

**Structural and Resonance
Techniques in
Biological Research**

Edited by

DENIS L. ROUSSEAU

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Chemical Physics Research Laboratory

AT&T Bell Laboratories

Murray Hill, New Jersey



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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

- TRUMAN R. BROWN¹ (1), Molecular Biophysics Research Department, AT&T Bell Laboratories, Murray Hill, New Jersey 07974
- D. P. E. DICKSON (245), Department of Physics, University of Liverpool, Liverpool, England
- C. E. JOHNSON (245), Department of Physics, University of Liverpool, Liverpool, England
- DANIEL J. KOSMAN (89), Department of Biochemistry, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214
- KEITH MOFFAT (363), Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853
- ROBERT A. SCOTT (295), School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801
- J. STAMATOFF (437), Celanese Research Company, Summit, New Jersey 07901
- KÄMIL UĞURBİL (1), Department of Biochemistry and Gray Freshwater Biological Institute, College of Biological Sciences, University of Minnesota, Navarre, Minnesota 55392

¹ PRESENT ADDRESS: Department of Radiology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111.

Series Introduction

With the appearance of "Structural and Resonance Techniques in Biological Research" and "Optical Techniques in Biological Research," both edited by one of us (D. L. R.), we mark the introduction of a new series of volumes, **Physical Techniques in Biology and Medicine**. This series is intended to replace a previous treatise, **Physical Techniques in Biological Research**, as many of the diverse physical methods used to address biological systems were in their infancy when the original works were published. For those techniques that had attained relative maturity, the earlier contribution has been cited in the present chapters, which provide an updated view and discuss contemporary developments.

The subject matter we hope to include in this series will form volumes of a topical nature. The biophysical sciences are populated by physicists who have become interested in living systems and by biologists who find they have a need for a particular physical technique in which they may have had no formal training. It is this duality of audience we hope to reach, making every attempt to ensure that each chapter is sufficiently methods-oriented and illustrative of a range of applications that the reader will be provided with an adequate entrée to the desired technique. We shall also stress the importance of a comprehensive bibliography to permit ready access to the literature.

As technology continues to advance our instrumental and computational capabilities, we look forward to a plethora of both novel and renewed topics to be covered by volumes in this series.

Denis L. Rousseau
William L. Nastuk

Preface

The first two books in this series are composed of chapters loosely organized into a volume on structural and resonance techniques and one on optical techniques. Included in this volume are discussions of nuclear magnetic resonance, electron spin resonance, Mössbauer spectroscopy, x-ray absorption spectroscopy, macromolecular crystallography, and small-angle x-ray scattering and diffraction.

The chapters in this volume are aimed at a level such that only a general understanding of chemistry and biology is required. The objective, which I believe has been largely achieved, is to present material in a way that allows the research worker to assess quickly the applicability, utility, and significance of the specific technique to his or her problem or field of interest. With these guidelines the authors have written chapters in which the diversity of the particular technique and its application to different types of problems have been stressed, occasionally at the expense of timeliness and excessive detail. Similarly, these chapters are not intended to be exhaustive reviews of all the literature, but instead include examples which most appropriately illustrate the application of the technique. Extensive derivations of the basic principles underlying the physics of each technique have been kept to a minimum and do not form the central theme of any chapter. However, the underpinning for each technique is outlined, and sufficient references have been provided for the reader who wants a more in-depth understanding.

Finally, I wish to thank all the authors for being willing and able to take time away from their other responsibilities in order to write these pedagogical chapters. I am especially thankful to those who submitted their manuscripts on time. I hope that they are sufficiently pleased with the final product that they are able to excuse the delays that went into the completion of these volumes.

