NUCLEAR MAGNETIC RESONANCE IN BIOCHEMISTRY

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PRINCIPLES AND APPLICATIONS

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PREFACE

In the past few years, nuclear magnetic resonance (NMR) has been successfully used to unravel some knotty problems in several areas of biological and physiological chemistry. As a result of these applications, many biological and biochemical researchers are now aware of the utility of nuclear magnetic resonance. However, no text currently exists which considers NMR phenomena from the viewpoint of the life scientist. The aim of this book is to correct that condition.

The text is intended primarily for biochemists, biophysicists, and molecular biologists. It is anticipated that many chemists will also be interested in learning those aspects of nuclear magnetic resonance most useful for studying biological systems. The text should be suitable for certain graduate courses. A recent trend has been to apply more sophisticated physical techniques to biological and biochemical problems. The graduate curriculum in many schools is beginning to reflect this trend with the introduction of courses covering these newer techniques.

Several texts on NMR are available, but they generally cover material of interest to the physicist or chemist. The features of greatest utility in biological applications often differ from those presented in the chemically oriented texts. For example, the use of NMR relaxation phenomena enjoys a more prominent place in biological applications than it does in chemical applications. Appropriately, this book covers NMR relaxation and its applications in more detail than will be found in chemically oriented texts.

This book will be of interest to people with varying backgrounds. Introductory material is given in Chapter 1 for those with little previous NMR knowledge. It may be sufficient for some readers to go directly to the

discussion of NMR applications in their field of interest after reading Chapter 1. It may, however, be necessary to read the appropriate sections of Chapter 2 or Chapter 3 for a better understanding of the theoretical basis for those applications. Chapter 2 presents the principles of nuclear magnetic resonance without preoccupation with derivation of equations. The emphasis is to convey an understanding of the equations, especially those which have found use in biochemical studies. In addition to a discussion of the basis for chemical shifts and spin–spin splitting, Chapters 3 and 4 include several examples of the use of these NMR parameters in studies of small molecule interactions and structure. Chapter 6 is concerned primarily with NMR spectral parameters of small molecules interacting with macromolecules, and Chapter 7 deals with the information obtainable from the spectra of biopolymers. Chapter 8 discusses NMR investigations of the state of motion of lipids in membranes and model membranes, water in macromolecular and cellular systems, and sodium ion in biological tissue.

Chapter 5 describes the experimental apparatus and procedures employed in NMR studies, with a strong emphasis on those aspects of greatest importance in biological applications, e.g., sensitivity improvement. A fairly comprehensive discussion of Fourier transform NMR is included since Fourier transform NMR is a relatively new, sophisticated technique which provides the sensitivity gain necessary for many biochemical studies as well as the capability for selective relaxation time measurements. Several promising new NMR techniques which have yet to be applied to biological problems are also briefly discussed.

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Thomas L. James

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CHAPTER 1

INTRODUCTION

The high degree of specificity for many biological reactions and processes depends on subtle differences in the structure and conformation of molecules. Nuclear magnetic resonance (NMR) spectroscopy is one of the few techniques available with the capacity to obtain detailed information about biomolecular phenomena. With NMR, an individual nucleus in a molecule can be "observed" by monitoring that nucleus' line in an NMR spectrum. The various NMR parameters of that line—frequency, splitting, linewidth, and amplitude—can be used to study the electronic and geometric structure of "simple" molecules or macromolecules, molecular motion and rate processes, and molecular interactions. Quite often the molecular information obtained is of a qualitative nature; however, in many cases NMR can provide quantitative information not obtainable by other means.

Data from many of the other physical methods used in studies of biological systems are often interpreted empirically because theory usually provides little aid in their qualitative interpretation. The usual procedure for those methods is to rely on compilations of data for comparison with present experimental results. A comparatively better understanding of theory is required for a satisfactory interpretation of NMR results. The

yield for this better understanding is usually unequivocal qualitative results, or often quantitative results.

