NMR MICROSCOPIC IMAGING OF THE SINGLE CELL:

Acetabularia mediterranea

by

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to the required standard

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ABSTRACT

NMR imaging studies performed in the microscopic realm using the cell organelles of the single celled marine green alga, *Acetabularia mediterranea*, are presented. The study had two main objectives. First, to attain microscopic spatial resolution. Second, to monitor development stages in the reproductive structure, the cap. The images of caps which are flat, oriented in the xy plane, have been obtained as 2-dimensional images.

Using a 270 MHz spectrometer and an imaging probe made in this department, a lower resolution of 40-50 μm is reported. The probable causes for the limitation of resolution are discussed in terms of the molecular diffusion of the vacuolar water and the magnetic field inhomogeneity caused by the susceptibility differences at the interfaces of the sample and the sample holder on which the sample was mounted. Proton density images of immature, mature and partly mature caps are presented in order to portray the attainable resolution. Images obtained at higher gradients (~50 Gauss cm\(^{-1}\)) are presented and compared with those obtained at moderate gradients (~15 Gauss cm\(^{-1}\)). The necessity to perform the imaging sequence at a shorter time, in order to minimize molecular diffusion, is highlighted.

\(^1\)T contrast imaging experiments were performed to investigate the different maturation stages. The main attention is given to the partly mature caps, which have a mixture of mature and partly mature rays. Images obtained by \(^1\)T contrasting, and the \(^1\)T values determined in a preliminary study, are discussed in relation to the probable changes in mobile and bound water during maturation. The variation of the \(^1\)T values from long (immature) to short (partly mature) is explained in accordance with the ultrastructural development of the cap. Results of contrasting using \(^2\)T values and paramagnetic ions (\(Mn^{2+}\)) are presented briefly. Imaging of the rhizoid has been done, demonstrating the possibility of monitoring the large nucleus during maturation.
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Magnetic nuclei are ones which possess an unpaired nuclear spin and hence an inherent angular momentum and a magnetic dipole moment. When placed in an external magnetic field, such nuclei can absorb energy from an external source in the radio frequency (rf) range. This phenomenon is known as Nuclear Magnetic Resonance. The first such Nuclear Magnetic Resonance (NMR) experiments were performed by Bloch et al [1] and Purcell et al [2] in 1945. The magnetic nuclei residing in different chemical environments in a molecule were observed to experience slightly different magnetic fields, and hence required a slightly different rf frequencies to induce resonance. This led to the observation of an important aspect of NMR, the chemical shift [3]. The NMR signal obtained is a function of the local chemical environment, and the intensity of the signal is proportional to the number of magnetic nuclei under investigation [4].

The first commercial NMR spectrometer was built in 1953, and the method implemented in the early stages (until about 1966) was the continuous wave (CW) technique [4].

The rapid development of the NMR technique and the sophistication of instrumentation brought many breakthroughs in NMR spectroscopy. Probably the three most important ones being;

a) Introduction of Pulse Fourier transform (FT) technique by Ernst and Anderson [5].

b) The development of high field superconducting magnet systems [6].

c) The advancement of computer technology and microcircuitry.

The pulse NMR technique uses a short burst (pulse) of strong radiofrequency power
to excite all the resonating nuclei in a sample simultaneously [4]. The response is recorded as a function of time (time domain signal), and it contains all the information about the magnetic environment of the nuclei under observation. Fourier transformation [7] of this time domain signal, known as the free induction decay (FID), yields the familiar NMR spectrum (frequency domain signal). This pulse FT method, not only enables one to perform a given experiment at a shorter time, but also makes it possible to increase the signal/noise ratio of the final spectrum by signal accumulation [4],[8]. The pulse FT technique has had substantial impact on chemistry (e.g. study of nuclei, such as $^{13}C$, $^2D$, $^{15}N$, which are less sensitive and isotopically dilute at the natural abundance level).

Probably one of the most significant breakthroughs in NMR was the extension of the 1-D conventional NMR spectrum into two dimensions. In 2-D NMR spectroscopy the spin behaviour is investigated with respect to two independent time variables [9],[10]. The response, after double Fourier transformation, is graphed on two orthogonal axes which portray two NMR parameters [11]. 2-D spectroscopy has made possible the study of many complex systems which would have been impossible with 1-D spectroscopy.

Recently, interest has been directed towards the possibility of monitoring biological processes using NMR spectroscopy. Among the valuable contributions from this area, the most important, which drew the attention of the biochemist and the clinician alike, was an observation made by Damadian in 1973 [12]. He observed a difference in relaxation times of the water signal between tumour cells and the analogous normal cells. The differences in relaxation times were attributed to the quantity and the environment of water in the tumour cell in contrast to normal cells [13].

Biological NMR studies have been mainly confined to performing studies on protons due to the following reasons [14],

(a) The high intrinsic sensitivity of protons (High $\gamma$ value).
(b) Protons are the most abundant NMR sensitive nucleus in a living organism (Over 80% of the “body” content is water).

(c) Relaxation times ($T_1$ and $T_2$) variation in the water signal were found to vary with various pathological conditions.

The early biological NMR studies were performed using spectrometers with small bore magnets. This imposed stringent limitations on the size of the sample. Thus, the biological samples essentially had to be either perfused organs, sections (in vitro study), small laboratory organisms (in vivo study) [15], or biological liquids [16],[17],[18].

Biological spectroscopy, especially in vivo spectroscopy which has advanced tremendously over the last decade, has shown great potential in biochemistry as well as medicine. Performing in vivo spectroscopic studies eliminates the need for biopsies and other cumbersome sample preparation procedures [19]. Many physiologically important biochemicals, and water, are shown to have distinct differences in NMR parameters (chemical shifts, intensity, line width, $T_1$ and $T_2$) in the normal and abnormal states [18],[19]. The study of drug behaviour, which might entail physiological changes which can be monitored by NMR, for instance, could open a whole new perspective in drug therapy (e.g. The use of cis-diaminoplatinum complexes in cancer therapy [20],[21]). The realization of the importance and the role that other nuclei (besides $^1H$) played in physiology, has expanded the arena of the familiar proton NMR studies to studies of nuclei which are physiologically less abundant (e.g. $^{23}Na$, $^{31}P$, $^{19}F$) [22].

Conventionally, a nuclear system in a polarizing magnetic field is made to interact with an external rf field and the response, the NMR signal, is measured [3],[8]. These experiments require a homogeneous magnetic field over the sample of interest to ensure uniform excitation [4]. Conversely, consider the case where either the magnetic field or the rf field is varied (i.e. a condition where a magnetic field gradient across the sample is
maintained). Here, different regions of the sample should experience a different magnetic field thereby resonating at different frequencies (because the field is proportional to the Larmor frequency.), hence making it possible to obtain spatial information across the sample and to construct an image [23]. An image is a graphical representation of an object which portrays its three dimensional structure. Thus, NMR imaging is the spatial mapping of the variation of some suitable NMR parameter of the spins (e.g. spin density, relaxation times).

Gabillard [24] in 1951 was the first to use the field gradient method on a water filled tube, to obtain a one dimensional projection which highlighted the spatial distribution of the proton spin system. Lauterbur in 1973 [25], used two orthogonal field gradients to obtain the first NMR image of two water filled capillaries. The image was obtained by joining, or coupling, the rf magnetic field with spatially defined magnetic fields. Using the Greek term Zeugma (which means joining), Lauterbur christened this technique NMR Zeugmatography.

NMR zeugmatography or NMR imaging subsequently witnessed a rapid development of an avalanche of techniques and instrumentation. Several methods [26],[27],[28], which effectively use a field gradient of one form or the other over the static magnetic field, have been developed. Initially, Lauterbur obtained NMR images using the projection reconstruction method. This method was already being used in the fields of computerized tomography and electron microscopy [26],[29]. Later the more convenient double and triple fourier transform (FT) methods were introduced in order to obtain 2-D and 3-D images [30]. Today, these methods are automated on the instruments in use, and have made NMR imaging a practical reality. Despite the initial doubts of maintaining magnetic field homogeneity over large volumes, NMR imaging instruments which are capable of scanning an adult human body have been developed [31]. The NMR imaging instrument has an added advantage in that an image can be obtained from virtually any direction leaving the patient and instrument in a stationary position.
Since the biological tissue is a heterogeneous system, the protons in this heterogeneous living system are in different micro-environments. Because spin relaxation times ($T_1$ and $T_2$) of atomic nuclei (water protons) are dependent on the local environment, the inherent relaxation time differences of the various water protons in these local sites, can be used in imaging methods to obtain contrast. This is known as $T_1$ and $T_2$ contrast imaging (section 3.7.). Basically, in these contrast imaging techniques, a part of an experiment which is used in the relaxation time determination is "coupled" to an imaging experiment. The net result is an image highlighting contrast between the volume elements (voxels) of the sample which is primarily dependent upon the relaxation times of the spins in individual voxels. These contrast images often have the capability of revealing more information from a heterogeneous system than the analogous proton density image [32]. The inherent relaxation differences in tissues eliminates the necessity for external contrast agents, which is an essential feature in most other imaging techniques. However, the addition of external contrasting agents, which are complexes containing paramagnetic ions, reduces the relaxation times, thereby providing better contrast and a reduction in the total imaging time [33].

$NMR$ imaging has shown promising potential in the identification of many abnormal biological conditions, such as cancer [19]. Therefore this technique is complementary to the existing diagnostic techniques such as X-Ray, Sonographic and radiotracer techniques. In some instances, $NMR$ imaging has shown itself to be superior to all other techniques, especially in discriminating between abnormalities in soft tissues [34]. Since $NMR$ uses nonionizable radiation from the radio frequency range to induce resonance and obtain the signal or image, it is believed to be a noninvasive and safe method to image biological systems. No harmful effects have yet been reported at the implemented frequency range of $NMR$ [26],[35],[36],[37],[38].

Selective excitation methods to study local regions of interest (viz. a point, a slice
or a volume element) within the whole system (localized imaging) have led scientists to focus on smaller and smaller regions. This is eventually leading to the lower spatial limit of NMR; NMR microscopy [39],[40]. A microscope is a device used in order to resolve and visualize objects which the naked human eye is unable to see clearly. The resolving power of a normal human eye is about 250μm. Observation of objects smaller than this lower limit requires a microscope. Various forms of microscopes have been in use for a long time. The optical microscope, for instance, has a history of over two centuries [41]. Its possibilities of spatial resolution seemed to have been realized by the early decades of this century, when many people believed that microscopy was reaching its limits. But the invention of the electron microscope changed the limit of spatial resolution by several orders of magnitude. This has led, from the 1940's to the present, to a previously unexpected concentration in biology on the study of increasingly fine details of ultrastructures. The biologist has become primarily interested in the world below 1μm. Hence it is not easy to interest large numbers of modern biologists in new techniques with spatial resolutions still only in the tens of μm; they hardly think of this as microscopic. Nevertheless, NMR imaging does have potential which should ultimately make it of interest at least to some biologists, especially those studying development. In the developing organism, whether in the higher or lower forms of life, morphological and metabolic differences between regions often first appear on a scale of about 10μm. If NMR microscopy could commonly resolve such differences, especially in vivo in hidden structures which cannot usually be seen without killing the organism, it could become a powerful tool in developmental biology.

The present work represents an intermediate step towards this objective. The level of spatial resolution achieved, of the order of tens of microns, is between the requirements of medical diagnosis and those of developmental biology. The present work, and the studies carried out elsewhere on similar spatial scales, makes such improvement appear a possibility
for the immediate future.

A general overview of the upper limit of resolution where different types of microscopic techniques can be used is given in Table-1 [42].

<table>
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<th>DIMENSIONS (mm.)</th>
<th>STRUCTURE OBSERVED</th>
<th>TECHNIQUE</th>
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<td>10 − 1</td>
<td>Organs</td>
<td>Eye, Simple lens, Imaging techniques</td>
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<tr>
<td>0.1</td>
<td>Tissues</td>
<td>Light Microscopy</td>
</tr>
<tr>
<td>0.01</td>
<td>Cell</td>
<td>Light Microscopy</td>
</tr>
<tr>
<td>0.001</td>
<td>Bacteria</td>
<td>Light and Electron Microscopy</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>Cell Organelles</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>Molecular Structures</td>
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In both optical and electron microscopy, it is well known that the limit of spatial resolution is given by the wavelength of the radiation (or electrons) used. This is because the image is acquired as the pattern of a wavefront of scattered radiation, in which all the photon or electron waves are mutually interactive according to their phases. The spatial resolution of NMR microscopy must be discussed quite differently. The wavelength of the radiation is almost irrelevant; it consists of radio waves, with wave length much greater than the size of the sample studied. Imaging depends upon placing a magnetic field gradient across the sample so that the conditions of interaction of the incident radiation with the sample depends upon position in the sample.

The attaining of better resolution in NMR microscopy is mainly restricted by :

1) Requirement of high field magnets with high homogeneity, the linearity of gradients and the necessity to use larger gradients [39].
2) The inherently weak NMR signal which becomes more critical by reducing the sample dimensions. This in turn reduces the number of magnetic nuclei per unit volume hence reducing the sensitivity, the chemical shift variation over the sample or volume of interest (e.g. water and fat), and random molecular motions of constituents in an organism [39].

3) Molecular diffusion of water is believed to be the limiting factor for attaining higher spatial resolution at sub-millimeter levels [42]. If, for instance, complications due to molecular motions were absent in an organism the sensitivity and probably the resolution could be increased by signal accumulation [39],[43].

The fact that approximately 80% of the tissue contents is water, which has made the water protons the most convenient choice for NMR studies of biological systems, has unavoidably become the only choice in NMR microscopy in order to compensate for the lower sensitivity restrictions at reduced voxel sizes. Besides being the convenient molecule for NMR, the tissue water is found to have a range of behaviour in living organisms. This behaviour of water will depend on whether the movement is relatively free, bound firmly to a particular site for a long time or influenced occasionally by an electrical charge during its random motion within the organism [42]. Collectively the living system is considered to possess over 80% of "liquid like" water and the rest as "bound" water [44]. In proton density imaging what one observes is an image which is a composite of these two types of water components (assuming that the bound water component has a longer $T_2$ value than the time taken for an imaging sequence). Besides being the major component of a biological form, water makes an attractive target for microscopic imaging. On the one hand, the site dependent nature of $T_1$ and $T_2$ makes contrast imaging ($T_1$ and $T_2$) possible, and on the other, it is possible to monitor flow and diffusion. This should lead to a better understanding of cell function and physiology. It is clear that NMR microscopy is applicable in systems
Introduction

other than biology such as hygroscopic fibers, ceramics, flow through porous media [42].

Microscopic imaging is being performed on small organisms, tissues and phantoms (systems built with artificial material to hold a particular spin system at a desired shape). Microscopic imaging of few different biological samples has been reported in the recent literature. Resolution of $50 \times 50 \times 1000 \, \mu m$ has been reported for studies done with rat brains [45]. Bottomly et al [46] have studied the roots of the bean, *Vicia faba* L, in different soils in order to monitor and image the water uptake by the roots at $1.5 \, T$. They have reported a resolution of $600 \, \mu m$. A similar study performed on maize, (*Zea mays* L), in a 200 MHz spectrometer, reports a pixel resolution of $50 \times 50 \, \mu m$ [47]. Eccles and Callaghan [48] have attained a resolution of $20 \, \mu m$ with a slice thickness of $1.5 \, mm$, at $60 \, MHz$ and by the projection reconstruction method [25], using the plant stem, *Alyssum tenium*. The most striking observation in the microscopic realm has been the *NMR* imaging of a single cell, the ovum of the African clawed toad *Xenopus laevis* [49] with a reported resolution of $10 \times 13 \, \mu m$ in the imaging plane. However, many authors [26][42] cite a limiting resolution of $10 \, \mu m$ with the current technology.

It is clear, that unless an unforeseen fundamental breakthrough takes place in *NMR* imaging, the superior quality and resolution of the optical and electron microscope is beyond reach for today’s *NMR* microscopic techniques. But, in contrast to the other microscopic techniques, *NMR* has its own advantages which are capable of extending the understanding of biological processes to the tissue or perhaps the cellular scale. They can be categorized into four features [42].

a) *NMR* microscopy has the capacity to portray function alongside morphology. This is in contrast to conventional microscopy where the outcome depicts only the structure. The phenomenological differences seen in *NMR* microscopic imaging are of major significance in relating what is seen and what is actually taking place within a biological system.
b) $NMR$ images properties of water which are caused by the inherent nature of water as well as by the interaction of water with sub-cellular and sub-molecular environments. The differences in relaxations times, diffusion rates, chemical shifts etc. are important processes inherent to $NMR$. In particular, the relaxation times are found to vary as a function of cellular growth state, metabolism, and respiration $[39]$.

c) Measurement by $NMR$ uses radiation from the radiofrequency range which implies lower power dissipation within the sample. No ionizing radiation is used which ensures that, at least during the imaging process, there is negligible perturbation in the sample.

d) Probably the most important and striking feature is that sample fixation, sectioning and other cumbersome sample preparation techniques are not called for in $NMR$ microscopy. The inherent natural contrasting sets no restrictions on the living condition of the organism, and permits in vivo imaging.

Conventional microscopy often uses dyes or stains and heavy metals to fix the sample and to enhance contrast. This process usually freezes the structural patterns which reveals morphology but has little or no relationship to the function. The dynamics of molecular motions, which are revealed by the influence of biological relaxation times and diffusion of water, are the primary source of functional information. Many components of physiological importance are usually lost, or are redistributed in the sample during the process of fixation $[42]$. A useful analogy (modified from reference $[42]$), where the difference between a fixed biological sample and the analogous natural sample may be compared to an aerial photograph of a burnt forest to its natural state, gives a good general comparison to show the effect of fixation.

In summary, $NMR$, spectroscopy and macroscopic imaging, has developed to become an important technique in many research fields. Because of the high resolution attainable by other techniques and technical limitations, $NMR$ microscopic imaging has received little
attention. The image quality is a complex function of several factors, the most important one being the gradient strength attainable from a particular instrument. The development of new instrumentation and more research on the field of NMR microscopic imaging might enable one to look beyond the limiting ~10 \( \mu m \) resolution barrier in the near future.

**General plan of the work and the thesis**

This study was undertaken with two objectives. First, to perform NMR imaging experiments in order to attain high spatial resolution from a biological sample. Second, to study the developmental stages of a biological sample by NMR imaging. This work did not involve new mathematical analysis of NMR nor new chemical structure elucidations. All studies were intended to compare and contrast biological systems from a chemist's point of view.

The spectrometer used consists of a 54 mm small bore vertical magnet interfaced to a Nicolet spectrometer operating at 270 MHz (for \( ^1H \)). This high resolution spectrometer (used for spectroscopy) was modified, with the necessary equipment, for NMR imaging of small objects. Most of the NMR microscopic imaging work outlined in this thesis was performed on this modified 270 MHz spectrometer. The probe with the appropriate gradient coils was built by Dr. Stanley Luck *et al.* Three gradient power supplies and a digital to analog converter (DAC) along with this probe were already being used to perform imaging experiments. The biological sample chosen in this study was the reproductive structure ("Cap") of the marine green alga, *Acetabularia mediterranea*. The discoid caps having a diameter of about 5-7 mm made them ideal objects for a narrow bore magnet. The presence of morphological details on spatial scales from hundreds to tens of \( \mu m \) with high water content throughout the cap made it a challenging object for the attempt to resolve fine details and highlight different water components by NMR imaging. Moreover the discoid
nature (flat) found in most of the caps implied that there was no need to perform slice selection. All images were obtained by using two orthogonal gradients (viz. x and y) and projecting the response on to the xy plane, i.e. two dimensional imaging.

A brief introduction to the basic theory of conventional NMR (1-D NMR) begins CHAPTER 1. Also the determination of relaxation times is treated in a classical sense. A brief discussion on two dimensional spectroscopy is presented in order to introduce the reader to the final section, two dimensional NMR imaging. Basic theory of NMR imaging is presented. Main concentration is focussed on Fourier Transform imaging and its performance at the microscopic level, with emphasis being placed on the resolution in a microscopic image.

In CHAPTER 2 the development of the NMR experimental procedure for obtaining proton density images is described. Initially, the spectrometer and probe are considered. The pulse sequences used in this study are discussed. The selection of parameters is described. The processing of the NMR data to obtain the final image is explained. The phantom studies carried out in order to assess the resolution attainable are discussed. Some of the relevant studies performed to monitor the instrument or the techniques implemented are described in the Appendicies for the interested reader.

CHAPTER 3 is discussed in two major sections. First, the proton density imaging experiments carried out on the biological sample, Acetabularia, to attain high spatial resolution are discussed. The studies performed under two different gradient power levels are discussed. Second, the contrasting imaging techniques, with emphasis on $T_1$ contrast imaging are discussed. Also two other types of contrasting methods are discussed briefly. The possibility of monitoring the development stages in Acetabularia are examined. Finally, results of imaging of other parts of the Acetabularia cells are presented.

Finally in CHAPTER 4 conclusions from the studies are summarized together with suggestions for future work.
CHAPTER 1

NMR THEORY

This chapter consists of three main sections in which the basic theory of NMR spectroscopy and NMR imaging is discussed. In section one the basic theory of conventional (1-D) NMR is given along with the relaxation time measurement techniques. Sub-section two of section one consists of a classical explanation of the common two-dimensional NMR experiment. The main intention of this is to introduce the reader to the third section, on NMR imaging. Neither a comparison nor an analogy between 2-D spectroscopy or 2-D imaging is presented. Because of the morphological features of the sample studied 2-D NMR imaging is considered extensively. A brief discussion on the projection reconstruction method is presented mainly because of its historical value. Double Fourier transform imaging methods are discussed. Special constraints in the microscopic realm such as the sensitivity and resolution are considered.

1.1 Conventional (1-D) NMR

Many nuclei in the periodic table possess spin angular momentum. Depending on the nucleus the spin can take a value of half integral or integral [4]. Of all nuclei with spin angular momentum, protons ($^1H$) with spin $1/2$ are by far the most important, because
they are the most frequently studied and also the most important in biological NMR studies. Therefore further discussions will always assume the proton nucleus. The magnetic moment \( \mu \) produced due to the spin angular momentum \( (P) \) is given by the following equation [50].

\[
\mu = \gamma P
\]  

(1)

where \( \gamma \) is known as the magnetogyric ratio which is a constant for a given nucleus. Also \( P = \hbar I \) where \( \hbar \) is the Plank constant/\( 2\pi \) and \( I \) is the spin quantum number.

When an ensemble of such nuclear spins (spin quantum number \( I \)) is placed in an external magnetic field, \( B_0 \), the nuclear spins are quantized along the field and they place themselves into \( 2I + 1 \) energy levels [8]. In the case of an ensemble of identical \( ^1H \) nuclei with spin \( \frac{1}{2} \), there will be two energy levels with the energy separation \( (\Delta E) \) between the two levels given by [8],

\[
\Delta E = \gamma \hbar B_0.
\]  

(2)

The nuclei will distribute between the two energy levels according to the Boltzmann equation [50]. At room temperature, the lower energy state (the nuclear magnetic moments which are aligned with the applied external magnetic field) will have a slight excess of nuclear spins over the higher energy state.

The exact features of nuclear magnetic resonance are understood by quantum mechanical considerations. Nevertheless, many features can be comprehended more easily by a classical vector approach.

When a magnetic dipole (a proton nucleus) is placed in an external polarizing magnetic field, \( B_0 \), the magnetic moment vector orients itself inclined to the magnetic field. In spin \( \frac{1}{2} \) nuclei (protons) the magnetic moments will take two orientations (corresponding to the two energy levels possible) with respect to \( B_0 \) [8]. Conventionally the main magnetic field is considered to be along the \( z \) axis. This main magnetic field exerts a torque upon the
nuclear magnetic moment vector in such a way as to make it precess about the external applied field $B_0$. (FIG-1A)

The frequency ($\nu_0$) at which this magnetic moment precesses about the $B_0$ field, commonly referred to as the Larmor frequency, is given by the Larmor equation.

$$\nu_0 = \frac{\gamma}{2\pi} B_0$$  \hspace{1cm} (3)

Consider a collection of magnetic moment vectors of identical protons. This will precess about the applied magnetic field as shown in FIG-1B.

Consider the application of energy into the spin system in the form of an electromagnetic field perpendicular to $B_0$ and oscillating at a radio frequency $\nu_r$. In the laboratory frame its magnetic component $B_1$ will rotate about the main magnetic field. In order to understand the behaviour of the magnetization vector easily, it is appropriate to introduce the concept of the rotating frame of reference at this point [4],[8]. The new coordinate system, which is designated as $z', y'$ and $z$ ($z = z'$) coordinates, is considered to be rotating about the $z$ axis at a frequency $\nu_r$ which is normally equal to the Larmor frequency. Observing the rotating nuclear magnetic moments in a frame rotating at the same frequency makes the individual magnetic moments appear fixed.

At thermal equilibrium the ensemble of all the nuclear magnetic moments gives rise to a macroscopic magnetization, $M_0$, aligned along the direction of the main magnetic field $B_0$. (FIG-1C)

The $B_1$ field applied along the $z'$ axis will rotate the magnetization vector, $M_0$, initially along the $z$ axis, towards the $y'$ axis [3],[8].

Because of the widespread applications and the relevance to this study, the rf field applied is assumed to be a short pulse. The angle $\theta$ by which the magnetic moment vector
is changed or flipped is given by the following equation:

\[ \theta = \gamma B_1 t_p \]  

(4)

where \( \theta \) is the flip angle, \( B_1 \) is the applied rf field and \( t_p \) is the pulse width.

For one particular value of the applied rf field, \( B_1 \), and at a fixed pulse width, \( t_p \), the flip angle \( \theta \) will be equal to 90°. This is called a 90° pulse. The magnetization vector will be flipped on to the \( xy \) plane. The result is a transverse magnetization component rotating in the xy plane (FIG-1D).

Once excited the magnetization returns to its equilibrium value as described by two first order time constants. They are the spin-lattice relaxation time (\( T_1 \)) and the spin-spin relaxation time (\( T_2 \)) [3], [4], [8]. Spin-lattice relaxation time \( T_1 \), denotes the exponential growth of the longitudinal magnetization \( M_z \) to its equilibrium value \( M_0 \). During this process energy is dissipated to the surroundings or the lattice. The spin-spin relaxation time \( T_2 \), denotes the decay time constant of the transverse magnetization components \( M_y \) and \( M_x \). Here the nuclear energy is redistributed among the other spins. The result is the dephasing of the nuclear spins and the loss of phase coherence to reach the equilibrium value, zero. Therefore when the pulse is turned off the transverse magnetization will precess freely about the \( xy \) plane relaxing with the time constant \( T_2 \). This forms the familiar free induction decay (FID). In practice the NMR receiver is designed to detect the voltage induced by this rotating and decaying transverse magnetization or FID.

Bloch et al explained this behaviour and the motion of the magnetization vector in an external magnetic field by a differential equation. The solutions for this Bloch equation are documented in almost every NMR text book [3], [8], [50].

