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Analytical Methods Section

NMR studies of non-freezing water in cellular plant tissue

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The distribution of water proton transverse relaxation times is used to monitor the amount and subcellular distribution of non-freezing water and ice in potato tissue. At subzero temperatures most of the non-freezing water is found to be associated with starch granules and cell wall material, and the amount of unfrozen water at each temperature has been determined. The potential of NMR relaxation techniques in food cryopreservation is discussed.

INTRODUCTION

The formation and distribution of ice crystals in cellular tissue during freezing is important in food preservation since it largely determines whether the cells and organelles retain turgor pressure and impart a 'fresh' texture on thawing (Morris, 1981; Grout & Morris, 1987; Bald, 1990). Cryomicroscopy is the most direct way of observing the distribution of ice crystals in cellular tissue but this technique usually requires thin tissue sections unless confocal microscopy is available (Morris, 1981) and it is qualitative, in the sense that volume fractions of ice and non-freezing water are not readily deduced from the micrographs. NMR has also been used extensively to measure the amount of unfrozen water in frozen materials from the reduction in the amplitude of the water proton free induction decay (FID) (Blanshard & Derbyshire, 1975; Derbyshire, 1982; Belton, 1989).

Unfortunately measurements of FID amplitudes alone give no information on the spatial distribution of the unfrozen water. In this paper we show how NMR water proton relaxometry has the potential of being able to monitor not just the amount of unfrozen water but also its spatial distribution in intact cellular tissue in a non-destructive and quantitative manner. The method is based on the observation that water in different subcellular compartments is often associated with different, and characteristic water proton transverse relaxation times (Hills *et al.*, 1990; Hills & Duce, 1992) determined mainly (but not exclusively) by fast proton exchange between water and exchangeable protons on biopolymers and dissolved metabolites found in the compartment (Hills *et al.*, 1990; Duce *et al.*, 1992). The distribution of water proton transverse relaxation times for a tissue therefore reflects the amount of water in each compartment as well as the extent to which water can diffuse between the compartments. In contrast to water, the proton transverse relaxation time of ice is only a few microseconds so that it is 'invisible' on the timescale of milliseconds appropriate to water proton relaxation in cellular tissue. This means that ice formation in a particular subcellular compartment is associated with a dramatic loss of signal from that compartment, in direct proportion to the amount of ice formed.

In principle, therefore, it should be possible to continuously monitor the distribution of non-freezing water and ice crystal formation in (sub-)cellular compartments in food tissue. To explore this possibility we choose potato tissue since this has a fairly uniform distribution of cell sizes and there are a variety of subcellular organelles easily visible in light micrographs such as membrane-bound starch granules, non-starch vacuoles, nuclei, cytoplasm and cell walls (Fig. 1).

MATERIALS AND METHODS

NMR measurements were carried out on a Brucker MSL100 spectrometer operating a 100 MHz, using a thermostated high-power probe with a 90° pulse length of 2 μs. Transverse relaxation times were measured using a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with a 90–180° pulse spacing fixed at 200 μs and a long recycle delay of 10 s to avoid saturation. Data were averaged over eight acquisitions before analysis as a continuous distribution of exponentials with the CONTIN program (Provencher, 1982). Purified potato starch granules were purchased from BDH chemical suppliers. They were used as a randomly packed water-saturated bed which was allowed to equilibrate for at least 24 h before measurement.

