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NMR SPECTROSCOPY of CELLS and ORGANISMS

Volume I

Raj K. Gupta

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Editor

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PREFACE

Applications of NMR spectroscopy in the study of cells and organisms represent an ever-expanding area of research actively pursued in many universities and research institutions. Notable advances have been made in recent years in the use of ^{1}H, ^{19}F, ^{31}P, ^{13}C, and ^{23}Na NMR to study tissue physiology and biochemistry in vivo. These volumes are aimed at describing the noninvasive use of NMR to study electrolytes and metabolites in living cells. NMR measurements of intracellular pH, free Mg^{2+}, free Ca^{2+}, and Na^{+} ions, membrane ion-transport processes, cell water content, and steady state rates of intracellular reactions by saturation transfer, as well as mapping of metabolic pathways for the processing of organic molecules by ^{13}C NMR, are described. In vivo ^{31}P NMR studies of the metabolic state of tumor tissue, nuclear relaxation studies of tissue water, as well as multinuclear spectroscopic imaging of intact organisms, are also included. The intent of these volumes is to familiarize the physiologist/biologist/chemist with the advances and progress in cellular applications of NMR and the future potential of this technique.

THE EDITOR

Raj K. Gupta, Ph.D. is a Professor of Physiology & Biophysics and a Professor of Biochemistry at the Albert Einstein College of Medicine of Yeshiva University in New York City.

Professor Gupta obtained his graduate education leading to a Ph.D. in chemical physics from the Indian Institute of Technology, Kanpur, India. He was a postdoctoral fellow in biophysics at the IBM Watson Laboratory, Columbia University, New York and at the IBM Thomas J. Watson Research Center, Yorktown Heights, New York from 1969 to 1972. Subsequently he spent a year as a visiting scientist at the National Institutes of Health, Bethesda, Maryland. In 1973, Professor Gupta joined the Institute for Cancer Research in Philadelphia as a member of its senior research staff and later also held a joint appointment as an associate professor of biochemistry and biophysics at the University of Pennsylvania. From 1976 to 1981, he was the recipient of a research career development award from the U.S. Public Health Service pursuing research on NMR spectral studies of the structure and mechanism of heme- and metal-containing proteins. He left Philadelphia in 1982 to become a Professor at Albert Einstein, where, along with some teaching and consulting, he is actively involved in NMR research on intact cells, tissues, and organisms. Professor Gupta has played a key role in the development and use of NMR techniques for the study of intracellular $^{23}Na^{+}$, $^{39}K^{+}$, Ca^{2+}, and Mg^{2+} ions and pH.

Professor Gupta is a member of the American Chemical Society, the American Physical Society, the Biophysical Society, and the American Society of Biological Chemists. He has served on numerous scientific review panels and on the editorial board of *Biophysical Journal*. Professor Gupta has published scores of research articles and reviews on NMR spectroscopy and its biochemical and physiological applications.

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I. INTRODUCTION

With increasingly spectacular success, NMR is being utilized for the study of diverse living systems. The development of this spectroscopic tool from its origins in the physics laboratories of Bloch and Purcell 40 years ago[1] to current applications with cells and intact organisms is an exciting history, spanning across a multitude of disciplines in chemistry, biochemistry, biology, physiology, and medicine. Current applications build upon a solid theoretical and experimental framework, much of which was developed in the 2 decades following the discovery of NMR. Since that time, the introduction of increasingly sophisticated instruments and new techniques has permitted extension of NMR methods into the realm of living cells and organisms.

In this chapter we first review some basic NMR concepts and techniques, essential to an understanding of the more complex biological applications of NMR discussed in this book. The history of NMR experimentation is then traced from early studies on small molecules of biochemical significance to the emergence of in vivo applications with cells, perfused organs, and intact animals and human beings. Although literature references are included to give examples of some of the earliest applications of NMR and also some of the more recent developments that facilitate modern experimentation, they are intended only as a guide for readers unfamiliar with the NMR literature and are not meant to confer credit upon particular individuals. Throughout this introduction, we intend to point out some of the major steps forward that NMR has taken in this field and the creativity in instrumental design and technique that has permitted them.