Denis L. Rousseau

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Nuclear Magnetic Resonance

TRUMAN R. BROWN

*Department of Radiology
Fox Chase Cancer Center
Philadelphia, Pennsylvania*

KÂMIL UĞURBIL

*Department of Biochemistry and
Gray Freshwater Biological Institute
University of Minnesota
Navarre, Minnesota*

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I. Introduction

The phenomenon of nuclear magnetic resonance (NMR) was discovered in 1946 (Block *et al.*, 1946; Purcell *et al.*, 1946). At first, the discovery was exploited primarily by physicists interested in understanding the structure of the nucleus. However, the discovery in 1950 that the exact resonance frequency depends on the details of the molecular environment (Proctor and Yu, 1950) led chemists to exploit the technique for probing the structure of molecules. As NMR techniques increased in sensitivity and sophistication, biologists and biochemists started to employ this form of spectroscopy as a research tool. The range of biological applications in which NMR has proven to be useful is too wide to cover adequately in a single chapter. Necessarily, we have restricted our discussion and, in doing so, have tried to emphasize the broad areas of application such as structure, dynamics, kinetics, and cellular metabolism. There are obviously numerous other specific applications, some of which are summarized in Section VI.

We would like to emphasize that this chapter is not meant to provide a review of the NMR work conducted in these areas; its aim is to help investigators decide whether it is worth the effort to learn the details of a complicated technique to aid their research. Therefore, the discussion is at an introductory level and is meant to provide only an intuitive picture. See Abragam (1961) or Slichter (1978) for a fuller treatment.

II. Introduction to Nuclear Magnetic Resonance

This section is intended to provide a qualitative understanding of the overall physical processes observed and the type of information provided in NMR spectroscopy. The ideas are presented heuristically and will be repeated later with greater detail and rigor.

The phenomenon of nuclear magnetic resonance arises as a result of interactions between magnetic fields and those atomic nuclei which possess magnetic properties. In the presence of an external field, such nuclei behave like microscopic bar magnets and experience forces which tend to align them parallel to the external field. As a result of these forces, the energy levels of these nuclei change and acquire new values which depend on their orientation. Unlike the bar magnets, however, these nuclear magnets must behave according to the laws of quantum mechanics. Consequently, they can acquire only a limited number of discreet orientations and energies. For example, in the case of "spin- $\frac{1}{2}$ " nuclei, such as ^1H , ^{13}C , and ^{31}P , the nuclear energy levels split in two in the presence of the magnetic field; each level corresponds to one of the two possible orientations these nuclei can have

relative to the direction of the field. Transitions between these two energy states can be induced by a secondary magnetic field oscillating in time with the appropriate frequency. These transitions are detected and presented as absorption peaks in an NMR spectrum (Fig. 1). In NMR spectroscopy one is generally interested in the values of four parameters which are directly or indirectly obtainable from the NMR peaks. These are the resonance frequency, the area of the NMR peak, and the spin relaxation times T_1 and T_2 . These parameters in turn yield a wealth of information about the atoms and the molecules being studied.

The resonance frequency of a magnetic nucleus is determined primarily by three factors; the properties intrinsic to the magnetic nucleus, the electronic environment of the nucleus within the atom or molecule, and the magnitude of the external field. The first is the reason why, at a constant magnetic field, NMR spectroscopy of different nuclei are performed at very different frequencies (Table I). This is a much larger effect than the one induced by the electronic environment (Fig. 2); however, it is the latter which provides chemical information and which renders NMR spectroscopy so useful.

The peak area is simply the integral of the absorption peak. It is proportional to the total number of spins contained in the sample giving rise to the observed peak. Combined with the sensitivity of NMR to chemical environments, measurement of peak areas tells us how much of a certain chemical species our sample contains.