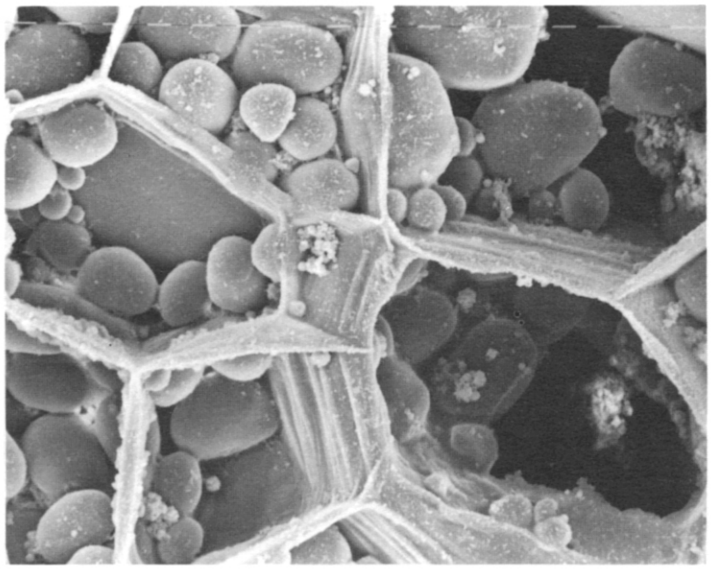

Fig. 1. An optical micrograph of a potato cell showing the presence of starch granules and cell walls. (Courtesy of Dr M. Parker.)

A potato cell wall extract was made by crushing a whole potato and washing the solid residue several times with water and ethanol to remove protein and lipid. After drying, the cell wall extract was rehydrated to 80% (w/w) with deionized, distilled water. Higher hydration levels gave multiple exponential water proton relaxation indicating the presence of excess bulk water.

Potato fluid, consisting mainly of cytoplasm and vacuolar fluid was prepared by crushing the potatoes, followed by filtering to remove cell wall fragments and starch granules. It was stored at 278 K to slow enzymic degradation prior to use.

RESULTS

Potato starch granule suspensions

To illustrate the principles of the NMR method we first analysed simple systems consisting of isolated fractions of potato tissue beginning with a randomly packed bed of water-saturated potato starch granules. These membrane-bound granules contain approximately 60% by weight of dry starch (DeWilligen, 1976) and vary in diameter between 5 and >100 μm. To avoid starch gelatinization the temperature of the granules was kept at or below 298K.

Figure 2 shows the relaxation time distributions acquired by first freezing the sample in the NMR probe at 238K and then raising the temperature in steps up to 298K, allowing at least 20 min at each temperature for sample equilibration before commencing NMR measurements. Evidently the relaxation peak at *c.* 20 ms corresponds to water outside the starch granules since it only appears above 273K. The remaining peak between 1 and 5 ms can therefore be assigned to water inside the granules. Non-exchanging starch CH protons would not be expected to contribute a measurable signal because their transverse relaxation times are only a few tens of microseconds in the rigid lattice conditions prevailing inside the partly frozen granules. The observation that the relaxation time inside the granules is the same (*c.* 5 ms) at both 271K, when there is ice outside the granules, and at 274K when the ice has melted, indicates that at these low temperatures the diffusion of water between the inside and outside of the granules is slow. The small relaxation peak that is seen at *c.* 4 ms below 248K most probably arises from mobile lipid in the starch granule membranes and/or from low molecular weight metabolites, which would only appear with significant signal intensity after the bulk of the water signal has been removed by freezing.

It is noticeable that the relaxation time of the non-freezing water in the granules becomes progressively shorter at lower temperatures down to 243K. This behaviour is expected if the amount of ice inside the granules in thermodynamic equilibrium with non-freezing water associated with the starch increases with decreasing temperature, much like solutions at low temperature. The fraction of non-freezing water at any temperature (F) can be measured from the reduction in the amplitude of the water proton free induction decay (FID) which is plotted, relative to the room temperature value, in Fig. 3. The marked increase observed on warming to 273K corresponds to the melting of the extragranular ice and this shows that *c.* 52% of water is extragranular in the packed bed, which agrees with the relative areas of the two peaks observed at 298K (Fig. 2(a)).

The F values below 273K show the relative amounts of the ice and non-freezing water inside the granules at each subzero temperature. This could be measured more accurately, if desired, by normalizing the receiver sensitivity to its optimum value at 272K when the signal from the extragranular water has been removed, rather than at 298K. We now apply the same protocol to potato cell wall extracts.