II. PHYSICAL BASIS OF NMR

NMR arises from the interactions between the magnetic moments of atomic nuclei and strong magnetic fields imposed in the laboratory. We shall not provide a detailed account of NMR theory here since such developments are available in a number of textbooks,[2] but we shall summarize the important results and equations. Some features of NMR spectra can be understood only by the use of quantum mechanics, while others can be explained equally well in classical terms. Both classical and quantum mechanical approaches lead to a fundamental equation, the principles of which were first derived classically by Sir Joseph Larmor (long before the discovery of NMR) to explain the Zeeman effect in optical spectroscopy:[3]

$$\nu_0 = [\gamma/2\pi]\, B_0 \tag{1}$$

where ν_0 is the resonance frequency; γ, the magnetogyric ratio, is a constant for the particular nucleus being studied; and B_0 is the magnetic field applied to the sample. The magnetogyric ratio relates two fundamental nuclear properties, the magnetic moment (μ), and the nuclear spin (I):

$$\gamma = \mu/I\hbar \tag{2}$$

where $\hbar$ is the reduced Planck's constant ($h/2\pi$). Both μ and I are important factors in determining the strength and fine detail of the NMR signal to be observed from a given nucleus. The magnetic nuclei with major applications in biological NMR are numerous (Table 1). The applicability of a nucleus to the study of a particular biological process often depends on the NMR sensitivity of the nucleus. Nuclei with very low sensitivities (see Table 1) demand greater sample concentrations and sizes, special experimental designs (with longer measurement times), and also often require isotopic enrichment. Among all stable nuclides, ^1H has the highest sensitivity and has historically been the most popular nucleus for NMR studies, particularly in early organic and biochemical applications.

Table 1
MAGNETIC NUCLEI OFTEN UTILIZED FOR BIOLOGICAL STUDIES

Nucleus	NMR frequency[a] (MHz)	Magnetic moment (μ)[b]	Spin (I)[c]	Natural abundance (%)	Relative sensitivity at natural abundance[d,e]
^{1}H	100.00	2.7927	1/2	99.98	1.00
^{19}F	94.08	2.6273	1/2	100.00	0.83
^{23}Na	26.45	2.2161	3/2	100.00	0.09
^{31}P	40.48	1.1305	1/2	100.00	0.07
^{39}K	4.67	0.3910	3/2	93.10	0.0005
^{13}C	25.14	0.7022	1/2	1.11	0.0002
^{17}O	13.56	−1.8930	5/2	0.04	0.00001
^{15}N	10.13	−0.2830	1/2	0.37	0.000004
^{2}H	15.35	0.8574	1	0.02	0.000002

[a] The frequency given corresponds to a magnetic field of 2.35 T (23.5 kG).
[b] The magnetic moment is given in multiples of the nuclear magneton.
[c] The nuclear spin is given in units of $h/2\pi$ where h is Planck's constant. Nuclei with $I > {}^{1}/_{2}$ are "quadrupolar" and have NMR spectral and relaxation characteristics that are distinctly different from nonquadrupolar nuclei.
[d] This value corresponds to the relative sensitivity for equal numbers of nuclei (at a given field strength) multiplied by the natural abundance.
[e] The relative sensitivity of nuclei such as ^{13}C and ^{15}N may be substantially increased by the use of heteronuclear double-resonance techniques.

For additional information, see Becker, E. D., *High Resolution N.M.R.*, 2nd ed., Academic Press, New York, 1980, Appendix B.

