METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 25

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Edited by **DAVID GLICK**

Life Sciences Division
Stanford Research Institute
Menlo Park, California

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PUBLISHER'S NOTE

From its inception in 1940, the Interscience publishing program emphasized the issuance of "Advances" series and the publication of books and series devoted to techniques and methods. It was natural, therefore, to invite David Glick, after publication of his *Techniques of Histo- and Cytochemistry* in 1949, to start *Methods of Biochemical Analysis*. The first volume appeared in 1954. This series has represented a fortunate combination of two of our editorial aims.

The 25-year history of the series has shown that we could not have made a better choice for its editor. Dr. Glick's own field of scientific endeavor, histo- and cytochemistry, was a focal point of biological, chemical, and physical methodology. In addition, he developed a special interest in all phases of methods and techniques applicable to the study of biochemical and biological problems and their instrumentation. In addition, he was able to publish many "firsts" in the methodology that introduced their application throughout the international scientific community.

His travels in Europe, with bases at Carlsberg Laboratory (Copenhagen), Karolinska Institute (Stockholm), and Stazione Zoologica (Naples) were especially devoted to the in-depth study of new developments of methodology. The connections he established there are reflected in the broad international flavor of the Advisory Board and the contributors to the series.

With the publication of the twenty-fifth volume of *Methods of Biochemical Analysis*, we welcome the opportunity to express our gratitude to Dr. David Glick for the splendid editorial guidance he has provided and look forward to many more volumes under his leadership. The long-standing acceptance of the series by life scientists is itself a tribute to his unerring judgment in the selection of topics at the frontiers of biochemical research. Thus Volume 1, published in 1954, had a chapter on luminescence assay and in Volume 24 field desorption mass spectrometry is reviewed. The current volume maintains the high standard for which the series is well known.

Special comment must be made on the international character of *Methods of Biochemical Analysis* as evidenced by the membership of the Advisory Board and the worldwide representation of the contributing authors.

If I may add a personal note, I am particularly honored to have been associated with Dr. Glick over these many years. It has been a relationship of continuing professional and personal gratification.

ERIC S. PROSKAUER

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CONTENTS

The Application of High Resolution Nuclear Magnetic Resonance to Biological Systems

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I. INTRODUCTION

Since the last review of nuclear magnetic resonance (NMR) spectroscopy in this series (1), there have been very considerable advances in instrumentation and in methodology. The magnetic field strengths available have increased by a factor of 4, and the sensitivity of instruments has increased by a

factor of 40 or more. This has led to a major change in the type of problem that can be tackled by high resolution NMR. For example, detailed studies of macromolecules have become possible, and recently the study of whole tissues (e.g., muscle or bacteria) has begun.

There are a large number of reviews (2–5) and books (6–8) that include the subject of this chapter. Our aim, however, is to cover *methods* suitable for the study of biological systems by high resolution NMR. The application of NMR to specific problems is not reviewed exhaustively. Rather, the examples chosen serve to illustrate the approach to a given problem.

To exploit fully the applications of NMR in biology, it is necessary to make use of sophisticated techniques, as discussed in this chapter both from theoretical and empirical points of view. However we hope that the reader who does not wish to go into detailed theory can omit these sections and still appreciate the biological consequences of NMR.

The theory has been covered in a condensed and somewhat unusual manner. The interested reader should see not only the origin of the rather complex equations that describe some aspects of the NMR phenomenon but also should be able to refer to the theory when reading subsequent sections.

Some of the instrumentation in current use is discussed, particular emphasis being placed on pulse methods. It is the improvement in instrumentation that has, above all, opened the door to the study of a wide range of biochemical problems. However there is also a wide range of different experimental techniques available that can be used in the study of a given system. This chapter discusses methods for increasing resolution and for assignment of the NMR spectra of a variety of systems including proteins, nucleic acids, and whole tissue. It is only when resolved signals in a spectrum can be assigned to specific nuclei in the system that the full specificity of NMR can be exploited.

Finally, some of the applications of NMR in biology are briefly reviewed. These are dealt with under the headings: analysis, thermodynamics, kinetics, structure, and motion. It is our intention to show the scope of NMR in biology by illustrating experimental approaches of different types.

II. BASIC CONCEPTS

This section briefly defines many of the concepts encountered in nuclear magnetic resonance. The material can be read at several levels; a first reading of Sections II.1 to II.4 should give some idea about the fundamental aspects of the method. Sections II.5 to II.7 deal in more detail with aspects that are important in many applications of the technique. Some attempt has been made to indicate how the various equations arise. For the newcomer to the subject it will probably be necessary to use additional material such as refs. 6, 9, and 10. The importance of understanding this subject is that many of the observed phenomena can be treated with rather precise theory in a meaningful

way. (In many other forms of spectroscopy, application of sophisticated theory is a less fruitful pastime because of the numerous assumptions involved.)

1. The Nuclear Magnetic Resonance Phenomenon

If a nucleus has a nonzero spin angular momentum $I\hbar$ (integer or half-integer in units of $\hbar$) and a magnetic moment $\boldsymbol{\mu} = \gamma\hbar\mathbf{I}$, then an assembly of such nuclei, where γ is the magnetogyric ratio, can exhibit the phenomenon of nuclear magnetic resonance.

The vectors $\boldsymbol{\mu}$ interact with an applied magnetic field B_0 and take up discrete energy values that are labeled by the quantum number m; m can have $2I + 1$ values (e.g., for $I = \frac{1}{2}$, $m = \pm\frac{1}{2}$). Transitions between the m states can be induced by an applied oscillating electromagnetic field. Large numbers of the vectors $\boldsymbol{\mu}$ can be made to change orientation in the field when the resonance condition

$$\omega = 2\pi\nu = \gamma B_0 \qquad [1]$$

is fulfilled. In this equation ω is the angular frequency of the oscillating magnetic field. Such changes in nuclear orientation, when detected, give a nuclear magnetic resonance spectrum.