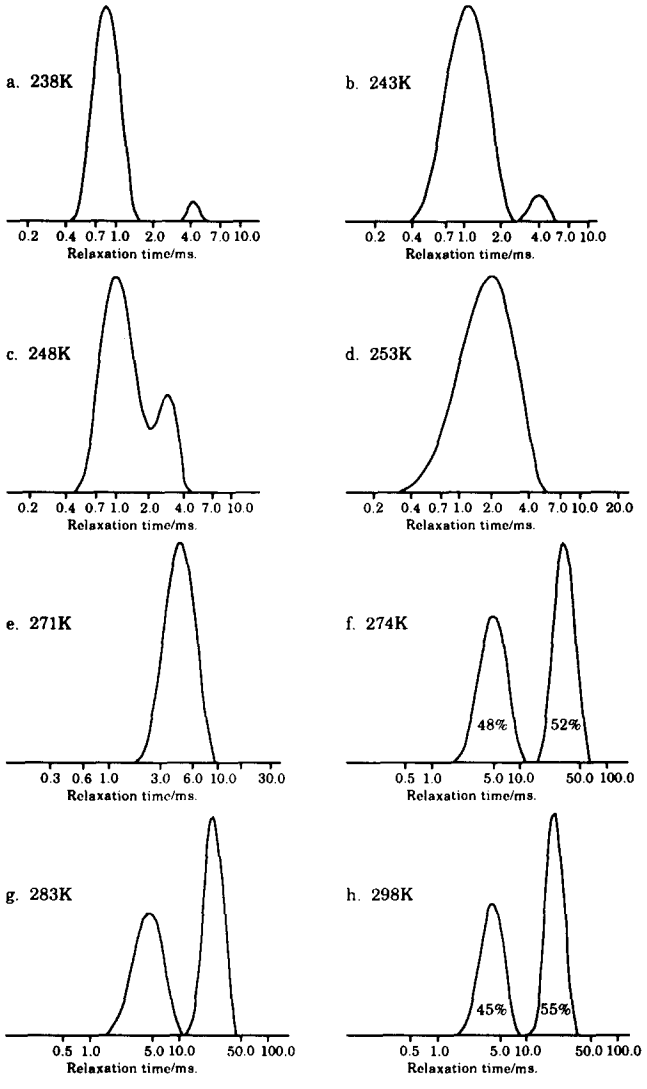

Fig. 2. Water proton transverse relaxation time distributions of a frozen bed of potato starch granules as it is warmed from 238K. Note the change in relaxation time scales.

Potato cell wall extract

Figure 4 shows the temperature dependence of the water proton transverse relaxation distributions for potato cell wall material rehydrated to 80% by weight with water as it is thawed from 233K. It can be seen that the relaxation is always unimodal and that the peak maximum shifts progressively between *c.* 1 ms at 233K to just over 10 ms at 298K. These relaxation times correspond closely with those of water inside the starch granules so we do not expect to always be able to resolve the relaxation peaks for starch granules and cell wall material in intact potato tissue. Figure 3 includes the temperature dependence of the water proton fractions deduced from the FID amplitudes for the cell wall material.

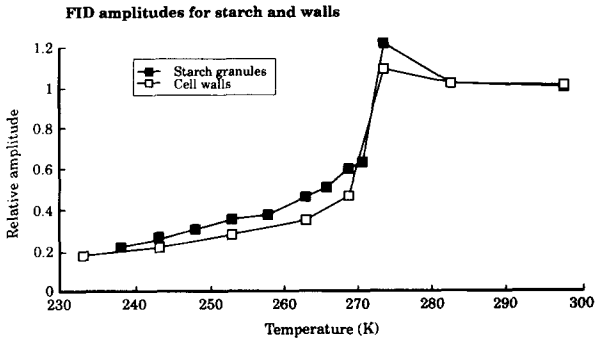

Fig. 3. The relative amplitudes of the free induction decays for a packed bed of potato starch granules and for a potato cell wall extract rehydrated to 80% (w/w) as the frozen material is warmed from 233K.

