practice in the country[32]. What seems to be lacking is a close interaction of scientists in the fields of electronics, computers and allied disciplines with clinical neuroscientists, which is crucial for the emergence of new developments in the field of clinical neurophysiology. Mechanisms need to be evolved to promote and foster such interactions in the country.

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Nuclear magnetic resonance: Molecules, cells and animals

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Since its discovery in 1945, nuclear magnetic resonance (NMR) has developed into one of the most important techniques utilized in almost all branches of science. In the field of life sciences itself, NMR applications cover problems at almost all levels: structure and function of biological molecules, cellular metabolism, morphology and chemistry of intact organs such as brain, heart and liver, microscopy of tissues, and as a diagnostic tool in medicine. I propose to give some information on the current status of the developments in diverse areas of biological NMR, with particular attention to interests of neuroscientists. The future trends in biological NMR will also be presented. For those interested in details, several general references have been appended.

What is NMR, MRI and MRS?

The principle of NMR is based on the fact that nuclei having non-zero spins I (e.g. ^1H, ^{31}P and ^{13}C, which have $I = 1/2$) act like tiny magnets with magnetic moments (μ_n) and have nondegenerate energy states in

an external magnetic field (B_0). For example, for spin 1/2 system, there are two states corresponding to the nuclear magnets aligned parallel and antiparallel to the magnetic field and characterized by $m_I = 1/2$ or $-1/2$. The energy difference between the two states (ΔE) is given by the relation

$$\Delta E = 2\mu_n B_n.$$

The resonance frequency ν, which is related to ΔE by $\nu = \Delta E/h$, is therefore given by

$$\nu = 2\mu_n B_n/h. \quad (1)$$

Equation (1) implies that the resonance frequency is proportional to the magnetic field at the site of the nucleus (B_n) and to the nuclear magnetic moment (μ_n), and is, therefore, different for different nuclei. Typical magnetic fields used in NMR experiments range between 1 and 17 T. At 1 T, the resonance frequencies of ^1H, ^{13}C and ^{31}P are 42.9, 10.9 and 17.2 MHz, respectively. The resonance frequencies thus lie in the radiofrequency (rf) range. NMR experiments are usually performed by placing the subject under investigation in a magnetic and a radiofrequency field, and placing a detector coil such that the three are in orthogonal directions. When the frequency (ν) of the rf field satisfies equation (1), resonance absorption of energy by the ensemble of nuclear spins takes place, which, in turn, induces a current in the pick-up coil of the detector system.

The second important feature of NMR arises from the fact that the samples of interest are not the nuclei themselves but nuclei embedded in molecules. In view of the magnetic fields produced by the electrons, the magnetic field at the site of the nucleus (B_n) differs somewhat from the applied magnetic field (B_0). One such difference arises from the direct screening of the magnetic fields by the electronic cloud surrounding the nucleus (σ_n):

$$B_n = B_0 (1 - \sigma_n) \quad (2)$$

Equation (2) gives rise to the phenomenon of chemical shifts. For example, for ethyl alcohol, one observes three lines in the ^1H spectrum since the screening constants (σ_n) corresponding to the CH_3, CH_2 and OH protons are slightly different. The order of magnitude of chemical shift ($\Delta\sigma_n$) is 10^{-5} for ^1H and ^{31}P, and 10^{-4} for ^{13}C. The chemical shifts are, therefore, often expressed in parts per million (ppm). The total spectral width for ^1H NMR is about 10 ppm, about 20 ppm for ^{31}P and around 200 ppm for ^{13}C. In terms of frequencies, however, the signals of chemically distinct nuclei are separated by $\Delta\sigma \cdot B_0$ and the ranges of spectral windows cover a few kHz.

The next point of interest is the signal intensity. Since during the NMR experiments we excite the ensemble of nuclear spins from the ground to the excited state, the intensity depend on the population difference of nuclear spins between the two states ($m_I = 1/2$ and $m_I = -1/2$), which, in turn is given by

$$n_-/n_+ = \exp(-\Delta E/RT) \approx 1 - 2\mu_n B_n/RT. \quad (3)$$

The signal intensity is proportional to $n_+ - n_-$, and since ΔE is very small, this difference is only of the order of 10^{-6}. Thus, by its very nature, NMR is not a very sensitive technique. It is obvious from equation (3) that sensitivity will be better if higher magnetic fields are used. At a constant magnetic field, the signal intensity is proportional to the total number of spins ($n_+ + n_-$). For example, in the case of ethyl alcohol the three signals mentioned above are in the ratio of 3 : 2 : 1.