Once the 90° pulse is terminated the transverse magnetization components, \( M_y \) and
(FIG-1) (A) The precession of a nuclear magnetic moment $\mu$ about the external magnetic field $B_0$ (B) The precession of an ensemble of proton nuclei in an applied magnetic field. (C) The behaviour of the non-equilibrium macroscopic magnetization vector (M) in the rotating frame. At equilibrium this $M_0$ is oriented along the $B_0$ field ($z$ axis). (D) Precession of M in the transverse plane following the application of a 90° pulse.
\(M_x\) and \(M_y\) respectively, are given by the following equations.

\[
M_x = M_0 \cos(\Delta \omega t) e^{-t/T_2} \tag{5}
\]

\[
M_y = M_0 \sin(\Delta \omega t) e^{-t/T_2} \tag{6}
\]

where \(M_0\) is the equilibrium magnetization, \(\Delta \omega\) is the resonance offset, \(M_x\) and \(M_y\) are the transverse magnetizations along \(x\) and \(y\) at time \(t\) and \(T_2\) is the transverse relaxation time.

The detected free induction signal is amplified, digitized and stored for processing. Acquiring the signal in the quadrature detection (QPD) mode makes it possible to detect both the transverse magnetization components \(M_x\) and \(M_y\). The important step is to regain the frequency information in the time signal or the FID and to obtain a spectrum. This is done by a mathematical analysis technique known as the Fourier transform [7]. It enables the determination of the periodicity of a property which varies with time by integration over all time values. Fourier transformation (FT) of the complex time domain signal obtained in QPD yields a complex frequency domain spectrum. The frequency signal \(S(\omega)\) is given by the following equation.

\[
S(\omega) = \frac{M_0 T_2}{(1 + T_2^2 \Delta \omega^2)} + \frac{i M_0 T_2^2 \Delta \omega}{(1 + T_2^2 \Delta \omega^2)} \tag{7}
\]

where \(i = \sqrt{-1}\).

The real part of this equation corresponds to the Lorentzian absorption line and the imaginary part designates the respective dispersion line.

### 1.1.1 Spin lattice relaxation time \((T_1)\)

Consider a case where the magnetization vector \(M_0\), has been flipped away from the \(z\) axis by an rf pulse. After the pulse the longitudinal magnetization grows exponentially to regain the equilibrium value \(M_0\). This exponential growth (relaxation) is characterized
by the time constant $T_1$. Several mechanisms are believed to be responsible for the $T_1$ relaxation time and the reader is directed to reference [8] for details. The important fact is that the $T_1$ relaxation times often portray the local molecular environment. This brings the necessity to study relaxation times to understand molecular behaviour, biological systems in particular, since the relaxation times are found to yield information about abnormal biological conditions [12].

During this study the method used to determine the $T_1$ value was the inversion recovery technique [51]. The pulse sequence used in this method is as follows,

Relaxation delay $- 180^0 - \tau$ (Delay) $- 90^0 -$ Acquisition

The behaviour of the magnetization vector during this pulse sequence is shown in FIG-2. At equilibrium the magnetization vector, $M_0$, is aligned along the $z$ direction (FIG-2A). The initial $180^0$ pulse along the $x'$ axis flips $M_0$ so that it lies along the $-z$ axis (FIG-2B). Once disturbed, the magnetization vector starts to relax exponentially to reach its equilibrium value $M_0$ which is along the $+z$ axis. At time $\tau$ the magnetization components have relaxed, so that the new magnetization component along $z$ ($M_z$) will be smaller than the respective component immediately after the inverting $180^0$ pulse (FIG-2C). Application of the $90^0$ pulse along the $x'$ axis will flip the magnetization vector, $M_z$ on to the $-y'$ axis (FIG-2D). The signal is acquired immediately after the $90^0$ pulse. A delay is imposed after each acquisition in order to make sure of complete relaxation or reaching equilibrium. A set of spectra obtained at several delay times is shown in FIG-2E.

The exponential growth of the non-equilibrium magnetization vector, $M_z$, following a $180^0$ pulse is described by the following equation. (FIG-2F)

$$M_z = M_0 \left( 1 - 2 e^{-\tau/T_1} \right)$$

(8)
FIG-2 Determination of $T_1$ by the inversion recovery sequence.

(A) The magnetization, $M_0$, at equilibrium is aligned along the +z axis. (Note the arrow head along $\mathbf{\sigma}$ denotes the application of a pulse)

(B) Application of an $180^\circ$ pulse along $\mathbf{\sigma}$ axis flips the magnetization $M_0$, on to the -z axis ($-M_0$).

(C) The magnetization is relaxing to its equilibrium value, $M_0$. After a time $r$ (=t) the magnetization along -z axis is $M_r$. Note that at time $r$, $M_r < M_0$.

(D) Application of a $90^\circ$ pulse along $\mathbf{\sigma}$ axis. This carries the remaining magnetization, $M_r$, along -z axis to the $-y'$ axis. This magnetization component is acquired as the NMR signal.

(E) A stacked plot of several NMR spectra obtained at different delay times ($r$). At short delay times the magnetization lies along the -z axis and therefore a $90^\circ$ pulse flips it to the $-y'$ axis which is $180^\circ$ out of phase with respect to a signal corresponding to a larger delay time.

(F) A plot between the intensity of the peaks (signal heights) versus the delay time, $r$, to obtain the $T_1$ value. The arrows show the relative growth of the magnetization during relaxation.
where $T_1$ is the spin-lattice relaxation time.

The magnetization is directly proportional to the signal intensity [4],[8] and in practice what is measured is the intensity of the NMR signal. Therefore equation (8) can be altered as follows,

$$\ln(A_\infty - A_r) = \ln 2 A_\infty - \frac{r}{T_1}$$

(9)

$A_\infty$ corresponds to the equilibrium magnetization while $A_r$ corresponds to the magnetization vector at time $r$.

Therefore a plot of $\ln(A_\infty - A_r)$ versus $r$ values yields the value of $T_1$.

### 1.1.2 Spin-Spin Relaxation ($T_2$)

Consider the application of a $90^\circ$ pulse along the $x$ axis to a spin system at equilibrium. As a result $M_0$ will be flipped on to the $xy$ plane. Once on the $xy$ plane, natural processes cause exchange or redistribution of energy between neighbouring nuclei. Thus, the transverse magnetization component $M_{xy}$, loses phase coherence (i.e. the magnetic moment vectors begin to spread out or dephase). This loss of phase coherence or decay is characterized by the $T_2$ relaxation time. Thus, following the $90^\circ$ pulse the NMR signal (FID) decays with a time constant $T_2$. The decay of the transverse magnetization $M_{xy}$, is given by the following equation.

$$M_{xy} = M_0 e^{-(t/T_2)}$$

(10)

As the time, $t$, increases the transverse magnetization vector tends to zero. At some point the $M_{xy}$ will decay to an undetectable level [3].

In practice, the $B_0$ magnetic field is never uniform over the sample of interest. This nonuniformity, more commonly known as the magnetic field inhomogeneity, causes the
transverse component to lose phase coherence (dephase) faster than it would under pure spin-spin relaxation. The FID decay time constant in the presence of magnetic field inhomogeneity is designated as \( T_2^* \) defined as,

\[
\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_{2\text{inhomogeneity}}}.
\]  

(11)

where \( \frac{1}{T_{2\text{inhomogeneity}}} \) represents the field inhomogeneity contribution to the decay of the FID.

In practice field inhomogeneity makes a significant contribution to the disappearance of the transverse magnetization \([52]\) and therefore to the lineshape of the \( NMR \) line \([8]\). Also during the spin-spin relaxation the spin system dissipates energy to the surroundings by the spin-lattice relaxation. The important point that should be noted is that the expression,

\[ T_2^* \leq T_2 \leq T_1 \]

generally applies \([8]\).

Hahn's discovery of spin-echoes \([53]\) demonstrated that one could overcome the effect of \( B_0 \) field inhomogeneity and observe the pure spin-spin relaxation decay. This experiment consists of a 90° pulse followed by a 180° pulse after a delay \( \tau \). The behaviour of the spin system during this pulse sequence is shown in FIG-3. Following the 90° pulse (FIG-3A and B) the spin system dephases during the period \( \tau \) (FIG-3C) because of the \( T_2 \) relaxation and the magnetic field inhomogeneity. The 180° pulse, which is applied along the \( z' \) axis after time \( \tau \), rotates the dephasing magnetization components about the \( z' \) axis by 180° (FIG-3D). Since the actual \( T_2 \) is a natural process the 180° pulse does not have any effect on this component. But more importantly the component due to the inhomogeneity is changed (FIG-3E). Therefore after a time \( \tau \) these components refocus along the \(-y'\) axis to form an echo (FIG-3F). This is known as a spin echo. The spin echoes at different \( \tau \) values will depict the loss in echo intensity which is primarily a function of the actual \( T_2 \) value given
FIG-3 Hahn spin echo sequence \( (T_2 \text{ measurement}) \).

(A) Application of a 90° pulse to a spin system which is at equilibrium.

(B) The magnetization vector, \( M_0 \), has been flipped to the \( xy \) plane.

(C) The individual spin vectors dephase primarily due to pure \( T_2 \) relaxation and magnetic field inhomogeneity collectively known as the \( T_2^* \). This dephasing is allowed to occur for a time \( r \). (The \( z \) axis is not shown in the rest of the figures for clarity.)

(D) A 180° pulse along \( z \) axis carries the dephasing spins on to the \(-y\) axis.

(E) The result is that the magnetization vectors which were dephasing in the rotating frame starts to converge towards one another.

(F) After another time \( r \) the magnetization vectors refocus the dephasing inhomogeneity component to form an echo (Hahn echo). The \( T_2 \) is a natural process and hence there is a finite loss in magnetization. Therefore the magnetization along \( y \), \( M_I < M_0 \). Repeating the experiment at different \( r \) values and observing the signal intensity, enables one to calculate the \( T_2 \) value.
in equation (11). Several modifications to this method, which effectively rely on the formation of an echo, have been suggested in order to measure the spin-spin relaxation time more accurately [8][54][55]. The basic idea of this Hahn spin echo sequence was used, firstly, in the imaging pulse sequence used in this study, and secondly in the determinations of the $T_2$ values. Some $T_2$ values were determined by using a modified Hahn pulse sequence (viz. CPMG) which is given below.

Carr-Purcell-Meiboom-Gill $90^\circ y - (\tau - 180^\circ y - 2\tau - 180^\circ y - \tau)_n - \text{Acquisition}.$

Nuclear spin-spin relaxation time is dependent on the molecular reorientation time and consequently upon the molecular diffusion [8]. Therefore on the one hand it affords a powerful method to study molecular motions and on the other, as will be seen later, it imposes a severe limitation on NMR microscopy. Molecular diffusion also facilitates the dephasing of the $M_{xy}$ component [8],[54] and will therefore obscure the true $T_2$ decay. In a Hahn experiment, taking molecular diffusion into account, the echo amplitude after time $2\tau$ is given by the following equation.

$$M(2\tau) = M_0 e^{(-2\tau/T_2)} e^{(-\frac{2}{3} \tau^2 G^2 D \tau^3)}$$

(12)

$M(2\tau)$ corresponds to the echo amplitude at time $2\tau$, $G$ is a linear field gradient, $D$ is the diffusion coefficient, $\tau$ is the time between the initial $90^\circ$ pulse and the refocussing $180^\circ$ pulse.

1.2 Two Dimensional (2-D) Spectroscopy

In pulse NMR, there are at least two time intervals separately under the control of the experimenter, as independent variables. Corresponding to these two time domains there are, after a double Fourier transform, two different frequency domains. NMR signal intensity
may therefore be depicted in two dimensions, as a function of either the two times or the
two frequencies. Any such experiment may be referred to as 2-D NMR [11]. Imaging, in
which the field gradients are used (see following section) so that the two dimensions may
be related to spatial positions, is a special case of 2-D NMR, with substantial differences
from the other technique covered by that term. In this section, 2-D NMR is discussed in
general as a preliminary to the discussion of NMR imaging.

Almost any pulse NMR experiment, regardless of the dimensionality (1-D, 2-D) or the
technique (spectroscopy, imaging), can be divided into three main time sequences. This is
shown in the following figure.

\[ \text{PREPARATION TIME} \quad | \quad \text{EVOLUTION TIME} \quad | \quad \text{DETECTION TIME} \]

\[ t_1 \quad | \quad t_2 \]

FIG-4 The time sequences in a pulse experiment.

In conventional (1-D) spectroscopy the evolution time \( t_1 \), is either zero, a constant or a
parameter which is not Fourier transformed. The response which is either a FID or a spin
echo (depending on pulse sequence used) is detected as a function of the detection time \( t_2 \).
As there is only one variable time the spectrum obtained is essentially a one dimensional
spectrum. In contrast, in 2-D NMR, the evolution period \( t_1 \) is varied as a parameter in
successive experiments [9]. Keeping all other parameters constant and varying \( t_1 \) yields a
2-D time signal, $S(t_1, t_2)$ (c.f. Fourier imaging method by Kumar et al [30]). That is, if a systematic variation in the evolution period $t_1$ results in a periodic change of the state of the spin system under investigation, in theory, one should be able to perform Two-Dimensional experiments [56]. These two independent time variables are Fourier transformed twice (double FT) to obtain a double frequency response [11],[57]. The resultant two frequency variables are usually plotted on two axes normal to each other. Depending on the interest the plot can be of two types. Generally, a series of spectra (similar to 1-D) corresponding to the number of increments of $t_1$ are stacked one behind another. This gives a 3-D appearance to the final spectrum (the 2-D images were also plotted in a similar manner). In this 2-D spectrum the behaviour of the spin system during both the evolution period ($t_1$) and the detection period ($t_2$) is shown simultaneously. Correlation between these two parameters is an extra source of information.

An important factor in performing 1-D or 2-D spectroscopy is that the applied magnetic field over the sample must be as homogeneous as possible. Presence of inhomogeneity or field gradients would essentially reduce the quality of the final spectrum mainly by broadening the NMR lines.

1.3 Effect of magnetic field gradients on the spectrum.

The magnetic field inhomogeneity is known to be the major cause for line broadening in high resolution NMR [8],[52]. Field inhomogeneity can be regarded as a random magnetic field gradient across the sample and little useful information can be obtained from such a situation. On the other hand, consider the case where the uniformity of the static magnetic field is degraded deliberately by applying a carefully monitored linear field gradient across a sample. This will cause the magnetization vectors at different local sites in the sample to
experience slightly different static magnetic fields. This is given by the following equation.

\[ B_z(y) = B_0 + (G_y \cdot y) \]  

(13)

where \( B_z(y) \) is the static magnetic field along the z axis at coordinate y in the presence of the static linear field gradient along the y axis, \( G_y \). This in turn implies that the nuclear spins at different locations will have different resonance frequencies (\( \omega \)) given by equation (14).

\[ \omega_y = \omega_0 + \gamma G_y \cdot y. \]  

(14)

where \( \gamma B_z(y) = \omega_y \) and \( \gamma B_0 = \omega_0 \). Therefore varying the amplitude of the field gradient, at different locations of the sample, will generate a distribution of precessional frequencies across the sample [23]. This application of the linear field gradient (say along y) will permit one whole plane perpendicular to the applied gradient direction (y dimension) to experience the same magnetic field and hence to resonate at the same frequency. It is this spin behaviour in an applied field gradient which lays the basis for NMR imaging.

1.4 NMR imaging

1.4.1 One dimensional (1-D) projections.

If a linear field gradient [26] is applied across a sample, immediately after a 90° pulse, the transverse magnetization components will experience different magnetic fields and hence will have correspondingly different precessional frequencies. This response, like a normal FID, can be acquired as a time signal and processed. If the natural linewidth due to spin-spin relaxation time and magnetic field inhomogeneity is small compared to the line width obtained in the presence of the gradient, the frequency spectrum corresponds to the spin density function of the system [23][58]. This is explained more rigorously by Kumar et al [30]. More generally, the spectrum (\( S(\omega) \)) obtained in the presence of a gradient along the
$y'$ axis (for e.g.) is given by [58],

$$S(\omega) = \int c(y) S(\omega, y) \, dy.$$  \hspace{1cm} (15)

where, $c(y)$ is the nuclear spin density projected onto the $y$ axis and $S(\omega, y)$ is the NMR signal from a plane of spins at a particular $y$ coordinate.

As indicated earlier, the transverse magnetization components which result in the NMR signal, can be Fourier transformed to yield a 1-D projection which reflects the spatial coordinates of the spin system. In practice, if the object is simple such as a disk filled with water (FIG-5A), assuming homogeneous distribution of spins within the system, the projected profile will resemble the cross sectional shape of the object (FIG-5A). However, realizing the shape of an object by a single 1-D projection becomes more and more difficult as the system gets complicated. Consider the square shown in FIG-5B. Assume the shaded areas of the 16 volume elements (voxels) to be uniformly filled with water. Application of a linear field gradient along the direction shown in FIG-5 will yield a 1-D projection profile as shown in FIG-5B. Theoretically, there are 96 different ways to fill these voxels to obtain the same 1-D projection [26]. This calls for more such 1-D projections from different orientations if one is to specify the spatial coordinates accurately. Generally, the higher the asymmetry the larger the number of such projections needed to specify the object. In this example the spin systems were assumed to be homogeneous. However, in reality, the system is often extremely heterogeneous (e.g. biological systems).

In general, the application of gradients in all three physical dimensions enables one to encode spatial information to the whole sample. The NMR signal obtained in the frequency domain $S(\omega)$ can be considered as a composite of the contributions from all the volume elements of the sample. This can be represented by the following equation [58].
(A) Application of a gradient along y, $G_y$, across a simple circular object uniformly filled with water. A single 1-D projection identifies the shape of the object.

(B) Application of the $G_y$ gradient to a more complicated system which is divided into 16 identical voxels. The shaded areas depict the spins and it is assumed that the distribution of spins within a voxel is uniform. There are 96 different ways that the spins can be arranged among the voxels to yield the same 1-D projection shown in FIG-5B. Therefore, as the system gets more complex, several such projections will be required to uniquely define its shape.
where $c(v)$ is the 3-D spin distribution function and $S(v, \omega)$ is the NMR signal from a volume element.

The method by which the spatial information is obtained in order to construct the final image will depend on the imaging technique used.

1.4.2 NMR imaging techniques.

Several imaging techniques have been developed since the first NMR imaging experiment. Three main imaging techniques are outlined in the following sections. The interested reader is referred to reference [26] and the original literature cited therein for a rigorous treatment of all the existing imaging techniques.

1.4.2.1 Projection reconstruction method.

This method was used by Lauterbur to obtain the first $NMR$ image [25]. The technique uses gradients along different directions to yield several 1-D projections of the object. The free induction decay after a $90^\circ$ pulse is allowed to evolve under the influence of a linear magnetic field gradient along a particular direction (say $y$). The direction of the gradient is then changed by applying a linear combination of two orthogonal gradients ($x$ and $y$) generated by two gradient coils. The final result is a series of 1-D projections obtained along different directions. The image is constructed by the projection reconstruction method [26]. The pictorial representation of this technique is given in FIG-6A. This method has now been replaced by the Fourier imaging methods discussed below. Besides being of historical importance, some authors point out some advantages of this method over the widely used Fourier imaging techniques [59].
1.4.2.2 Fourier imaging (Fourier zeugmatography.)

This method was first described by Kumar et al. [30]. It has a very close resemblance to the familiar 2-D spectroscopic technique. Basically, instead of letting the spin system evolve freely during $t_1$, in Fourier imaging, this evolution is accompanied by a linear magnetic field gradient. The pulse sequence used in a typical Fourier imaging experiment is shown in FIG-6B. Here, after the 90° pulse, the linear field gradients $G_x$ and $G_y$ are applied to the system in succession. The evolution period $t_z$ (where $G_x$ is applied) is incremented by constant intervals. The resulting signal for each increment is acquired as a function of time $t_y$ when the gradient $G_y$ is on. This yields a 2-D data matrix [23][30]. Double FT of the time domain data matrix $S(t_z,t_y)$ yields the respective frequency function $S(\omega_z,\omega_y)$ which corresponds to the spatial coordinates of the spins in the xy plane [23][58]. In order to understand the pulse sequence shown in FIG-6B, assume a small region of interest (voxel) in the sample located at coordinates $x,y$. Application of the $G_x$ gradient will alter the angular precessional frequency of the magnetization within this region to $\omega_z = \omega_0 + \gamma G_x x$, where $\omega_0$ is the offset frequency when $G_x = 0$. Thus after the evolution time $t_z$, the transverse magnetization, will have acquired a phase angle $\phi_z$.

$$\phi_z = (\omega_0 + \gamma G_x x) t_z$$

(17)

During the signal acquisition time $t_y$, where the $G_y$ is on, the precessional frequency is given by $\omega_y = \omega_0 + \gamma G_y y$. The phase of the signal reflects the position of the voxel in the x dimension. The final signal which is a function of $t_z$ and $t_y$ is given by the following equation [23].

$$S(t_z, t_y) = M_0 \rho(x, y) e^{i[(\omega_0 + \gamma G_x x) t_z + (\omega_0 + \gamma G_y y) t_y]} e^{-\left(\frac{t_y}{T_2}\right)}$$

(18)

where $\rho(x, y)$ is the spin density at coordinates $x,y$, $t = t_z + t_y$.

In the above sequence, during the time $t_z$, the magnetization acquires different phases
depending on the x coordinate, while during time $t_y$, the precessional frequency of the transverse magnetization components depends on the y coordinate. Therefore the gradients applied during these time periods, $t_x$ and $t_y$, are known as the phase encoding gradient and frequency encoding gradient respectively. Double FT of this time signal will yield a frequency domain signal which is related to the spatial coordinates and the spin density of the system [23]. By increasing the time $t_x$, for which the gradient (constant magnitude) is applied, several such signals can be obtained. Double FT of these signals will yield the corresponding frequency signals. An appropriate plot of these signals will correspond to a 2-D image representing the spin density of the system. This method has virtually been replaced by the more versatile spin warp imaging technique. Nevertheless this method should be appreciated from a standpoint of introducing the familiar multidimensional FT method to obtain images.

### 1.4.2.3 Spin warp imaging

The fundamental basis of this technique is similar to the Fourier imaging technique introduced by Kumar et al. The significant difference is that the time, $t_x$, for which the phase encoding gradient is applied is kept constant in this method. The effect of varying the phase encoding time, $t_x$ (Kumar’s method) is brought about by incrementing the magnitude of the phase encoding gradient in successive experiments [23][60]. The basic behaviour of the spins in the spin warp sequence is very similar to that of the Fourier imaging technique. Now the 2-D data matrix obtained corresponds to $(G_x, t_y)$ where the final signal can be given by equation (18) [23]. The obvious advantage of this method is that, because the evolution period is constant (less loss in signal due to $T_2$ process when compared to Fourier imaging), the sensitivity of this technique will be higher [23]. Here the signal is acquired as an echo by the use of a 180° refocussing pulse. The basic pulse sequence used in a spin warp experiment is shown in FIG-6C.
FIG-6A Projection reconstruction method.

(a) A combination of x and y gradients are used to obtain several 1-D projections which portrays the spin distribution in the respective gradient direction. (From reference [25])

(b) The simplified diagram of the rf and gradient pulses in a series of experiments each combination of a rf, $G_y$ and $G_z$ giving a single 1-D projection. (From reference [61])

FIG-6B Fourier imaging.

(a) The basic pulse sequence used in fourier imaging. The phase encoding gradient, $G_z$, is applied during the evolution period. The time for which it is applied is incremented for each data set (the incrementing of the gradient is shown in the time axis). The gradient magnitude is kept constant. The frequency encoding gradient, $G_y$, is applied during acquisition (the open end of the gradient means that the gradient is on through out the acquisition).

(b) A pictorial elaboration of the Fourier imaging technique. The phase encoding gradient, $G_z$, is increased in the time axis but note that its amplitude is constant. The process is shown for simplicity but note that one rf pulse, one $G_z$ and the frequency encoding pulse combine to form one data set.

FIG-6C The spin warp imaging technique.

(a) The pulse sequence used in a typical spin warp sequence. The phase encoding gradient amplitude is incremented (c.f. The Fourier imaging technique). The signal is acquired as an echo after the refocussing $180^0$ pulse. The frequency encoding is on during acquisition. (b) The gradient incrementation in this study was done from $-G_z$ to $+G_z$. 
1.5 Sensitivity and Performance time

As in conventional NMR, the sensitivity of a NMR imaging experiment can be increased by acquiring a large number of scans. However in practice the time scale lays severe constraints on NMR imaging techniques. Imaging experiments are extensively performed on biological samples. In a living organism biological processes are continuous. Therefore it is clear that in order to monitor such a system the imaging time (performance time) should be as short as possible. That means that if NMR imaging is to be used in medicine and biology it is necessary to obtain images in a short time and also obtain the maximum S/N.

The sensitivity and the performance time for various imaging techniques are reviewed elsewhere [23], [26], [62], [63]. In general the relationship between S/N (Ψ) and the number of scans is given by,

$$\Psi \propto \sqrt{N}$$  \hspace{1cm} (19)

where,

$$\frac{t_{total}}{t_1} = N$$  \hspace{1cm} (20)

and Ψ is the S/N ratio, N is the number of scans, t_{total} is the total time taken to perform an imaging experiment and t_1 is the time taken for one scan.

It is clear that the increase in N would increase the signal to noise (Ψ) but the total time taken will also be increased accordingly. The efficiency of the imaging technique lies on obtaining the maximum S/N in the shortest possible time (smallest number of scans, N). This shows that the increase in the total imaging time will not necessarily increase the efficiency of the technique. One way to circumvent this problem is to reduce the flip angle (i.e. use less than a 90° pulse). With a reduced flip angle, the system will return to equilibrium faster [59]. Therefore the repetition time can be shorter and more scans can be acquired in a given time. However, one should be able to tolerate a certain amount of loss in S/N during this procedure. It has been shown that the maximum sensitivity is obtained
by functioning at the Ernst angle \[10\].

Optimizing the necessary conditions to obtain the maximum S/N will reduce the performance time. This will also increase the quality of the image and the resolution. The signal from a given volume sample is more or less constant. But the noise sources in a NMR instrument are numerous. It is fair, however, to consider noise generated at the probe and the preamplifier to be by far the most important in determining the final S/N ratio \[59\]. The topic of S/N in biological samples has been treated by many authors in detail \[62\], [64]. The S/N is shown to be mutually coupled to several instrumental and experimental conditions \[64\]. One important expression which is noteworthy is given below.

\[
\Psi = K \frac{\omega^{7/4}}{\Delta f^{1/2}} V_s T_s^{-1/2}
\]  

where \(\Delta f\) is the sweep width, \(\omega\) is the resonance frequency, \(V_s\) is the sample volume, \(T_s\) is the sample temperature and \(K\) is a function of several parameters; e.g. Coil Q, coil temperature, filling factor \[64\].

The control of the parameters in the above equation is clear. For instance, S/N will be higher in higher frequency spectrometers (\(\omega\)). But other mechanical problems \[26\] such as power dissipation and winding of rf coil to low inductance arise. This is because of the complex dependence of S/N on several factors. The important point is the direct relationship between the S/N and the sample volume. The NMR signal is proportional to the number of protons in a given volume which lies in the rf coil. Although it appears that increasing the volume will give better S/N, other constraints will always limit this possibility. However, assuming that the other factors are in favour, the voxel size plays an important role in the S/N ratio.