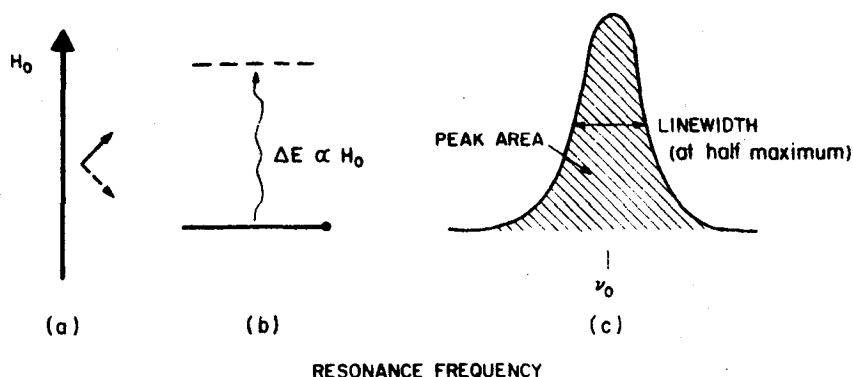


Fig. 1. Schematic representation of spin orientations (a) and energy levels (b) in the presence of a magnetic field, and the resultant absorption peak (c) that would be detected as a result of transitions between these levels. The peak-area linewidth and frequency are the experimental parameters determined by such a measurement.

Table I Magnetic Properties of Some Nuclei

Nucleus	Spin	Natural abundance (%)	Resonance frequency at 70.46 kG (MHz)
^1H	1/2	99.99	300.00
^2H	1	0.015	46.05
^{13}C	1/2	1.1	75.43
^{14}N	1	99.63	21.67
^{15}N	1/2	0.37	30.40
^{17}O	5/2	0.037	40.67
^{19}F	1/2	1.00	282.23
^{23}Na	3/2	100	79.35
^{25}Mg	5/2	10.13	18.36
^{31}P	1/2	100	121.44
^{35}Cl	3/2	75.53	29.40
^{37}Cl	3/2	24.47	24.47
^{39}K	3/2	93.1	14.00
^{41}K	3/2	6.9	7.68
^{43}Ca	7/2	0.15	20.18
^{111}Cd	1/2	12.75	63.62
^{113}Cd	1/2	12.26	66.55
^{199}Pt	1/2	33.8	64.50
^{199}Hg	1/2	16.84	53.48
^{205}Tl	1/2	70.5	173.12

Spin-relaxation parameters T_1 and T_2 are generally not directly measurable from the NMR peaks and require execution of other experiments, which are discussed in Section IV.B. Qualitatively, the nature of T_1 and techniques for its measurements can be understood if one considers the fact that the NMR sample is not a single nucleus but consists of many such nuclei. These nuclei distribute themselves between the allowed energy states; ultimately, the distribution is determined only by the sample temperature and the energy of the allowed states. When this time-independent distribution is reached, the spins are said to be in thermal equilibrium. The rate constant for the approach to thermal equilibrium is T_1^{-1} .

At thermal equilibrium, there is a net difference between the spin populations of the allowed nuclear-energy states, with more of the spins residing in the lower energy state. This difference is the source of the NMR absorption peaks. The distribution of spins between the energy states can be perturbed by the application of radio frequency (rf) radiation of appropriate power and frequency. For example, it is possible to invert the spin populations (Fig. 3). If an NMR spectrum is taken immediately after such a perturbing pulse, one observes a negative or emission peak as shown in the lower part of Fig.

3. This new population state, however, is unstable. The spins must return to the distribution dictated by the temperature. They do so at a rate determined by the rate constant T_1^{-1} . Thus, one can measure T_1 by perturbing the spin populations and monitoring the relaxation back to the stable state.

T_2 is connected with an analogous but somewhat more complicated relaxation which is explained in Section III.F. In the event the laboratory magnetic field is perfectly homogeneous over the sample, T_2 is proportional to the inverse of the resonance linewidth. If, however, the external field is not sufficiently homogeneous, the observed linewidths contain contributions from this inhomogeneity as well as the relaxation process associated with T_2 .

