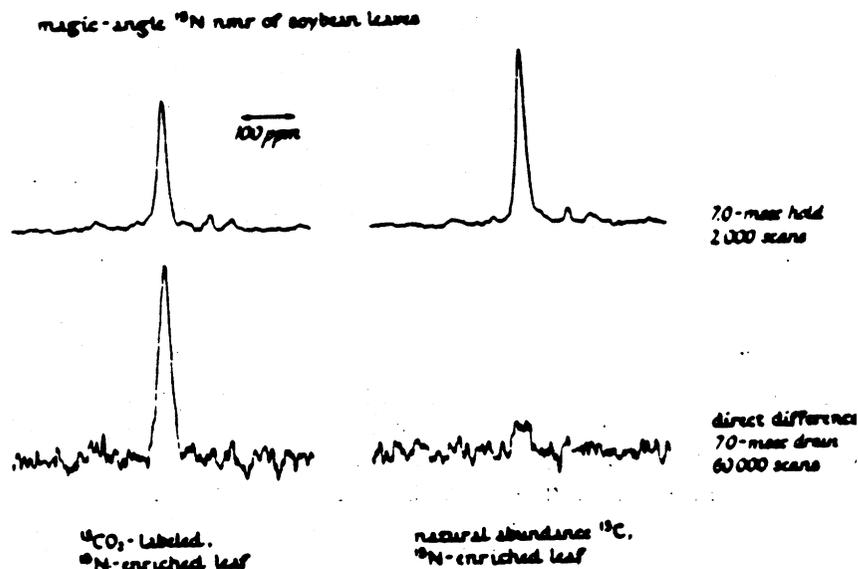


Figure 16. Magic angle cross-polarization  $^{15}\text{N}$  NMR spectra of  $^{15}\text{N}$ -enriched lyophilized soybean leaves exposed to  $^{13}\text{CO}_2$  for 7 days (left) and exposed only to normal  $\text{CO}_2$  (right) during active photosynthesis. The spectra at the top of the figure were obtained using the pulse sequences with a nitrogen spin lock of 7 ms and the carbon rf field off-resonance by 60 kHz. The spectra at the bottom of the figure are the result of double cross-polarization direct difference experiments in which the carbon rf field is first off-resonance and then on-resonance. This procedure was repeated 30 000 times. The positive signal which accumulates is a measure of the concentration of  $^{15}\text{N}$ - $^{13}\text{C}$  pairs in the labeled leaf protein. Schaefer et al., (1981).

manding represent an excellent complement to the high resolution *in vivo* techniques for tissue studies as well as the fractionation-dependent *in vitro* experiments necessary for examining solubilized polymeric matrices.

### Magnetic resonance imaging

In contrast to conventional NMR spectroscopy, magnetic resonance imaging (MRI) is concerned with applications to heterogeneous specimens, for example parts of the human body, whole plants, etc., which are not small, and which are placed deliberately in a nonuniform magnetic field. The purpose of the nonuniform field, as described by Dr. Lauterbur in his chapter is to label different parts of the specimen with different field strengths so that they respond with recognizably different NMR frequencies, enabling the structure and internal processes of the specimen to be derived and displayed. Moreover, besides giving morphological information MRI gives additional diagnostic insights

through relaxation parameters, which are not available from other imaging methods. Excellent reviews of the imaging techniques and applications have been given by Bottomly (1982) and Andrew (1983).