Consider an object with 2-D volume element (voxel) \(N \times N\) (the voxel is assumed to have an infinitesimally small thickness). Assume the spins within each voxel to be homo-
geneous. Accurate determination of the spatial coordinates by a Fourier imaging technique will require $N$ different experiments \[23,26\]. Let the time taken for one experiment be $t_r$. The time taken to obtain a given $S/N$ and to resolve all the $N \times N$ voxels will be $t_r N$. Assume that the spatial resolution is increased by a factor of four in both dimensions. The new image will be represented by $4N \times 4N$ voxels. The voxel size that the signal is received from will be reduced by a factor of 16. That is, the $S/N$ of the image with high spatial resolution will be reduced by a factor of 16. Since the $S/N \propto \sqrt{\text{total time}}$, it is clear that in order to obtain the same $S/N$ as in a voxel $N \times N$ the imaging time must be increased by a factor of $16^2$. Hence, attempting to reach higher spatial resolution seems to encounter a unique set of obstacles in NMR imaging.

1.6 Imaging in the microscopic realm

Ideally a microscope is required to observe an object which is not clearly visible to the naked human eye. Any technique which will observe small objects (or small details of the structure of large objects), beyond the resolution of the human eye, may be called microscopy. The eye has an angular resolution of about $10^{-3}$ radians, or 0.25 mm (250 \(\mu m\)) at the minimum distance of distinct vision (about 25 cm). Optical microscopes are used to extend this range down to about 1 \(\mu m\). This permits observation of the larger organelles within biological cells, such as the nuclei.

The modern biologist has become accustomed to observation, with the transmission electron microscope, of intracellular features down to a few nanometres, i.e. a further factor of 100 to 1000 beyond the limit of resolution of the optical microscope.

The improvements in the NMR imaging technique are now giving resolution in a range between approximately 1 mm resolution which is good enough for most medical applications and the resolution $10^{-3}$ to $10^{-6}$ times better which biologist nowadays think of as
microscopy. The range of applications for the intermediate range of resolutions is limited (but might include much of developmental biology). But it is also important to note that, at any spatial scale, NMR "sees" different features from optical or electron microscopy.

*NMR* imaging on a microscopic scale imposes certain problems which will be discussed in the following sections. One important point is that the detection of the signal from smaller volumes is often a problem in spectroscopy as well as in imaging. The *NMR* technique generates signals which are inherently weak [4],[39]. When the dimensions of interest (voxel size) decrease this become more and more critical. This is because the number of protons per unit volume is decreased. This not only reduces the S/N ratio but also the detectability or resolution [39]. Usually the quality of a microscope is assessed by the resolution attainable by the particular microscopic technique. Hence resolution is of prime importance to any microscopic technique.

### 1.6.1 Resolution

The word resolution in *NMR* imaging does not have a universal definition. The ambiguity may be partly due to its imprecise use. There may be several definitions for resolution. Two such definitions are considered in this thesis [42].

One method defines resolution in terms of the dimensions of the smallest object which can be detected in the imaging procedure. Consider a spin warp experiment (with 256 phase encoding gradient increments) performed on a block size of 512. The final image obtained will be a square digitized by 65536 points. This is usually known as a $256 \times 256$ image. Each of these points is assumed to designate the intensity of the spin density of the volume element or voxel. This image is plotted on an appropriate TV-graphics screen. Each of these point is now known as a picture element or pixel. Hence the image described above will be defined by $256 \times 256$ pixels. Therefore an image, from the standpoint of a
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computer, can be considered as a representation of a set of 3-D voxels by an arrangement of 2-D pixels. If the length of the object or the excited area is known, the length of a single pixel can be calculated. For instance let the length of the object be 5.12 mm. Assuming it covers the whole image displayed, the pixel width in a $256 \times 256$ image would be $5120/256 \mu m$, which is about $20 \mu m$. This is known as the pixel resolution. Features smaller than the pixel size cannot be seen or more precisely cannot be distinguished from one another. Many authors seem to use this method to indicate the resolution of NMR micrographs.

The second method of defining resolution (from optical microscopy) relies on defining the minimum limit of observing a system isolated from the adjacent surroundings. This definition is used to indicate the resolution of the images presented in this thesis. It seems more logical to consider resolution as to involve at least two sites than one single site. Resolution hence, can be defined as the ability to uniquely observe a certain structure of a system or the system itself which is separated by a real or an imaginary boundary from the rest of the system or the surroundings.

Consider two points which are spatially resolved. Since any physical measurement is imprecise, these two points will be represented by a distribution function which can be designated as a point broadening function of half height width $\Delta A$ [42]. Let the actual separation between two such points be $\Delta B$. (FIG-7). This generalized idea can be extended to NMR imaging where $\Delta A$ corresponds to the half height linewidth of the peak and $\Delta B$ is the spatial separation between two positions of interest. Assume a condition where these peaks are brought closer together or $\Delta B$ being reduced. The resolution limit is considered to be the point at which $\Delta A = \Delta B_0$ (FIG-7C). That is, the smallest resolvable width is the half height line width of the NMR line.

The resolution defined by this method cannot be predetermined. This means that knowing the sample dimensions and the image size will not necessarily yield the true
FIG-7 The diagramatic interpretation of the resolution limit [42]. Two distribution functions from adjacent voxels are considered. (A) The individual line width of the peak $\Delta A$ is smaller than the separation between the peaks (voxels) $B_1$ (B) Consider the two distribution functions as having origins at a closer distance when compared to the peak width $\Delta A$. (C) The case where $\Delta A = \Delta B_3$. This is known as the resolution limit. (Modified from reference [54])
resolution in the image. The true resolution in this case will depend on the sample being studied and on instrumental factors.

1.7 Factors that affect resolution in NMR microscopy

The factors affecting resolution in microscopic images were first discussed by Mansfield et al [65]. Since then several authors have addressed this topic in considerable detail [59],[63]. The discussion in this section will be a general overview of some of the important factors.

The contributions to differing signal intensities from two adjacent voxels, and hence to the possibility of resolving the two from each other, may be discussed under six categories;

1) Variation in the spin density from voxel to voxel.

2) Chemical shifts from two or more entities from a voxel.

3) Signal digitization and processing.

4) Relaxation times, as influencing linewidth.

5) Relaxation times, as effecting sensitivity.

6) Motion of the voxel and within the voxel.

1.7.1 Variation in spin density from voxel to voxel

Some biological samples have small "wet" areas immediately adjacent to extensive dry areas. That is, there is good contrast between two adjacent voxels because of the drastically different relative water content. In such a case, the minimum voxel size detectable by the sensitivity of the instrument (pixel resolution) is most probably the spatial resolution. Plant stems are promising samples in this respect [48]. More commonly, however, variations in water proton density from voxel to voxel in a biological sample will not be as clear cut
as mentioned above. In that case, if the detection limit (pixel resolution) is a 10\(\mu\)m voxel length, the resolution might not be as good as 10\(\mu\)m.

### 1.7.2 Chemical shift differences within a voxel

Considering a biological sample the two major constituents will be water (chemical shift \(\sim 4.8 \text{ ppm}\)) and fat (chemical shift \(\sim 1.2 \text{ ppm}\)). Therefore in a 270 MHz spectrometer these two peaks will be separated by about 972 Hz. If the applied gradient is not large enough to encompass these two peaks the response would be a composite of the proton densities of the two components [39],[43],[63]. This will probably lead to image artifacts, because the chemical shift is interpreted as a spatial shift in the data processing. This problem was not crucial to this study because, firstly, the gradient used was quite high (\(\sim 15 \ \text{G cm}^{-1}\)) and, secondly the system studied comprised more than 80% water.

### 1.7.3 Signal digitization and processing

Optimization of the experimental parameters is essential in order to obtain better images. In an imaging experiment the observation bandwidth (sweep width) is chosen such that the outer limits of the bandwidth exceed the dimensions of the object of interest. This full width is known as the field of view (FOV). If the image is to have the same spatial scale in all directions, the FOV in the frequency encoding dimension has to be equal to the FOV in the phase encoding dimension.

The field of view (FOV) in the frequency encoding dimension is given by the following equation.

\[
FOV_y = \frac{2\pi}{\gamma G_y DW}
\]  \(\text{(22)}\)

where \(FOV_y\) is in cm, \(G_y\) is the frequency encoding gradient, and \(DW\) is the dwell time.
In theory, higher resolution can be achieved by the application of larger gradients. This is because larger gradients increase the difference in precessional frequencies between adjacent points. In this case the sweep width should be increased accordingly in order to encompass this larger frequency spread. This process will reduce the digital resolution if the block size is not increased.

The FOV in the phase encoding dimension is given by the following equation.

\[ FOV_x = \frac{2\pi}{\gamma G_{z_i} \cdot t_\phi} \]  

(23)

where \( G_{z_i} \) is the phase encoding gradient increment and \( t_\phi \) is the phase encoding time.

The resolution in the phase encoding dimension will be directly proportional to the number of phase encoding gradient increments [23],[66]. This is discussed further with a sample calculation in Appendix-B.

1.7.4 Relaxation times, as influencing line width

In order to achieve the required spatial resolution it is necessary to separate each voxel. Thus, in the frequency domain, frequency separation (caused by the gradient) between adjacent voxels should be larger than the line broadening caused by other factors in the same voxel. A quantitative approximation can be obtained as follows. The line width at half height can be denoted as [8],

\[ \nu_{1/2} \geq \frac{1}{\pi T_2^*} \]  

(24)

This can be altered as follows,

\[ \gamma G \delta x \geq \frac{2\pi}{\pi T_2^*} \]  

(25)

G is the applied gradient and \( \delta x \) is the resolution limit.

\( T_2^* \) is the line broadening contribution from many factors. This can be generalized by
the following expression [26].

\[
\frac{1}{T_2^*} = \frac{1}{T_2 \text{ natural}} + \frac{1}{T_2 \text{ mag.fld.inhom.}} + \frac{1}{T_2 \text{ mag.suscep.}} + \frac{1}{T_2 \text{ diffusion}}
\]  

(26)

Each of these factors will be considered briefly.

a) Natural spin spin relaxation and magnetic field inhomogeneity.

Many authors have shown that the line broadening due to these factors is not a critical factor in NMR microscopy [26],[63]. It can be shown that, according to equation 25, if the gradient was $10 \text{ G cm}^{-1}$ and the $T_2^*$ for a biological sample was 25 ms, the attainable resolution will be 2.9 $\mu$m. It is clear that low $T_2$ values will make the transverse magnetization decay faster. This fast decay can reduce the S/N attainable in a given time which might effect the resolution indirectly. It should be mentioned at this point that adding paramagnetic contrast agents broadens the NMR line which might consequently degrade the resolution.

b) Effect due to magnetic susceptibility differences

The field changes that occur at biological interfaces as well as external interfaces can generate inhomogeneity and therefore reduce resolution. Hedges has treated this subject extensively by considering magnetic susceptibility values at different conditions such as solid/liquid, solid/gas, liquid/liquid interfaces [63], where it is shown that the susceptibility differences can correspond to an inhomogeneity of about 6 ppm for an air/solid interface. During this study, it was predicted, by studying the line broadening caused by the interfaces of NMR tubes, that the susceptibility could have a considerable effect on image quality.
c) Effects due to molecular diffusion

Random translational motion through a voxel is found to cause randomization of phase \([26,39,42]\). This can be regarded as a factor that causes dephasing of the transverse magnetization and hence causes line broadening. The molecular diffusion is thought to be by far the most important factor that limits the attaining of high spatial resolution in NMR microscopy \([26,42]\). The effect of molecular diffusion is expressed by the following equation.

The echo amplitude after time \(2\tau\) in a Hahn spin echo (from equation (12)) sequence can be written as follows \([26,54]\).

\[
\text{Decay} \propto e^{-\left(\frac{2\tau}{T_2}\right)} e^{-\left(\frac{2}{3} g^2 D \tau^3\right)}
\]

If molecular diffusion is the dominant factor for the decay of the signal, from the above equation and equation 25, it can be shown that \([26]\),

\[
(\delta x)^3 \geq \frac{D}{12 \gamma G}
\]  
(27)

where \(\delta x\) is the resolution limit, \(D\) is the diffusion coefficient of water and \(G\) is the applied gradient.

For instance, if the \(D\) for water in a biological sample is \(10^{-5} \text{cm}^2\text{sec}^{-1}\) and the applied gradient \(G\) is \(10 \text{Gcm}^{-1}\) the resolvable width would be \(\sim 6 \mu\text{m}\). Unlike the other line broadening effects, where the resolution has a simple inverse proportionality to the gradient (eqn. 25), when molecular diffusion dominates, the relationship between the gradient power and resolution involves a third power (eqn. 27). It can be expected that in attempting to resolve microscopic structures the gradient required can be prohibitively large. For instance, if the resolution of interest is 1 A, \((D = 2.19 \times 10^{-5} \text{cm}^2\text{sec}^{-1})\) the gradient required to attain this resolution is about \(4 \times 10^{14} \text{Gcm}^{-1}\).
1.7.5 Relaxation times, as affecting sensitivity.

Consider the case where two adjacent voxels have the same intensity or S/N. The chances of observing them apart from one another is minimal. This is often the case in a pure proton density image. But it is possible that these two voxels have different relaxation times (because the relaxation times depend on the microenvironment of the spin system). Therefore if the difference in relaxation times (say $T_1$) is sufficiently large, it can be used to obtain images capable of resolving the two respective voxels. In fact this is what is done in contrast imaging. (This is discussed further in chapter 3). The purpose of this is to create a difference in signal intensities between two adjacent voxels to give better contrast.

1.7.6 Motion of a voxel or motion within a voxel.

Using larger gradient pulses which switch on and off quickly can cause relative mechanical movement of gradient coils and the sample. This is a potential source of resolution degradation [67]. These movements are thought to cause blurring as well as sidebands in the final image. The use of larger gradient will not have any affect on resolution if this type of motion prevails.

Another type of motion in a biological system is the motion of the cytoplasm as a bulk. This is often known as cytoplasmic streaming. Within a given imaging time if the bulk movement is greater than the voxel dimensions, it will affect the final image. Here, unlike molecular diffusion where the effect is dependent on the time for each single gradient increment, the effect is a function of the total imaging time. Because the biological sample used in this study was amputated from the rest of the cell it was assumed that the cytoplasmic streaming has been halted or is negligible.
2.0 Experimental

In this chapter the organization of the instrument and other necessary experimental conditions to perform NMR imaging are summarized. Details are given in the appendices. The choice of a particular condition or a result is highlighted by a sample calculation, NMR spectra or photographs where necessary. However, some inferences are made without any pictorial elaborations.

Firstly the spectrometer, the probe and the gradient power supply are considered. A detailed discussion on the properties and the evaluation of the probe is cited in references [58],[68],[69]. The two different spin-echo pulse sequences used in this study are discussed. Selection of experimental parameters is discussed with special attention to performing imaging experiments at shorter performance times. The main steps followed in the data processing in order to obtain the final image are given. Finally the phantom studies which were performed in order to visualize the possible limits of resolution are discussed. The resolution with respect to the real biological sample, the Acetabularia cap, is discussed in chapter 3.
2.1 Spectrometer and Probe

The NMR spectrometer used in this study is comprised of a 54 mm narrow bore, vertical magnet from Oxford instruments operating at 6.3 Tesla. (1 Tesla = $10^4$ Gauss). Therefore for protons, the operating frequency is 270 MHz. This instrument was first installed in this department in August 1977. The magnet was interfaced with a Nicolet 1180 computer and a 293B pulse programmer. Also a disk drive was installed in order to enable one to use magnetic discs to increase storage capacity. Originally this spectrometer was confined to performing high resolution 1-D and 2-D NMR experiments. It was later modified to incorporate the facilities to perform NMR imaging experiments. Most of the early studies were done with phantoms.

In 1986 Stanley Luck†, Tom Markus§, and Emil Matter¶ built a new probe, with x, y and z gradient coils, which was especially designed for NMR imaging. Details of the probe are given in Appendix-A and reference [58]. The probe's inner liner, a glass tube of 8 mm inner diameter, defines the maximum sample diameter. This tube is vertical and its axis is the z-direction. Hence for disc-shaped samples, the plane of the disc is the xy plane.

2.2 Pulse sequences

A series of rf and gradient pulses designed to encode spatial information from an object is known as an imaging pulse sequence. Two such pulse sequences were used in this study. Both the sequences were effectively similar to the one used in the spin warp technique. These two pulse sequences had the three main time delays common to any multidimensional NMR technique (viz. the preparation period, the evolution period and the detection or acquisition period respectively). The preparation time in this study was a comparatively long delay to

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¶ Mechanical shop. Dept. of Chemistry. U.B.C. Canada.
allow the spins to reach equilibrium from previous perturbations. The phase encoding was
done during the evolution period and finally the frequency encoding gradient was applied
during acquisition. In the imaging pulse sequences implemented in this study the frequency
encoding was always along the y direction, \( G_y \), and the phase encoding along the x direction,
\( G_x \). Since the phase encoding gradient is increased in magnitude in the spin warp technique
(chapter 1) the phase encoding gradient increments in this thesis will be designated as \( G_z \).

### 2.2.1 Pulse sequence 1

This pulse sequence was used in the early stages of this study and also by the previous
user [69]. The block diagram of the pulse sequence is given in FIG-8A. After a considerable
delay the protons in the biological sample were excited (flipped on to the \( xy \) plane) by a \( 90^\circ \)
pulse. This was followed by the application of the frequency encoding gradient, \( G_y \), which
was usually between 1-2.5 msec (Note that the rf \( 90^\circ \) pulse is 15 \( \mu \)sec). At this point the
effect of this gradient is to translate an echo. That is, this gradient dephases the transverse
magnetization components to different extents along the applied gradient direction. The
subsequent application of a \( 180^\circ \) pulse will interchange the magnetic components and they
will finally refocus to form an echo. Soon after the \( 180^\circ \) pulse the phase encoding gradient,
\( G_x \), was applied. Finally during the acquisition period the frequency encoding gradient,
\( G_y \), was turned on. As seen in FIG-8A the gradient pulses are separated from each other.
This was especially designed to avoid possible coupling, if any, between the high gradient
power pulses used in the frequency encoding and phase encoding dimensions.

### 2.2.2. Pulse sequence 2

The pulse sequence is illustrated in FIG-8B. The major difference from the former pulse
sequence (FIG-8A) is that the phase encoding, \( G_x \), was applied before the refocusing \( 180^\circ \)
pulse. At the same time the frequency encoding gradient, $G_y$, was turned on. Finally the frequency encoding was done during the acquisition time. The time per acquisition in this pulse sequence is reduced by about a factor of half as compared to pulse sequence 1. Longer evolution times in theory yield less intense and probably poorly resolved images. A comparative study was performed mainly to assess the image quality obtained. The results are discussed in Appendix-A. Based on the results obtained pulse sequence 2 was used in the later part of the study. Also many other authors [23],[66] have successfully used similar forms of the pulse sequence 2 in NMR imaging experiments.

It should be mentioned at this point that small delays were incorporated in both the pulse sequences (FIG-8A and 8B) between the rf and gradient pulses. These delays are not shown in FIG-8A and FIG-8B for clarity. The intention was to minimize any coupling between the rf and gradient coils.

2.2.3 The gradient pulses

In this study the gradients were applied as gradient pulses. The gradient power used was considerably higher, for instance, than that used in the NMR body scanner in the U.B.C. hospital (~ 0.5 Gauss cm$^{-1}$). In contrast, in this study the minimum gradient utilized was about 15 Gauss cm$^{-1}$. Gradients as high as 60 Gauss cm$^{-1}$ were attempted in certain imaging experiments. Therefore it is clear that the coupling between the pulses should be minimized. This was the reason for the use of pulse sequence 1 by the previous user and also at the early part of this study.

2.3 Choice of experimental parameters

Selection of the proper parameters is the most important operator controlled factor which is used to yield an image which resembles the original object. As discussed in chapter
FIG-8 The two pulse sequences used to obtain NMR images during this study.

A Pulse sequence 1 The 90° pulse is followed by the frequency encoding gradient ($G_y$). Note that the phase encoding gradient ($G_z$) is applied after the 180° pulse. The open end of the frequency encoding gradient is to denote that it is on throughout the acquisition time. Note that the separation of pulses has caused the evolution time to be long. The separation between pulses were maintained to avoid any interferences by either gradient coils. Typical pulse lengths of $G_y$ and $G_z$ were between 1.2 msec to 2.5 msec (The 90° pulse was about 15 μsec).

B Pulse sequence 2 The phase encoding ($G_z$) and the frequency encoding ($G_y$) gradients are applied simultaneously before the 180° pulse. The frequency encoding gradient is turned on again during acquisition. Note that the evolution time is shorter compared to the pulse sequence shown in FIG-8A.

* Although not shown in the diagram small delays were maintained between each gradient and rf pulse in order to minimize any possible interactions.
one it is necessary to optimize several experimental conditions to obtain a distortion free image. In relation to this study it is important to consider three main parameters.

a) The block size and digital resolution.

b) The field of view (FOV).

c) Number of experiments or the number of gradient increment values ($G_z$).

These are discussed in detail in Appendix-B. Primarily, for a biological sample which deteriorates when out of water the total imaging time is important and therefore the number of gradient increments is important (any living sample, even if quite healthy out of water, may have significant internal movements on the microscopic scale in times of minutes or hours).

In a microscopic region the number of protons is small. Therefore two important aspects had to be considered. First was to acquire at least 8 to 12 scans for each gradient increment to compensate for the lesser number of protons in the microscopic scale. However, it should be clear that excessive acquisition of data would not make the technique practical due to the time taken and also it was thought that the increase in image quality would not be substantial if molecular diffusion prevailed and hence deteriorated the resolution. Secondly, sufficient time was necessary during the preparation time in order to make sure that the most of the spins in the microscopic region were completely relaxed because saturation could not be tolerated from the already hampered microscopic environment. Therefore a relaxation delay of 1-2 sec was almost always used in this study. Thus in order to acquire 12 scans with a relaxation delay of 1.5 sec and to perform a 128 block (128 gradient increments) experiment a total time of about 40 minutes was required. As discussed in Appendix-D, in order to suppress the artifacts which sometimes appeared on images it was necessary to acquire about 20 scans. This resulted in the performance time of the experiment being longer. Using sample holders (FIG 12B, 12C, 12D, 12E) which exposed the *Acetabularia*
cap, the moisture content in the sample was in doubt. This became more critical when at least 4 contrast images and a regular proton density image were required for comparative studies.

The obvious choice for reducing the performance time was to reduce the number of blocks or the number of phase encoding gradient increments. Therefore some studies on *Acetabularia* caps were performed using 64 gradient increments. The data obtained were zero filled (Appendix-B) twice during image processing, to obtain a 256 × 256 image. However, the images obtained using 64 blocks showed a serious deterioration in image quality and resolution. Experiments were performed to study the loss in resolution with reduced block sizes and other experimental conditions. These are discussed in Appendix-B. These studies proved that a higher number of gradient increments (more than 64 gradient increments or blocks) had to be used in order to attain the maximum possible resolution. As for this study, the maximum number of blocks that could be implemented was 256.

If the relaxation delay was 1.5 sec. and the number of scans acquired was 16, for an imaging experiment with 256 blocks (gradient increments) a total time of 1 hour and 42 minutes was required. This is too long for most biological samples to stay unchanged down to microscopic scale, especially a sample which is not viable indefinitely out of water. In order to obtain 1 proton density image and 3-4 $T_1$ contrast images one would require a total experimental time of about 8 and 1/2 hours. Therefore most of the experiments in this study were performed with 128 blocks (gradient increments). The total time taken to perform an experiment with 128 blocks hence was about 40 minutes.

2.4 Data processing to obtain an image

Once the FOV and the gradient were known the imaging experiment was performed and the acquired *NMR* signal (an echo) which was digitized was saved by storing on discs.
Several such echoes corresponding to the specified number of phase encoding gradient increments were acquired and stored. These data were finally processed to yield the final NMR image. A descriptive account of image processing is given in appendix-B.

The image processing procedure was somewhat similar to the familiar 2-D spectroscopic data processing technique. The sets of echoes acquired were Fourier transformed for the first time. Prior to the 1-FT, the data was base line corrected and apodized (Appendix-B) by a sine function multiplication. In order to perform the second Fourier transformation the partially processed data were transposed. The transposed data were Fourier transformed for the second time to obtain a 2-D image. All the data obtained after the 2nd-FT were projected as positive quantities by a software controlled absolute value generation procedure called the magnitude calculation (MC). The images were either obtained as stacked plots or were transferred, via the departmental 1280 computer, to an IBM-PC which was interfaced with a 256 x 256 gray scale graphic screen and obtained as photographs.

2.5 The limits of resolution attainable

Resolution of small morphological structures was of primary interest in this study. Optimization of the experimental conditions was done mainly to gain highly resolved images. As a systematic approach for this, initially phantoms of different diameters were studied. The spin system used in all phantom studies was water. The water was doped with MnCl₂ in order to reduce the $T_1$ relaxation times of water thereby enabling the repetition time per experiment to be made shorter.

Four melting point capillary tubes (i.d. 1.2 mm) were filled with paramagnetically doped water and NMR images were obtained after placing them in a 5 mm NMR tube. The images showed clearly resolved projections corresponding to the 1.2 mm tubes. Since the biological sample (the *Acetabularia* cap), has a discoid shape another phantom was made
from delrin with a thickness of 2 mm and a diameter of 6.5 mm. Eight holes were bored in this disc with different diameters. The smallest orifice was \( \sim 100 \mu m \) and the largest was \( \sim 800 \mu m \). These openings were filled with water and the images were obtained. The practical difficulty was to fill and then maintain the water in these small holes during the time of the experiment. Despite these limitations it was possible to observe the water even in the smallest orifice. Another set of phantoms was developed with melting point capillary tubes. The 1.2 mm (i.d.) capillaries were drawn out to finer structures using a Bunsen flame. The diameters of these flame drawn structures were not known. The imaging experiments were performed as follows. First, three capillary tubes were filled with a 5 mM \( MnCl_2 \) solution and the flame drawn structures were placed in them. Four such capillaries, with one empty, were placed in an empty NMR tube. The image obtained is shown in FIG-9A. In another experiment a 5 mm NMR tube was filled with 5 mMol \( MnCl_2 \) solution. Four melting point capillaries were filled with 1 mM, 2 mM \( MnCl_2 \) and 5 mM, 10 mM \( CuSO_4 \) solutions. The finer flame drawn structures were placed in the larger 1.2 mm melting point tubes. It was sometimes observed and sometimes assumed that the finer structures were filled with the respective paramagnetic solutions due to capillary action. Finally all four melting point tubes were placed in the 5 mm NMR tube. All the tubes were filled with solutions, firstly to minimize the number of interfaces in the rf coil region, and secondly, to observe a clear demarcation between liquid and glass. The image obtained is shown in FIG-9B. The diameters of the finer structures were calculated with respect to the image diameter of the 1.2 mm diameter of the melting point tubes. It was found that the diameter of some of these fine structures was about \( \sim 90 \mu m \). The thickness of the glass wall, which is also distinguishable, was calculated to be about \( \sim 30 \mu m \) (The pixel resolution of the image was \( \sim 20 \mu m \)). The potential resolution capabilities shown with phantoms led to the study of the biological sample \textit{Acetabularia}. Scientifically significant results on this sample are discussed in the next chapter.
FIG-9 Phantom studies performed to visualize the resolution limits attainable by the spectrometer.

(A) An NMR image of three 1.2 mm i.d. melting point capillary tubes filled with MnCl$_2$ doped water (5 mM). One capillary and the NMR tube (which is not seen) was empty.