Both T_1 and T_2 are sensitive to molecular motion. This dependence is generally well understood and is used to extract detailed information on molecular dynamics. The most apparent manifestation of this sensitivity to motion is the increase in observed resonance linewidths with increasing molecular weight. Small molecules, such as those generally encountered in organic chemical applications, execute rapid random rotations in solution and yield NMR spectra with very narrow linewidths which are typically less than 1 Hz for spin- $\frac{1}{2}$ nuclei. NMR peaks from biological macromolecules,

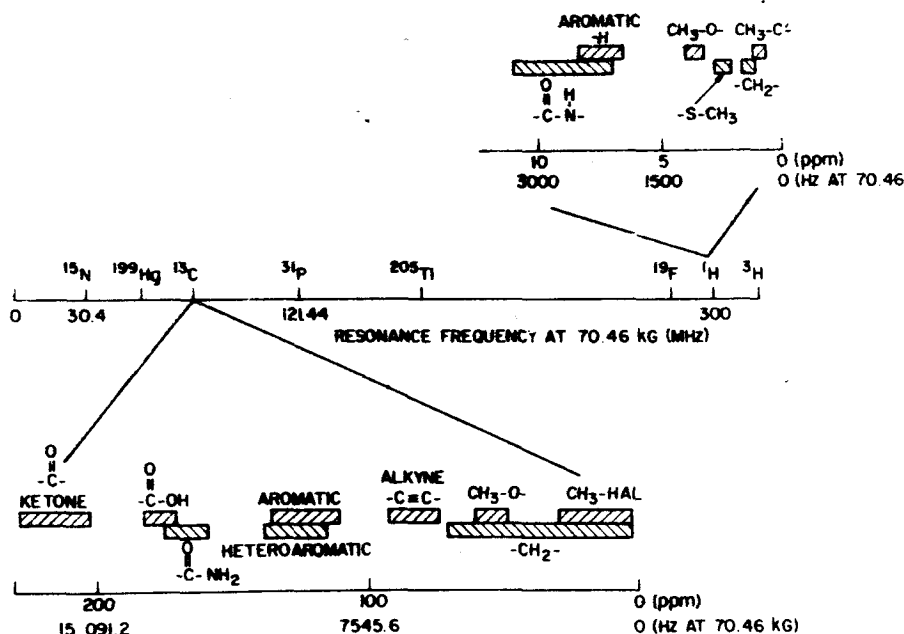


Fig. 2. Nuclear resonance frequencies of several magnetic nuclei at 70.46 kG and the chemical shift range ^{13}C and ^1H nuclei.

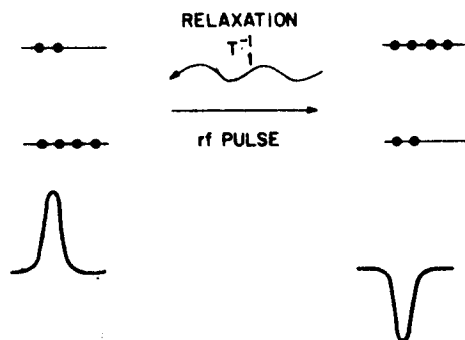


Fig. 3. Spin populations at thermal equilibrium (left) and after they are inverted by a pulse of oscillating magnetic field (right).

on the other hand, are much broader due to the slower rotation of these larger molecules. For example, proton resonances from lysozyme can have line-widths greater than 5 Hz. In cases where the T_1 and the T_2 are dominated by magnetic interaction among the various spins in a molecule, they are a function of the internuclear distances as well as the molecular motion. This distance dependence is the source of most of the structural studies with NMR spectroscopy.

In the following sections, we will discuss in greater detail the physical basis of these parameters and the information that can be extracted from them. As we have done in this section, we will limit our examples, except when stated otherwise, to spin- $\frac{1}{2}$ nuclei, which constitute the simplest and generally most useful set of nuclear spins.