Many monographs are available that treat the NMR phenomenon with varying degrees of rigor. Some texts, in order of difficulty, are Abragam (1), Slichter (2), Carrington and McLachlan (3), and Becker (4). Reviews on various aspects of NMR studies of biological molecules have been written by pioneers in the field: Kowalsky and Cohn (5, 6), Roberts and Jardetzky (7, 8), and McDonald and Phillips (9).

1.1. Magnetic Properties of Nuclei

Roughly half of the known nuclei behave as though they were spinning like a top. The magnitude of the angular momentum

$$J' = \hbar \sqrt{I(I + 1)} \tag{1-1}$$

of this spinning motion depends on the nuclear spin quantum number I, which differs for different nuclei ($\hbar$ is Planck's constant divided by 2π). The value of the nuclear spin quantum number I is determined by the mass number and atomic number according to the following tabulation.

MASS NUMBER	ATOMIC NUMBER	SPIN NUMBER, I
Odd	Odd or even	Half-integer: $\frac{1}{2}, \frac{3}{2}, \frac{5}{2}, \ldots$
Even	Even	0
Even	Odd	Integer: 1, 2, 3 . . .

Nuclei of interest having spin $\frac{1}{2}$ are ^{1}H, ^{13}C, ^{31}P, and ^{19}F. Nuclei of interest having spin 0 are ^{12}C, ^{16}O, and ^{32}S. ^{2}H (or D) and ^{14}N have $I = 1$ and ^{23}Na and ^{39}K have $I = \frac{3}{2}$. A complete list is available in Appendix 2.

Because nuclei are positively charged, a spinning nucleus gives rise to a magnetic moment

$$\vec{\mu} = \gamma \vec{J}' = \gamma \hbar \vec{I} \tag{1-2}$$

where γ is the gyromagnetic (or magnetogyric) ratio and $\vec{I}$ is a dimensionless angular momentum. As a physical picture, the nucleus with $I > 0$ is equivalent to a tiny bar magnet the axis of which is coincident with the spin axis.

Nuclei with spin $I = \frac{1}{2}$ behave as spherical entities possessing a uniform charge distribution. However, the charge distribution within a nucleus with $I \geq 1$ can be described as a prolate (cigar-shaped) or oblate (flattened)

spheroid. A measure of the nonsphericity of the nuclear charge distribution is embodied in the electric quadrupole moment, which depends on I. Only nuclei with $I \geq 1$ possess an electric quadrupole moment. Therefore, when a charged species (e.g., an electron) approaches a nucleus with an electric quadrupole moment, the nucleus experiences an electric field the magnitude of which depends on the direction of approach. Possession of an electric quadrupole moment will critically affect the relaxation time of a nucleus and the coupling of that nucleus' spin with spins of neighboring nuclei. Appendix 2 lists electric quadrupole moments for those nuclei with $I \geq 1$.

1.2. Magnetic Resonance

When a nucleus with magnetic moment μ is placed in a strong, uniform magnetic field H_0 (oriented in the z direction), the magnetic dipole is quantized into a discrete set of orientations. This is referred to as "nuclear Zeeman splitting." Each one of these orientations corresponds to a nuclear energy state or level with energy

$$E = -\mu_z H_0 \tag{1-3}$$

where μ_z $(= m_I \gamma \hbar)$ is the z component of the nuclear magnetic moment. The magnetic quantum number m_I, characteristic of each nuclear energy level, depends on the nuclear spin quantum number and may take on the values