In the classical picture of NMR, the magnetic moment, $\boldsymbol{\mu}$ (a vector quantity since it has both magnitude and direction) can be viewed as precessing, like a spinning top, about a fixed magnetic field $\mathbf{B}_0$ (also a vector quantity) at the frequency ν_0, as illustrated in Figure 1a. Detection of the NMR signal is achieved by the introduction of a second (radiofrequency, RF) field, $\mathbf{B}_1$, perpendicular to $\mathbf{B}_0$ and rotating at the same Larmor frequency. It is the interaction between $\mathbf{B}_1$ and the magnetic moment which produces a signal in the NMR receiver coil. In a real sample there is, of course, a very large number of nuclei. Such an ensemble of identical nuclei all precessing at the same frequency about an applied field $\mathbf{B}_0$ is depicted in Figure 1b. Nuclei with a component of the magnetic moment (magnetization) parallel to $\mathbf{B}_0$ are in the lowest energy state and are in very slight excess in the sample due to the Boltzmann distribution. The nuclei have no preferred orientation in the x or y directions, and thus their components of magnetization along these axes average to zero. The result is a "macroscopic magnetization" $\mathbf{M}$, directed at equilibrium along the z axis parallel to $\mathbf{B}_0$. As we shall see later, this classical mechanical treatment provides a useful picture for understanding many aspects of the NMR experiment.

In the first few years after the discovery of NMR in 1946, major attention was focused on obtaining accurate values of γ for various nuclei, in understanding the interactions between the nuclear magnets and the RF waves used to study their resonance properties, and in elucidating the mechanisms by which nuclei relax back to equilibrium after being disturbed in an NMR experiment. As the stability of NMR spectrometers and the homogeneity of magnets improved, physicists studying NMR were surprised to find that the resonance frequencies of nuclei depended on the chemical compounds in which the nuclei resided. This "chemical shift", initially a source of annoyance in limiting the precision of the measurement of γ, soon became the cornerstone for the development of NMR as a chemical

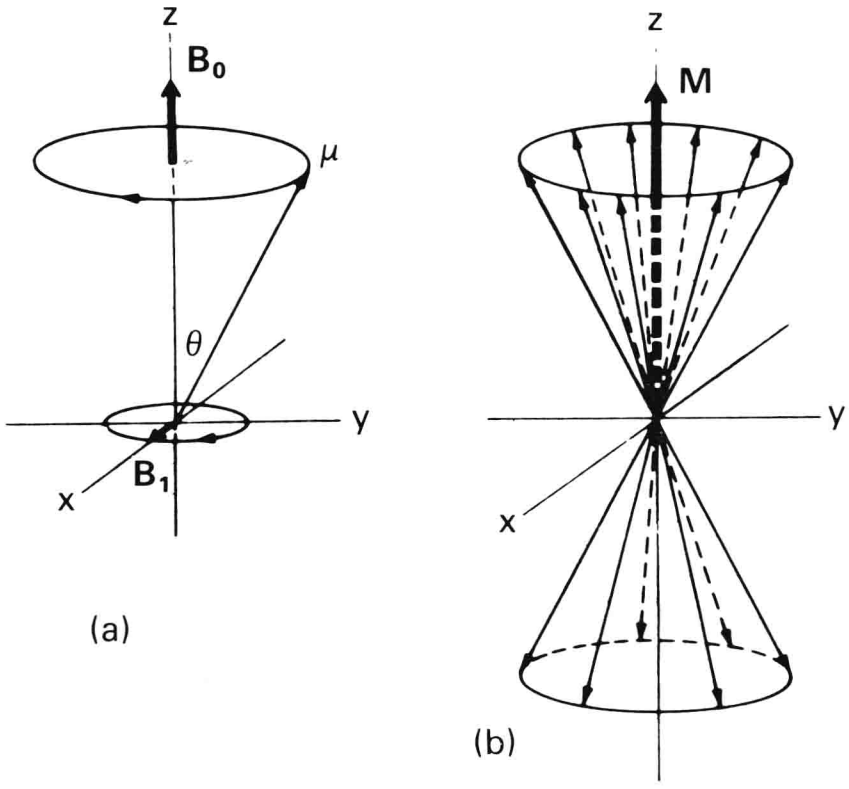

FIGURE 1. (a) Precession (at the Larmor frequency, ν_0) of a proton magnetic moment, $\boldsymbol{\mu}$, about a fixed magnetic field, $\mathbf{B}_0$. (b) Precession of an ensemble of magnetic moments of spin quantum number $I = {}^1/_2$ (e.g., ^1H, ^{13}C, ^{31}P).

tool, as it was realized that nuclei could serve as probes of electronic structure in molecules. The Larmor equation was thus modified to take into account the electronic shielding, σ, around each nucleus:

$$\nu_0 = [\gamma/2\pi]\, B_0(1 - \sigma) \tag{3}$$

In particular, the observation in 1951 of three lines in the proton NMR spectrum of ethanol (Figure 2) demonstrated the utility of NMR in the structure elucidation of organic molecules.