III. Theory

A. Zeeman Energy

In the presence of a magnetic field, magnetic dipoles experience a force which tends to align them along the direction of the field. Consequently, the dipole acquires a potential energy which depends on its orientation relative to the field. This dependence is expressed by the equation

$$E = -\boldsymbol{\mu} \cdot \mathbf{H} = -H\mu \cos \theta, \quad (1)$$

where μ and H represents the magnitudes of the magnetic dipole moment and the magnetic field, respectively, and θ is the angle between the direction of the magnetic field and the direction of μ . By convention, the direction of H is taken as the z axis and $\mu \cos \theta = \mu_z$, the projection of μ along the

direction of H . Thus, the energy of the dipole varies between $-\mu H$ and $+\mu H$, and the projections of μ along H which correspond to the minimum and maximum energy are those for which θ is equal to 0 and 180 degrees, respectively. Note that the azimuthal orientation with respect to the magnetic field has no effect on the energy.

A macroscopic magnetic dipole, such as a circular loop of wire carrying a current, can assume all possible orientations relative to a magnetic field. Therefore, its energy can vary continuously between $-\mu H$ and $+\mu H$. For electrons and atomic nuclei, however, quantum mechanics dictates that the angular momentum and the dipole moment can have only a limited number of discreet projections along a particular direction. The angle θ and therefore the energy acquired by a magnetic nucleus in the presence of the magnetic field become discrete as well. These different projections correspond to the different energy levels mentioned in the introduction.

The number of possible different projections for a particular kind of particle is fixed and determined by its spin quantum number I , which characterizes both the spin angular momentum and magnetic moment of the particle. For any particular nucleus, I is an invariant quantity and must be an integer or half integer. For spin- $\frac{1}{2}$ nuclei such as ^1H , ^{13}C , and ^{31}P , I is equal to $\frac{1}{2}$. For deuterium, I is 1 (spin-one), and for ^{23}Na , a magnetic isotope of Na, I is $\frac{3}{2}$ (see Table I for other nuclei).

The number of possible projections the dipole moment can have is limited to $2I + 1$; each projection is described by specifying another spin quantum number, m_I , which can vary between I and $-I$ in integer steps. The magnitude of the overall magnetic moment and its z component depend on I and m_I in the following way:

$$\mu = \gamma \hbar \sqrt{I(I+1)}, \quad (2)$$

$$\mu_z = \gamma \hbar m_I, \quad (3)$$

where \hbar is Plank's constant divided by 2π and γ the gyromagnetic ratio, so called because it relates the angular momentum of a particle to its magnetic moment. The energy of the nucleus is given by

$$E = -\mu \cdot H = -\mu_z H_0 = -\gamma \hbar m_I H_0, \quad (4)$$

where the last equality follows from Eq. (3).

For spin- $\frac{1}{2}$ nuclei, m_I can be $+\frac{1}{2}$ or $-\frac{1}{2}$, corresponding to a parallel or antiparallel orientation of the spin (and hence the magnetic moment) with respect to the magnetic-field direction. Figure 4 represents these two possibilities for a spin- $\frac{1}{2}$ particle. For higher spin particles, μ can acquire $2I + 1$ allowed orientations.

For spin- $\frac{1}{2}$ particles, the energies of the two orientations are $-\frac{1}{2}\gamma \hbar H_0$ and

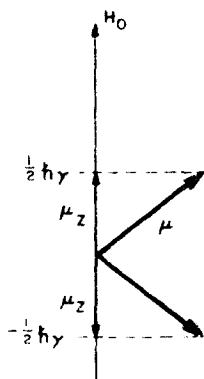


Fig. 4. Allowed orientations for a spin- $\frac{1}{2}$ nucleus, where $|\mu| = \hbar \gamma \sqrt{\frac{1}{2}}$.

$+\frac{1}{2}\gamma\hbar H_0$. The energy separation between these states is then $\gamma\hbar H_0$. For higher-spin particles, the separation between adjacent levels will still be $\gamma\hbar H_0$ since μ_z can change only in steps of $\hbar\gamma$. Because of the H_0 factor, the energy separation will increase with increasing magnetic field and, at a fixed field strength, will be higher for nuclei with the higher gyromagnetic ratio. For example, the γ of the ^1H nucleus is approximately fourfold higher than that of the ^{13}C nucleus. Thus, at a fixed magnetic field strength, the energy separation between the adjacent spin states of the ^1H nucleus is approximately fourfold larger than that of the ^{13}C nucleus.