$$m_I = I, (I-1), (I-2), \ldots, -(I-2), -(I-1), -I \tag{1-4}$$

Energy levels are shown in Fig. 1-1 for nuclei with spin quantum numbers

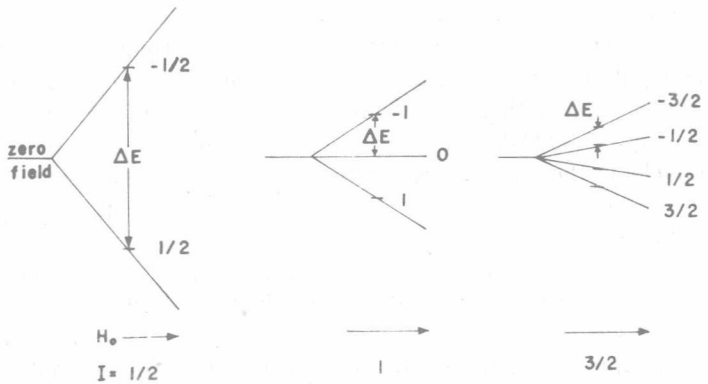

FIG. 1-1. Nuclear Zeeman splitting of energy levels in a magnetic field for various nuclear spin quantum numbers.

$I = \frac{1}{2}$, 1, and $\frac{3}{2}$. For each nuclear spin, the energy levels are equally spaced. The NMR experiment is to induce transitions between the levels by absorption or emission of a photon with the requisite energy. The selection rules of quantum mechanics prescribe that transitions between neighboring levels are the only allowable transitions. The energy of the photon required to induce transitions is just the energy separation between adjacent levels,

$$\Delta E = \gamma \hbar H_0 \tag{1-5}$$

The Bohr condition ($\Delta E = h\nu$) permits us to give the frequency of the nuclear transition

$$\nu = \gamma H_0 / 2\pi \tag{1-6}$$

which is in the radiofrequency (rf) portion (e.g., 100 MHz) of the electromagnetic spectrum. Therefore, for any particular nucleus in a given magnetic field, the NMR frequency will be characteristic, depending primarily on the gyromagnetic ratio peculiar to that particular nucleus.

The theory of electromagnetic radiation states that the probability of a photon inducing a transition from a higher energy level to a lower one is the same as the probability for induced transition from a lower to a higher energy level. Therefore, in a large ensemble of spins (such as a sample in an NMR tube), a net absorption or emission depends only on the difference between the number of nuclei in the upper and lower energy levels.

The distribution of nuclei in the various possible energy states is given, under conditions of thermal equilibrium, by the Boltzmann equation:

$$\frac{N_{\text{upper}}}{N_{\text{lower}}} = e^{-\Delta E/kT} = e^{-h\nu/Tk} \tag{1-7}$$

where N_{upper} and N_{lower} represent the population (i.e., number) of nuclei in upper and lower energy states, respectively. With radiofrequencies used in NMR, the population ratio might typically be 1.000035 (at $\nu = 220$ MHz) for hydrogen nuclei in thermal equilibrium at room temperature. That means for every 1,000,000 nuclei in the upper energy state there are 1,000,035 nuclei in the lower energy state. Without this small excess in the lower energy state, there could be no nuclear magnetic resonance phenomenon.

This small excess does permit NMR to thrive, but it also presents an unfortunate sensitivity problem. The relatively low sensitivity of NMR compared with some other spectroscopic techniques is perhaps the greatest limitation for application of NMR to biochemical systems. One of the motivating factors for use of stronger magnetic fields is the increased sensitivity. From Eqs. 1-6 and 1-7, it is seen that nuclei in a stronger

magnetic field have a larger Boltzmann factor and, consequently, a larger NMR signal.

1.3. NMR Relaxation

A nuclear spin system in a stationary magnetic field H_0 may be considered. At equilibrium, the spin populations of the various Zeeman energy levels will be described by the Boltzmann distribution (Eq. 1-7), giving the lower energy levels a slightly greater spin population, as just discussed. If a radiofrequency field at the resonance frequency is applied to the system, the probability of an upward transition is equal to the probability of a downward transition. Because there is a greater spin population in the lower energy levels, there will be more upward transitions than downward transitions, resulting in a nonequilibrium spin distribution. If this process continues, the excess of nuclei in the lower energy state will continually diminish with consequent decrease in the NMR signal intensity. Under certain circumstances the two spin populations may be equal and the NMR signal may disappear completely. This phenomenon is referred to as "saturation" and, in practice, can occur if strong rf fields are applied. For an NMR signal to persist, some mechanism must be available for replenishing the number of nuclei in the lower energy state.