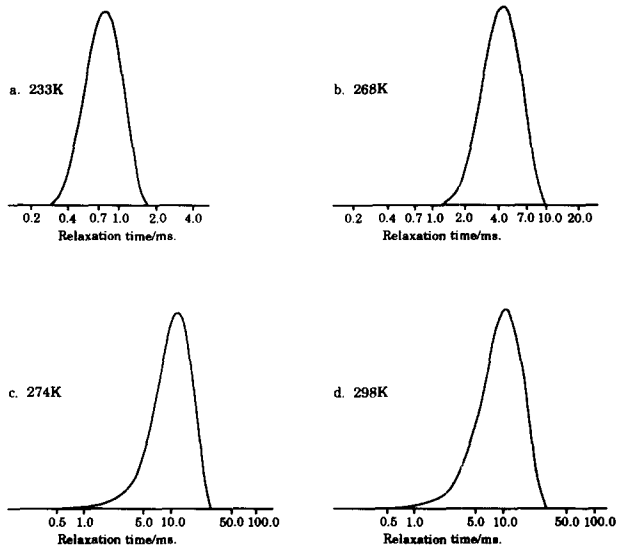

Fig. 4. Water proton transverse relaxation time distributions for a potato cell wall extract rehydrated to 80% (w/w) as the frozen material is warmed from 233K.

Filtered potato fluid

Figure 5 shows the temperature dependence of transverse relaxation time of filtered potato fluid as it is cooled from 298K. Between 298K and 273K the relaxation time falls from 500 ms to 300 ms. By 265K the sample has frozen causing a dramatic drop in FID amplitude (Fig. 5(e)) and the appearance of a non-freezing water peak centred at *c.* 1–2 ms. This non-freezing water is presumably located as a surface layer a few molecules thick around dissolved macromolecules (Kuntz & Kauzmann, 1974).

Thawing frozen potato tissue

Having examined the relaxation behaviour of the major individual components of potato tissue we are now in a position to understand the freeze–thawing behaviour of whole potato tissue. Figure 6 shows the temperature dependence of the distribution of water proton transverse relaxation times for frozen potato tissue as the temperature is raised in steps from 258K to 298K. Assigning these various relaxation peaks to the particular subcellular compartments is not straightforward because, as seen, several contributions may overlap, and diffusive exchange of water between the subcellular compartments may, to some unknown extent, average the relaxation so that a simple one-to-one correspondence between the T_2 peaks with individual subcellular

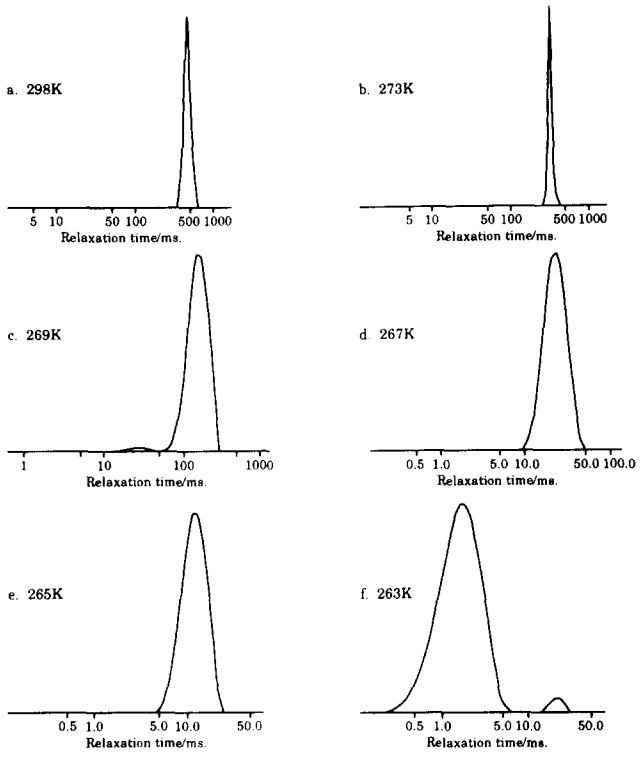

Fig. 5. Water proton transverse relaxation time distributions for filtered potato fluid as it is cooled from 298K.