If a *totally isolated* magnetic dipole is in one of the energy states specified by the quantum number m_I , it will stay in that state forever. For this particle to undergo a transition to another state with a different m_I value, it has to interact with another system and either gain or lose energy to that system. Such an interaction can occur with magnetic fields whose magnitudes are oscillating in time. From time-dependent perturbation theory it can be shown that an oscillating magnetic field will cause transitions between different energy states of a magnetic dipole, provided the oscillatory magnetic field is perpendicular to the main static field and the frequency of oscillation is equal to γH_0 , the energy separation between adjacent states divided by \hbar . This frequency is the natural resonant frequency of the spin system and is referred to as the Larmor frequency. Time-dependent perturbation theory also dictates that $\Delta m_I = \pm 1$; in other words, transitions can be induced only between adjacent states which differ in the quantum number m_I by 1. The probability of a transition is the same whether the quantum number changes from m_I to $m_I + 1$ or $m_I + 1$ to m_I . Thus, in a sample with an equal number of nuclei in each state, no net absorption of energy would occur. Fortunately, in real samples at finite temperatures the lower energy states are

populated in excess, and a net absorption of energy does occur. This is discussed in greater detail in Section III.B.

In reality, the energy levels of the spins in the presence of an external field are never infinitely sharp. Consequently, a spread of frequencies centered about γH_0 can induce transitions. The transition probability is highest when the frequency of oscillation ω is equal to γH_0 ; it decreases as the difference between ω and γH_0 increases. As a result one observes an NMR peak centered about γH_0 with a finite linewidth which comes from the widths of the energy levels (Fig. 1).

In contemporary NMR spectrometers H_0 is typically 20–120 kG (kilogauss) (10 kG = 1 T). The Larmor or resonance frequencies of several nuclei at 70.46 kG are given in Table I. Note that γH_0 has units of radians per second; however, traditionally, frequencies are expressed as $\gamma H_0/2\pi$ using units of megahertz (MHz).

B. Boltzmann Distribution and Bulk Magnetization

In an NMR measurement, as in many other forms of spectroscopy, one does not deal with a single nucleus; instead, the sample contains an extremely large number of spins. For example, a typical sample for ^1H NMR determinations is ~ 0.5 ml, and its solute concentration is ≈ 1 mM; thus, it contains $\approx 3 \times 10^{17}$ spins.

In the absence of external perturbations which induce transitions, these spins distribute themselves between their different energy states. As in any system at thermal equilibrium, the probability of finding a spin with a certain energy E is proportional to $e^{-E/kT}$, where k is Boltzmann's constant and T the absolute temperature. Therefore, at a given instant, a larger fraction of the spins in the sample will occupy the lower energy state. It is this difference in the spin population which gives rise to an NMR signal.

Let us consider the spin- $\frac{1}{2}$ case. There are two energy states characterized by $m_I = \pm \frac{1}{2}$. As previously discussed, these two energy states correspond to two different orientations of the magnetic dipole with $\mu_z = \gamma \hbar m_I$. Since more spins will occupy the lower energy state, the sum of magnetic moments along the z direction will not cancel out. There will thus be a net bulk magnetic moment along the z axis whose magnitude will be $(\frac{1}{2}\gamma \hbar \Delta n)$, where Δn is the population difference between the two states. Calculations for the general case of spin I nuclei yield

$$M_z^0 = [\gamma^2 \hbar^2 I(I+1) H_0 N] / 3kT,$$

where M_z^0 is the bulk magnetization along the z axis, H_0 the magnetic field strength, N the total number of spins in the sample; and the term $I(I+1)$ arises from the quantum mechanical nature of the spin states. This equa-