Finally, we talk of relaxation of nuclear spins. Once the system is excited, the nuclear spins transfer their energy to the surroundings in a complicated manner and return to thermal equilibrium as defined by equation (3). The relaxation behaviour of nuclear spins depends on a number of factors, one of which is the molecular motion.

While there are a number of other factors which are of interest to NMR physicists, the above four are of paramount interest from the viewpoint of biologists. A word about terminology. Chemists generally use the term NMR to distinguish this phenomenon from other techniques in magnetic resonance such as ESR, NQR, etc. Unfortunately, the biomedical community has dropped 'nuclear' and one talks of magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). The three abbreviations are, at best, dialects of the same basic language.

Problems in NMR studies of biological systems

The co-discoverer of NMR, Felix Bloch had observed proton NMR signal from his finger when it was inserted in the NMR probe. Thus, in a way the study of living systems by NMR is as old as the field of NMR itself. However, it took almost three decades for NMR to become an indispensable tool in life sciences. Even in the early seventies, when we were making a case for the purchase of an NMR instrument at TIFR for bio-molecular studies, some of the professional molecular biologists felt that NMR could never become a tool for studies in life sciences. What were the reasons for this pessimism, and how have these problems been surmounted?

The entire development of NMR is related to advancement in sensitivity. In the early sixties it was usual for chemists to work with neat liquids or solutions of concentrations in molar (M) ranges. Obviously, it is difficult to obtain such large quantities of biological materials. Due to the development of a new technique in NMR, called the Fourier transform (or FT, for which Richard Ernst was awarded the 1991 Nobel prize),

coupled with better magnet technologies, and the use of higher magnetic fields and better electronics, millimolar (mM) concentrations of samples can be used today without any difficulty. This means that mg quantities of proteins and nucleic acids are sufficient for NMR work. Further, several molecules are present in living systems in mM concentrations and these can now be detected using *in vivo* NMR.

The next problem is related to resolution. In any biological macromolecule, there are thousands of protons all resonating in a small spectral window of 10 ppm. The problem is further complicated by the fact that the line widths in biological systems are usually larger than those in organic molecules in biochemical problems. The problem of resolution has been countered through the use of very high magnetic fields. Here we make use of the greater dispersion of signals at higher fields. For example, the 10 ppm spread of ^{1}H frequencies is equivalent to about 430 Hz at 1 T but 4300 Hz at 10 T. Another major development is the emergence of what is called multidimensional FT NMR. Using such techniques, the spectral information is spread in two or three dimensions of spectral space. Such techniques also provide valuable information on structural parameters.

The third problem relates to the assignment of spectral peaks to specific nuclei in the system. This problem is also manageable today, thanks to the new developments in multidimensional NMR and the ease with which biomolecules can be labelled with ^{13}C and ^{15}N using gene cloning and microbiological techniques.

Finally, we have the problem of undesirable signals. The milieu of biology is water. Thus, signals arising from mM concentrations of biological molecules have to be viewed against the background of huge water signal (about 110 M, since water has two identical protons). In living systems, another undesirable signal is that arising from the CH_2 groups of fatty acids present in different forms. Today, solvent suppression techniques have been developed which are able to eliminate undesirable signals so that problems of interest can be studied conveniently.