(B) An NMR image of four magnetically doped water filled melting point capillary tubes (i.d. 1.2 mm). The smaller structures seen are fine glass structures flame drawn using the melting point tubes. The four capillary tubes were placed in a 5 mm NMR tube. The whole system was filled with water to, firstly, reduce the number of interfaces and, secondly, to enhance demarcation between glass and water.

$G_y = 27.93 \, G \, cm^{-1}$, $FOV_y = 0.526 \, cm$, $G_z = 0.213 \, G \, cm^{-1}$, $\Delta FOV = 2.33 \times 10^{-3} \, cm$, $t_\phi = 2.1 \, msec$, $NS = 8$, $DW = 16 \, \mu sec$. Number of gradient increments=128.
In this chapter a biological description of the test sample Acetabularia mediterranea is given. The life cycle of the organism is discussed with special attention to properties which could be relevant to NMR studies. The selection of the Acetabularia caps and the photomicrography is discussed. The different sample holders developed to localize the test sample in the probe are discussed and compared. The selection of parameters and image processing is discussed in chapter 2. The $^1H$ density images (will be referred to as normal or proton density images in this discussion) of the reproductive caps are discussed from the standpoint of the spatial resolution attainable. Images obtained at moderate gradient power, high gradient power and "enlargement" of images are discussed. Cellular water in a biological system is summarized. The study of the maturation or development of Acetabularia caps is discussed in detail based on the information obtained by $T_1$ contrast images. $T_1$ contrast images of immature, mature and partly mature caps are presented. $T_1$ relaxation times of individual caps, which were determined in order to explain the results, are considered briefly. These relaxation data are of a preliminary nature. The results are discussed in terms of the ultrastructural changes that occur during the maturation of the cap. The other contrasting methods that can be used, such as $T_2$ contrast and contrasting with paramagnetic ions, are discussed briefly with a few results obtained. Also the imaging
experiments performed on other parts of the cell, mainly the rhizoid, are discussed briefly.

3.1 *Acetabularia*: A GIANT SINGLE CELL

The organism selected for this *NMR* imaging study is an unusual one with two special advantages for this work. First, it produces a discoid array of elongated structures, arranged spokewise, which taper from hundreds to tens of $\mu$m in diameter (FIG-10). This array provides an ideal test pattern for the achieved limits of spatial resolution. Second, the culmination of the life cycle is a major cytoplasmic reorganization within this structure. Within a few days it changes from nucleus-free continuous cytoplasm, via a multinucleate syncytium, to an assembly of a large number of cysts, each with a heavy cellulosic wall (see below), packaging the formerly continuous cytoplasm in small separated regions. This developmental sequence is likely to entail changes in ordering, binding and compartmentation of much of the water in the system. These might be expected to produce changes in water proton relaxation times, on a large enough spatial scale to show the possibility of monitoring intracellular development by *NMR* imaging at the spatial resolution now achieved.

*Acetabularia* (especially the species *Acetabularia mediterranea*, also known as *A. acetabulum*) is not studied by a very large number of biologists, but is very well-known for particular kinds of study, for which it offers unique advantages. Through most of its life cycle (FIG-10) it is a single cell, uninucleate. The nucleus is at the opposite end of the cell from the growing tip where extensive morphogenetic activity occurs in a species-specific manner, and the cell grows to a length of about three to four centimetres. This magnificent gift of nature therefore has the special property where the functions of the nucleus and the cytoplasm can be monitored separately and easily. Hämmerling used this organism in the 1940s to study the rate of transport of nuclear information through the cytoplasm. The cultures from which the samples for the present study were taken have been used in this
university for two main purposes: studies of chloroplast DNA free from contamination with nuclear DNA [70], and studies of complex morphogenesis in regions containing no nuclei [71].

Acetabularia is a marine alga classified in the order Dasycladales (Greek: Hairy branches) of the siphonous line (Greek siphon: a tube or pipe) of the green algae (Chlorophyta), has a vertical growth of a few centimetres, but remains essentially as one single cell. The entire dasyclad order inhabits subtropical marine waters, the Mediterranean being the coldest waters in their range. The habitat is at a depth of a few metres, below the intertidal zone. This is a disadvantage for the present study. In nature, the algae are never out of water and they are not adapted to remaining alive for long periods out of sea water.

The life cycle begins with a more or less spherical cell which is formed as a result of sexual reproduction (FIG-10E). This cell grows to become a mature plant. Generally this process takes about sixty days where the environmental conditions play an important role. The cell elongates by tip growth at one end and the other end develops into the “root like” rhizoid (Fig-10G*). This growth pattern resembles higher plants where the apical growth forms the stem and branches and the rest forms the root system. Therefore, although a single cell, Acetabularia shows a distinct division of labour as any other higher plant. The rhizoid contains the nucleus (diameter ca. 100\(\mu\)m) which directly and indirectly [72] controls all activities of the cell. Also the rhizoid helps attach the cell to the ocean bed and prevents it from being washed away.

The apical growth develops a stem or stalk (FIG-10F). The cell usually grows to a length of about four centimeters with a diameter of about 400\(\mu\)m. The cell wall of the stalk has an average thickness of 10\(\mu\)m and is thicker close to the rhizoid. About 90% of the stalk diameter is occupied by the vacuole [73] (an enclosed pouch usually filled with lifeless liquid). Therefore the cytoplasm is confined to the outer 5-10\(\mu\)m of the cell. After about 2 – 3 months, the vegetative growth stops and the cell develops its reproductive structure
(refer to [74] for a detailed discussion). Until the reproductive structure is fully grown the cell is considered to be in its growth period. In Acetabularia, unlike some other plants in the order Dasycladales, the nucleus does not divide until the growth is completed. That is, it remains strictly a single cell in the sense of being uninucleate.

The mediterranean species, Acetabularia mediterranea, at the end of the growth period develops a discoid shaped reproductive organ. Hence, it is sometimes referred to as the “mermaids wineglass” (Some authors now prefer the name A. acetabulum. Acetabulum was the ancient roman vinegar bowl). The reproductive organ is called “The Cap” (Fig 10-G). The Cap consists of 50–100 radial rays tapering towards the central apex. Although they are separated from one another by the cell wall, all the rays have connections to the apex and therefore to the cell. The average diameter of a cap varies from about 5 to 7 mm. Therefore the width of a ray at the outer edge (periphery) is around 250-400μm, narrowing down to about 40μm at the centre. In an immature stage, before the nucleus starts dividing, the rays are similar to the central stalk. The rays contain a continuous cytoplasm which has a considerable number of chloroplasts, hence appearing green (See Fig 14A). Beneath this is the vacuole connected with the vacuole in the stalk. The formation of the cap is thought to be triggered by a chemical messenger released from the nucleus. Hämmerling and others [72] have shown by various experiments that reproduction is triggered by the cap formation.

When the cell is fully grown (including the cap) the nucleus starts to divide. By cytoplasmic streaming, large numbers of daughter nuclei are transported to all the rays in the cap. While the cytoplasm in these rays is still continuous, these daughter nuclei become distributed in a fairly orderly array. The cytoplasm then starts to “pull in” around each nucleus. At this stage, it cannot be seen whether plasma membranes have yet formed around each clump of cytoplasm. Soon, however, thick cellulosic cell walls form around each nucleus.
and associated cytoplasm (This is a major switch in carbohydrate synthesis. Unusually, the outer envelope of the entire organism is a mannose polymer, mannan, not cellulose). These structures are known as cysts (diameter ca. 50\(\mu\)m). Thus the living contents of each ray are packaged into cysts, each of which contains, eventually, many biflagellate gametes (because the nuclei continue to divide), chloroplasts, cytoplasm etc. Each ray can consist of several dozens of such cysts (FIG 10-A).

For reproduction, when the environmental conditions are favourable, the cysts are first released by the rupturing of the cap and releasing the cysts to the water (FIG-10A*). In nature, this occurs by bacterial breakdown of the cap; in sterile laboratory cultures, it is done with scissors. With the aid of sea currents these cysts gets dispersed.

The cyst has a lid like structure which opens (FIG-10B) and releases thousands of isogamous gametes arising from further nuclear divisions within the cyst (length 5-8 \(\mu\)m and width 2-3 \(\mu\)m). These gametes, which are equivalent to germ cells in higher organisms, do not have any gross morphological difference between the male and female. Hence, reproduction is called isogamous. But they do have two different cell surface features for mutual recognition and therefore are called (+) and (−). These gametes have two separate structures for movement in water, called flagella (FIG-10C). While moving, if two gametes of opposite kind, one (+) and one (−), come in contact they fuse to form a quadriflagellate zygote containing the fertilization nucleus (FIG-10D). The zygote sheds its flagella and settles in the ocean bottom. This forms the more or less spherical cell, (FIG-10E) which grows to a mature plant.

In summary, the remarkable properties of Acetabularia species which are of interest to the biologist [72] as well as to this study are,

a) Growth into a giant but single cell.

b) Complex shape of the cell surface (A useful “test pattern” for NMR imaging).
FIG-10 Life cycle of *Acetabularia mediterranea* (modified from [72])

A  A mature *Acetabularia mediterranea* cell. Some of the cysts are seen.

A*) The Cap of a mature cell and the release of cysts after rupture of cell wall. Although each ray has a cell wall the rays are inter-connected by the stem or stalk.

B  A cyst (diameter ca.50μ) opening at the lid and releasing gametes.

C  Two biflagellate isogamous gametes.(approx. dimenions 5 × 3μ).

D  Two gametes fused to form a quadriflagellate zygote.

E  Germination of the zygote to form the initial *Acetabularia* cell.

F  Formation of the stalk by apical growth and the rhizoid (morphological differentiation).

G*) Structure of the rhizoid which bears the nucleus and helps attach the cell to the sea bed.

G  Formation of the cap. This terminates the growth period.
c) The nucleus does not divide until the cell is fully mature.

d) Different *Acetabularia* species have different cap shapes.

e) Location of the nucleus with respect to the rest of the cell.

f) *Acetabularia* has a very efficient capability to regenerate cell parts.

g) The nucleus can be handled less carefully than for many organisms. (*e.g.* The nucleus of *Ameoba* cannot come in contact with the culture medium. In contrast, an *Acetabularia* nucleus can be removed, washed several times and reimplanted if desired).

h) Very rapid cytoplasmic streaming, and the orderly disposition of nuclei early in cyst formation, suggest that cytoplasm-cytoskeleton interactions may be very strong and extensive. This could have *NMR*-observable effects on water relaxation, and there is a chance that such effects could in some way maximize at the most active stage of cap maturation.

Marine Chlorophyta (green alge) were not easy to culture in the laboratory until suitable procedures were developed in the 1950's. This was because of the lack of understanding of the exact environment of the alga, especially their need for vitamins [75].

Cultured species still show some differences from the same species growing in the natural environment, *e.g.* *Acetabularia* and most other Dasycladales show calcification on the outer wall of the rhizoid and stalk while none of the *Acetabularia* grown in the laboratory have calcified walls.

An important and major problem is the invasion of bacteria into the medium in which *Acetabularia* is cultured. This often destroys the whole culture if it is not handled under sterile conditions, and bacterial infections treated with antibiotics. This was also developed in the 1950's, [75] and is now a routine procedure in the culturing of *Acetabularia*.

Caps of *Acetabularia* cultured in the laboratory sometimes possess infertile or immature
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rays (See FIG-14B and C). The author is not aware whether this occurs in a natural environment or not. Its occurrence in the laboratory, however, provides useful test samples for various kinds of contrasting in NMR imaging.

Currently Acetabularia is cultured in artificial sea water [75] and the culture medium is known as Shepard’s medium (Shep). The major chemical constituents of the Shep are given in Appendix-C. The Acetabularia cells used for this study were originally collected from the bay of Naples. They have been maintained in culture for many generations in Europe and later in Dr. B. R. Green’s (Botany, U.B.C.) laboratory. Currently they are being cultured in the laboratory of Dr. L. G. Harrison (Chemistry, U.B.C.). The cells are grown in Shep containing sterile flasks which are placed in growth chambers. The temperature of these chambers is maintained at 20°C while they are illuminated at 300-500 ft. candles at a light (day) and dark (night) cycle of 12:12 hours. Each flask contains many such cells of Acetabularia.

3.2 Preparation of Acetabularia caps for NMR imaging

3.2.1. Selection and photomicrography of Acetabularia caps

A few appropriate Acetabularia cells were picked out from an ensemble of cells growing in culture flasks. The choice of the caps of interest was determined by the morphological features of the cap.

1) Because of the sample mounting constraints the cap had to have a diameter of less than about 7 mm.

2) The images were obtained by projecting the spin density on to the xy plane. Slice selection was not used in this study. Therefore, in order to obtain an image which represented the original sample, the Acetabularia cap had to be more or less flat. If
a cap has a high curvature that the utilized gradient could not resolve, two or more adjacent regions will be projected on to one point and it will appear brighter in the final image. Such an image is shown in FIG-11.

![image of Acetabularia cap](image)

FIG-11 An image of an *Acetabularia* cap showing the necessity to select caps which are flat. The bright areas are the total spin density, of areas which are not "flat", projected on to the xy plane.

3) Three main morphological appearances were of primary interest. They were,

(a) Immature caps which had "continuous cytoplasm" in all the rays with chloroplasts distributed "evenly" among them, hence appearing green (FIG-14A).

(b) Mature caps where arrays of cysts were visible (FIG-14D).

(c) Partly immature caps where some of the rays remain "immature" [sterile] while the other rays have reached maturity [fertile] (FIG-14B/C). These type of caps were mainly selected for performing contrast imaging experiments in order to study the developmental stages of a cap.

The samples were handled in a hood with high outward laminar flow to minimize bacterial invasion in the working area. All metal equipment used in handling the cells were
disinfected over a nude flame. The glass petri dishes have been autoclaved and the plastic ones were fresh. The contents of the culture flasks were first emptied to a larger glass sterile container. The cells used in imaging were picked and placed in plastic containers. The cells not used were transferred to a new sterile flask followed by the Shep. A flame was used to sterilize the flask openings when required.

Once the selection of the cells, from the culture flasks, was done the remaining caps were restored in the growth chambers. Therefore the initial selection of the cells had to be performed under sterile conditions to prevent bacterial infections. After the caps were selected, the imaging experiments were performed within two to three days. Therefore these cells were not handled under strict sterile conditions. The caps were severed from the stalks prior to photomicrography. This cut probably leaves a permanent unhealed opening to the intracellular space of the cap.

The caps were placed under a dissecting microscope which was connected to an Olympus 35 mm camera body (model PM 10 especially designed for microscopes, 160 ASA colour slide film). The exposure time was determined by an external light meter which was also interfaced with the camera. The photomicrographs of caps were usually obtained with a ten times magnification. At the time of photography the Shep was used so that it barely covered the cap because it was found that excess liquid often produced blurry photographs. The caps were submerged well in the Shep after photography.

3.2.2. Sample holders

3.2.2.1 Developing a new sample holder

The biological sample, the *Acetabularia* cap or any other part of the cell studied, needed to be localized in the rf coil region of the NMR probe. Special sample holders were developed
in order to locate the sample in the correct area of the probe. The first sample holder used was a simple 5 mm (i.d.) NMR tube cut from one end to a length of 90 mm leaving both ends of the tube open. A 7.8 mm diameter teflon ring was fixed to one extreme of this “NMR” tube (FIG-12A). The other two teflon rings were to minimize mechanical movements, if any, during the experiment. The larger teflon ring could be moved to maintain the correct height between the sample and the rf coil centre. This was also used to support the holder by resting it on the teflon plug of the probe (see Appendix-A, FIG-24, structure A). The sample, which is disc shaped, was made to adhere itself to the teflon ring by capillary action. However, this holder had four disadvantages.

(a) The sample very often fell inside the probe during insertion into the probe. This was often a cumbersome process and required great care in placing the sample. Also it was time consuming when the sample was found to have fallen during an experiment.

(b) The sample shape that was studied had to be essentially circular or disc shaped (FIG-13B). A variety of different shaped samples could not be mounted in this sample holder.

(c) Although the sample was “blotted” to remove extracellular water from the surface before placing in the holder some times, especially in $T_1$ contrast images, some water was seen to have been entrapped between the teflon ring and the glass NMR tube (FIG-12A* and FIG-13A).

(d) The long time exposure of the biological sample away from its natural culture medium was suspected to dehydrate the sample, thereby altering the proton density.

In order to circumvent some of these problems a new sample holder was developed. This consisted of a portion made out of delrin which bore a platform to accommodate the sample (FIG-12B). The upper rings were made out of teflon. Delrin was chosen for this purpose. Because of the much harder nature of the substance (compared to teflon), it was amenable to finer mechanical craftsmanship. Besides being able to prevent the sample from
FIG-12 The different sample holders used to mount the *Acetabularia* cap. The centre tube was a 5 mm *NMR* tube. The upper rings of all the holders were made out of Teflon.

(A) The Teflon ring is attached to the bottom of the *NMR* tube. The sample was made to adhere to the teflon ring by capillary action.

(A*) The view from the bottom of the holder A, showing the gap between the teflon ring and the glass tube.

(B) The flat bottom platform built out of delrin to localize the biological sample.

(C) The round bottom delrin sample holder developed in order to study the effect of sample holder shape on image quality.

(D) / (E) Flat bottom and round bottom teflon holders built to monitor possible effects which the material of the holder had on an image.

(F) A closed holder made from delrin to prevent the sample from dehydrating. The sample was placed in the lower part and was secured to the upper part.
drying, this design eliminated the other three problems cited earlier (FIG-13A, FIG-13B and FIG-13C and FIG-13D). A disadvantage of this holder was that the finite thickness of the walls holding the platform (FIG-12B) limited the maximum sample dimensions from about 7.8 mm (old holder) to about 7.4 mm. Use of a flat bottom sample holder required the attempt to shim the magnet under the appropriate conditions. The results are discussed in appendix-A. However, during this study the magnet shimming was done on a 5 mm NMR tube filled with water so as to obtain a half-height line width of 2-3 Hz. The gradient power supply was turned on during the process of shimming but no gradients were applied. The shims were adjusted to yield the maximum FID (computer) which was also relatively smooth (visual). Shimming on the sample was not done during this study because the time taken for this process was comparatively long (could lead to changes in the sample).

3.2.2.2. Other sample holders

The difference in line shapes observed in different shapes of NMR tubes (Appendix-A) and some observations made by others [76],[77],[78] led to the attempt to observe whether the shape of the sample holder had an affect on the image quality. Another sample holder was built which consists of a delrin round bottom (FIG-12C). The main difference in this holder, besides the external shape, was that the sample was placed by rotating it by 180° (up side down). Also in order to study whether the material from which the holder is made, Delrin in this case, will affect the final NMR image, two more holders, one a round bottom and the other a flat bottom, were built out of teflon (FIG-12D and FIG-12E). The images obtained using the four different sample holders are presented in Appendix-D.

The sample holder designed in order to minimize the sample from drying was considerably superior (FIG-12F). Imaging experiments which lasted for about seven hours were performed using this holder. Attempts were also made to obtain images by placing the cap
FIG-13 The sample shapes that can studied.

A) An image of an *Acetabularia* cap obtained by adhering the cap to sample holder A in FIG-12. The semicircular protrusion in the centre of the image corresponds to the extracellular water entrapped between the teflon ring and the *NMR* tube (FIG-12A*).

B) An image of an immature *Acetabularia* cap obtained using the sample holder A in FIG-12. The sample had to be essentially circular for maximum contact with the teflon ring for adhering.

C) /D) Irregular shapes of *Acetabularia* could be imaged using the sample holder shown in FIG-12B (also 12C-F).
in water. These results are also discussed in Appendix-D.

Caps which lay within the dimensions of a sample holder were removed from the culture medium, “blotted” to remove extracellular water, and were placed in the sample holder of interest prior to the placement in the probe.

3.2.2.3. Sample holders and image quality

As explained in Appendix-A the line broadening seen in different NMR tubes can be attributed to the change in magnetic field susceptibility at the interfaces of air/glass/water/air which could result in a considerable magnetic field inhomogeneity. This type of line broadening was also observed in a different probe and even in a different spectrometer.

In the four images obtained using the four sample holders, (FIG-33, Appendix-D), there is no apparent change in image quality. This showed that the effect of interfaces of different shapes or different materials, which can cause inhomogeneities in the field, and hence the resolution, has probably affected all four images to the same extent.

3.3 $^1H$ density imaging of Acetabularia caps: Achievement of microscopic resolution

The imaging experiments were performed by using either one of the pulse sequences described in chapter 2 (FIG-8). The data were processed by double FT to obtain the final image (Appendix-B). The final images were displayed on the 256 x 256 gray scale screen and most of the pictures presented in this thesis are the photographs obtained from the screen. Imaging experiments were performed at two different frequency encoding gradient power levels.

(a) At moderate gradient power ($G_y$ between 10-20 $G cm^{-1}$).
(b) At relatively high gradient power ($G_y$ between 30-40 $G \text{ cm}^{-1}$).

A few attempts to use gradients of the order of 65 $G \text{ cm}^{-1}$ resulted in the disconnection of one lead in the probe. Hence gradients larger than about 60 $G \text{ cm}^{-1}$ were not attempted during the rest of the study. The proton density imaging experiments were mainly done to attain highly resolved images and to determine the limits of resolution attainable as far as the spectrometer and the Acetabularia caps are concerned. Experiments were not performed to investigate or verify the factors which limits the resolution attained with Acetabularia caps. However, some possible effects will be discussed.

3.3.1. $^1H$ density imaging at moderate gradients

Almost all the studies were performed with moderate gradients. That is, the frequency encoding gradient was between 10-20 $G \text{ cm}^{-1}$. The sweep width used was 30 KHz with a block size of 512. The acquisition time corresponding to this frequency was 4.11 msec. The frequency encoding time and the phase encoding times were 2 msec each. This made the echo time (time between the 90° pulse and the echo) for pulse sequence 1 (FIG-8A) about 8 msec and for pulse sequence 2 (FIG-8B) about 4 msec. Most of the experiments were performed with delays incorporated between rf and gradient pulses to avoid any interaction. Pulse sequence 2 was used extensively during this study (Appendix-A).

Four sets of images of mature, immature and partly mature caps with their respective photomicrographs are shown in FIG-14. The individual experimental parameters are given alongside the figure. The resolution obtained in terms of resolving two neighbouring structures and in terms of pixel sizes are discussed in section 3.4. In general, the resolution of all four images shown in FIG-14 was better than 125 $\mu \text{m}$.
FIG-14 $^1H$ density NMR images of four different Acetabularia caps from three morphologically different stages. The images were obtained at moderate gradients.

A) Immature cap. Calculated resolution $\sim$65 $\mu$m. Pixel resolution = 35 $\mu$m.

$$G_y = 16.51 \text{ G cm}^{-1} (LT = 3), \text{ FOV}_y = 0.889 \text{ cm}, G_{z_i} = 0.12 \text{ G cm}^{-1} (LT = 0.028),$$

$$\Delta \text{FOV} = 6.7907 \times 10^{-5} \text{ cm}, t_\phi = 2.2 \text{ msec}, DW = 16 \mu\text{sec}, NS = 20. \text{ Number of gradient increments} = 128.$$

B) A mostly mature cap with two immature rays. Note that the immature rays are resolved better. Calculated resolution $\sim$40 and 70 $\mu$m. Pixel resolution = 36 $\mu$m.

$$G_y = 15.96 \text{ G cm}^{-1} (LT = 2.9), \text{ FOV}_y = 0.92 \text{ cm}, G_{z_i} = 0.116 \text{ G cm}^{-1} (LT = 0.027),$$

$$\Delta \text{FOV} = 2.2 \times 10^{-3} \text{ cm}, t_\phi = 2.2 \text{ msec}, DW = 16 \mu\text{sec}, NS = 20. \text{ Number of gradient increments} = 128.$$

C) Image of a cap with mixed rays. Also note that the immature rays are well resolved.

Calculated resolution $\sim$40 and 85 $\mu$m. Pixel resolution = 37 $\mu$m.

$$G_y = 15.41 \text{ G cm}^{-1} (LT = 2.8), \text{ FOV}_y = 0.953 \text{ cm}, G_{z_i} = 0.112 \text{ G cm}^{-1} (LT = 0.023),$$

$$\Delta \text{FOV} = 3.72 \times 10^{-3} \text{ cm}, t_\phi = 2 \text{ msec}, DW = 16 \mu\text{sec}, NS = 16. \text{ Number of gradient increments} = 128.$$

D) Mature cap. Calculated resolution $\sim$125 $\mu$m. Pixel resolution = 36 $\mu$m.

$$G_y = 15.96 \text{ G cm}^{-1} (LT = 2.9), \text{ FOV}_y = 0.92 \text{ cm}, G_{z_i} = 0.116 \text{ G cm}^{-1} (LT = 0.027),$$

$$\Delta \text{FOV} = 2.2 \times 10^{-3} \text{ cm}, t_\phi = 2.2 \text{ msec}, DW = 16 \mu\text{sec}, NS = 16. \text{ Number of gradient increments} = 128.$$


3.3.2. Imaging at higher gradients

In order to compensate for the increase of the gradient it was necessary to increase the sweep width (section 2.3. and Appendix-B). Therefore at higher gradients a sweep width of 62.5 KHz were used (this is the highest SW applicable (QPD) in the 270 MHz spectrometer). This reduced the acquisition time from 4.11 msec to 2.05 msec. In order to maintain more or less the same FOV as compared to moderate gradients it was also necessary to reduce the time for which the gradient is applied, from 2 msec, to 1.1 msec. The main difference that should be highlighted is that the time for one sequence, i.e. the time between the initial 90° pulse and the end of the acquisition, was reduced by approximately half. However, because high gradient power (~ 50 G cm⁻¹) was used, long relaxation delays (2 sec) were enforced in order to reduce the duty cycle of the gradient pulses.

Two sets of images of a mature cap and an immature cap, obtained at higher gradients, are shown in FIG-15. The images of the same cap obtained at moderate gradients are given for comparison.

3.3.3. Image "enlargement"

This effect was obtained by using high gradients and maintaining moderate sweep widths. Strictly the image obtained was a foldover of the image because of the smaller window specified. However, some interesting observations were made. Two such "enlargements" of the mature and immature caps which are shown in FIG-15 are given in FIG-16. The sweep width used was 30 KHz. Note that the outer edge of the caps are being folded over to the centre of the image. Clearly the long-range organization of such images is untrustworthy. However, it can be seen that finer components of the cap are made visible under these conditions.
FIG-15 The $^1$H density images obtained of a immature cap and a mature cap at high gradient power. The images obtained at moderate gradients are given for comparison.

A) Immature cap

1) Image obtained at a moderate gradient. Calculated resolution $\sim 60 \mu m$. Pixel resolution $= 33 \mu m$.

$G_y = 17.45 \text{ G cm}^{-1} (LT = 2.5)$, $FOV_y = 0.841 \text{ cm}$, $G_z = 0.133 \text{ G cm}^{-1} (LT = 0.0258)$,

$\Delta FOV = 1.442 \times 10^{-3} \text{ cm}$, $t_\Phi = 2.1 \text{ msec}$, $DW = 16 \mu sec$, $NS = 20$. Number of gradient increments = 128.

2) Image of the immature cap obtained at a higher gradient. Calculated resolution $\sim 50 \mu m$. Pixel resolution $= 31 \mu m$.

