Susceptibility, Diffusion and Relaxation Contrast in NMR Microscopy at High Resolution

A thesis presented in partial fulfilment of the requirements

for the degree of Master of Science in Physics

at Massey University

Lucy Catherine Forde

1994

To my mother, Margot,
who set me on the road to science

Abstract

An integrated approach to the functional NMR imaging of plant tissue at moderately-high transverse resolution (23 μm) was undertaken. Attention was paid to all the possible commonly-known influences, such as sources of nuclear spin relaxation or of artefacts, relevant to the final image intensity of the different tissues.

While it was not clear at the outset which influences might prove to be significant, two phenomena in particular, susceptibility inhomogeneity and correlated diffusion effects, were selected for detailed investigation using simple model systems constructed from small glass tubes and rods combined with aqueous solutions, before continuing on to more complex plant samples. Simulated images compared well with the experimental results in these studies.

Preliminary images of a stem of an intact *Stachys sylvatica* L. plant showed that the apparent T_2 relaxation time is much less (an order of magnitude) than the T_1 relaxation time in all tissues. A range of diagnostic pulse sequences was then carried out on this and similar stems in order to reveal the signatures for different models of T_2 relaxation which might explain this fact (assuming that the water protons imaged fall within the extreme-narrowed region of Bloembergen, Purcell and Pound theory). It was found that measures were necessary to avoid the complicating factor of attenuation due to diffusion in the applied read gradient, specifically the use of Carr-Purcell-Meiboom-Gill (CPMG) refocusing pulses. Susceptibility inhomogeneity seemed important in sensitive gradient echo images, but further experiments at different B_0 strengths revealed that it (and chemical shift exchange) does not contribute significantly to the spin echo image contrast. The Brownstein-Tarr model of relaxation at boundaries and surfaces (without local field offsets) was also considered as a possibility, but was ruled out for at least some of the tissues (those which display a CPMG pulse-spacing dependence). Another alternative explanation is short-range dipole interactions between water protons and protons of more slowly-moving molecules, which should be abundant in the particular cells which escape the other hypotheses, but it is difficult to confirm this within the scope of the pulse sequences used here. More progress might be possible with proper multicomponent T_2 analysis and improved knowledge of subcellular structure of our particular tissues.

Acknowledgements

Firstly, I would like to thank my supervisor, Professor Paul Callaghan, for extensive theoretical and practical support during this project, and for a belief in the value of these experiments.

I would also like to thank the following people for their involvement in this work.

Dr Yang Xia, a fellow student in the early stages of this study, for instruction on the advantages, pitfalls and handling of the plant *Stachys sylvatica* as a specimen for NMR imaging.

Karen Tyrrell, for experimental assistance when I was far from home.

The Department of Plant Biology, for providing glasshouse space for the cultivation of plant specimens, and in particular Dr Izabela Konczak-Islam, for painstaking effort taken over the microtoming of my plant stems.

Dr Al Rowland and Dr George Ionas, for their kind services contributing to the photomicroscopy in this thesis, and their respective departments, the Department of Plant Biology and the Department of Microbiology and Genetics, for supplying the necessary facilities.

Walter Köckenberger (University of Bayreuth), Dr Iain Taylor (University of British Columbia) and Dr Jim Pope (University of New South Wales), for expert advice on matters botanical and otherwise while they were visitors to this laboratory.

The Ministry of Research, Science and Technology, for financial assistance in the form of a generous scholarship in 1991 and 1992.

The New Zealand Lotteries Board and the Foundation for Research, Science and Technology, for grants supporting the Nuclear Magnetic Resonance laboratory at Massey University.

Finally, I sincerely thank my friends in the NMR research group and in the Department of Physics and Biophysics as a whole, for valuable moral support throughout.

Contents

Chapter 1
Introduction

Nuclear Magnetic Resonance (NMR) imaging has widely been recognised as an invaluable tool for the non-invasive exploration of materials and organisms at the macroscopic and microscopic level *(1,2)*. The most commonly encountered applications are in medicine *(3)*, where patients are routinely scanned for soft-tissue abnormalities signalling tumours and a variety of other conditions. The size of instrument appropriate to accommodate human bodies or limbs precludes an in-plane resolution of much less than a millimetre. However the technique may be extended to a resolution on the order of a few microns with a corresponding reduction in the size of the sample to be inspected.