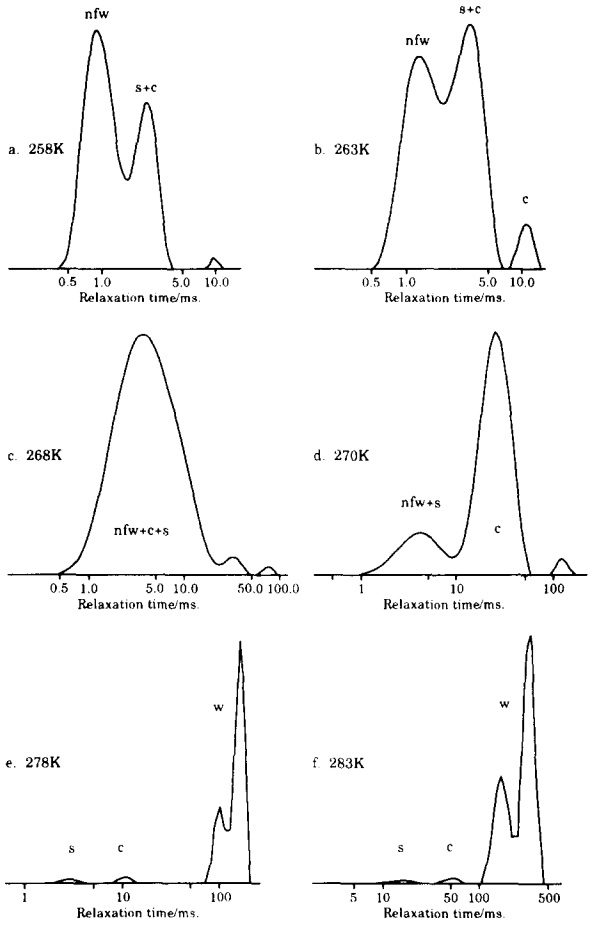

Fig. 6. Water proton transverse relaxation time distributions for whole potato tissue as the frozen material is warmed from 258K. Tentative peak assignments are shown. s, starch granules; c, cell walls; nfw, non-freezing water found as a layer a few molecules thick at macromolecular interfaces; w, water in the cytoplasm, non-starch vacuoles.

compartments may not exist. Ignoring these complications, a tentative interpretation of the results in Fig. 6 can be made. At 258K (Fig. 6(a)) the peak at *c.* 1 ms most probably corresponds to non-freezing water associated with macromolecules in the frozen cytoplasm, non-starch vacuoles and extracellular water (cf. Fig. 5(f)). Comparison with Fig. 2(d) and 4(b) suggests that the peak at *c.* 3–4 ms is associated with water inside partly frozen starch granules and cell wall material. As the temperature is raised these peaks shift to longer relaxation times (cf. Figs 2, 4 and 5) and by 272K the water in the starch granules and cell walls has probably all melted as Figs 2(e) and 4(c) and the steady increase in FID amplitudes suggests.

At 273K the ice in the cytoplasm, non-starch vacuoles and extracellular regions melts, creating large peaks around 100 ms which dwarf the relaxation peaks of the starch granules at *c.* 5 ms and of the cell walls around 10 ms (see Fig. 6(e)). As the temperature is raised to 298K all four peaks shift to longer relaxation times as the molecular correlation times shorten and diffusive exchange of water between the compartments becomes faster.

The relative areas of the peaks around 100 ms (arising from cytoplasm, non-starch vacuoles, etc.) to those at 5 ms (starch granules) and 10 ms (cell wall water) is 93%:7% which is very roughly the ratio of the amount of water in these compartments (84%:16% respectively) calculated from literature estimates that potato tissue consists of *c.* 80% by weight water, 16% by weight dry starch and *c.* 2% by weight lipid (DeWilligen, 1976; Cone & Walters, 1990), with the starch granules containing *c.* 60% by weight of dry starch. The changes in amplitude of the FIDs for the whole potato tissue during a freeze-thaw cycle (Fig. 7) support these assignments and show that the amount of non-freezing water at 263K is *c.* 10% of the total water in the tissue. An hysteresis loop is seen in Fig. 7 because the cooling curve is kinetically controlled, ice nucleating about 5°C below the observed melting point.