$G_y = 37.29 \text{ G cm}^{-1} (LT = 4)$, $FOV_y = 0.787 \text{ cm}$, $G_z = 0.271 \text{ G cm}^{-1} (LT = 0.031)$,

$t_\Phi = 1.1 \text{ msec}$, $DW = 8 \mu sec$, $NS = 20$. Number of gradient increments = 128.
B) Mature cap (is a mirror image of the two NMR images).

3) Image obtained at a moderate gradient. Calculated resolution $\sim 90 \mu m$. Pixel resolution $= 32 \mu m$.

$$G_y = 17.71 \, G \, cm^{-1} \, (LT = 1.9), \, FOV_y = 0.829 \, cm, \, G_z = 0.135 \, G \, cm^{-1} \, (LT = 0.0153),$$

$$\Delta FOV = 2.372 \times 10^{-4} \, cm, \, t_{\phi} = 2.1 \, msec, \, DW = 16 \mu sec, \, NS = 20. \, \text{Number of gradient increments} = 128.$$

4) NMR image of the mature cap obtained at a higher gradient. Calculated resolution $\sim 80 \mu m$. Pixel resolution $= 30 \mu m$.

$$G_y = 38.22 \, G \, cm^{-1} \, (LT = 4.1), \, FOV_y = 0.768 \, cm, \, G_z = 0.228 \, G \, cm^{-1} \, (LT = 0.0315),$$

$$\Delta FOV = 1.51 \times 10^{-4} \, cm, \, t_{\phi} = 1.1 \, msec, \, DW = 8 \mu sec, \, NS = 20. \, \text{Number of gradient increments} = 128.$$
Chapter S: Imaging Acetabularia

B

3)  

4)
FIG-16 Image "enlargement" by using high gradient power and smaller FOV. The pixel resolution of both the images shown was 15 μm.

A The "enlarged" image of the immature cap shown in FIG-15A.

\[ G_y = 37.29 \text{ G cm}^{-1} (LT = 4), \text{FOV}_y = 0.394 \text{ cm}, G_x = 0.248 \text{ G cm}^{-1} (LT = 0.035), \]
\[ \Delta \text{FOV} = 1.263 \times 10^{-3} \text{ cm}, t_\Phi = 2.1 \text{ msec}, DW = 16 \mu\text{sec}, NS = 20. \]
Number of gradient increments = 128.

B An image of the mature cap shown in FIG-15B.

\[ G_y = 37.29 \text{ G cm}^{-1} (LT = 4), \text{FOV}_y = 0.394 \text{ cm}, G_x = 0.284 \text{ G cm}^{-1} (LT = 0.0322), \]
\[ \Delta \text{FOV} = 1.369 \times 10^{-5} \text{ cm}, t_\Phi = 2.1 \text{ msec}, DW = 16 \mu\text{sec}, NS = 20. \]
Number of gradient increments = 128.
3.4. Results and discussion

The main importance of this study from the standpoint of microscopic imaging technique was the resolution. Highly resolved images would certainly bring about the possibility of the better understanding of the structure and even the function of the biological system which in this study was the *Acetabularia* cap. As mentioned in the introduction many authors [26],[39],[42] have discussed a lower limit for the resolution attainable with the existing NMR facilities which is about 10 μm. In this study the maximum resolution attained by $^1H$ density imaging was between 40 and 50 μm. The literature values of resolution which were about 10 μm were never achieved as far as the actual visible spatial resolution is concerned. However, the pixel resolution of the images obtained were much better than the resolution limit, of 40-50 μm, attained in this study.

**Estimating the resolution from NMR images**

The resolution limit was considered to be the ability to resolve two adjacent points or regions from one another (See FIG-7, chapter 1). The *Acetabularia* caps investigated provided a good test sample with the radial rays narrowing down to about 30 or 40 μm. The resolution limit was calculated by tracing the point at which two adjacent rays became indistinguishable. The procedure is as follows. Let the radius of the photomicrograph of a circular *Acetabularia* cap be $R$ mm (measured using a micrometer). Therefore the perimeter of the cap would be $2\pi R$ mm. If the cap has N radial rays the peripheral width (arc) of a single ray is $2\pi R/N$ mm. Let this quantity be $A$ mm. In the image obtained, assume that the rays are not resolvable after a distance of $r$ mm from the center of the cap. If the distance between two rays at distance $r$ mm is, $a$ mm, this is the resolution limit of the image obtained. This is clarified in the following simple diagram.
And the resolution, \( a \), can be calculated from,

\[
\frac{A}{R} = \frac{a}{r}.
\]

Some authors seem to use the pixel resolution to designate the resolution attained. The author does not wish to comment or compare this method of defining the resolution with the former but opens the opportunity of doing so to the reader. However, the pixel resolution can be calculated as follows. If the FOV in a particular 256 × 256 image is known this FOV in one dimension will be defined by 256 pixels. Therefore the pixel resolution is FOV/256 length units.

The perimeters of the caps were measured by two methods. For circular caps the formula \( 2\pi R \) was used while for caps with irregular shapes a twine was aligned along the periphery of the cap (photomicrograph) and the length of this twine was measured. An important assumption made during this process was, that the rays in a selected cap were spaced equally (same arc width).

The resolution calculated and the pixel resolution of the images shown in FIG-14 is given below.
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<table>
<thead>
<tr>
<th>Image Description</th>
<th>Resolution ($\mu$m)</th>
<th>Pixel resolution ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Immature cap</td>
<td>$\sim 70$</td>
<td>35</td>
</tr>
<tr>
<td>B Partly immature cap</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Immature ray</td>
<td>$\sim 40$</td>
<td>36</td>
</tr>
<tr>
<td>2 Mature ray</td>
<td>$\sim 70$</td>
<td>36</td>
</tr>
<tr>
<td>C Partly mature cap</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Immature ray</td>
<td>$\sim 40$</td>
<td>37</td>
</tr>
<tr>
<td>2 Mature ray</td>
<td>$\sim 90$</td>
<td>37</td>
</tr>
<tr>
<td>D Mature cap</td>
<td>$\sim 125$</td>
<td>36</td>
</tr>
</tbody>
</table>

In the images shown in FIG-14 it should be clear that the resolution of immature caps or "immature" rays in partly mature caps was better than the resolution attained in mature caps or mature rays. This observation is explained by the behaviour of water in the cap. Two factors should be reiterated at this point. First, in microscopic imaging diffusion of water in a living system and the time for which the water is allowed to diffuse are found to be the critical process which determines the resolution. Second, as mentioned in section 3.1, the immature rays have a continuous superficial cytoplasm while during maturation the cysts are formed by the cytoplasm concentrating to form regular clumps leaving the tapering end of each ray "empty" (i.e. full of non-living aqueous vacuolar solution).

Most of the members in the plant kingdom are found to bear a cell vacuole [73]. *Acetabularia* in its growth process develops a vacuole in the rhizoid which eventually extends to the stalk and also through each ray in an immature cap. Therefore the "empty" space created during maturation, in a mature cap, is connected with the vacuole of the stalk.
Previous work done on plant cells [79] indicates that the cell water in plants has a higher diffusion coefficient for cellular water than that in animal cells. There are two widely accepted postulations for the behaviour of water in a biological system. Briefly, one predicts that some water is bound to the macromolecules of the cell hence retarding the motion of water; the other water component is relatively free. These two components are known as bound and free water respectively. The other postulates that some of the water is ordered or "structured" in the cell, limiting its movement within the cell. Regardless of the way the behaviour of cell water is explained the result accepted by many is that there are at least two kinds of water in a live organism. Based on this idea one can expect the rays of an immature cap, which has a continuous superficial cytoplasm, to have the macromolecules in an ordered manner resulting in a fairly large fraction of the intracellular water being structured or bound. Therefore the free diffusion of the cytoplasmic water in an immature ray is probably impeded by the continuous cytoplasm. Also, the underlying vacuolar water must be influenced to a certain extent by the cytoplasm. On the other hand, the mature caps or rays will have bound or structured water within the cyst or in the immediate vicinity. The centre area of each ray should have more free water which has a higher diffusion rate. Therefore, the inability to resolve the centre of mature caps was thought to be due to the relatively high free water component, which has a higher diffusion rate, in this area. This can be explained further as follows.

The resolution can be shown to be related to the gradient applied and the diffusion coefficient by the following equation (equation 27, chapter 1 and [26]).

\[(\delta z)^3 \geq \frac{D}{12 G \gamma}\]

This equation clarifies an important point; that is, the inverse cube root dependence of the gradient required to attain a particular resolution when molecular diffusion is dominant.
For instance, if \( D = 2 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \) and the gradient is 15 Gauss cm\(^{-1}\), the resolution attained will be \( \sim 3 \mu\text{m} \).

The resolution limit can also be expressed by the following equation [42].

\[
(\delta x) \geq 3 \sqrt{\frac{2}{3} D t}
\]

where \( t \) is the total time for one experiment, or the total time that diffusion occurs, which is between the initial 90\(^0\) pulse and the end of acquisition.

Two important points are assumed in this equation. First, in the spin-echo sequence, a phase difference is thought to occur for only 1/3 of the total time, \( t \). (note that, in this study, for a SW of 30 KHz the total time was about 6 msec where the phase encoding gradient was applied for only 2 msec). Second, the other factors which limit resolution are ignored.

Alternatively, a similar equation can be obtained by considering the line broadening or the randomization of phase due to molecular diffusion. By the random walk treatment of the spins [42],[54] this phase difference during diffusion can be given by the following expression.

\[
(\Phi)^2 = \frac{2}{3} \gamma^2 G^2 D t^3
\]

where \((\Phi)^2\) is the mean square difference in phase when the spins are allowed to diffuse for a time \( t \). The reader is directed to reference [42] and the references cited therein for a detailed discussion.

The distribution of this function can be given as a Gaussian function, where the standard deviation of this distribution is given by \( \sqrt{\frac{2}{3} D t} \). Hence, the line width of the Gaussian line is \( 2 \sqrt{\frac{2}{3} D t} \). Considering two adjacent Gaussian lines which are to be resolved, the separation has to be larger than \( 2 \sqrt{\frac{2}{3} D t} \) which results in an expression similar to equation (28) (Note that Gaussian lines have to be separated to a larger extent than Lorentzian
lines in order to be resolved). According to equation (28), with $D = 2 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$ and $t = 6 \times 10^{-3} \text{ sec}$, the resolution, $\delta z$, will be $\sim 8.5 \mu\text{m}$. Equation (28) shows the direct relationship between the resolution and the imaging time of one sequence.

Consider the centre of a mature cap. It has more free water which will give rise to a higher diffusion coefficient, $D$. Therefore, from equation (28), it can be realized that at a given time the mature ray centre will have a lower resolution when compared to an immature ray. However, it should be mentioned that even the immature ray has only a superficial cytoplasmic layer where the water may be less mobile. Beneath this cytoplasmic layer is the vacuole connected to the stalk. Therefore the greater fraction of free water, with relatively high diffusion coefficient, must be at least one of the reasons which hindered attaining better resolution.

Using either equation, (viz. (27) and (28)) the resolution limit approximates to less than 10 $\mu\text{m}$. This does not explain entirely the resolution limit of 40-50 $\mu\text{m}$ attained during this study.

As mentioned earlier, the decapitation of the cell to obtain the cap probably, at least in the mature cap (because the mature cap has aborted its vegetative growth), leaves a permanent opening at the point of incision. Placing the severed caps, especially the mature caps, in the Shep medium facilitates the diffusion of the medium into the cellular area of the cap. This was one reason that the imaging experiments were performed within a day or two after selecting the caps (the others were to prevent bacterial invasion and to minimize developmental changes occurring within the cap). Stanley Luck [58] has reported that when the caps were placed in the Shep enriched with $D_2O$, the $D_2O$ diffused into the cap within a short time. Experiments were not performed to monitor any effect on resolution which might result in allowing the cap to stand in the Shep for a longer time.

The $T_2$ values found, using the Hahn spin echo sequence, for most of the caps were
within the range 15-40 msec (see section 3.7.1.). Using equation (25), and a $T_2$ value of 25 msec as an average value for the *Acetabularia* caps, it can be shown that the resolution limit is about 1 $\mu$m. This is about 2% of the resolution limit reported in this thesis. Therefore the effects due to $T_2$ can be neglected as being less critical to the resolution attained during this study.

The one-pulse experiments performed with all types of *Acetabularia* caps resulted in broader lines with half height linewidths over 1000 Hz. Based on the one-pulse spectra a categorization of mature, immature or partly mature caps was not obvious because the spectra were quite different from one another. Therefore attempts were not made to identify the nature of a cap with the aid of the one-pulse spectra obtained.

Consider the following equation (equation (24) in section 1.7.4),

$$\nu_{1/2} \geq \frac{1}{\pi T_2}.$$ 

Substituting a lower $T_2$ value of 15 msec in the above equation the half height linewidth should be about 20 Hz. The observed linewidth, which is over 50 times the expected value, was assumed to be, besides molecular diffusion, mainly due to the magnetic field inhomogeneity generated due to the susceptibility differences of the interfaces of the sample and the sample holders. The possible effect of line broadening by placing interfaces at the rf coil region was studied using NMR tubes. The results are presented in Appendix-A.

The NMR tube had air/glass/water/air interfaces in the rf coil region. This was found to yield a water peak with a half-height line width of about 250 Hz (on a 270 MHz spectrometer this is about 1 ppm). On the 6.3 T magnet this difference will correspond to about 0.063 Gauss. Since the moderate gradient power used in this study is about 15 Gauss cm$^{-1}$, in a spatial scale this difference can be approximated to be about 40 $\mu$m. As for the real sample and the holder the number of interfaces are much higher (viz.
air/solid[holder]/air/“water”[sample]/air). Hence, with the heterogeneous biological sample the loss in spatial resolution can be expected to be worse (considering a half height line width of 1000 Hz). Based on these observations the magnetic susceptibility changes at different interfaces were concluded to be the other major cause, besides diffusion, for the loss in resolution of the images obtained. Moreover, Stanley Luck who studied the same biological sample reported similar line broadening [58]. Hedges [63] has shown the water/air interface to correspond to an inhomogeneity of about 6 ppm. This, in theory will correspond to a maximum loss in spatial resolution of hundreds of microns.

However, these calculations assume the most severe conditions. In practice, the inhomogeneity due to susceptibility changes can be small, as the present results show. This effect is, however, most probably the main factor limiting resolution in this study.

As seen in FIG-15 images obtained at higher gradients had better image quality. In theory, higher gradients will resonate adjacent voxels at larger frequency separations, yielding better resolved images. However, in this study, the sweep width (SW) was increased along with the gradient. Therefore ideally, the FOV remains more or less the same which implies that the resolution has to be almost the same. One important fact is that by increasing the SW, one decreases the acquisition time. Also the gradient time has to be reduced as the gradient is increased. This is done to maintain the proper FOV (Appendix-B). This procedure eventually reduces the overall evolution period. This in turn has the capability of increasing the signal intensity (less signal lost due to $T_2$ process) and also reducing the effect due to molecular motions within the cell during the evolution period (equation (28)). Therefore, the improvement in image quality at higher gradients was attributed to the reduction of evolution period rather than to the direct utilization of higher gradients.

Careful observation of the image obtained using higher gradients in FIG-15 (viz. FIG-15A) shows that the image is slightly distorted when compared with the photomicrograph or
the corresponding lower gradient image. This is probably due to the improper recognition of the gradient increment value ($LT \ G_{xi}$) specified in the software. The value specified through software for the high gradient value was 0.031 (calculated value 0.03074). Perhaps the specification of decimals of .031 was too large compared to 0.0307, which might have caused a visible error in one dimension of the image.

The resolutions attained by using higher gradients (FIG-15) were calculated as explained earlier and are given below.

<table>
<thead>
<tr>
<th></th>
<th>Resolution ($\mu$m)</th>
<th>Pixel resolution ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Immature cap</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B Moderate gradient</td>
<td>~60</td>
<td>33</td>
</tr>
<tr>
<td>C High gradient</td>
<td>~50</td>
<td>31</td>
</tr>
<tr>
<td>2) Mature cap</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E Moderate gradient</td>
<td>~90</td>
<td>32</td>
</tr>
<tr>
<td>F High gradient</td>
<td>~80</td>
<td>30</td>
</tr>
</tbody>
</table>

The “enlargement” procedure was not considered to be of acceptable quality or the technique to obtain a better resolved image. However, as seen in FIG-16A and FIG-16B, which are high gradient images of the immature and mature caps shown in FIG-15, probably better but crude microscopic insight to the cap is possible. Here the gradient was increased and the sweep width was kept constant. The result was the decrease in FOV. Careful observation of the folded images of the mature cap shows orderly arrays of dark points which closely resemble the arrangement of nuclei at the onset of cyst formation. As expected this
type of structure was not seen in the immature cap. The diameter of the suspected cysts was estimated to be about 30 \( \mu \text{m} \). The pixel resolution for FIG-16A and FIG-16B was 15 \( \mu \text{m} \). Confirmation of the identity of these structures as cysts needs a biological approach of staining caps with a nuclear stain and monitoring the staining regions of the cysts. This appears feasible but has not yet been done.

Almost all the images presented in this thesis have a loss in resolution and sometimes even in signal intensity at the upper and lower centre regions of the images. Several experiments were performed to estimate and monitor the magnetic field homogeneity over the sample. These are discussed in Appendix-D. Based on these experiments this loss in image quality was attributed to the rf inhomogeneity over the sample.

\section*{3.5 Water and the cell}

Water is essentially the most important nutrient that a cell requires for life. Deficiency of water will influence various levels of biological activities of the organism directly or indirectly. Water, which can probably be regarded as one of the building blocks of the cell, has been under scientific investigation for a long time. From a \textit{NMR} point of view, water in different microenvironments of the heterogeneous organism is bound to have different \textit{NMR} properties, such as \( T_1 \), \( T_2 \), molecular diffusion.

The state of water has been broadly categorised into two main components. First, the water which is bound to macromolecular arrangements (cell organelles) or macromolecules, having restricting movement and a somewhat structured nature. Second, the relatively free water which is remote from, and not directly influenced by, the relatively immobile macromolecular surfaces [44],[79],[80],[81]. (Recall that the resolution differences in immature and mature caps which were discussed in section 3.4. were based on this idea of bound and free
NMR relaxation times of the organism are functions of the molecular environment in which the water is localized. $T_1$ and $T_2$ curves obtained from biological samples very often have a multi-exponential decay providing evidence of different water components in a biological system. A large number of NMR studies performed on biological systems have shown the dependence of relaxation times and molecular diffusion on particular sites of water [79], [80].

NMR studies reported on plants are few [80], in contrast to the immense amount of research done on animals. However, the limited literature reveals important information about plant cells. More work could undoubtedly make beneficial contributions to forestry (lumber industry), agriculture and even indirectly to medicine.

Relaxation studies on plant cells have shown, that in general, the average $T_1$, $T_2$ and diffusion coefficient values of water are higher than in animals [79], [80] which has been attributed to the presence of a vacuole in many plant cells. The studies performed on dried yeast [81], beans and maize [79], cucumber [82] and wood [83] support the idea of at least two, or sometimes three [80], [83], different water components in plant cells. The reader is directed to the references of interest for further discussions on plant cellular water.

The Acetabularia mediterranea cell, which is a marine green plant, has distinct morphological changes during maturation which might be portrayed by the change in the environments of water thereby affecting the $T_1$ values of the cap. $T_1$ value for total water content (that is with one exponential fit) for a mature cap was found to be about 500 msec. This led to the interest in contrasting the different regions of the cap as a function of their respective $T_1$ values or $T_1$ contrast imaging.
3.6 $T_1$ contrast imaging of Acetabularia mediterranea caps

3.6.1. Pulse sequence

This was essentially a combination of the inversion recovery pulse sequence (FIG-2) and the spin echo imaging pulse sequence 2 (FIG-8B). A $180^\circ$ pulse was applied to the proton spin system of the Acetabularia cap which inverted the spins. The spin system was allowed to relax for a time $t (= r)$. This was followed by a $90^\circ$ pulse which carried the magnetization components to the $x'y'$ plane. This spin system then evolved in the presence of the phase and frequency encoding gradients. A final $180^\circ$ pulse, to refocus the spins, was applied and the echo was acquired in the presence of the frequency encoding gradient.

The calculation of the $T_1$ values was done using the inversion recovery technique. Only one curve fitting routine was used in this process. The composite peak heights of water, generated from all the components of water in the cap, were plotted against the interpulse delay and a single average $T_1$ value was calculated. Since the software was not capable of handling a multi-exponential decay the plots for peak height ($A_\infty - A_t$) versus the interpulse delay, $t$, were done manually. The fitting of data into straight lines, if two or more decays were conspicuous, was done by visual approximations.

3.6.2. $T_1$ contrasting

If the water at different voxels in the Acetabularia cap has different $T_1$ values, one may obtain a $T_1$ contrast image. For instance, assume that there are only two different kinds of water in the cap which correspond to two $T_1$ relaxation times. Let them be $T_{1m}$ (mature rays) and $T_{1y}$ (immature or young). After a $180^\circ$ inverting pulse, the exponential growth of the two magnetization vectors, $M_m$ and $M_y$, can be expressed as follows (from equation 8).

$$M_m = M_0 \left( 1 - 2 e^{-\frac{t}{T_{1m}}} \right)$$

(29)
and

\[ M_y = M_0 (1 - 2 e^{-\frac{t}{T_{1y}}}) \]  \hspace{1cm} (30)

where \( M_0 \) is the equilibrium magnetization, \( t \) is the time between the inversion 180° pulse and the 90° pulse, \( M_m \) and \( M_y \) are the magnetization components of mature and immature rays after time \( t \) and \( T_{1m} \) and \( T_{1y} \) are the spin lattice relaxation times for the water in the mature and immature rays respectively.

Since, \( T_{1m} \neq T_{1y} \), after time \( t \), also \( M_m \neq M_y \). Hence the result after the 90° pulse will be the NMR signal proportional to the \( T_1 \) relaxation time of the respective ray. When this is followed by the imaging pulse sequence the final result is an image corresponding to different \( T_1 \) values of the mature and immature rays or a \( T_1 \) contrast image. Similarly, this equation can be applied to each voxel provided they have different \( T_1 \) values. In a condition where each voxel has different \( T_1 \) values, the final image mapped will have different intensities which are a function of individual \( T_1 \) times. It is clear, from equation (29) and (30), that a series of such images are obtainable by varying \( t \), the interpulse delay time, which will probably have different intensities.

The data obtained were processed as in a normal proton image. However, when a series of NMR images which consisted of 3-4 \( T_1 \) contrast images, all the images were scaled with respect to, the absolute intensity value of, an image.

\( T_1 \) contrast images of three different Acetabularia caps, a) immature, b) mature and c) partly mature, are shown in FIG-17, FIG-18 and FIG-19 respectively. The relevant proton density image and the photomicrograph are given with the respective \( T_1 \) contrast images for comparison. The interpulse delays (time between the inverting 180° pulse and the 90° pulse) were varied between 200 msec and 500 msec (e.g. 200 msec, 300 msec, 400 msec, 500 msec).
FIG-17 $T_1$ contrast images of an immature *Acetabularia* reproductive cap.

A) Photomicrograph of the cap. (x 10)

B) Normal proton density image.

$T_1$ contrast images.

C) Interpulse delay 200 msec.

D) Interpulse delay 300 msec.

E) Interpulse delay 400 msec.

F) Interpulse delay 500 msec.

$$G_y = 16.51 \text{ G cm}^{-1}, \text{ } FOV_y = 0.889 \text{ cm}, \text{ } G_{z_1} = 0.12 \text{ G cm}^{-1}, \text{ } \Delta FOV = 9.269 \times 10^{-3} \text{ cm},$$

$$t_g = 2.2 \text{ msec, } DW = 16 \mu\text{sec, } NS = 128. \text{ Number of gradient increments}=128.$$

* The interpulse delay is the time that the spin system is allowed to evolve before detection or the time between the inverting $180^\circ$ pulse and the $90^\circ$ pulse.
FIG-18  $T_1$ contrast images of a mature cap.  A) Photomicrograph of the cap. ($\times$ 10)
B) Proton density image. C) $T_1$ contrast image with an interpulse delay of 400 msec.
D) Image with the interpulse delay of 500 msec.

$G_y = 17.45 \ G \ cm^{-1}$, $FOV_y = 0.92 \ cm$, $G_z = 0.116 \ G \ cm^{-1}$, $\Delta FOV = 3.264 \times 10^{-1} \ cm$,
$t_\phi = 2.2 \ \mu sec$, $DW = 16 \ \mu sec$, $NS = 16$. Number of gradient increments = 128.

* Note the orderly arrangement of cysts seen with more intense inner areas.
FIG-19 $T_1$ contrast images of a mixed cap which had mature immature and partly mature rays.

A) The photomicrograph of the cap. The partly mature rays show developing cysts as dark spots (a late stage of development; at very early stages the nuclei appear as small light spots). ($\times 10$)

B) Proton density image.

$T_1$ contrast images.

C) Contrast image. Delay of 200 msec.

D) Contrast image. Delay = 300 msec.

E) Contrast image. Delay = 400 msec.

F) Contrast image. Delay = 500 msec.

$G_y = 17.45 \, \text{G cm}^{-1}$, $FOV_y = 0.841 \, \text{cm}$, $G_z = 0.133 \, \text{G cm}^{-1}$, $\Delta FOV = 9.438 \times 10^{-5} \, \text{cm}$, $t_\varphi = 2.1 \, \text{msec}$, $DW = 16 \, \mu\text{sec}$, $NS = 16$. Number of gradient increments = 128.

* In D and E, most of the rays which give a very intense signal are the partly-mature ones.
3.7. Results and discussion

During $T_1$ contrast imaging, 2-4 contrast images and a proton density image were obtained. This process extended the total experimental time to over three hours. Therefore the closed sample holder (FIG-12F) was always used in order to minimize loss of water by evaporation. Furthermore, the almost universal property of a plant cell, the presence of a cell wall, was a natural gift exploited during this study in maintaining the moisture contents within the cell.

Initially, one value of $T_1$ for a mature cap was found which was 520 msec. Based on this single value and the $T_1$ value quoted by Stanley Luck (580 msec.)[58], the interpulse delay times (the time between the inverting $180^\circ$ pulse and the $90^\circ$ pulse) were chosen between 200 msec. and 500 msec. The idea put forward by many authors [80], that biological systems contain two or more components of water, was extrapolated to the Acetabularia cells. Under this assumption a cap was thought to have at least two different $T_1$ values corresponding to two different water components and hence yielding a bi-exponential decay curve. The 270 MHz spectrometer software was not capable of defining more than one exponential curve. As a result, the absolute $T_1$ values of different caps were not of primary interest at the commencement of this study. The study was oriented in a direction to obtain 3-4 $T_1$ contrast images and then; firstly, to extract an absolute $T_1$ image from the contrast images obtained and secondly, to monitor the development or maturation stages of the cap by NMR imaging. The former was thought to be of considerable use in either highlighting or suppressing an area of interest by manipulating the interpulse delay time (since the $T_1$ values are known). A computer programme‡ was written for this purpose. The theoretical aspect of this programme was to determine the intensities of a pixel of the $T_1$ contrast images and to plot them against the interpulse delay, $t$, in order to calculate the $T_1$ value.