One must not make the mistake of equating NMR and optical images, because they do not depict the same characteristics and indeed rarely resemble each other closely. Instead of relying on reflection of incident light from the required object, NMR measures the radio frequency responses of target nuclei, receiving an equally strong contribution from nuclei in the interior of the object as from those at the surface. Thus we may examine the interior hidden from our eyes without significant disturbance to the specimen. Although somewhat unfamiliar in interpretation to a brain accustomed to processing optical data, the NMR image is of course no less valid a representation. Ideally the picture element (pixel) intensities should imitate the concentration of target nuclei in the specimen volume elements (voxels), but many other parameters may modify the intensity observed, and these may be used to indicate various properties of interest. It is this range of parameters and nuclei-specific concentration which material scientists and biologists utilise, especially where the lack of physical penetration and consequent detrimental effects is crucial, as in medicine.

There is a wealth of literature devoted to the use of NMR imaging in medical applications, which are wide-ranging, and may be non-invasive or instead undertaken in the laboratory. For example, in the former case the technique may be utilised to directly observe tumours, transplants and spinal cord injury or to follow metabolite levels in the brain *(4,5)*, to discriminate between skin, muscle, marrow and cartilage tissue in a finger by separate examination of fat and water components *(6)*, and to possibly enable early detection of osteoarthritis in the knee joint through changes in calcification and the cartilage matrix. In the latter case it is possible to study cancer processes using tumour spheroids or by inspection of inflammation and necrosis using the histology of excised tumours, to monitor the osmotic pressure in blood *(7,8)* and so on. Parallel research may be carried out on animal subjects, often (but not always) with a view to the advance of

medical aims. Examples include studies of muscle characteristics in barnacles and rats *(9-12)*, brain anatomy in rats and dogs *(13,14)*, metabolism in 16 different rat tissues *(15)* and, more remarkably, *in vivo* imaging of a healthy beating rat heart viewed at chosen points in the beat cycle *(6)*. In this way defects like holes in the heart may soon be diagnosed safely in humans.

NMR imaging is actually ideal for a great many biological applications beyond medicine, due to the ease of detection of the hydrogen nuclei (protons) in water, and the roughly 75% water content of most biological tissue. It is capable of rapid histology without artefacts from fixation, dehydration, embedding, sectioning or staining which can plague the ability of conventional histology to reflect the true state of tissues *in vivo*. An added advantage is the capacity for three-dimensional imaging to aid visualisation of whole structures, their positions with respect to others and any links between them. Yet more dimensions may be added if measurement of the concentration of a specified chemical component is desired. These variations in technique together can be put to good use by industries such as horticulture, food and forestry.

Non-destructive quality evaluation of fruit and vegetables is possible, for example observing bruising in apples, worm damage in asian pear, seeds in grapes, dry regions in oranges and hollow heart in potatoes *(16-18)*. The spread of botrytis (grey mould fungus) mycelium across a single raspberry drupelet and a whole strawberry fruit has been monitored in the hope of breeding resistant cultivars *(19)*. Sugar content and other ripening processes have been followed in grape, raspberry and barley seeds *(20-22)* enabling optimum storage conditions to be determined *(18,23)*. Other postharvest processes are also studied, from the senescence of mushrooms to the moisture transfer and shrinkage in an ear of corn during dessication *(24)*. The effects of chilling and freezing (e.g. cell wall rupture) are seen in vegetables such as zucchini *(17,25,26)*, and water distribution during the steeping of corn kernels in water is elucidated *(27)*. Simple morphological studies can be useful *(28-30)*, especially where vascular architecture is complex as for the spiral arrangement of drupelets and vascular traces in a raspberry receptacle, or where there are changes with development and maturation *(31)*. It is possible to follow the differentiation of separate tissues which arise from different ontological origins.

Other fully-processed foods are complex and interesting systems, often containing dispersions of lipid and water, or liquid and air. Knowledge of the structure and collapse of food foams, as found in whipped cream or egg white or in beer, helps in the design of required foam stability in each food product *(32)*. Air spaces in cheeses such as gruyere

are easily seen, as well as components in composite foods like chocolate bars, without even removing the wrappers *(17)*. Cocoa butter, the main matrix of chocolate, occurs in more than one crystalline form, and these may be studied if the chocolate is melted and cooled at different rates to generate the various polymorphic states *(33)*.

It is evident that NMR imaging is here to stay in the commercial world, pervading a fairly wide cross-section of industries, but it is also employed in more basic research as a tool for greater understanding of non-commercial species. The contrast afforded by NMR, apart from chemical and nuclear specificity, mainly arises from the NMR relaxation rates present in the sample which modulate the intensity observed. These are sensitive to molecular dynamics such as diffusion and binding state *(25,26,34-39)* and therefore tend to reflect morphology more effectively than the molecular density alone. In particular relaxation times T_1 and T_2 are often discussed in this context, and will be explained in more detail later, but for the moment it is enough to know that T_2 measurement is the method most commonly used to probe the chemical dynamics of a sample or to distinguish between variations in anatomy. Relaxation times could be likened to inherent proton 'stains'.