Since hydrogen is the most abundant element in all living organisms, proton NMR is used as the principle nuclide for all images. Applications in agricultural area such as studies of plants and animal embryology are just beginning. The first images of an intact plant, (bean *Vicia faba*), from root to top in soil, with a spectral resolution of  $0.56 \text{ mm} \times 0.56 \text{ mm}$  was recently described by Bottomley, Rogers and Foster (1986). Their images were able to detect water transport in roots with light stressed foliage using water doped with a paramagnetic NMR contrast agent. In addition the basic root structure and pathology as evidenced by partial decay of hypogeal cotyledons were also indicated. Figure 17 shows the  $^1\text{H}$  images of the *faba* root system in which the effects of light stress are evident. Cure (in press) has examined the same plant system but with much greater spatial resolution ( $0.15 \text{ mm} \times 0.15 \text{ mm}$ ). These improved images allow for discrimination between tissues of the main root and visualization of the vascular connection of branch roots to the central stele. Application of proton imaging with simultaneous spatially resolved high resolution NMR has been carried out during the embryonic development of the desert locust (Gassner and Lohman, in press). These images taken over a period of 8 days show the dynamics of pattern formation, compartmentation, and biochemical changes within and between compartments within the embryo. Submicroliter cube resolution allowed for the assessment of distribution changes in water and lipid during development. Figure 18 shows the changes in lipid water concentrations in two distinct sections of the embryo in addition to the overall image. Other reports of spatially resolved high resolution NMR for examining the *in vivo* chemistry of living organisms by  $^{31}\text{P}$  NMR have been reported by Belton et al. (1982) for embryonic development of hen's eggs and by Storey et al. (1984) for gall fly larvae. A full review of topical magnetic resonance is given by Gordon, Hanley and Shaw (1982).

### Perspectives

While high speed computers have made 2-D NMR a more or less routine technique for establishing the shift assignments and structure of small to medium sized organic molecules, an investment of as much as 1-2 years time is often required to analyze the complex data derived from the spectra of relatively small protein molecules. Future developments in the area of computer assisted data analysis and molecular modeling promise to shorten this time appreciably.