‡ Dr. Sarath Abayakoon. Faculty of Civil Engineering. Univ. of Peradeniya. Sri Lanka.
for that pixel. Similarly to perform (e.g. for a $256 \times 256$ image) $T_1$ calculations for each pixel and finally to reassemble these values to form a $T_1$ image. However, this could not be implemented during this study due to instrumental limitations.

Therefore the study was confined to the latter, investigating the maturation stages of the caps by $T_1$ contrast imaging. This will be of more importance, especially to the developmental biologist, because often many morphological changes in development are found to occur on a scale of tens of micron which is resolvable by NMR imaging.

In order to explain the contrasting, consider a partially mature cap with mature rays (relaxation time = $T_{1m}$) and immature rays ($T_{1y}$) where $T_{1m} \neq T_{1y}$. As shown in FIG-20A there will be two distinct exponential curves corresponding to $T_{1m}$ and $T_{1y}$ (In practice on the 270 MHz spectrometer, one observes a composite of these two values). From the equations given in section 3.6.2. (equation (29) and (30)), the contrast can be denoted [58] as a ratio as follows,

$$\frac{A_m}{A_y} = \frac{(1 - 2 e^{-\frac{t}{T_{1m}}})}{(1 - 2 e^{-\frac{t}{T_{1y}}})},$$

where $A_m$ and $A_y$ are the peak heights corresponding to the magnetization components of the mature and immature caps respectively at delay time $t$. It was assumed that the equilibrium value of the magnetization ($M_0$) is the same in both types of rays. However a plot of $\ln \frac{A_m}{A_y}$ against $t$ using the above equation to observe the variation of contrast has an infinite value. Therefore, in order to circumvent this problem the following method can be used to express the contrast [84].

$$A_m - A_y = 2 A_0 \left( e^{-\frac{t}{T_{1y}}} - e^{-\frac{t}{T_{1m}}} \right)$$

A plot of this equation is shown in FIG-20B. Note that the these plots were obtained by using arbitrary units in order to explain the results. From FIG-20B one can obtain the time at which the contrast is maximum.
The image contrast in the $T_1$ contrast imaging scheme used in this study can be explained by the same equation but the plots have to be discussed in a different manner. Recall that the imaging protocol produced NMR signals only in the absolute mode (magnitude calculation in Appendix-B). That is regardless of the sign, all signals are rectified to yield a positive quantity. Under these circumstances a plot of signal versus the interpulse delay would take the form shown in FIG-20C rather than the plot shown in FIG-20A [85]. This was one obstacle for the incorporation of the computer programme written to calculate (see above) the $T_1$ values for pixels of an image (the other reason was that the software available did not permit the transfer of negative data to the image display system [from the 1280 computer to the IBM-PC]). The plot to monitor the variation of contrast of the plot shown in FIG-20C is given in FIG-20D. Note that FIG-20B and FIG-20D are quite different although they were obtained from the same equation. The important point is that, from FIG-20C, a component which has very short $T_1$ as well as long $T_1$ values yields a positive signal. In a NMR image both these components will appear as intense regions which generated a certain ambiguity during this study. Therefore the results had to be analysed bearing this point in mind.

The $T_1$ contrast images obtained using an immature cap are shown in FIG-17. The four interpulse delays used were 200 msec, 300 msec, 400 msec and 500 msec respectively. The images did not show a drastic change in contrast. This was expected because all the immature rays in the cap were almost identical, each having a more or less continuous cytoplasm. The $T_1$ value for an immature cap was not known. Therefore, it was assumed that the $T_1$ values of immature and mature caps were in the same range when the interpulse delays were chosen. In the following discussion one would realize that this assumption was not exact. The band of noise appearing over some of the images is an artifact. The types of artifacts and the probable causes for them are discussed in Appendix-D.
FIG-20 $T_1$ and $T_2$ contrast plots of two magnetization components (e.g. mature and immature rays) which have different $T_1$ values. These plots are drawn in arbitrary units in order to explain the observations.

A) Exponential growth of the two magnetization components.

B) Contrast between two exponential functions obtained by subtracting one another.

C) The rectification of all the negative data (in imaging) because of the intensity of all the negative peaks is made positive by the magnitude calculation.

D) The contrast curve generated from functions shown in C).
Two $T_1$ contrast images obtained from a mature cap are given in FIG-18. The two images correspond to interpulse delays of 400 msec and 500 msec. Clearly the contrasts in these images are much superior to the respective proton density images. The contrast images highlight the presence of clusters of cysts in mature rays (FIG-18C and 18D). The cysts appear more intense than the rest of the cap. This can be explained as follows. The high intensity means the cyst contents has a very long or very short $T_1$ (FIG-20C). The cyst diameter is about 50 $\mu$m. Within this minute structure it has a dense cytoplasm, numerous daughter nuclei, chloroplasts, food storage granules etc. Therefore the water entrapped within the cyst should be strongly bound or highly influenced by the neighbouring macromolecular arrangement giving rise to a very short $T_1$ component for water.

The most interesting caps for $T_1$ contrast imaging and also which could provide insight into the development processes were the ones which contained a diversity of rays, some immature, some partly mature and some completely mature. Such samples were found frequently in the cultures of Acetabularia. As mentioned earlier, the author is unaware of its presence in nature. However, without more biological study, it is not possible to conclude whether the lack of synchrony in development of the rays is fairly normal in reasonably healthy cells, or whether the sample chosen for this purpose is somewhat unhealthy.

Fig-19 shows a photomicrograph of a cap with three distinctly different types of rays, a normal $^1H$ density image and four $T_1$ contrast images obtained at delay times 200 msec, 300 msec, 400 msec and 500 msec. The images obtained at delay times 400 msec and 500 msec yields good contrast. Three distinct observations were made from these images.

a) Shorter delay times, such as 200 msec, yielded images which were not much different from the proton density image.

b) The mature rays appeared less intense (darker) than the partly mature or immature rays.
The partly mature rays (the partly formed dark structures, which are cysts, are seen in these rays) yielded stronger signals when compared to a totally immature ray.

The second observation (b) can be explained as follows. The water which is not entrapped within the cysts will have a longer $T_1$ value than the water in the cyst. But the close packed nature of the cysts in a ray will cause the water $T_1$ values in the vicinity to be shorter than free water, that is, to an intermediate $T_1$ value. Therefore the mature ray will appear comparatively less intense in these images. Since the immature and partly mature rays appeared more intense (c) and because of the image processing technique, this implies that these two types of rays will either have very long or very short $T_1$ values in comparison to a mature ray (FIG-20C). These observations appeared to portray an important maturation stage with distinct structural changes which directly or indirectly affected the state of water and therefore the $T_1$ contrast images.

The ambiguity and inability to explain the final observation (c) called for the necessity to measure the absolute $T_1$ values for caps with different developing stages.

The $T_1$ values for a few caps of different maturation stages were measured. Approximation of the $T_1$ values for mature rays in a partly mature cap was done by measuring the $T_1$ values for fully mature caps. $T_1$ values were also determined for partly mature caps. During the performance of these measurements, some intrinsic and external problems were found to make these $T_1$ measurements questionable.

(a) The development stage of maturation appeared to be short lived. Selection of the caps at the right stage was extremely difficult. A strenuous investigation with great patience was done by Mr. Keith Graham, by observing many caps through the optical microscope, before the suspected ones used in the $T_1$ measurements were provided.

(b) At the time of these measurements, the only culture available which was actively capping (cap forming) was in a doubtful state of health, having suffered a bacterial infection
which had apparently been effectively controlled with Penicillin.

(c) Short interpulse delays between 5-40 msec could not be used due to spurious noise peaks appearing on the water resonance peak. These delays were important in recognizing any short component of $T_1$.

(d) The $T_1$ values obtained directly from the spectrometer were composite values representing all the protons in the caps. Clearly these values were not of direct use to this study. As mentioned earlier the software of the spectrometer has not been designed to fit multiexponential decays to the data.

Because of the drawbacks of this nature, the study and the results obtained were considered to be quite preliminary. However, some $T_1$ values are given below.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>One fit $T_1$(msec)</th>
<th>Components of $T_1$(msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature</td>
<td>977</td>
<td>362 1500</td>
</tr>
<tr>
<td>Mature</td>
<td>1071</td>
<td>390 1625</td>
</tr>
<tr>
<td>*Mature</td>
<td>1040</td>
<td>280 1400</td>
</tr>
<tr>
<td>Partly mature</td>
<td>1135</td>
<td>320 1750</td>
</tr>
<tr>
<td>Partly mature</td>
<td>1130</td>
<td>580 1775</td>
</tr>
<tr>
<td>*Partly mature</td>
<td>1215</td>
<td>380 1040</td>
</tr>
<tr>
<td>Partly mature</td>
<td>1289</td>
<td>350 1775</td>
</tr>
<tr>
<td>*Partly mature</td>
<td>1376</td>
<td>One component</td>
</tr>
<tr>
<td>Immature</td>
<td>1403</td>
<td>375 2040</td>
</tr>
<tr>
<td>Immature</td>
<td>1551</td>
<td>340 -</td>
</tr>
<tr>
<td>*Immature</td>
<td>1430</td>
<td>150 1720</td>
</tr>
</tbody>
</table>

* The infinite-time intensity was considered to be as the peak height at an interpulse delay of 20 sec. The $A_\infty$ value for the other $T_1$ values were considered to be the peak height at a delay of 4 sec.
Clearly the $T_1$ values obtained had quite large discrepancies, which calls for careful curve fitting on many well-characterized healthy caps. However, two points were clear from this study. First, the immature cap had a longer $T_1$ value when compared to partly mature or mature caps. Second, most of the plots appeared to yield a bi-exponential curve, suggesting the presence of at least two different components of water. A short component which was in the range of 150-350 msec and a long component which fell in the range 850-1700 msec were identified in most of the caps. The plots which showed only one component or no component at all, were probably due to the improper use of the infinite peak height value at a delay of 4 sec. The individual components were not studied further because of the preliminary nature of the results.

As observed in the images given in FIG-19 the partly mature rays were more intense than mature rays and also the totally immature rays. One intention of the $T_1$ value determination was to attempt to extract, from a partly mature cap, the information necessary to explain the above observation. Definitive results have not yet been obtained. Therefore based on these observations it was only possible to conclude that the partly mature or "developing ray" had a very short or very long $T_1$ component, in contrast to both a totally immature or mature ray, because as shown in FIG-20C, either way, the final image will have a intense signal corresponding to a partially mature ray. A selective determination of $T_1$ values of partly mature rays was not possible.

Since it was not possible to evaluate the $T_1$ value of a partly mature cap directly, an experiment was performed to observe the relative intensity variation of the partly mature rays upon saturation. The experimental pulse sequence was very much similar to the one shown in FIG-8B, which was used to obtain all the images presented in this thesis. The difference was that the experiments were performed by varying the repetition time. Four imaging experiments were performed on the same *Acetabularia* cap by only varying the
repetition rate of the different experiments from 2 sec to 1 sec to 500 msec and finally to 250 msec. That is, the preparation period or the relaxation delay, which is the time that was permitted for the perturbed magnetization vector to reach equilibrium, was varied as mentioned above. The four images obtained and the photomicrograph are given in FIG-21. The effect of reducing the repetition time is that this process saturates any signal which arises due to a long $T_1$ component. This is because of the inadequate time permitted for the components with long $T_1$ values to reach equilibrium. Therefore, in an image the areas with long $T_1$ values will appear as less intense regions. This is essentially the basic idea of the saturation recovery technique used by some authors in obtaining contrast images [94]. Note that this type of contrasting was not suitable for microscopic imaging because signal saturation was hard to tolerate in an already dilute proton environment.

In the images obtained (FIG-21) the partially mature rays appeared more intense. If these rays had longer $T_1$ relaxation times, as in totally immature caps, then in the images with short repetition rates the partially mature rays should appear less intense. Therefore the partially mature rays which appeared brighter in the $T_1$ contrast images (FIG-19) cannot have long $T_1$ values. This suggested that the partially mature ray have a very short relaxation time. That is during maturation the $T_1$ relaxation time of a cap will vary from a long $T_1$ to a very short and then to a moderate $T_1$ value. Biologically, this means that there is a major structural change during the cap maturation process which affects the state of the water in the maturing rays. These can be conveniently explained by, first looking at the ultrastructural changes of the cap during maturation in more detail.

3.7.1. The development; Ultra structure

When the *Acetabularia* cap is fully formed but does not yet contain nuclei, each ray is occupied by continuous cytoplasm as a cylindrical shell surrounding a central cylindrical
FIG-21 The NMR images obtained by varying the repetition time or preparation time. This saturates components with long $T_1$ values. Performed to monitor the behaviour of the partly mature rays.

(A) The photomicrograph.

(B) Repetition rate between two successive excitation 90° pulses were 2 sec.

(C) Repetition time = 1 sec.

(D) Repetition time = 500 msec.

(E) Repetition time = 250 msec.

$G_y = 16.51 \text{ G cm}^{-1}$, $FOV_y = 0.889 \text{ cm}$, $G_z = 0.12 \text{ G cm}^{-1}$, $ΔFOV = 9.27 \times 10^{-3} \text{ cm}$,

$t_g = 2.2 \text{ msec}$, $DW = 16 \mu\text{sec}$, $NS = 16$. Number of gradient increments = 128.
vacuole, i.e. the same arrangement as the main stalk of the cell. During maturation, cytoplasm containing daughter nuclei flows into each ray and the vacuole is expelled into the main stem [74]. Thus the fraction of the total water in the cap rays which is associated with living biological material increases, while that of simple aqueous solution decreases.

When the nuclei have entered the cap, they become arranged at equally-placed intervals in a regular array in each cap. They first become visible under the optical microscope as small colourless spots, which then enlarge [86]. The regular geometrical ordering of the nuclei appears to be brought about by the cytoskeleton; a very large number of microtubules appear in the cytoplasm near to the nuclei [86]. The cytoplasm then starts to contract around each nucleus. At some point, which has not been observed precisely in vivo, a membrane forms around each developing cyst, and a heavy cellulosic wall forms. These stages of cyst formation involve also the re-entry of aqueous solution, not cytoplasm-associated, into each ray. At the early stages of their development, the cysts appear large and green. Later, they are somewhat smaller and sufficiently densely-packed with living contents to appear black under a microscope. In some instances, abnormal cyst development is seen, in which these are many large enclosed regions of elongated shape, instead of smaller spherical cysts. Such abnormality can be induced by the use of colchicine, which inhibits the production of microtubules [87].

With the understanding of maturation given above, the observation of partially mature rays having a short $T_1$ value and hence appearing as an intense area in the image can be explained easily. The immature ray has a centre vacuole where the cytoplasm only coats the periphery. The $T_1$ value which represents the whole ray appears brighter because the overall $T_1$ value is larger (closer to that of free water because the rays are extensively occupied by the vacuole, which has free water). During maturation the cytoplasm and nuclear materials expel the vacuole and the whole ray in a partly mature cap should be densely packed with
cytoplasm. Therefore the water associated is highly influenced or bound to the neighbouring macromolecules resulting in a drastic drop in the $T_1$ value. After the cysts are formed the centre of the cap is filled with free water (long $T_1$) but the water associated with the cysts should in theory have a short $T_1$ value. However, since the value obtained is a composite of these two components the final $T_1$ value will be higher than a partially mature stage. Therefore it is clear that the partially mature rays will appear as high intensity signal in an image.

The formation of cysts occurs once only over a period of few days in a life cycle of several months. In any culture, the details of this process are likely to vary somewhat from time to time. This stage of development has not been a particular study of this laboratory. Therefore, correlation of $T_1$ contrast imaging and $T_1$ measurement with developmental stage as reported here, is a very preliminary investigation. The results suggest that a much more extensive project, with careful characterization of the developmental stages, could be fruitful. From the above account, it seems likely that changes in relaxation times (resolvable components of a multi-exponential relaxation) could arise from:

a) Changes in fractions of "cytoplasm-associated water" and "aqueous solution" during development;

b) Changes in cytoplasmic organization during cyst formation.

3.8 Other types of contrast imaging

3.8.1. $T_2$ contrast imaging

The spin-spin relaxation time, $T_2$, is also being used for contrast imaging [88] because, like the $T_1$ relaxation time, $T_2$ also depends on the molecular environment of the biological system. The pulse sequence used for this purpose was the spin echo imaging pulse sequence
shown in FIG-8B. In order to vary the echo time, the time that the spin system is allowed to evolve or the time between the initial $90^0$ pulse and the echo, two delay times were incorporated in this familiar pulse sequence. This delay could be varied and a series of $T_2$ contrast images could be obtained.

During this study, $T_2$ contrast imaging was given little attention because of the following reasons.

a) The $T_1$ contrast imaging was the major goal in this work. In $T_2$ contrasting there is loss in signal due to $T_2$ relaxation, especially at longer delays, therefore at the initial stages of this study this method was considered to be less fruitful in microscopic imaging.

b) As discussed earlier the molecular diffusion of water in *Acetabularia* caps and the susceptibility changes caused at the interfaces of the sample and the holder had a significant effect on the loss of signal. This suggested that the $T_2$ contrast images would reveal little information because they are also dependent on the factors given above.

The $T_2$ values were determined for almost all the caps that were used to calculate the $T_1$ values. The pulse sequence used was a simple Hahn echo sequence. From the values obtained, an approximate demarcation of the developmental stages was possible. The mature caps had $T_2$ values of 18-24 msec (agrees with the value quoted in reference [58]) while the values obtained for immature caps were between 30-40 msec. The $T_2$ values for partly mature caps showed a certain overlap but most had values between 20-30 msec.

In the simple Hahn sequence, the effects due to diffusion are not minimized, especially at long delay times [8]. This appeared to have a significant effect in the decay of the transverse magnetization component, when the $T_2$ values for a few partly mature caps were determined using the CPMG pulse sequence. The $T_2$ values were found to be between 60-70 msec. These values obtained are of a preliminary nature which calls for several such $T_2$ values in order to come to a fruitful conclusion. However, based on these $T_2$ values,
it appears that there is a three fold increase in the $T_2$ values obtained when the CPMG sequence was used.

### 8.8.2. Contrasting using paramagnetic reagents

Paramagnetic ions are known to reduce the relaxation times of water in any system. This idea has been used in various forms of biological tissues to improve contrast [89],[90]. The reader is referred to these references for more detail. A study performed elsewhere [91] led to the attempt, in the present work, to contrast structures in a mature cap with cysts using a solution of $MnCl_2$ as a paramagnetic relaxation time enhancer.

In nature the cysts are dispersed into the sea. Until further development these cysts must survive in the external environment which can change drastically over time. Hence the cysts, with the thick cellulosic wall which is evolved in nature for such hardships, were thought to resist the absorption of a paramagnetic substance such as $MnCl_2$, at least for a considerable amount of time. A mature cap was submerged in a 2 mM solution of $MnCl_2$ for one hour. An imaging experiment was then performed on this paramagnetically doped cap. As expected the cysts in the mature cap appeared as brighter regions. That is, the cyst walls have prevented the flow of the ion during the allocated time. The image obtained is given in FIG-22. The respective proton and $T_1$ contrast images of this cap obtained during other experiments are given in FIG-14D and FIG-18 respectively.

Similar images have been obtained [58] using $D_2O$ as the contrasting agent. These images have been obtained by, first, immersing the cap in heavy water for a few minutes. The images produced by Deuterium contrasting [58] also highlight the cysts which had resisted the diffusion of $D_2O$ into them during the experiment.
FIG-22 A NMR image of a mature cap obtained after being doped for one hour in a 2 mM MnCl₂ solution. The gray scale and the false colour images are given. Note the cysts which are clearly visible.

This contrasting technique was not continued in this study although it showed great potential as a contrasting technique. Despite the possible biological disturbances introduced by using these external contrasting media, it appears that this technique could be used to monitor the maturation stages of a cap.

3.9 Imaging other parts of the Acetabularia cell

Imaging experiments were performed on isolated cysts as well as the rhizoids. The experiments done on isolated cysts (diameter ca. 50 μm) need more careful studies. Therefore it is excluded in this discussion. The imaging experiments done on rhizoids are of a preliminary nature which suggest an area for an extensive study with much support from a biological standpoint besides NMR.
3.9.1. Imaging the rhizoids of *Acetabularia*

The rhizoid, like the roots of higher plants, helps attach the cell to the surface of its habitat. In *Acetabularia* the rhizoid also bears the nucleus [72], [74]. It is important to briefly recall the biological structure of the rhizoid in order to explain the reason for studying this organelle. At an immature stage the rhizoid is full of cytoplasm. The large nucleus, which has a diameter of about 100 \( \mu \text{m} \), is embedded in this cytoplasm. Because the growth of the cell begins from the rhizoid area, the cell wall in the stalk close to the rhizoid is thick. The thickness of the wall is about 10-30 \( \mu \text{m} \). As the cell grows, the vacuoles which are initially generated in the rhizoid, gradually extend to the stalk. This process forces the cytoplasm and the nucleus to the periphery of the cell. As the cap is formed and the cell matures, the nucleus divides into numerous daughter nuclei which get transported to the cap. A residual portion of the nucleus which is smaller in size remains in the rhizoid.

*NMR* imaging experiments were performed on rhizoids, firstly, to attempt to resolve the thick cell wall and secondly, and more importantly, to study the change in the nucleus during maturation. The samples were obtained by cutting the stalk a few millimetres away from the rhizoids of freshly selected cells. The cut end was essentially in contact with the environment. The sample was placed in the sample holder shown in FIG-12F and the imaging experiments were performed with 128 gradient increments.

Since the formation of the vacuole in the rhizoid and the immediate stalk area confines the cytoplasm to the outer layer of the cell wall, the images obtained showed the demarcation of the "cell wall", which was concluded to be a composite of the cell wall and the cytoplasm which is forced against the wall by the vacuole. Also what one observes is a projection of the cylindrical stalk on to one plane. This obviously gives a misleading sense about the resolution attained. Slice selection, in a plane perpendicular to the xy plane, across the stalk is a better method of assessing the exact thickness of the wall. However, slice selection was
not done in this study. Also the $T_1$ values of the cell wall and the adjoining cytoplasm (i.e. $T_1$ contrasting), might to a certain extent, replace the necessity for slice selection.

One major problem in using the rhizoid was the loss of moisture from the amputated end of the rhizoid. Therefore the imaging experiments had to be performed at shorter times and only 2-3 images were obtainable. Three images, one proton image and two $T_1$ contrast images with delays 200 msec and 300 msec, were obtained from a mature and immature rhizoid. The three false colour encoded images along with the respective photomicrographs are shown in FIG-23.

The pixel resolution of these images was about 30 μm. Recalling that the lowest resolution attainable is the pixel dimensions, the structure observed in the stalk (mature, left) which appeared as the cell wall, was most probably a composite of the cell wall and the cytoplasm. However, further studies with higher gradients, to reduce the FOV and hence the pixel dimensions, were not done during this study. The main interest was to observe the nucleus which has a distinct change during maturation. In the images shown, the brighter area (green) is seen clearly in the immature rhizoid while it is less dense in the mature rhizoid. This appeared to be the nucleus of the respective cells. However, justification of this observation requires a biological approach of staining the rhizoid to monitor the changes in the nucleus. As mentioned earlier this could open up an interesting study in monitoring the behaviour of the nucleus during maturation.
FIG-23 NMR images of the rhizoid of *Acetabularia*

A) The photomicrograph of two, left = mature and right = immature, rhizoids.

B) Proton density images of the two rhizoids obtained by placing both on the holder in FIG-13F. The colour encoded images are shown to highlight the centre bright area which was suspected to be the nucleus.

C) $T_1$ contrast image of the rhizoids. Delay = 200 msec.

D) $T_1$ contrast image. Delay = 300 msec.
CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

The work reported in this thesis had two aspects;

a) Demonstration of the achievement, for $^1H$ imaging of an actual biological sample, of a spatial resolution at the threshold of microscopic range;

b) Demonstration that it may be possible to monitor a major cytoplasmic reorganization during a developmental event, because of the fact that reorganization produces a changing distribution of relaxation times $T_1$ and $T_2$ for water protons.

It was shown that, in terms of resolving two adjacent structures, a resolution of 40-50 μm could be achieved by using the 270 MHz spectrometer, the imaging probe (Appendix-A) and gradients in the range of 10-20 $G/cm^1$. As an achieved resolution of visible features in the images this is at least equal to the best previously-reported examples in the literature.

The principal sources of limitation of resolution in this work are probably,

(a) Susceptibility change across interfaces, and

(b) Diffusion of water.

By simulating similar conditions to the sample height and the position where the sample and the holder are localized in the probe, using NMR tubes filled with water, it was shown that there is a significant line broadening of the NMR line obtained under these
conditions. This line broadening was attributed to the presence of interfaces and hence the inhomogeneity generated in the rf coil region. This sample closely matched the positioning of the biological sample and the sample holder in the probe. Therefore, the fact that the line broadening (or loss in image resolution) was caused by the field inhomogeneity due to the presence of several interfaces, was safely extrapolated to the *Acetabularia* cap which has an air-liquid-air interface in the rf coil region (ignoring the sample holder). Using an NMR tube, which was filled with water, it was observed that this type of line broadening also occurred in a different probe and in a different spectrometer.

The $T_1$ contrast images of the partly mature caps yielded information which correlated with development. The partly mature rays appeared more intense than either mature or immature rays. This highlighted a probable major cytoplasmic reorganization within the cap accompanying maturation which influenced the $T_1$ values of cellular water. The images obtained at shorter repetition times yielded intense signals corresponding to the partly mature rays suggesting shorter $T_1$ values for them. From the $T_1$ contrast imaging experiments and the preliminary $T_1$ values obtained, it was found that the $T_1$ values of the immature caps were long. Similarly the $T_1$ values of mature rays appeared to be shorter than immature rays but were longer than those of partly mature rays. Therefore, it was concluded that during maturation the $T_1$ value of the cap changed from a long to a short and finally to a moderate value.

From a biological point of view, during maturation, the cytoplasm gets concentrated with numerous bio-materials which are used to form the cysts. Also, the formation of a new special structure, known as microtubules, adds to this dense nature of the cytoplasm at an intermediate state of development. These structures eventually disappear as the cysts are formed. Therefore, probably the highest concentration of bio-materials which will affect the cap water, by binding or otherwise, can be expected to be at the intermediate stage. The
observations made by NMR, where the cap $T_1$ values were concluded to vary from, long (immature) via a short value (intermediate) to an intermediate (mature) value fits into the biological description of maturation given above.

**Suggestion for future work**

Both aspects, as reported here, are rather preliminary indications, and very extensive future work is possible on both. Much could be done on the same organism, *Acetabularia*. For extension to other organisms, it would probably be best to concentrate on the plant kingdom, in which cells are often in the 50-100 μm size range. In the animal kingdom, most interesting developmental events occur in multicellular arrays in which the cell size is $\sim 10$ μm. Hence the animal development biologist tends to regard 10μm as the upper limit of the useful microscopic range.

In regard to spatial resolution, it would be useful to use higher gradients (provided all the instrumental conditions could be optimized) than those used in the present work in an attempt to push the resolution from the presently achieved $\sim 40$ μm down to $\sim 10$ μm, using plant specimens as test objects. In the one very high gradient experiment reported here (FIG-17) on an *Acetabularia* cap, a pattern was seen which might be the pattern of distribution of nuclei at the beginning of cyst formation. This could be a suitable sample for future study. The NMR work would have to be combined with good biological characterization of the event of cap maturation under the optical microscope. Nuclear staining would be needed to confirm that the pattern observed by NMR imaging is indeed that of the nuclei.