There are various mechanisms leading to radiationless transitions that cause the perturbed system to return to the equilibrium spin distribution. These radiationless transitions are called "relaxation processes." There are two kinds of relaxation processes: spin–lattice (or longitudinal) relaxation and spin–spin (or transverse) relaxation. The spin–lattice relaxation time is designated by T_1 and the spin–spin relaxation time is designated by T_2. T_1 is a characteristic time describing the rate at which the nonequilibrium spin distribution $(N_{\text{lower}} - N_{\text{upper}})$ exponentially approaches equilibrium $(N_{\text{lower}} - N_{\text{upper}})_{\text{equil}}$ following absorption of rf energy:

$$(N_{\text{lower}} - N_{\text{upper}}) = (N_{\text{lower}} - N_{\text{upper}})_{\text{equil}} (1 - e^{-t/T_1}) \qquad (1\text{-}8)$$

The lattice is the environment surrounding the nucleus—the remainder of that molecule as well as other solute and solvent molecules. Spin–lattice relaxation occurs by interaction of the nuclear spin dipole with random, fluctuating magnetic fields caused by the motion of surrounding dipoles in the lattice that happen to have components fluctuating with the same frequency as the resonance frequency described in Eq. 1-6. The energy of the radiationless transition is transferred to the various energy components of the lattice as additional rotational, translational, or vibrational energy (with total energy unchanged) until the nuclear spin system and the lattice

are in thermal equilibrium. There are several possible mechanisms contributing to spin–lattice relaxation, which will be discussed in Chapter 2. In solids or viscous liquids, the T_1 relaxation may be several hours. In most nonviscous liquids and solutions, T_1 is usually on the order of 0.001–100 sec.

Spin–spin relaxation processes also have a relaxation time, T_2, which characterizes the rate of these relaxation processes. The sources of the random magnetic fields giving rise to T_1 relaxation will also lead to T_2 relaxation. However, spin–spin relaxation has other relaxation mechanisms that may contribute to T_2. Simply, the additional contribution to spin–spin (or transverse) relaxation is the result of chemical exchange or mutual exchange of spin states by two nuclei in close proximity. The distribution of energy among the spins in this manner is an adiabatic process and, although it decreases the lifetime for any particular nucleus in the higher energy state, it does not change the number of nuclei in the higher energy state.

Spin–spin relaxation is caused by random magnetic fields (usually from neighboring nuclei) in the sample that are not fluctuating. These random local fields will cause shifts in the resonance frequencies for individual nuclear spins in the sample. There will then be a distribution of resonance frequencies for any sample depending on the variation in the random local fields. The greater the variation in local fields, the greater the linewidth of the peak in the absorption spectrum will be. The spin–spin relaxation time T_2 can then be related to the resonance linewidth $W_{1/2}$ in the absorption spectrum by

$$T_2 = 1/\pi W_{1/2} \tag{1-9}$$

Equation 1-9 is valid in the absence of instrumental instability and magnetic field inhomogeneity. Variation of the stationary magnetic field H_0 over the area of the sample will cause inhomogeneity broadening just as variations in the microscopic local fields within the sample are the cause of the NMR linewidth. High-quality instruments often enable inhomogeneity and thus instrumental considerations to be overcome, with the result that Eq. 1-9 is a justifiable expression of the true T_2. However, with large values for T_2 ($\gtrsim$1.0 sec), Eq. 1-9 may not accurately relate the observed linewidth to T_2.