The ethanol lines shown in Figure 2 represent NMR signals that differ only very slightly in their Larmor frequencies, ν_0. Their discovery was a major achievement at the time, requiring a magnetic field B_0 that was uniform to one part in 10^7 over the entire sample volume. Even so, further spectral detail was still masked by B_0 inhomogeneity and was revealed only when the continued improvement in NMR instruments permitted the resolution of electron-coupled spin-spin interactions (coupling) that cause splittings of chemically shifted NMR lines into multiplets. Thus, in the years that followed, the high-resolution proton NMR spectrometer (typically operating with 5-mm sample tubes at 60 to 100 MHz) became the chemist's workhorse, producing a voluminous amount of fundamental data on all manner of chemical systems. The rapid development of theory to account for the details of the sometimes complex NMR spectra and the resultant correlation of NMR properties with features of molecular structure and chemical exchange processes led to the widespread use of NMR and its acceptance as one of the most powerful physical methods available in the chemistry laboratory.

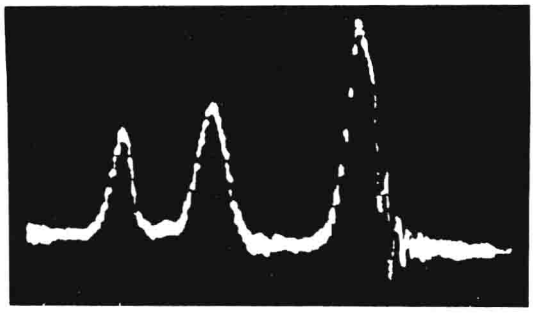

FIGURE 2. Oscillograph trace of the 32-MHz NMR signal from ethanol obtained in 1951. The peaks from left to right represent OH, CH_2, and CH_3, respectively. (Reprinted by permission from Arnold, J. T., Dharmatti, S. S., and Packard, M. E., *Chem. Phys.*, 19, 507, 1951. Copyright 1951, American Institute of Physics.)

III. EARLY BIOCHEMICAL APPLICATIONS

The roots of modern NMR experimentation in biochemistry and biology lie in the late 1950s and early 1960s. Examples of proton spectra for molecules of interest to nearly every area of biochemistry may be found in articles published during that decade. Although the spectra of more complex substances were often more tantalizing than informative because of the limitations in resolution and sensitivity in early instruments, a wealth of fundamental data on smaller molecules was published, and these studies provided the foundation needed for the applications to complex in vivo biological systems that were to come much later. Contributions from NMR spectroscopists were made in all areas of structural and conformational analysis for a wide range of biochemically important substances. For example, early observations on ring current effects and substituent effects on chemical shifts of porphyrins[4] laid the groundwork for later studies of heme proteins. The early development of techniques for structure determinations of alkaloids based entirely on NMR data[5] ushered in the modern era of natural product chemistry. Empirical correlation of NMR data with known steroid structure had its origins in early studies which focused on points of unsaturation and on angular methyl groups appearing as sharp, intense signals superimposed upon a broad, characteristic "fingerprint" spectrum from the steroid backbone.[6] Pioneering work established NMR techniques as a tool for the studies of conformation and intermolecular interaction in nucleic acid bases, nucleosides, and nucleotides.[7] Other early studies highlighted the applicability of NMR for the study of the hydrogen-bonded protons participating in base-pair interactions[8] and the association of bases and nucleosides in aqueous solution (base stacking).[9] The large body of literature which has developed on carbohydrates began in extensive studies first performed at 40 MHz.[10] Observation of the sensitivity of chemical shifts and coupling constants to the axial or equatorial positioning of substituents was an important step toward establishing NMR as a useful tool for conformational studies. The first report investigating amino acid chemical shifts appeared nearly 30 years ago.[11] Since that time, NMR has been widely utilized for the determination of relative populations of different conformers in flexible systems, and the elucidation of preferred conformations in more rigid, often cyclic, peptides.