Structure and dynamics of biological macromolecules

One of the most fruitful areas of NMR continues to be molecular structure, dynamics and function. The problems of sensitivity, resolution and assignments have been largely resolved through the use of recently developed two- and three-dimensional FT NMR. Proteins, nucleic acids and other biomolecules up to molecular weights of 15 kD can be conveniently handled with 5–10 mg of pure material. The basic structural parameters obtained from NMR studies are the chemical shifts, coupling constants (which are related to the torsional angles) and the nuclear Overhauser effect (which provides information on interatomic distances) Computer algorithms are available to transform this information (often with the help of distance geometry algorithms, molecular dynamics simulations or energy minimization techniques) into three-dimensional structure of the molecule. Thus, there exists a possibility for neuroscientists to use NMR for structural determination of neurotransmitters, neural receptors and their interactions. Since both NMR and X-ray crystallography are used for the purpose of structure determination, some comparative points may be mentioned NMR provides structure in aqueous solutions, the milieu of biological systems. Parameters such as pH, ionic strength, substrate concentration, etc., can be easily adjusted. The structure is, therefore, not affected by environmental forces. X-ray diffraction is used in solid state, where the molecules are constrained by crystal forces. One of the rate-determining steps in X-ray crystallography is the availability of suitable crystals. In NMR, once a suitable amount of the pure material is available, work can start without further delays. Solution conformations are generally found to be more flexible than the crystal structures. On the other hand, in terms of the size of molecule, the limits for X-rays is much higher. Also, NMR builds up the structure from local conformations while X-ray looks at it from a global angle and generally provides more precise information.

Good facilities are available for biomolecular NMR both at TIFR, Bombay, and IISc, Bangalore. In this field, our country has done as well as any other reputed laboratory in the world.

Cellular NMR

Cells derive their energy by oxidation of fuels such as sugars, fats and amino acids. In the process of glycolysis, the end product is lactate. Under aerobic conditions, the final products are CO_2 and water. The energy generated in these processes is used in the synthesis of ATP from ADP and in the production of phosphocreatine (PCr). Thus, ^{31}P NMR can be used to detect levels of ADP, ATP, PCr, etc., and hence also the metabolic activities of the cells. The position of inorganic phosphate peak provides a convenient handle in the measurement of cellular pH. Use of ^{13}C NMR using ^{13}C-labelled glucose has provided valuable information on products of cell metabolism. Finally, water-suppressed ^{1}H NMR can provide information on metabolic levels of lactate, precursors of lipid and protein synthesis, and certain neurotransmitters. Cellular NMR can thus provide information on the chemistry of neuronal cells as influenced by a variety of conditions: malignancy, presence or absence of oxygen, pH, blockers of metabolic pathways, drugs and effect of toxic compounds.

In India, work on different cellular systems is going on at TIFR, Bombay, and CDRI, Lucknow. Since this is an active area of research globally, our success depends on the choice of unique cell lines and asking questions not addressed earlier.

Magnetic resonance imaging (MRI)

In 1973, a new technique, commonly known as MRI, was proposed by Lauterbur. Here one looks at a single strong resonance signal, often that due to ^1H in water. Thus, the problems of sensitivity, resolution and assignment do not arise. The spatial resolution of water present in different parts of a body (e.g. brain) can be achieved by placing it in a magnetic field, the strength of which is varied in a predetermined way in different parts of the body. The ^1H resonance of water, therefore, occurs at different frequencies [equation (1)]. If water in different regions has different characteristics (as reflected, for example, in relaxation rates) then such properties can be utilized to obtain an image of the body. NMR imaging techniques have revolutionalized biomedical research as well as diagnostics. Unlike the techniques of CT and PET scans, NMR methods do not use ionizing radiations or chemicals injurious to health. Using NMR, pictures of brain can be obtained from any desired direction (e.g. saggital, transverse, axial or coronal). In the past few years, the contrast of such images has improved significantly. NMR is particularly suited for viewing soft tissues (c.f. from CT scan, which is better for skeleton regions), and for distinguishing between grey and white matter in the brain. The NMR imaging technique is now well established as a diagnostic tool in neurosurgery, complex fractures, arthritis, cardiovascular diseases, malignancy, ischaemia, infarction and a variety of other clinical disorders. MRI makes it possible to monitor the efficacy of novel drugs and their toxicology and is thus an important tool in drug development.

The equipment for such techniques is now available in all major cities in India. In addition to clinical work, such facilities have also been utilized in biomedical research.