AGRICULTURAL/BIOLOGICAL APPLICATIONS OF NMR

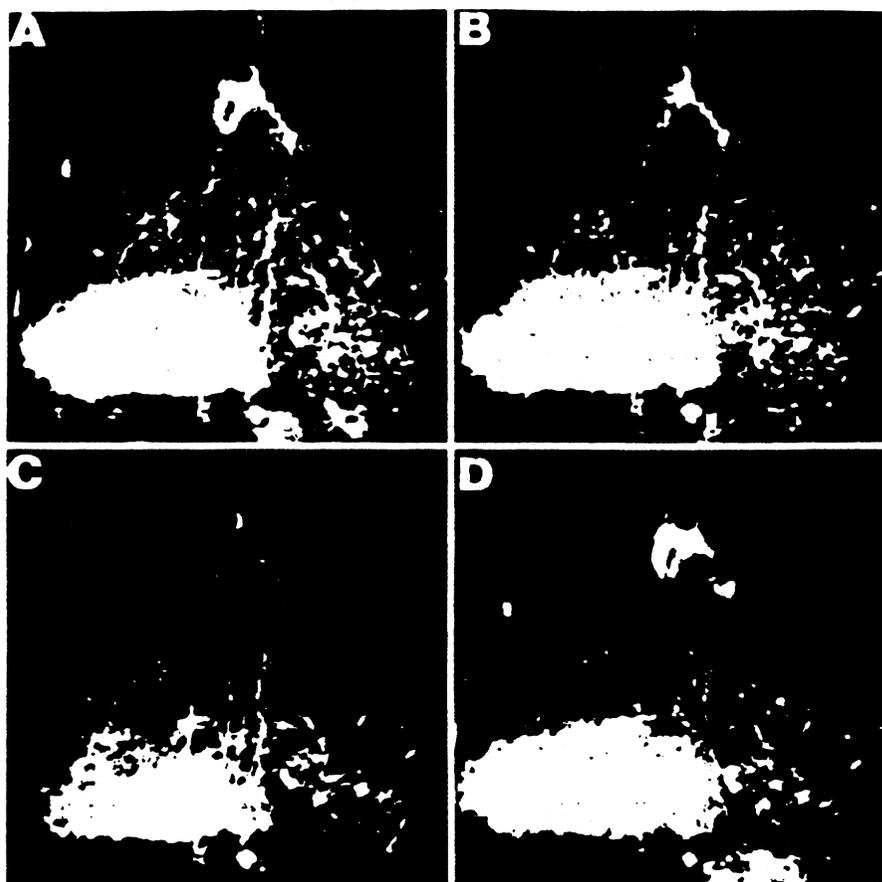


Figure 17. (a) Image of the *V. faba* root/soil system, with a 20-ml bolus of water injected into the soil at the base. (B) The root system image after 87 min of exposure to light stress shows water leaving the cotyledonary area. (c) After 200 min of light exposure, water has almost cleared from this area. (d) The image after 275 min, light having been fully removed at 200 min, shows recovery (return of water) in the cotyledonary area. All images were obtained in 3.4 min, with a 0.2-s pulse repetition period. (Scale = 1:2.7). Bottomly et al., (1986).

*In vivo* work on plant materials is advancing at a rapid pace and intact tissue studies ( $^{31}\text{P}$ ,  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ) of whole unexcised plant sections will be expanding as large bore magnets become more accessible. This approach to spatially resolved or topical spectroscopy will be conveniently coupled with imaging methodology in a single commercial instrument. Presently, a few of these prototypes have been utilized with relatively good success. Images based on  $^{23}\text{Na}$ ,  $^{39}\text{K}$  and  $^{31}\text{P}$  nuclei offer promise as an alternate to the established  $^1\text{H}$  images because their differences in relaxation behavior and diverse distribution yield a perspective on cellular structure.

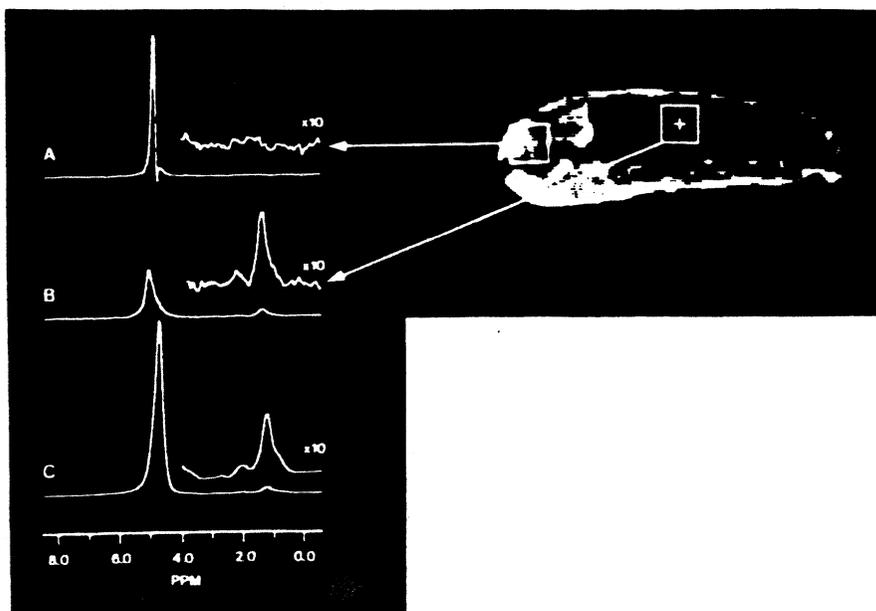


Figure 18. Image selected *in vivo* spectroscopy (ISIS) spectra from the image of locust shown, indicate that the submicroliter volume dorsal to the stomatodeum (a) at 83% development is essentially void of lipid while the haemocoel (b) contains lipid. Note the overall spectrum (c). Gassner and Lohman, (1986).

Two-dimensional methods are expanding into *in vivo* and solid state spectroscopy. Its principal application will be in the area of determining metabolic exchange processes, (unidirectional flux between metabolites), in living tissue as well as molecular diffusion rates in solid materials. Greatly enhanced resolution in solid state spectra of heterogeneous materials is also envisioned through the development of a new technique called zero field NMR.

All of the above innovations promise to expand the capabilities of NMR and its impact on all biologically oriented disciplines including agriculture.

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