It might be helpful to assess further the factors given as the reasons for being unable to attain better resolution than $\sim 40$ μm (*viz.* Molecular diffusion of water and the inhomog-
geneity caused by the interfaces of the sample and sample holder in the rf coil region).

The development event of cap maturation in *Acetabularia* occurs once only in a life cycle of several months, and occupies a few days. To assure an adequate supply of well-characterized samples, much attention must be given to the biological side of the work. New cultures must be started at frequent intervals, and the course of the maturation event must be observed closely.

The indication from $T_1$ contrasted imaging that, with suitable choice of delay time, an intermediate stage of cytoplasmic reorganization may be highlighted, comes from one sample (FIG-19). This had three kinds of rays, identified as "immature", "mature", and "partly mature". The last mentioned are the rays of particular interest and the description of them as "partly mature" is tentative in the absence of more extensive study of maturation stages. For the $T_1$ measurements which this observation led to, the only culture available at that time was one which had been penicillin-treated. There seemed to have been substantial recovery, but the behaviour of this culture is not necessarily typical of healthy cells. The $T_1$ components which seem to be present also requires confirmation with attention to acquiring (a) several data points in the 1-200 msec time range; (b) several data points in the 1-3 sec range.

The few studies reported here on the rhizoid end of *Acetabularia* cells show some promise that both the nucleus and the very thick cell wall might be imaged. Further work on this needs attention to sample handling: either a good ligation procedure for the cut stalk, or some good way of arranging intact cells in a sample holder so that the rhizoid can be imaged. This sample could be a useful one on which to try slice selection procedures, provided that slice thickness can be no more than 10's of $\mu$m.

The *Acetabularia* cell is big enough that the major parts of it can be readily separated. It would be useful to perform $T_1$ measurements on such parts, *e.g.* a number of ripe cysts,
cytoplasm, vacuole contents, cell wall etc. Also very high gradients can be used on small structures such as the cysts in order to attain smaller resolution levels.

Despite its advantages for this work, *Acetabularia* has one great disadvantage: it will not stay healthy very long out of water. Plant samples which are more stable in air might usefully be studied. In higher plants, wound recovery, *e.g.* in tomato plants, involves the formation of undifferentiated callus tissue which, over a few weeks, differentiates with the formation of a new growing layer (cambium) and new vascular bundles. Cell diameters start at ~100 μm and decrease to ~50 μm in a few days after wounding. The best kind of study of this kind, however, would involve following the process of the recovery or maturation *in vivo* day by day or weekly, and would need a means of introducing the intact plant into the spectrometer.
REFERENCES


39 J. Morrow, *J. Histochem. and Cytochem.* 34 [No.1], 75 (1982).


References


93 *Nicolet 1180 or 1280 Operational Manual*. Nicolet magnetics.

References


APPENDICES

These appendices contain detailed material on experimental procedures likely to be of interest chiefly to future experimenters using the same or similar equipment.

Appendix-A

The probe.

The gradient calibration.

Line broadening at interfaces—"magnet shimming".

The pulse sequences used.

Appendix-B

Experimental parameters.

Data processing.

Appendix-C

Constituents of Shephards medium; The culture medium for *Acetabularia*.

Appendix-D

Sample holders and image quality.

Field inhomogeneity of the imaging probe.

Image artifacts.
A.1 The Probe, Gradients coils, Shimming and pulse sequences

A.1.1 The probe and the gradient coils

A detailed discussion on the evaluation of the probe is given in reference [58][68]. The following is an overview of the structure and some evaluations done by the author in order to perform NMR imaging experiments. The probe is enclosed in a 37 mm (o.d.) Al tube. 3mmx5cm slots are cut in this Al tube to minimize effects due to eddy currents. The sudden variations of the gradient pulses in an external magnetic field generate these undesirable currents on the surface of the probe. The Al shield is fitted into a 5 mm thick perspex cylinder whose o.d. is 34 mm. In this perspex cylinder, grooves are cut which are 3 mm deep and 3 mm wide in order to localize the x,y, and z gradient coils. A high precision glass tubing (inner diameter 8 mm) is fitted into the centre of the probe. This forms the area in which the sample is placed. This means that the maximum diameter of an unforced sample must be less than 8 mm. The 11.5 mm diameter saddle shaped transmitter and receiver coil is supported on this glass tube (FIG-24). The centre of the rf coil is about 86 mm below the top end of the probe. This probe has also been designed to study flow effects. Therefore the centre of the probe is uninterrupted space. Hence, neither was it possible to blow air to spin the sample nor was it intended to. In order to mount the sample the probe had to be removed completely from the magnet and replaced after placing the sample in the probe. No air could be blown in order to lower the sample, as it is done in normal spectrometers, because of the continuous opening in the probe. Also placing the sample at the exact height.
FIG-24 A diagramatic representation of the imaging probe (not drawn to scale).

Structure A is made out of teflon and this localizes the glass tube on which the rf coil is wound and also helps support the sample at the correct height. The inner glass tube has a 8 mm i.d. and therefore the unforced sample has to be less than 8 mm in diameter. The outer Al shield is not shown in this diagram. The 27 gauge insulated Cu wire gradient coils are localized in the perspex cylinder. The diameter of the rf coil is 11.5 mm.
was essential and removing the probe, although cumbersome, was done when required during this study. The probe was fixed into the magnet by just one screw mainly because of this sample localization method adopted.

A.1.2. Gradients

The gradients in these experiments are generated by a set of three gradient coils corresponding to the three main geometrical axes, x, y and z. They consist of 27 gauge insulated Cu wires. The x and y gradient coils have 12 turns and the z gradient coil consists of 20 turns [58]. A digital to analog converter (DAC) is used to enable one to define the required gradient power by a digital input from the computer. The DAC output is controlled by the control lines of the 293B pulse programmer. This allows software control of the gradient amplitudes. The gradient magnitude generated by each coil was calculated by Stanley Luck [58][69]. The calculated values are given in table-2 and these values were used in this study.

TABLE-2

Gradient magnitudes of the x, y and z gradient coils (from [58])

<table>
<thead>
<tr>
<th>COIL</th>
<th>No. of turns</th>
<th>G (Gauss cm(^{-1})A(^{-1}))</th>
<th>Resistance (Ω)</th>
<th>G (Gauss cm(^{-1})V(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>12</td>
<td>4.83</td>
<td>3</td>
<td>1.61</td>
</tr>
<tr>
<td>Y</td>
<td>12</td>
<td>5.04</td>
<td>3</td>
<td>1.681</td>
</tr>
<tr>
<td>Z</td>
<td>20</td>
<td>6.55</td>
<td>2</td>
<td>3.275</td>
</tr>
</tbody>
</table>
An Amcron M-600 (Crown Intnl. Corp.) amplifier was connected to the y gradient coil and Tecron 7560 amplifiers (Crown Intnl. Corp.) were connected to the x and z gradients. At the time of installation, only coarse variable gain settings of the gradient power supplies were available. A slight deviation caused high changes in the gradient magnitudes from the expected calibrated value therefore affecting the FOV (field of view) and hence the final image. These were later replaced by more fine gain controls. Each of the modified power supplies was re-calibrated at several gain settings. As mentioned earlier, further control of the gradient magnitude is achieved by software. The software command which is used to define the gradient is known as the LT value. This command enables one to specify a numerical value between ±9 for the x and y gradients. Although the z power supply was calibrated, since it was not used during this study it is ignored in the following discussion.

The calibration of the power supplies was done by measuring the voltage across the resistor which is in series to the respective gradient coils. For different LT values defined by the teletype, the voltage across the 3Ω resistor which is between the amplifier and the probe (gradient coils) was measured. Initially a voltmeter was used for this purpose but in order to read the voltmeter accurately the gradient pulse had to be considerably long (~1 sec). The heat generated melted the connections to the resistor at higher gradients. Therefore further measurements were done using an oscilloscope, where gradient pulses of about 2 ms were used. Two computer programmes† were written:- First, to plot the LT value against the corresponding voltage across the resistor for several gain settings; The plots were straight lines depicting the linear variation of the voltage with the LT value. Second, to obtain the slope of each plot by a linear regression method. The amplifier gain and the corresponding slope of the plots of Voltage vs LT value for the y and x coils are given in table-3 and table-4 respectively.

† Dr. Sarath Abayakoon. Dept. of Civil Engineering. Univ. of Peradeniya. Sri Lanka.
## TABLE-3

Amplifier gain and the slope of the plots of Voltage vs LT value for the y gradient coil

<table>
<thead>
<tr>
<th>AMPLIFIER GAIN</th>
<th>GRADIENT (volts $LT^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.5589</td>
</tr>
<tr>
<td>100</td>
<td>1.0700</td>
</tr>
<tr>
<td>150</td>
<td>1.5276</td>
</tr>
<tr>
<td>200</td>
<td>1.9989</td>
</tr>
<tr>
<td>250</td>
<td>2.4269</td>
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<tr>
<td>300</td>
<td>2.8533</td>
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<td>3.2734</td>
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<tr>
<td>375</td>
<td>3.4871</td>
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<td>400</td>
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<tr>
<td>425</td>
<td>3.9156</td>
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<td>450</td>
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<td>500</td>
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<td>6.0613</td>
</tr>
<tr>
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<td>6.5861</td>
</tr>
<tr>
<td>750</td>
<td>7.1594</td>
</tr>
<tr>
<td>800</td>
<td>7.8053</td>
</tr>
</tbody>
</table>
Amplifier gain and the slope of the plots of Voltage vs LT value for the x gradient coil

<table>
<thead>
<tr>
<th>AMPLIFIER GAIN</th>
<th>GRADIENT (volts $LT^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.5466</td>
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<tr>
<td>100</td>
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<td>150</td>
<td>1.4938</td>
</tr>
<tr>
<td>200</td>
<td>1.9083</td>
</tr>
<tr>
<td>225</td>
<td>2.1054</td>
</tr>
<tr>
<td>250</td>
<td>2.3055</td>
</tr>
<tr>
<td>275</td>
<td>2.4756</td>
</tr>
<tr>
<td>300</td>
<td>2.6634</td>
</tr>
<tr>
<td>325</td>
<td>2.8509</td>
</tr>
<tr>
<td>350</td>
<td>3.0142</td>
</tr>
<tr>
<td>375</td>
<td>3.2068</td>
</tr>
<tr>
<td>400</td>
<td>3.3738</td>
</tr>
<tr>
<td>425</td>
<td>4.0599</td>
</tr>
<tr>
<td>450</td>
<td>4.1509</td>
</tr>
<tr>
<td>475</td>
<td>4.3719</td>
</tr>
<tr>
<td>500</td>
<td>4.6044</td>
</tr>
<tr>
<td>525</td>
<td>4.8088</td>
</tr>
<tr>
<td>550</td>
<td>5.0252</td>
</tr>
<tr>
<td>600</td>
<td>5.4795</td>
</tr>
<tr>
<td>650</td>
<td>5.9696</td>
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<td>700</td>
<td>6.5398</td>
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<td>750</td>
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<tr>
<td>800</td>
<td>7.7571</td>
</tr>
<tr>
<td>900</td>
<td>9.4512</td>
</tr>
</tbody>
</table>
These tables yield the value of the voltage per unit LT value. The strength of the gradient for each coil was known per unit voltage from table-2 (viz. 1.61 Gauss cm\(^{-1}\) V\(^{-1}\) for the x coil and 1.681 Gauss cm\(^{-1}\) V\(^{-1}\) for the y coil). Therefore for a given amplifier gain, multiplying the slope \((V/\text{LT})^{-1}\) by the gradient magnitude \((\text{Gauss cm}^{-1} \text{V}^{-1})\), one obtains the gradient magnitude per LT value. For instance, if the gain of the y gradient is set at 500 (table-3), the voltage per LT value (slope) is 4.5753 V LT\(^{-1}\). Since the gradient magnitude (from table-2) for the y coil is 1.681 Gauss cm\(^{-1}\) V\(^{-1}\) the magnitude of the gradient per LT value will be \(4.5753 \times 1.681 = 7.691 \text{ Gauss cm}^{-1} \text{LT}^{-1}\). If a LT value of 5 is chosen the gradient generated is \(7.691 \times 5 = 38.46 \text{ Gauss cm}^{-1}\). This value is important because it is used to calculate the FOV of an imaging experiment (see page 160). The gradient power supplies were operated in constant voltage mode (as opposed to the constant current mode).

A.1.3. The gradient pulses

Ideally the gradient pulses “delivered” to the sample are assumed to be exactly square. But in reality the pulses are hardly ideal. The pulses are shown to have a finite rise time (time taken for the pulse to reach the expected power level) and a fall time (the time taken for the power level to reach the zero level). During this study the rise and fall times of the gradient pulses were found to be about 120 \(\mu\)sec. An oscillogram of the two gradient pulses, frequency encoding (\(G_y\)) and phase encoding (\(G_x\)), is given in FIG-25. The photograph was obtained by connecting the leads of the two channels of the oscilloscope to the x and y gradient power supplies. The rise and fall time in both the pulses can be seen in FIG-25. If delays were used in this study they were chosen to be as short as possible and within the range of the fall time of the pulses. Furthermore, the delays were manipulated so as to obtain the maximum echo at the centre of the acquisition time.
FIG-25 An oscillogram of the frequency encoding gradient ($G_y$) [top] and the phase encoding gradient ($G_z$) [bottom]. The rise and fall time of the gradient is about 120 $\mu$sec.

A.2 Magnet shimming

In a NMR experiment, whether spectroscopy or imaging, it is essential to maintain the best possible magnetic field homogeneity during the experiment. In practice this is done by fine alterations of the magnetic field using “shim” coils to obtain an uniform magnetic field over the sample. This process is known as shimming. A convenient practical method often used is to alter the field (using the shim coils) in order to obtain a narrow linewidth of the final spectrum, and in high resolution studies shimming is done to obtain line widths in the order of 1 Hz.

The sample holder, used in this study, had a flat bottom and also the biological sample studied rarely had a height of more than 2 mm. Therefore, the shimming procedure was attempted by using a flat bottom NMR tube with water filled up to about 2 mm and
positioning this part of the tube in the rf coil region of the imaging probe. The 1-pulse spectrum obtained under these conditions had a line width of about 250 Hz (FIG-26A). A similar experiment was performed with a round bottom NMR tube. A similar line broadening was observed (FIG-26B). The following sequence of experiments was performed with the flat bottom NMR tube. The tube was filled with water and the tube was lowered so that the rf coil would experience a more uniform spin distribution. That is the number of interfaces were taken further apart from the rf coil. As shown in FIG-26C,D,E and F the water peak became narrower as the tube bottom was moved away from the rf coil. In order to make sure that this observation was independent of the probe a similar experiment was performed in the high resolution probe. The results are shown in FIG-27. When the flat bottom or the round bottom tube was placed at its regular position the FID was smooth and the water peak was narrow. When the two tube bottoms were localized in the rf coil region, the FID’s showed a marked change and so did the final spectrum, yielding broader peaks (FIG-27C and D). This observation was further justified by performing the same set of experiments, as shown in FIG-27, in the departmental Bruker 400 MHz spectrometer where similar line broadening was observed when either the flat bottom or the round bottom NMR tube was placed in the rf coil region with a water layer of about 2 mm.

Since the imaging experiment utilizes gradients the shimming of the magnet was always done with the gradient power supply on. However, the gradients were not applied during this procedure. FIG-28A and FIG-28B shows the spectra obtained from the flat bottom tube while the gradient power was turned on (FIG-28A) and the power turned off (FIG-28B). Note that although there are no conspicuous differences in the spectra, a significant change is observered in the decay of the FID. Therefore it was concluded that, although a defined gradient was not applied during shimming, a finite current was flowing in the gradient coils when the gradient amplifiers were on.
FIG-26 One-pulse experiments performed in the imaging probe using flat bottom and round bottom NMR tubes containing water.

A) The NMR spectrum of a flat bottom NMR tube filled with water up to 2 mm and the flat bottom placed in the rf coil centre (line width ~250 Hz).

B) A NMR spectrum obtained under similar conditions as in A but using a round bottom NMR tube line width ~250Hz).

C) The water level was elevated to ~1 cm above the rf coil and the flat bottom was placed in the rf coil centre. The gradient amplifier was turned on when this spectrum was obtained.

D) The NMR spectrum obtained under similar conditions as in C, but the gradient power supply was turned off during the experiment. C and D was done to observe any affect of line broadening with respect to the gradient power supplies.

E) A one pulse spectrum obtained using the water filled flat bottom NMR tube. The flat bottom was placed ~0.5 cm below and the upper water level was placed ~1.5 cm above from the rf coil centre.

F) The NMR spectrum of a flat bottom tube filled with water up to a height of ~3 cm. The rf centre was placed in the centre. Note that as the number of interfaces (air/glass/water/air) were moved away from the rf coil region the line width of the NMR peak got narrower.
FIG-27 One-pulse spectra from the high resolution probe.

(A) *NMR* spectrum and the respective FID of a water filled round bottom *NMR* tube placed at the regular position in the high resolution probe.

(B) A one pulse spectrum and the FID of a water filled flat bottom *NMR* tube at the regular position.

(C) The spectrum and the FID of the round bottom *NMR* tube where the water level was reduced to 2 mm and the bottom placed in the rf coil region.

(D) The *NMR* spectrum and the corresponding FID of the flat bottom *NMR* tube obtained under similar conditions as C.

* Similar results were observed when the same sequence of experiments shown above was performed in the departmental Bruker 400 MHz spectrometer.
FIG-28 One pulse *NMR* experiments performed in the imaging probe with a water filled flat bottom tube at the normal position. This experiment was done to observe any effect from the gradient power supplies.

(A) The spectrum and the FID obtained when the gradient power supply was turned off.

(B) The *NMR* spectrum and the FID in the presence of the gradient amplifier turned on.

* Although the two spectra do not show an appreciable change the FID has a faster decay when the gradient was turned on. There was a finite current flowing in the probe even when a gradient was not specified. Therefore, magnet shimming was always performed when the gradient power supply was on.
Consider the results presented in FIG-26. The line width when the holder is at the regular position is about 3 Hz. The line width when the flat bottom is placed in the rf coil region is about 250 Hz. This difference (~ two orders of magnitude) shows that the field inhomogeneity caused by the differences in interfaces can be quite significant in the resolution of an image. The real sample and sample holder will have more interfaces than the NMR tube filled with water. This is elaborated in the following diagram. Here, since about 80% of the cell contents of the *Acetabularia* cell is water, the cap is considered as a liquid layer.

\[\text{Flat b. NMR tube} \quad \text{Sample and holder}\]

\[S = \text{Solid}, \quad L = \text{Liquid}, \quad A = \text{Air}, \quad R = \text{rf coil}.\]

Therefore, probably the effect of field inhomogeneity due to the number of interfaces with different magnetic susceptibilities in the rf coil region will be greater in the sample and sample holder. However, this factor was not investigated further during this study.

In the imaging pulse sequence, a 180° refocussing pulse was used to reduce the effects due to field inhomogeneities. However, a significant magnetic susceptibility difference, such as the one reported in this thesis (~ two orders of magnitude), might not be refocussed completely as far as the whole echo is concerned. That is, the decay due to the magnetic susceptibility differences might be incorporated in the frequency encoding dimension.
because the frequency encoding is done during the acquisition time. Also, some authors have reported [92] that a simple Hahn echo sequence (which was used in the imaging pulse sequence) is less efficient in refocussing the inhomogeneity due to susceptibility changes. However, these predictions need extensive study before fruitful conclusions can be made.

A.3. Comparison of the two imaging pulse sequences

Experiments were performed to compare and contrast the images obtained by using the two pulse sequences discussed in section 2.2. The two $^1H$ density images obtained using the same *Acetabularia* cap, are shown in FIG-29. Considering the two images obtained and also the centre echo (echo number 64) acquired, there are considerable differences between the two pulse sequences. Theoretically, the first pulse sequence (FIG-8A), where the pulses are separated from one another, has an extra added delay. This makes the total evolution period longer. This can have two main disadvantages. First, when the delays are longer there is greater signal loss due to $T_2$ processes. In a microscopic scale this can not be tolerated. Second, molecular diffusion which is a significant factor at the microscopic level can affect the resolution of the final image if the spin system is allowed to evolve for a longer time. Therefore in theory, one could expect the pulse sequence 2 (FIG-8B), where $G_z$ is applied before the 180° pulse to have a higher sensitivity as well as better resolution. Here it is assumed that complications such as gradient pulses coupling with each other etc. are absent. According to the results obtained (FIG-29) the pulse sequence 2 (FIG-8B) was observed to yield better images with higher signal intensity in contrast to the image obtained using pulse sequence 1 (FIG-8A).
FIG-29 NMR imaging using the two pulse sequences shown in FIG-8.

A Stacked plots of two different Acetabularia cap images obtained using the pulse sequence shown in FIG-8A.

B The plots of the same two caps obtained by using the pulse sequence shown in FIG-8B.

* Note that the pulse sequence 2 (FIG-8B) yields a better resolved image with higher intensity.
APPENDIX B

B Choice of Experimental Parameters and Data Processing

B.1 Choice of experimental parameters

B.1.1 Block size and digital resolution

 Digitization of a NMR signal is a function of the block size chosen in an experiment. For instance if the block size is 512, as used during this study, the NMR signal will be characterized by 512 points (256 real and 256 imaginary in Quadrature Phase detection, QPD). The time between two data points is known as the dwell time (A data point corresponds to a single sample of the voltage induced by the transverse magnetization). The dwell time is related to the total sweep width in QPD by the following equation.

\[
\text{Dwell Time} = \frac{1}{\text{Sweep Width}}
\]

The dwell time (DW) can be reduced by increasing the sweep width [66]. This was done in this study when larger gradients were used. As mentioned earlier the block size was limited in this study. Moreover, the maximum sweep width that can be used during this study was 62500 Hz (Dwell time 8 \( \mu \text{sec} \)). Therefore, when the sweep width was increased the same number of points (512) were used to specify a larger frequency dispersion. That is, in an image a given dimension is defined by a smaller number of points and therefore two points are separated from one another to a larger extent. This causes a loss in digital resolution. Therefore the increase of SW indefinitely (when larger gradients are applied) with constant block size will lead to image foldover and hence will degrade the final image.

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B.1.2 Field of view (FOV)

The FOV in both dimensions (frequency and phase) has to be identical [23]. If the FOV's are not compatible, the dimensions of the image get distorted when compared to the original object. As a result, sometimes one extreme of the image gets folded over to the other end of the image. Such image foldover is shown in FIG-30.

![FIG-30](image)

FIG-30 A NMR image of an Acetabularia cap showing image fold over due to improper selection of the FOV.

Parameters for FIG-30; $G_y = 16.22 \, G \, cm^{-1} \, (LT = 2.6)$, $FOV_y = 0.905 \, cm$, $G_z = 0.12 \, G \, cm^{-1} \, (LT = 0.0227)$, $\Delta FOV = 1.88 \times 10^{-3} \, cm$, $\tau = 2.1 \, msec$, $NS = 20$, $DW = 16 \, \mu sec$. Number of gradient increments = 128.

Since the FOV is an important parameter in an imaging experiment a sample calculation which was used to obtain the image shown in FIG-14D is given below.

The FOV in the frequency encoding dimension is given by the following equation.

$$ FOV = \frac{1}{G_y \cdot \gamma \cdot DW} $$

where the FOV is in cm, $G_y$ is the frequency encoding gradient, $\gamma$ is the gyromagnetic ratio
and DW is the dwell time.

The FOV in the phase encoding dimension is given by the following equation.

\[
\text{FOV} = \frac{1}{G_z \cdot \gamma \cdot t_\phi}
\]

where \( G_z \) is the phase encoding increment and \( t_\phi \) is the time for which the phase encoding gradient is applied.

In order to obtain a distortion free image the following condition has to be satisfied.

\[
\frac{1}{G_y \cdot \gamma \cdot DW} = \frac{1}{G_z \cdot \gamma \cdot t_\phi}
\]

\( G_y \), the frequency encoding gradient was calculated using the LT value defined by the operator. This is shown below.

\[
\text{LT value} = 2.9
\]

Gradient magnitude for the y coil (table2) = 1.681 \( \text{Gauss cm}^{-1} \text{ V}^{-1} \)

Voltage per LT value (Amplifier gain 350) [From table3] = 3.2734 \( \text{V LT}^{-1} \)

Therefore the gradient applied,

\[
2.9 \times 1.681 \text{ Gauss cm}^{-1} \text{ V}^{-1} \times 3.2734 \text{ V LT}^{-1} = 15.9575 \text{ Gauss cm}^{-1}
\]

The sweep width used in order to obtain this image was 30000 Hz or the dwell time was 16 µsec. Therefore,

\[
\text{FOV} = \frac{1}{15.9575 \text{ Gauss cm}^{-1} \times 4257.71(\gamma/2\pi) \text{ Gauss}^{-1} \text{ sec}^{-1} \times 16 \times 10^{-6} \text{ sec}} = 0.9199 \text{ cm}
\]

In this study before the FOV in the phase encoding dimension was found, the gradi-
ent increment was evaluated. The phase encoding time was 2.2 msec ($t_\phi$). This can be substituted in the above equation to obtain the phase encoding gradient.

$$\frac{1}{0.9199\text{ (cm)}} = 2.2 \times 10^{-3} \text{ sec} \times 4257.71 \text{ Gauss}^{-1} \text{ sec}^{-1} \times G_{x_i}$$

$$G_{x_i} = 0.1161 \text{ Gauss cm}^{-1}$$

As mentioned earlier this value could not be defined directly by the software. First this value had to be converted into a LT value which could then be specified. In order to calculate the LT value the gradient per volt value for the x gradient coil was required, which was 1.61 $\text{Gauss cm}^{-1} \text{ V}^{-1}$ (Table-2). Also the voltage per LT value was known for the amplifier gain 300 (table-4) which was 2.6634 $\text{V LT}^{-1}$. Therefore,

$$\text{LT}_{\text{phase}} = \frac{0.1161 \text{ Gauss cm}^{-1}}{1.61 \text{ Gauss cm}^{-1} \text{ V}^{-1} \times 2.6634 \text{ V LT}^{-1}}$$

$$= 0.02706$$

Ideally this value should have been specified on the teletype. However due to instrumental limitations, only three or four decimals were specified. Therefore the teletype defined LT value was 0.027.

The FOV in the phase encoding dimension was evaluated by a reverse calculation using the LT value 0.027. It should be clear that the FOV in the phase encoding dimension (0.9199 cm), calculated using the value 0.027, will not be equal to the FOV in the frequency encoding dimension (0.9221 cm). Nevertheless, the image obtained did not show any drastic difference in the two dimensions. However, attempts were not made to determine the cutoff point of safely obtaining good images versus distorted ones.

### B.1.3 Number of gradient increments and performance time

In a spin-warp imaging experiment, the higher the number of gradient increments, the better will be the image quality and the resolution. In practice the gradient is incremented
in multiples of $2^n$. In this study several increment values corresponding to 16, 32, 64, 128 and 256 were used. Most of the work presented in this thesis were performed on a 512 block size and with either 128 or 256 gradient increments. Whatever the number of gradient increments implemented they were zero filled (Appendix-B.2), during image processing, to yield an image of $256 \times 256$.