Thus the function of the assorted organs of plants can be investigated *in vivo*. The stems of plants have a range of very specialised cell types, combining to form the tissues which perform the vital processes of support and of transport of water and nutrients. An improvement of these processes can always be used to improve the yield of commercial crops. The stems of geraniums, runner beans, celery, cucumbers, horsetails, ferns and mosses have undergone examination *(30,34,35,40-44)*, sampling a variety of morphologies. Other studies of plant parts have looked at disease or growth of roots *(45,46)*, leaves *(47)*, seed pods *(5)*, germinating seeds *(41,48)*, bark *(49)* and flower buds *(50,51)*. The buds were from fruiting species such as apple and blackcurrant, and were subjected to chilling to simulate the outcome of an unexpected frost. Root function was probed under varied conditions of water stress and recovery, or different soil types, using the uptake of water doped with a paramagnetic ion such as Mn^{2+} which decreases T_1 *(41,52-54)*. Even such large specimens as trees do not escape attention, and the problems of incompatible graft unions in apple *(55)* and blight in citrus *(46)* have been considered as well as the normal growth rings, heartwood and knots found in aspen and spruce wood *(56)*. At the other extreme of plant life the development of the fascinating alga *Acetabularia mediterranea* is investigated through T_1 changes which are thought to relate to the changing concentrations of microtubules inside *(57)*. The entire alga consists of a single giant wall-less cell which is several millimetres in size and an elaborate fluted-

umbrella shape. Without the support of walls the organism must necessarily regulate shape and expansion via intracellular microtubules.

Clearly the understanding of plant physiology may be enhanced with these methods, and the facility of velocity and diffusion measurement is a particularly valuable aspect of NMR for this. Plant water relations have always proven tricky to analyse with confidence, and in the past studies have relied on the movement of a tracer molecule introduced often by invasive means. Now transpiration can be monitored directly in combination with changes in environmental parameters such as humidity and sunlight, for example during the course of a day, and this has been carried out for the case of greenhouse cucumbers *(30)*. Taken a step further velocity measurement may be combined with imaging giving a map showing spatially-resolved velocity, and this has been done using plants from different parts of the evolutionary tree, a moss, horsetail and a flowering plant, in order to compare the vascular structures which conduct water *(44)*. Transpiration may be studied using less direct methods as well, by examining water content and relaxation times in roots and stems for slow and active transpiration conditions *(34,58)*. Of course velocity mapping touches other fields too, promising to have a fruitful future in angiography (visualisation of the circulatory system) for heart disease and stroke problems in humans *(4)*. Diffusion coefficient mapping also has a part to play here, as diffusion is seen to drop in areas of the brain damaged by occlusion of an artery, and the mapping allows rapid estimation of the nature and seriousness of damage in stroke victims. Returning to plants, diffusion mapping in wheat grains helps distinguish between the major structural features, and the addition of velocity detection gives insight into the circulation inside the grain which loads nutrients from the plant's photosynthetic sinks into the endosperm *(59,60)*.

Closer analysis of relaxation times sometimes reveals further information in the form of multiple relaxation components *(11,54,61)*, arising from the separate populations of water within a heterogeneous sample which experience different environments. Examples of such populations might be intra- and extracellular water, that inside organelles like chloroplasts and mitochondria or water associated closely with phospholipid membranes, proteins, polysaccharides or other macromolecules. In this way the relative population sizes present in these sites may be estimated and observed during experimental treatments, although it is not always possible to be certain of the correspondence between a relaxation fraction and a cellular location *(10,12,48,62)*. Each plant species and part appears to exhibit its own characteristic 'fingerprint' of relaxation components. In ivy bark two components were found, relating to extracellular water in the cell wall and to the bulk inside the cell *(49)*. Plant tissue, unlike animal tissue,

typically contains air between the cells with just a little water hydrating the wall biopolymers outside the cell. In apple fruit there were three components, relating to the extracellular, cytoplasmic and vacuolar water *(63)*. Some plant cell types contain only a small amount of cytoplasm and instead a large and very dilute vacuole occupies most of the volume, separated from the cytoplasm by a membrane called the tonoplast. It is easy to see that the dynamics of the vacuolar contents will most closely resemble those of free water, while the dynamics of the hydration water will be much more restricted. In imaging, some hydration or 'bound' water will be so immobile as to be rendered effectively invisible, possessing a T_2 value below the minimum needed to enable detection during finite imaging times.