Freezing potato tissue

Figure 8 shows the changes in the relaxation time distributions on slowly cooling whole potato tissue in the NMR probe. It is apparent that undercooling occurs until *c.* 266K when the extracellular water, cytoplasm and non-starch vacuoles suddenly freeze by heterogeneous nucleation and create a new peak at *c.* 1 ms from non-freezing water associated with macromolecules in the frozen regions. The starch granules and cell walls are, however, still only partly frozen at 266K, as evidenced by the relaxation peaks at *c.* 4 ms and 10 ms respectively (Fig. 8(c)). Further cooling to 248K freezes the cell wall and starch granules leaving only a small residual amount of non-freezing water characterized by a relaxation time of *c.* 2 ms (cf. Figs 2(c) and 4(c)). The corresponding FID amplitudes are plotted in Fig. 7.

It is interesting to observe that the relaxation time

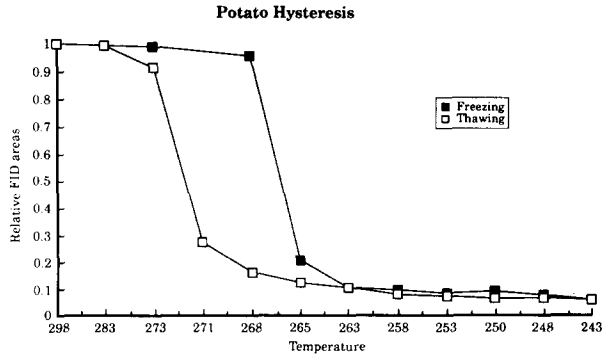

Fig. 7. Hysteresis in the freeze–thawing behaviour of whole potato tissue, monitored with the relative amplitudes of the water proton FID.

distributions for the undercooled tissue at 263K (no ice) and 283K show only minor differences even though the self-diffusion coefficient of water at 263K is estimated to be roughly half its value at 298K. This suggests that diffusive averaging of the intrinsic compartment magnetization is not a major effect.

Other assignment techniques

These exploratory low-temperature investigations have been restricted to the simplest protocol of direct measurements of the FID amplitudes and the transverse relaxation time distributions. There are, however, a wide range of other potentially useful NMR techniques that can be used to assign the relaxation time peaks, and determine the possible significance of diffusive averaging between subcellular compartments. In this section allusion is made to these techniques only briefly since, as far as we are aware none has yet been applied to cellular tissue at subzero temperatures. The techniques can be broadly classified as those which manipulate only the NMR pulse sequence and those which manipulate the sample.

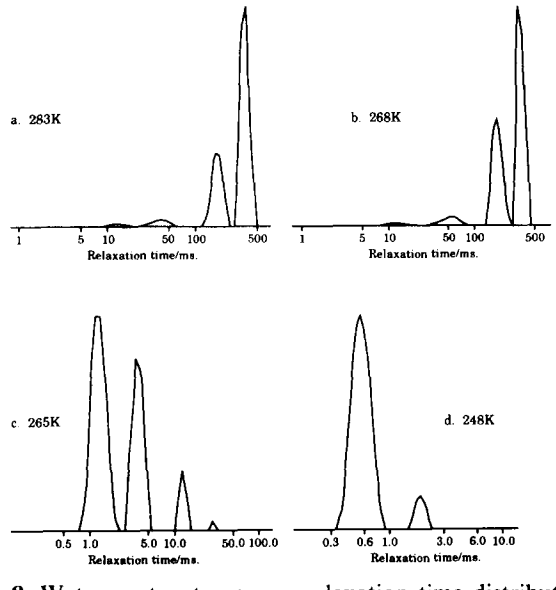

Fig. 8. Water proton transverse relaxation time distributions for whole potato tissue as it is cooled from 298K. Note the change of relaxation time scales.