Magnetic resonance spectroscopy (MRS)

It is now possible to obtain spectroscopic pictures of organs such as brain. It is desirable not to have spectra from the entire brain, which may not be very useful, but use small selected volumes. Localized spectroscopy allows volumes of interest as small as 1 cm^3 to be selected to obtain ^1H and ^{31}P spectra from such regions. Using ^{31}P NMR, one can obtain estimates of ATP, ADP, PCr and pH of such selected areas. Using water-suppressed localized ^1H NMR, molecules such as lactate, alanine, *N*-acetyl aspartate (NAA), glutamate, glutamine, aspartate, γ-amino butyric acid, creatine, phosphocreatine, phosphatidylcholine and inositol phosphates can be studied using spectral accumulation times of a few minutes. Even though the precise role of NAA (the most abundant biochemical in the central nervous system) is not known, it is generally agreed that its level is related to the density of healthy neurons. On the other hand, a normal brain contains a very small amount of lactate and a strong lactate signal generally indicates disturbed metabolism. Phosphomonoesters (PME) signal is prominent in situations where cells divide fast, e.g. the brain of growing babies or in case of malignancies. Thus, MRS is now advanced enough to allow a study of the chemistry of the brain in healthy and diseased states. A further development is the technique of chemical shift imaging, whereby water images can be supplemented with images corresponding to ATP, ADP, lactate, NAA, choline, PCr, etc.

MRS is perhaps one of the most promising areas for future research. Unlike PET, NMR provides complete information on the chemistry of the brain, not just changes in glucose levels during metabolism. Unfortunately, we still have to develop strong activities in this area in our country.

NMR microscopy

A technique which seems to have a future is the microscopy of tissues using NMR. Such techniques may prove useful in studying myelin morphology, nerve endings and related problems. Typically, samples of 0.01 mm^3 can be studied by NMR microscopy. However, the resolution in NMR microscopy has not developed as fast as was expected a few years back, mainly because of the problem of sensitivity. At the same time, new techniques such as confocal Raman microscopy, use of fluorescent probes, tunelling electron microscopy, low-angle X-rays and others provide a better resolution.

Future trends

New multidimensional NMR techniques coupled with the ability to produce large quantities of ^{13}C- and ^{15}N-labelled proteins through genetic methods has brought biomolecular and cellular NMR to a stage where it has become a technique of choice. Magnetic fields as high as 17 T are commercially available for such studies and higher-field spectrometers are under development.

The future directions in human and animal NMR will be closely linked to further advances in sensitivity. One of the ways in which this can be achieved is, of course, through the use of better electronics and higher magnetic fields so that smaller localized volumes can be used and better dispersion in spectroscopy can be

obtained. However, this raises the question of safety, particularly, while dealing with humans. As far as MRI is concerned, the use of fields beyond 2 T are anyway not very useful as water relaxation times tend to flatten out at higher fields and the contrast is thus lost. However, magnetic fields as high as 4 T are being used for animals and humans in MRS. At these high fields, subjects feel discomfort and nausea (possibly due to eddy currents from field gradient coils), but, otherwise, there are no clinical symptoms. A more serious problem, however, is the heating due to the use of high rf powers. Fat is a good absorber of rf radiation and the use of high powers can burn the tissues. The decision of doing imaging at 2 T and spectroscopy at 4 T is also influenced by the costs of having two different machines.

Quantitative estimation of metabolites from the NMR intensities poses certain problems in *in vivo* studies. Better spectral editing techniques are being developed to overcome such difficulties.

Attempts are being made to develop fast images. This problem is particularly important when one wants to obtain dynamic images of moving organs, such as the heart, so as to obtain a time profile. Time resolution of about 100 ms can be obtained at present.

Recently, NMR imaging has been extended to other nuclei such as ^{19}F and ^{23}Na. The former is useful in studies of molecular pharmacology of fluorinated drugs. The use of ^{23}Na NMR monitors alteration in regional Na contents and pathophysiology of neoplasia and stroke.

The first NMR Group in India was established by Prof. S. S. Dharmatti and Prof. H. J. Bhabha in 1953. Since then, NMR techniques have come a long way in biology. While developing future research programmes in our country, several points should be kept in mind. While the clinical relevance of a problem requires the use of non-invasive clinical model, experimental controls and monitoring are not so easy. On the other hand, NMR studies of cell cultures and perfused organs can be done at very high fields and experimental controls are much better. Expertise in NMR methods and electronics is as important for the success of a biomedical NMR research programme as the choice of the right problems. This requires a collaborative effort between NMR spectroscopists, biochemists and neuroscientists. Will we succeed in developing such multidisciplinary teams? I do hope that our country, which has a long tradition in NMR, will be able to initiate challenging problems in biomedical NMR.

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