Unless there are impermeable barriers, molecules are exchanged between the populations to a certain extent, and this leads to a partial blending of the relaxation components *(4,61,64-72)*. In apple species this exchange was observed and from it the permeability coefficients of both the cell membrane and tonoplast were calculated. Studies on the exchange between cell interior (lumen) water and the cell surface layer water in western red cedar wood show that lumen T_2 scales with surface-to-volume ratio and hence with cell diameter *(73,74)*. This method was able to distinguish between earlywood tracheids, formed first in the annual growth season and seen just outside the growth rings, and latewood tracheids which are generated at the cooler end of the season and have on average only a third of the lumen diameter found in earlywood. Other investigations of compartmentation at the tissue, cellular or intracellular level include size determination of erythrocytes in blood and separation of the cornea into its cell and collagen stroma constituents *(7,8,75-77)*.

Thus through sophisticated relaxation analysis we are able to partly explore the interior of cells which usually cannot be seen directly in NMR imaging. The resolution of NMR imaging is limited by the need for an adequate signal-to-noise ratio (related to the number of nuclei in each voxel) and is inferior to light microscopy. Only the upper end of the range of biological cell sizes may be identified individually in an image *(40,41,78,79)*. To make matters worse, diffusion effects (namely signal loss) become very unfavourable when the pixel size is small enough so that molecules may diffuse the order of the pixel during imaging times, and this can hinder efforts to image close to the fundamental resolution. In such adverse conditions only molecules with sufficiently slowed or restricted diffusion will be seen. Hence optimal resolution is at times elusive due to its special complications.

One way to accentuate the borders between cells or tissues is to use the gradient echo form of imaging as opposed to the spin echo *(19,22,27)*. These forms will be outlined in Chapter 2 but it is sufficient here to say that the gradient echo is very sensitive to stray magnetic fields induced in a sample by an external applied magnetic field, whereas the spin echo is more successful at refocusing the same effects, provided the extra fields are stationary. The origin of the induced fields is the range of magnetic susceptibilities found in a heterogeneous sample. The magnetic susceptibility of a material determines the size of any induced field, and the boundary between two differing susceptibilities will generate a whole distribution of fields in the vicinity of the discontinuity. Biological tissue would be expected to have many such boundaries, but the extent of the effect on imaging varies. These susceptibility effects might be considered a nuisance in some instances and a useful tool in others. Certainly the gradient echo sequence is used when maximum contrast between tissues is desirable.

Recently a very special application of susceptibility was discovered in the field of human psychology and neurology. Neural activity results in a mild local enhancement of oxygen consumption, which is overcompensated by a larger increase in regional blood volume, and after a few seconds a transient hyperoxemia arises. Thus the amount of paramagnetic deoxyhaemoglobin increases and this tissue oxygenation is detectable by an accompanying shift in susceptibility. If rapid acquisition techniques are used then brain activation may be monitored during the application of various stimuli involving the visual *(80-84)*, motorsensory *(85)*, language processing and other systems. A comparison of consecutive gradient and spin echo images *(86-88)* yields what appears to be a map of neural activity in the brain, directly visualising the cerebral compartments responsible for the thought or movement being studied. This capability opens the way for a whole host of psychological investigations (termed functional brain imaging) which are considered safe for the volunteers concerned.

Although the spin echo sequence of imaging is not influenced by strictly static field variation on top of the normal imaging gradients, the migration of molecules from one position to another in these variations will lessen its refocusing ability and hence additional relaxation will result. In this way susceptibility effects may also shorten T_2 itself although this depends on the rate of diffusion as well as the magnitude of the variations *(8,9,14,39,54,78,89,90)*. With such a diversity of relaxation mechanisms, as mentioned earlier, existing on top of the susceptibility effects, it can be an intricate task indeed to unravel the mechanisms which are at work in any new sample *(4,15,26,28,34,61,63,75,91)*.

The versatility of NMR imaging and its consequent role in so many branches of science makes continual improvement of understanding in this area worthwhile. The purpose of this thesis is to take an integrated approach to the imaging of plant tissue at fairly high resolution, looking closely at all the possible relevant influences (i.e. sources of relaxation and artefacts) which vie for dominance in determining the final image intensity, while keeping an open mind as to which of them will prove significant. The exhaustive NMR study of biology outlined above demonstrates more than anything the multiplicity of phenomena which occur for different biological specimens, and it is wise not to disregard any mechanisms available in the interpretation of the resulting image contrast. At the same time we hope to further the understanding of susceptibility effects in particular, as these are often alluded to but seldom pursued thoroughly.

A system providing a variety of challenges was necessary for this work, and plant stems seemed suitable in that they contain a range of very different cell types with wide-ranging functions. There is also the facility to demonstrate the measurement of very delicate biological flow *in situ*, which will actually pinpoint the active transport locations rather than assuming that all the cells differentiated for this purpose are so. The use of simple model systems will be utilised to increase awareness of two of the phenomena involved, susceptibility and diffusion and their correlation, before venturing on to truly complex examples. This route may enable us to build upon recent research by others into plant tissue contrast in NMR microscopy.