The total imaging time (performance time) was reduced by decreasing the number of gradient increments to 64. This reduced the imaging time to about 20 minutes. As expected the images obtained under these conditions yielded poorly resolved images. The deterioration of image quality when the number of gradient increments are reduced was studied by the following experiment. A set of NMR images were obtained by using the same Acetabularia cap with various number of gradient increments, from 16, 32, 64, 128, 256. The five images and the respective photomicrograph are given in FIG-31.

A series of experiments were performed in order to investigate whether any other factors contributed to the loss in resolution of the images obtained with 64 gradient increments.

(a) Images were obtained at various phase encoding gradient times, ($t_\phi$), varying from 1.2 msec to 3 msec. In theory, longer encoding gradients can cause a loss in signal and shorter gradient times should result in better quality images. Images obtained under the these conditions with 64 gradient increments did not show a significant improvement in image quality. However, these experiments were not repeated with higher number of gradient increments such as 128 or 256.

(b) Experiments were done by respecifying the x and y gradient in the computer memory so that the frequency encoding gradient was the x (usually it was always y in this study) and the phase encoding gradient as y gradient. This was done to observe any effects due to the gradient coils. However, the change of the gradients from x(phase) to x(frequency) and vice versa for the y gradients did not show a difference in image resolution.
FIG-31 The loss in resolution of an image with reducing the number of gradient increments (number of blocks).

\[ G_y = 16.51 \, G \, cm^{-1} (LT = 3), \quad FOV_y = 0.89 \, cm, \quad G_{z_1} = 0.12 \, G \, cm^{-1} (LT = 0.025), \]
\[ \Delta FOV = 9.27 \times 10^{-3} \, cm, \quad t_\Phi = 2.2 \, msec, \quad NS = 12, \quad DW = 16 \, \mu sec. \]

A) Photomicrograph of cap,
B) Gradient increments = 16,
C) Gradient increments = 32,
D) Gradient increments = 64,
E) Gradient increments = 128,
F) Gradient increments = 256.
From the above experiments it was concluded that the loss in resolution was due to the reduction of the block size.

**B.2. Data processing to obtain an image**

The procedure followed to obtain the image shown in FIG-14D is discussed to clarify the data processing procedure. In this experiment 128 different blocks were acquired (i.e. 128 different phase encoding gradient increment values). This yielded 128 echoes. The spin warp technique used in this study, implemented the gradient increments in the phase encoding dimension from $+G$ to $-G$ (128 increments $[G_{z}]$ from $+G$ to $-G$). Therefore the block 64 will have no applied phase encoding gradient and hence yields the maximum signal [23],[66]. This 64th echo was apodized and baseline corrected. Apodization is a procedure in which the signal is multiplied by a certain mathematical function in order to improve S/N and remove truncation effects due to Fourier transformation of a finite time-range signal [93]. In this study apodization was done by multiplying the signal by a sine function. This apodized signal was then Fourier transformed. This procedure was repeated to all 128 blocks and scaled relative to block 64. A selected number of blocks, prior to and following First FT, are shown in FIG-32A and 32B respectively. These Fourier transformed data were rearranged in order to make the second Fourier transformation possible. This process is known as transposition. The transposition procedure can be explained as follows. The first column of points in each of the 128 blocks (rows) are extracted and placed as the first row in a new data set. Similarly, each column of the source data set is reoriented to a row in the new data set. The real and imaginary parts of the transposed data set are shown in FIG-32C. This data could now be Fourier transformed for the second time. Since this transposition process interchanges the two axes, once the 2-FT is done the horizontal axis will depict the phase encoding dimension which was the vertical axis in the original data set.
The most intense block of the transposed data was apodized, base line corrected and zero filled [10] to a data size of 256. The zero filled data was Fourier transformed for the second time. This data set then subjected to magnitude calculation to yield a positive data set. The entire data set of 256 blocks were then subjected to the same procedure and the final data set corresponded to a 2-D image (FIG-32D). The image was then displayed as stacked plots (FIG-32E). The images were also displayed using a graphics monitor controlled by an IBM-AT computer (FIG-32F).

The procedure to obtain the final image on the graphics screen involved several transferring and formatting processes. The approximate time taken during each key process is given below.

A Data acquisition ~ 40 mins (for 128 gradient increments).

B Data processing ~ 06 mins.

B* Stacked plot-Zeta ~ 36 mins.

C translating a 2-D file (DAT file) to a set of serial files ~ 20 mins.

D Data transfer 1180 [data station] to 1280 [department] ~ 25 mins.

E Transfering to floppy ~ 10 mins [includes formatting].

F Transfer to 1280 [imaging room] ~ 2 mins.

G Transfer from 1280 to IBM-PC ~ 4 mins.

H Reformating image transfered ~ 5 mins.

I Image display ~ few seconds.
FIG-32 Data processing procedure to obtain an NMR image.

A The original data set acquired as an spin echo for 128 different phase encoding \((G_z)\) gradients. Blocks numbers 32,40,48,56,64,72,80,88 and 96 are shown. The total plot has been scaled on block 64.

B The real part of the data set after the 1-FT. In practise the highest intense block was processed by BC:MS:FT:AI and then automated or linked to obtain the total data set.

C The real and imaginary data after being transposed into a block size of 256. Now the respective blocks corresponds to 64,80,96,112,128,144,160,176 and 192. Note that in this process the phase encoding \((G_z)\) and the frequency encoding \((G_y)\) dimensions are interchanged.

D The real data set after the second Fourier transformation. The most intense block was processed by MS:BC:ZF:FT:MC:AI and automated to obtain a 256 x 256 data set.

E A stack plot of the image obtained.

F A photograph of the same plot after transferring to the IBM-PC 256 gray/color scale screen.

Abbreviations 1) BC= base line correction. 2) MS= Apodization by multiplying the echo by a sine weighting function. 3) FT=Fourier transformation. 4) AI= Scaling to the absolute intensity. 5) TP= Transposition. 6) MC=Magnitude calculation. 7) ZF=Zero fill data.
**APPENDIX C**

Constituents of the Shephard's medium

**TABLE-5**

<table>
<thead>
<tr>
<th>MAJOR NUTRIENTS</th>
<th>CONCENTRATION[^/mol l⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COMPOUND</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>4.11 × 10⁻¹</td>
</tr>
<tr>
<td>MgSO₄. 7H₂O</td>
<td>4.87 × 10⁻²</td>
</tr>
<tr>
<td>TRIS</td>
<td>8.26 × 10⁻³</td>
</tr>
<tr>
<td>CaCl₂. 2H₂O</td>
<td>6.80 × 10⁻³</td>
</tr>
<tr>
<td>KCl</td>
<td>1.01 × 10⁻²</td>
</tr>
<tr>
<td>NaN₃</td>
<td>4.71 × 10⁻⁴</td>
</tr>
<tr>
<td>Na₃PO₄</td>
<td>7.04 × 10⁻⁶</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MICRO NUTRIENTS</th>
<th>CONCENTRATION[^/mol l⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COMPOUND</strong></td>
<td></td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>3.22 × 10⁻⁵</td>
</tr>
<tr>
<td>ZnSO₄. 7H₂O</td>
<td>6.96 × 10⁻⁶</td>
</tr>
<tr>
<td>Na₂MoO₄. 2H₂O</td>
<td>4.13 × 10⁻⁶</td>
</tr>
<tr>
<td>FeCl₃. 6H₂O</td>
<td>1.85 × 10⁻⁶</td>
</tr>
<tr>
<td>MnCl₂. 4H₂O</td>
<td>1.01 × 10⁻⁶</td>
</tr>
<tr>
<td>CoCl₂. 6H₂O</td>
<td>8.41 × 10⁻⁹</td>
</tr>
<tr>
<td>CuSO₄. 5H₂O</td>
<td>8.01 × 10⁻⁹</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1.62 × 10⁻⁵</td>
</tr>
</tbody>
</table>

The solution obtained is sterilized by heat treatment (Autoclaved) and Na₂CO₃ [1.11 × 10⁻³ mol l⁻¹] and vitamins are added through bacteriological filters. The vitamins are added in extremely small amounts, usually as a mixture of Thiamine Hydrochloride, p-Aminobenzoate, Calcium Pentothenate and Vitamin B12.
APPENDIX-D

D Sample holders and image quality, magnetic field homogeneity and image artifacts

D.2 Sample holders and image quality

Fung [85] studying the frog gastrocnemius muscle showed that the orientation of the muscle affected the relaxation times of the muscle. Also Walter and Hope [86] showed that the shape of the slime mould plasmodia, *Physarum polycephalum*, in the magnet caused changes in the magnetic field homogeneity. Burnell *et al* [87] have studied the Barnacle muscle, *Balanus nubilus*, and its effect on the NMR line shapes. Based on these observations, made by different authors, it appeared necessary to study the effect the shape of the sample holder and the material it is made out of will have on the image quality.

Four imaging experiments were performed with the same sample, the *Acetabularia* cap, by differing the four sample holders (FIG-12B, 12C, 12D, and 12E). The sample was turned over when placed in the round bottom holders. The four different images obtained are given in FIG-33. The experimental parameters were identical and the condition of the sample was assumed to be more or less the same. It should be remembered at this point that the mounting of the sample from one holder to another was done by removing the probe from the magnet. Therefore the author does not wish to refer to the conditions as being absolutely identical. In the four images shown in FIG-33 there is no clearcut differences in image quality. Therefore the effect of the shape, or the material which the sample holder is made out of, was neglected during this study. However, no experiments were performed in order to verify whether or not the resolution limit imposed in this study was due to the inhomogeneity caused by the interfaces of the sample holders used.

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FIG-33 NMR images of the same *Acetabularia* cap obtained using the four different sample holders shown in FIG-12B, 12C, 12D, and 12E. $G_y = 16.51 \, G \, cm^{-1}$, $FOV_y = 0.889 \, cm$, $G_x = 0.12 \, G \, cm^{-1}$, $\Delta FOV = 9.27 \times 10^{-3} \, cm$, $t_\phi = 2 \, msec$, $NS = 16$, $DW = 16 \, \mu sec$. Number of gradient increments = 128. A) Round bottom teflon holder. B) Delrin round bottom holder. C) The *Acetabularia* cap flipped by $180^\circ$ and placed in the flat bottom teflon holder. D) Under similar conditions as C) but the holder is flat bottom delrin.
Although there was no significant difference in the four images obtained as shown in FIG-33, there was a marked change in the diameters of the images obtained with the round bottom holders and the flat bottom holders. The round bottom holder, irrespective of the substance it is made out of, yielded images with smaller diameter in contrast to the flat bottom holder. Since the 2-D NMR images were projections of the caps on to the xy plane, these differences in image diameter were attributed to the total area which can be projected on the xy plane. In the round bottom holder (FIG-12C and 12E) the sample takes the shape of the inner surface of the holder. This restricts the area that the Acetabularia cap can occupy. On the other hand, the flat bottom holders (FIG-12B and 12E) do not have an external surface to limit the sample shape. Because of the greater diameters of the images obtained, and also because of the easy mounting capabilities of the sample into holders, flat bottom holders were used extensively.

In order to prevent the biological sample from drying, a sample holder was developed out of delrin which could be sealed after placing the sample. The external shape of this holder is shown in FIG-12F. Here the sample is placed, in its most stable position, in the lower cup shaped portion of the holder. This is then secured tightly to the upper part of the holder. It was found that using this closed sample holder, a series of experiments \((T_1\) or \(T_2\) contrast) which lasted for over six hours could be performed without any visual morphological changes in the cap. The main disadvantage of this holder is that, because of the finite thickness of its wall, the sample accommodation capacity was reduced.

In order to compare the image quality, a similar holder was built with teflon and two different experiments were performed using the same Acetabularia sample under similar conditions. As shown in FIG-34A, the two images obtained using these two holders did not have any difference.

Another method to prevent the Acetabularia cap from dehydrating was to perform an
FIG-34A Two images and the respective photomicrograph of the same *Acetabularia* cap, obtained using the closed sample holders, delrin and teflon, built to prevent the sample from drying (FIG-12F). As seen in the two images there is no visible change in image quality.

\[ G_y = 16.51 \, G\, cm^{-1}, \, FOV_y = 0.889 \, cm, \, G_z = 0.12 \, G\, cm^{-1}, \, \Delta FOV = 9.27 \times 10^{-3} \, cm, \]
\[ t_g = 2 \, msec, \, NS = 16, \, DW = 16 \, \mu sec. \] Number of gradient increments=128.

FIG-34B A NMR image of an *Acetabularia* cap mounted on a closed delrin holder (modified form the holder shown in FIG-12F) which was filled with water. Note the residual signal from the extracellular water.

\[ G_y = 13.76 \, G\, cm^{-1}, \, FOV_y = 1.07 \, cm, \, G_z = 0.096 \, G\, cm^{-1}, \, \Delta FOV = 8.83 \times 10^{-3} \, cm, \]
\[ t_g = 2.3 \, msec, \, NS = 8, \, DW = 16 \, \mu sec. \] Number of gradient increments=128.
imaging experiment by immersing the *Acetabularia* cap in water [94]. Here the *Acetabularia* cap was placed in the closed holder (FIG-12F) which was then filled with water and secured tightly. Water was chosen because it has a long $T_1$ value (when compared to the $T_1$ of a cap) and by performing the experiment at shorter repetition times, the author was hoping that the external water protons would get saturated and hence would not contribute to the final image. An image obtained following this procedure is shown in FIG-34B. However, besides observing the remnants of the external water in the image, some other disadvantages were believed to accompany this method. First, the osmotic pressure in the biological system and water can be considerably different and this can cause the cap to rupture. Second, short repetition times can also reduce the signal intensity from the sample, especially with components with long $T_1$ values. When imaging in the microscopic scale this type of loss in signal is intolerable. The Shep (Appendix-C) was not used for mounting the *Acetabularia* cap because several paramagnetic salts were used in the culture medium and it was suspected that this would lower the $T_1$ value of the external water protons.

**D.2. Field homogeneity over the sample**

It is important that the sample experience a uniform magnetic field. Field inhomogeneity will lead to line broadening and hence to the loss in resolution and signal intensity. Since the most important factor in microscopic imaging is the resolution, this discussion will be oriented with respect to the loss in resolution and sensitivity. The rf field homogeneity in the xy plane was investigated by Stanley Luck [58]. According to these studies, the rf field homogeneity in the xy plane was considered to be considerably uniform. The rf homogeneity in the z dimension was estimated as follows. A new sample holder, similar to the one that is shown in FIG-12F, was made out of delrin. But here the bottom part was only about 3 mm deep (cf. the depth of the one used to place the *Acetabularia* caps was
FIG-35 The rf field homogeneity along the z dimension (the axis parallel to the $B_0$ and the axis of the rf coil). The sample was a disc of water secured in the holder similar to the one shown in FIG-12F. The disk of water was about 2 mm thick. Six different images were obtained by placing the disk at different z-positions inside the rf coil. The images obtained are displayed on a 8 gray scale display. This display even shows the field inhomogeneity in the xy plane. The most uniform rf excitation was observed at the rf coil centre. The homogeneity was seen to drop drastically as the sample was moved away from the centre.

A) sample placed 2 mm above rf centre.

B) sample placed 1 mm above rf centre.

C) The disk of water placed in the rf coil centre.

D) Sample located 1 mm below the rf centre.

E) system located 2 mm below the coil centre.

F) Sample located at 3 mm below the rf coil centre.

$G_y = 16.22 \, G \, cm^{-1}$, $FOV_y = 0.905 \, cm$, $G_{z1} = 0.124 \, G \, cm^{-1}$, $\Delta FOV = 2.24 \times 10^{-3} \, cm$, $t_b = 2.1 \, msec$, $NS = 8$, $DW = 16 \, \mu sec$. Number of gradient increments=128.
about 7 mm). An aqueous solution doped with 5 mMol $MnCl_2$ was added to the bottom part of the holder and the upper part was secured tightly. It was assumed that this procedure formed an approximately 2 mm thick disc of water. This disc of water was placed at the centre of the rf coil and an NMR image was obtained. Similarly five more images were obtained by lowering and lifting (1 mm at a time) the disc of water with respect to the centre of the rf coil. The six images obtained are shown in FIG-35. From FIG-35 it is clear that the rf homogeneity drops sharply along the z dimension. This is in agreement with the observation made by the previous user using another method [58]. Therefore the best possible location which had considerable field homogeneity was the rf coil centre. All attempts were made to maintain the sample in the centre of the coil. This was one reason why the probe was removed completely from the magnet for the installation of a new sample.

Even when the sample was placed at the centre of the rf coil a loss in signal intensity and resolution in the upper and lower centre areas of the image was observed. This was more prominent when the sample was moved away from the centre (see FIG-15B).

It was necessary to verify whether this loss in intensity was due to the inhomogeneity of the static magnetic field or the applied rf field. First an NMR experiment was performed on an *Acetabularia* cap by placing it in the regular position (FIG-36A and FIG-36D). The experiment was repeated under similar conditions but the probe was rotated by 120° clockwise with respect to the regular position (FIG-36B and FIG-36E). Another experiment was done, with the sample corresponding to images in FIG-36A and B, where the probe was fixed in its regular position but the sample (sample holder) was rotated by 90° (FIG-36C). From the above results it was clear that the loss in spatial resolution occurred irrespective of the probe orientation. If the static field ($B_0$) was inhomogeneous the rotation of either the probe or the sample should demarcate the loss in resolution at different sites on the image. But, because the less intense areas were unaltered by the above manipulations it
FIG-36 NMR images of *Acetabularia* caps used in order to determine whether the loss in resolution in the upper and bottom centre areas of all the images obtained during this study was due to rf ($B_1$) inhomogeneity or $B_0$ inhomogeneity.

A The NMR image of an *Acetabularia* cap obtained placing the probe at the regular position.

B The stack plot of the same cap obtained by rotating the probe by 120° (gradient coils, rf coil and sample rotated).

C The image obtained by placing the probe at the regular position and rotating the sample (sample holder) by 90°.

$$G_y = 17.69 \, G \, cm^{-1}, \quad FOV_y = 0.83 \, cm, \quad G_{z_1} = 0.14 \, G \, cm^{-1}, \quad \Delta FOV = 3.22 \times 10^{-3} \, cm,$$

$$t_\phi = 2 \, msec, \quad NS = 8, \quad DW = 16 \, \mu sec. \quad \text{Number of gradient increments}=64.$$

D An image of an immature *Acetabularia* cap obtained by placing the probe in the normal position.

E The image of the same cap obtained after rotating the probe by 120°.

$$G_y = 16.51 \, G \, cm^{-1}, \quad FOV_y = 0.889 \, cm, \quad G_{z_1} = 0.12 \, G \, cm^{-1}, \quad \Delta FOV = 3.16 \times 10^{-3} \, cm,$$

$$t_\phi = 2.2 \, sec, \quad NS = 20, \quad DW = 16 \, \mu sec. \quad \text{Number of gradient increments}=128.$$

* Note that the areas with low resolution remain unaltered with these experiments.
was believed that this observation was most probably due to the $B_1$ field inhomogeneity.

\textit{D.8 Artifacts on images}

Four types of artifacts which degraded the image quality were observed during this study. They are shown in FIG-37.

A) A vertical line of spike artifacts appeared over the centre of some images (phase encoding dimension) (FIG-37A).

It appeared that the apodization before the 2nd FT was necessary to reduce this spike (artifact) across the centre of the image. This is shown in FIG-38A and FIG-38B. Here the first image (FIG-38A) was obtained without apodization where as the second image (FIG-38B) was obtained after apodizing the data set two times. The next set of experiments was done in order to locate the position of the apodization in the image processing sequence if the artifact is to be eliminated. The results obtained are shown in FIG-38C and FIG-38D. In FIG-38C the base line correction was done before the apodization in contrast to the image in FIG-38D where this sequence was reversed. The latter did not have any artifact. This was thought to be because of a noise peak, which was probably at the end of each echo, giving rise to an overestimated DC offset \cite{93} which was suppressed by performing the apodization first. The maximum number of apodizations done was two and they were always used after base line correction.

B) A horizontal band of noise often appeared over some of the images (FIG-37B).

The band of noise which sometimes appeared across the image was moved away from the \textit{Acetabularia} image (bottom area of the image) by altering the sweep width (FIG-37B). Therefore this noise was thought to have being generated at a constant frequency range. Most images obtained before 8 A.M. did not have this band of noise. The images obtained
FIG-37 Image artifacts appearing during this study.

A) The centre noise peak appeared along the phase encoding dimension. Cause = an overestimated DC offset.

\[ G_y = 18.66 \, G \, cm^{-1}, \, FOV_y = 0.839 \, cm, \, G_{z_i} = 0.187 \, G \, cm^{-1}, \, \Delta FOV = 4.87 \times 10^{-3} \, cm, \]
\[ t_{\phi} = 1.5 \, msec, \, NS = 12, \, DW = 15 \, \mu sec. \] Gradient increments = 128.

B) A band of noise appeared over some images. Source - a rf pick up from the departmental 270 MHz spectrometer when \(^{19}F\) is being studied using the broad band decoupler for \(^1H\).

\[ G_y = 15.96 \, G \, cm^{-1}, \, FOV_y = 0.92 \, cm, \, G_{z_i} = 0.12 \, G \, cm^{-1}, \, \Delta FOV = 3.26 \times 10^{-3} \, cm, \]
\[ t_{\phi} = 2.2 \, msec, \, NS = 16, \, DW = 16 \, \mu sec. \] Gradient increments = 128.

C) The image of a cap where two noise peaks appeared over the image. The cause is not known.

\[ G_y = 17.45 \, G \, cm^{-1}, \, FOV_y = 0.841 \, cm, \, G_{z_i} = 0.19 \, G \, cm^{-1}, \, \Delta FOV = 3.41 \times 10^{-3} \, cm, \]
\[ t_{\phi} = 1.5 \, msec, \, NS = 8, \, DW = 16 \, \mu sec. \] Gradient increments = 128.

D) An NMR image of a cap showing one spurious noise peak over the image. Cause unknown.

\[ G_y = 17.61 \, G \, cm^{-1}, \, FOV_y = 0.834 \, cm, \, G_{z_i} = 0.14 \, G \, cm^{-1}, \, \Delta FOV = 1.24 \times 10^{-3} \, cm, \]
\[ t_{\phi} = 2 \, msec, \, NS = 8, \, DW = 16 \, \mu sec. \] Gradient increments = 128.

E)/F) A normal image of an Acetabularia cap (E) and an image of the same cap showing the "ghost image" artifact.

\[ G_y = 16.51 \, G \, cm^{-1}, \, FOV_y = 0.889 \, mc, \, G_{z_i} = 0.115 \, G \, cm^{-1}, \, \Delta FOV = 1.78 \times 10^{-3} \, cm, \]
\[ t_{\phi} = 2.3 \, msec, \, NS = 12, \, DW = 16 \, \mu sec. \] Gradient increments = 128.
later in the afternoon sometimes had this band. After a series of experiments done with the help of the U.B.C. Chemistry Department electronics shop\(^\dagger\), this was found to be a rf pickup from the departmental 270 MHz spectrometer (Distance between the two spectrometers was about 30 feet separated by one solid wall). The noise peak was observed only when \(^{19}\text{F}\) NMR experiments were being performed using the broad band decoupler for \(^1\text{H}\). Therefore instead of attempting to shield the magnet from any external interferences, images were obtained when the broad band decoupler was not being used by the departmental spectrometer.

C) A spurious set of noise peaks appeared in most of the images obtained during this study. They can be categorized as follows.

**Noise peak No 1**

Several one-pulse experiments were performed in the absence of a sample at different sweep widths from 15 KHz to 35 KHz. A single noise peak was seen which moved with the variation of the sweep width. This peak was seen to appear at one end of the spectrum when the band width was 30 KHz (FIG-38). Similar observations were made when performing an imaging experiment. Hence rather than attempting to locate the cause for this noise peak, a sweep width of 30 KHz was used for most of the imaging experiments.

**Noise peak No 2**

One or more noise peaks appeared across the image (FIG-37C and D) often degrading its appearance. Several experiments were performed to locate the possible cause for these noise peaks. But to date the author has been unable to infer the probable cause for these noise peaks.

1) In microscopic imaging experiments attempts are made to obtain as much signal as possible. Hence, during this study, data were acquired with minimum attenuation at the

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FIG-38 Effect and position of the apodization (MS) during image processing in order to suppress the vertical line of noise spike in the image centre (FIG-37A) (see FIG-32 for abbreviations).

A) The image was obtained with no apodization (no MS) during the 2nd FT process. Only BC:ZF:ZF:FT:MC.

B) The data was apodized two times (2 × MS) followed by BC during the 2-FT process. That is MS:MS:BC:ZF:ZF:FT:MC.

\[ G_y = 20.71 \, \text{G cm}^{-1}, \quad FOV_y = 0.709 \, \text{cm}, \quad G_z = 0.166 \, \text{G cm}^{-1}, \quad \Delta FOV = 4.82 \times 10^{-4} \, \text{cm}, \]
\[ t_\phi = 2 \, \text{msec}, \quad NS = 8, \quad DW = 16 \, \mu\text{sec}. \quad \text{Number of gradient increments}=64. \]

C) A NMR image obtained by first performing the base line correction (BC) followed by two apodizations (2 × MS).

D) The image of the same cap obtained by first performing two apodizations (2 × MS) followed by BC in the second FT process.

\[ G_y = 17.10 \, \text{G cm}^{-1}, \quad FOV_y = 0.859 \, \text{cm}, \quad G_z = 0.137 \, \text{G cm}^{-1}, \quad \Delta FOV = 4.72 \times 10^{-4} \, \text{cm}, \]
\[ t_\phi = 2 \, \text{msec}, \quad NS = 8, \quad DW = 16 \, \mu\text{sec}. \quad \text{Number of gradient increments}=64. \]

* The vertical line spike artifact.
receiver (receiver gain 2 or 4). It was suspected that some noise was produced by the receiver. Experiments were done with more signal attenuation (receiver gain 8, 16 and 32) but the appearance of these noise in some images was unavoidable.

2) Subsequently the gradient amplifiers were investigated. First, images were obtained with and without the low frequency filters of the gradient amplifier. Second, the compatibility of the amplifier gains were studied. Several images were obtained with $G_y(Freq.)$ and $G_x(Phase)$ at the same amplifier gain 400, $G_y(600) > G_x(400)$ and $G_y(400) < G_x(600)$. The occurrence of the noise peak appeared to be independent of the conditions used.

However, in the experiments performed with phantoms, which were capillary tubes filled with water, these spikes never appeared on the images. This was thought to be due to the high signal intensity. Therefore, when the *Acetabularia* caps were studied the generation of spikes were suppressed by acquiring more scans.

D) The images were sometimes distorted by "ghost images" artifacts.

As shown in FIG-37F at the later part of this study some images were obtained with "ghost image" artifacts (FIG-37E shows an image of the same cap). It was noticed at this point that due to the constant removal of the probe for sample insertion the screw which held the probe in the magnet provided limited but free movement to the probe. The mechanical vibrations during the switching on and off of the high gradient pulses were suspected to be the cause for these type of artifacts [95], [96], [97], [98]. In subsequent studies, the probe was fastened into the magnet with two screws. The frequency of appearance of this artifact was reduced but it was unavoidable. Although the exact reason was not ascertained it was believed to be most probably due to mechanical movements of the probe or of the sample during the experiment.