The first spectrum of a protein obtained in 1957[12] left much to the imagination, with only four broad bands resolved at 40 MHz. In subsequent studies, protein spectra were often recorded under denaturing conditions (high temperature, urea, or TFA). Also high concentrations were needed (for example, 20% solutions) leading to further degradation in spectral resolution from molecular association effects and high solution viscosities. By the mid-1960s

higher field instruments and time-averaging techniques (discussed later) resulted in significantly improved resolution and sensitivity. Protein spectra were obtained in which aromatic protons arising from histidine, phenylalanine, and tyrosine could be resolved.[13] This observation led to numerous subsequent studies where the histidine chemical shifts of a protein were recorded as a function of the solution pH, and thus the microscopic pK_a values determined. Nucleic acids also began to be studied successfully during this time, with the introduction of NMR melting curves to follow order-disorder transitions in single-stranded polynucleotides.[14]

Some of the very first studies of biological materials were in fact studies of water. As early as 1950, spectra were obtained for proteins, carbohydrates, and vegetable tissue to measure water content.[15] In an early study of DNA hydration, broadening of the water line due to interaction with solute DNA was detected, whereas proteins such as egg albumin caused no measurable effect.[16] In 1961 the proton relaxation enhancement (PRE) technique was introduced.[17] This technique still involved measurement of the relaxation properties of water in the presence of biopolymers, but rather than being limited to a study of the water-biopolymer interaction itself, the water served as a probe for the interactions of paramagnetic ions with biopolymers. The first system studied was the binding of transition metal ions to DNA. Subsequent work established the PRE technique as a probe for the investigation of active sites in enzymes.

The experiments discussed above report early observations of proton NMR. Although sensitivity was obviously a limitation with other nuclei, substantial contributions using ^{13}C and ^{31}P NMR were made on molecules of biological interest. By 1960, the ^{31}P NMR spectra of ADP and ATP had been obtained.[18] The ^{31}P chemical shifts were shown to be sensitive to the pH and ionic environment, an observation that foreshadowed current intense research activity monitoring metabolic changes in in vivo systems; ^{13}C NMR was extremely difficult with early spectrometers, yet a large body of useful data was collected in several laboratories. The first studies of ^{13}C chemical shifts and coupling constants in organic compounds in 1957 were carried out with neat liquids or in saturated solutions.[19] Extreme sensitivity problems prevented studies at lower concentrations. Thus, it was not until more than a decade later that the first molecules of biochemical interest were studied by ^{13}C NMR. For example, comprehensive surveys of natural abundance ^{13}C data for nucleosides and nucleotides[20] were published and ^{13}C enrichment was used to develop relationships between C-H vicinal coupling constants and conformation.[21] Initial ^{13}C observations of amino acids were accomplished with double resonance techniques (INDOR) and with enriched samples.[22] Later studies designed to determine characteristic chemical shifts of amino acids in peptides largely relied upon pulse Fourier transform techniques discussed below.

IV. DEVELOPMENTS IN INSTRUMENTS AND TECHNIQUES

The exploitation of NMR in biology and biochemistry has depended markedly on the phenomenal improvements over the years in NMR instrumentation. As higher magnetic fields of excellent homogeneity have become available, it has been possible to unravel features of complex molecules that could scarcely be envisioned at lower fields. It is often not appreciated that along with an order of magnitude increase in spectrometer frequency over the last 20 years has come an improvement in instrumental sensitivity of about a factor of 600, as illustrated in Table 2. This arises both from the higher field and observation frequency (including a more favorable Boltzmann distribution) and from improved electronics and better probe design.

Three other developments in NMR technique have also played a vital role in permitting the application of NMR to the typical low concentrations of molecules and to the insensitive nuclei found in biological systems. The first is the principle of time averaging, first utilized