1.4. Chemical Shift and Shielding

Historically, NMR entered the realm of chemistry when it was discovered that all protons do not have the same resonance frequency. The application of the nuclear magnetic resonance phenomenon to chemical problems depends on the fact that the field experienced by a nucleus is not exactly the

same as the applied magnetic field. The small variations in the field at the nucleus are caused by diamagnetic shielding by electrons within the molecule. The extent of this diamagnetic shielding depends on the chemical environment of the nucleus; i.e., each nucleus in a collection of chemically equivalent nuclei is shielded to the same extent, but the extent of shielding is different for any other collection of chemically equivalent nuclei. For example, the three methyl protons of ethyl bromide will form an equivalent set with resonance frequency ν_{CH_3} and the two methylene protons will form another equivalent set of nuclei with resonance frequency ν_{CH_2}.

The shielding of a nucleus is caused by the motion of electrons in the molecule induced by application of the stationary magnetic field H_0. The induced motion of those electrons sets up a local magnetic field opposed to the H_0 field. The magnitude of the effective field perceived by a set of equivalent nuclei will be proportional to the stationary field:

$$H_{\text{eff}} = H_0 - H_0\sigma = H_0(1 - \sigma) \tag{1-10}$$

where σ is a nondimensional screening or shielding constant. $H_0\sigma$ is the induced field caused by the motion of surrounding electrons. The resonance frequency will therefore be decreased by the shielding, as illustrated in Fig. 1-2.

It can be seen that the exact resonance frequencies for nuclei in a magnetic field depend on the strength of the magnetic field and on the precise frequency of the applied radiofrequency field. The nuclear magnetic resonance spectrum can be generated in either of two ways: (*1*) field sweep—the frequency of rf field is maintained at a fixed value and the strength of the applied H_0 field is slowly varied over a small range, or (*2*) frequency sweep—the strength of the applied H_0 field is maintained at a fixed value and the rf frequency is slowly varied. The positions of the resonance lines in the field sweep mode can be related to the positions in the frequency sweep mode simply by applying Eq. 1-6, $\nu = \gamma H_0/2\pi$, which expresses the resonance condition. For liquid samples, the usual practice is to describe the sweep in frequency units even if the field sweep method is being used.

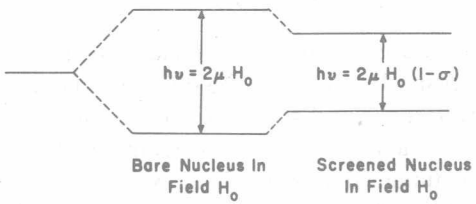

FIG. 1-2. Effect of shielding (screening) on the resonance frequency.

In most compounds, protons absorb at frequencies ranging over about 2500 Hz at a field strength of about 51,000 Gauss (or 5.1 Tesla). At this field strength, the protons resonate at a frequency of about 220×10^6 Hz. Knowledge of proton resonance frequencies provides valuable information about molecules. Therefore, it is desirable to make accurate measurements of the proton resonance positions. If the resonance position is to be determined with an accuracy of about ± 1 Hz, the 220 MHz instrument must be able to distinguish between frequencies of the order of 1 part in 10^8. The strength of the magnetic field cannot be determined with such a degree of accuracy, so the absolute resonance frequency cannot be determined to ± 1 Hz. However, the relative positions of two lines in the proton NMR spectrum can be determined to less than ± 1 Hz. The difference between a proton resonance position in the NMR spectrum and the position of some arbitrarily chosen reference is called the "chemical shift."

Typical reference compounds are 85% phosphoric acid for ^{31}P; trifluoroacetic acid or carbon tetrafluoride for ^{19}F; carbon disulfide or tetramethylsilane for ^{13}C; and cyclohexane, tetramethylammonium ion, tetramethylsilane (TMS), hexamethyldisiloxane (HMDS), or the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), $(CH_3)_3$-$Si(CH_2)_3SO_3^-Na^+$, for ^1H. For proton NMR, TMS has become the ultimate reference in nonaqueous solutions and DSS the reference in aqueous solutions. The resonance frequency of the methyl protons of DSS and TMS are nearly identical.