Examples of the first category include selective excitation and/or the application of pulsed field gradients. Whenever there is a significant difference in transverse relaxation times, as in unfrozen potato tissue, it is possible to selectively remove water proton relaxation from the short relaxation time peaks while leaving it in the long relaxation time peaks. Subsequent sampling of the relaxation after increasing delay times then permits the transport of water magnetization from the long relaxation time compartment into the shorter relaxation time compartment. This method has been successfully used to measure pore connectivities in granular beds (Hills *et al.*, 1993) but has not yet been applied to cellular tissue. The application of pulsed field gradients causes the water proton signal to decay not just by transverse relaxation but also by self-diffusion. This method finds its most elegant expression in 'q-space microscopy' (Callaghan *et al.*, 1991) which has recently been used to deduce the tonoplast water permeability in apple tissue at room temperature (Hills & Snaar, 1992). So far this technique is in its early developmental stages and has not yet been applied to cellular tissue at subzero temperatures.

In addition to manipulating the NMR pulse sequence it is also possible to assign relaxation time peaks by manipulating the sample itself. For example, immersing the sample in a solution of manganese ions should cause diffusion of the ions, first into the extracellular region associated with the cell walls, then, more slowly into the cytoplasm and then the vacuoles and starch granules. Since manganese ions act as potent relaxation sinks for water proton magnetization this should lead to progressive loss of signal and a dramatic shortening of the relaxation time from each of these compartments as they are entered by manganese (Snaar & Van As, 1992). Instead of a manganese solution, the sample could also be immersed in D_2O and the progressive loss of signal from each compartment when D_2O replaces H_2O can be monitored. At the same time proton NMR could be complemented by deuterium NMR. We will be reporting the results of some of these experiments in forthcoming papers.

DISCUSSION

In many ways the distribution of water proton transverse relaxation times is analogous to a one-dimensional NMR spectrum in that the position of a peak is characteristic of non-freezing water in a particular subcellular compartment and can be assigned by comparison with peaks from isolated cell fractions; the peak area is also proportional to the amount of non-freezing water in that compartment which can be measured independently from the changes in the relative FID amplitude. Diffusive exchange of water between compartments can complicate the assignment of peaks to particular subcellular compartments by causing peak shifting and averaging. Fortunately this does not appear to be a major problem in potato tissue though

it means that not every subcellular organelle, such as the nucleus and mitochondria, will give a separate relaxation peak.

The results for potato show that most of the non-freezing water is associated with the cell walls and starch granules and the amount of non-freezing water has been determined at each temperature. This is not an unreasonable result since the polysaccharide content of these structures is very high.

Some comment is needed about the physical state of the unfrozen fraction in the starch granules and cell walls. If T_g' is the glass transition temperature of the freeze-concentrated solute matrix inside the unfrozen fraction of the starch granules and cell walls then, at temperatures below T_g', this unfrozen fraction is expected to exist in an amorphous, solid glassy state (Franks, 1982; Levine & Slade, 1986). At temperatures above T_g' (but below the melting point of the ice) this unfrozen fraction undergoes a phase transition to a viscous 'rubbery' state. Since T_g' for high molecular weight starch is $\geq -8°C$ (Levine & Slade, 1986) and the water proton relaxation time observed for the frozen starch granule suspension in Fig. 2 is still very long ($\geq$ 1 ms) even at 238K, the NMR data strongly suggest that the water molecules, even in the glassy state at low temperature, are still extremely mobile.

The relatively long relaxation time of the non-freezing water in the frozen potato tissue (Fig. 6) shows that it may be possible to use NMR microscopy to image the distribution of ice and water in the frozen material on a distance scale of a few tens of micrometres. On a macroscopic distance scale magnetic resonance imaging has already been used to follow the kinetics of the freeze-drying of frozen potato tissue (Rutledge *et al.*, in press). As an alternative to NMR microscopy it might be possible to directly observe ice crystal formation with cryomicroscopy. Unfortunately in potato tissue, observation of non-freezing water in the starch and cell wall compartments by cryomicroscopy could be technically difficult because of the surrounding ice matrix.

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