Chemical shifts can be expressed in terms of the number of Hertz a resonance peak is from a reference peak. However, because the chemical shift is dependent on the strength of the applied magnetic field, it is advantageous to express the chemical shift as a function of field strength necessary to achieve the resonance condition:

$$\delta = \frac{H_{\text{ref}} - H_{\text{samp}}}{H_{\text{ref}}} \tag{1-11}$$

$$\delta = \frac{\Delta\nu \times 10^6}{\nu_{\text{instr}}} \tag{1-12}$$

where H_{ref} and H_{samp} are the resonance field strengths for the reference and sample nuclei, respectively; $\Delta\nu$ is the difference between the resonance frequencies of the reference and sample (in Hz); and ν_{instr} is the oscillator frequency (in Hz) characteristic of the instrument. The chemical shift is then obtained as a dimensionless number, expressed as parts per million (ppm), and chemical shifts are determined using spectrometers operating at different frequencies can be directly compared. For example, the chloro-

form proton resonates at 436 Hz downfield from the TMS reference on a 60 MHz (60×10^6 Hz) instrument and 1598 Hz on a 220 MHz instrument. In terms of δ units, the chemical shift is 7.25 ppm for either instrument.

Proton magnetic resonance has been more highly developed and conventions for presenting data are more uniform. In the literature, chemical shifts are given in any of three ways: (*1*) Hertz—the reference compound must be listed and the instrument frequency given, (*2*) δ (ppm)—the reference compound must be listed, and (*3*) τ (ppm)—TMS or DSS is assumed to be the reference compound with a value of $\tau = 10$ ppm. If TMS is used as the reference in (*2*), the τ and δ scales are related: $\tau = 10 - \delta$. Very few protons have resonance positions at a higher field than TMS and only a few resonances for protons in very electronegative environments or quite acidic protons are found below 10 ppm downfield from TMS. Therefore, the majority of proton chemical shifts can be found between 0 and 10 on the τ scale. Figure 1-3 shows the spectrum of adenine in D_2O using DSS as an external standard, i.e., the standard is in a capillary inside the NMR tube.

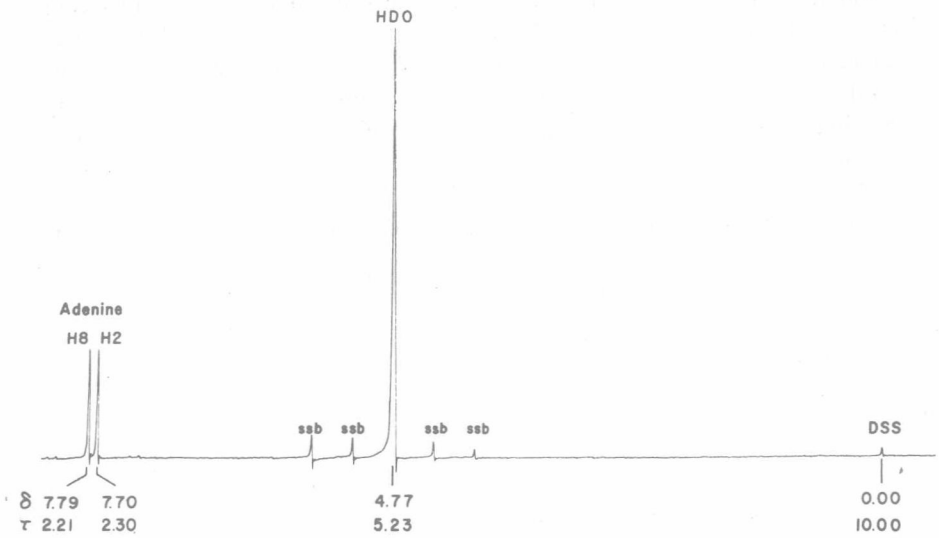

FIG. 1-3. 220 MHz proton NMR spectrum of 0.40 *M* adenine in 1 *M* NaOD with D_2O as solvent and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an external reference present as a D_2O solution in a coaxial capillary tube. The spinning side bands (designated ssb) flanking the residual HDO peak occur at integral multiples of the sample spinning frequency. The spinning side bands result from the sample spinning through a slightly inhomogeneous field.