Many important nuclei possess a spin (see Table I), and each is characterized by a value of γ and I. In some cases, when $I > \frac{1}{2}$, there may also be a quadrupole moment associated with the nucleus (see Section II.5). The

TABLE I

Properties of Some Magnetic Nuclei

Nucleus	Spin (in units of $\hbar$)	Q	Frequency at 10 T (MHz)[a]	Approximate shift range (ppm)[b]	Natural abundance (%)	Relative sensitivity for equal number of nuclei
^{1}H	$\frac{1}{2}$	—	425.8	15	100	1
^{2}H	1	2.8×10^{-3}	65.4	—	1.5×10^{-2}	9.7×10^{-3}
^{13}C	$\frac{1}{2}$	—	107.1	200	1.1	1.6×10^{-2}
^{14}N	1	7×10^{-2}	30.8	—	99.4	1.0×10^{-3}
^{15}N	$-\frac{1}{2}$	—	43.2	400	4	1.0×10^{-3}
^{17}O	$-\frac{5}{2}$	4×10^{-3}	57.6	—	3.7×10^{-2}	2.9×10^{-2}
^{19}F	$\frac{1}{2}$	—	400.1	—	100	0.83
^{23}Na	$\frac{3}{2}$	0.1	112.6	—	100	9.3×10^{-2}
^{31}P	$\frac{1}{2}$	—	172.4	30	100	6.6×10^{-2}
^{35}Cl	$\frac{3}{2}$	8×10^{-2}	41.7	—	75.5	4.7×10^{-3}

[a] The value of γ can be obtained from the formula, $2\pi \times \text{frequency} = \gamma B_0$.

[b] Only quoted for spin $\frac{1}{2}$ nuclei that occur naturally in biological systems. Note that for ^{31}P, biological molecules occur as phosphates; thus the range is only 30 ppm instead of the more usually quoted 600 ppm.

nuclear magnetic resonance, arising from the induced changes in nuclear orientation, is characterized by five parameters: (1) a chemical shift δ, which is a position on an energy scale, measured in hertz (or in parts per million of the applied field) from some suitable standard resonance; (2) a multiplet structure characterized by a spin-spin coupling constant J; (3) a relaxation rate $1/T_2$ related to the linewidth of the resonance; (4) a relaxation rate $1/T_1$, which characterizes the rate at which the resultant magnetization of the spin system returns to equilibrium along the field direction, after a perturbation from equilibrium; and (5) the intensity or the area of the resonance. These parameters are discussed below and are illustrated in Figure 1.

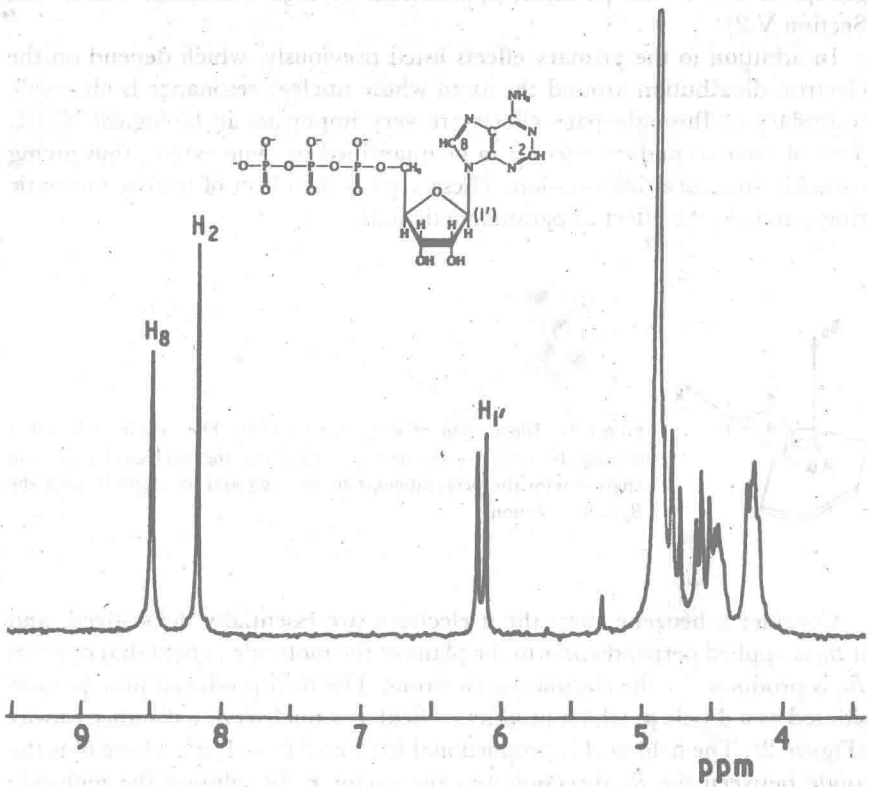

Figure 1. The 90-MHz ^{1}H NMR spectrum of 30m*M* adenosine triphosphate in 99.8% D_2O. Each resonance has a characteristic chemical shift value on the horizontal scale and has unit intensity for each proton. The resonances of H_8 and H_2 are singlets but the resonance of H_1' is a doublet because of coupling to the H_2' proton. The linewidths of different resonances (e.g., of H_2 and H_8) are different because of different relaxation times. The resolution and assignment of the remaining protons (H_2' to H_5') is difficult, and this general problem is discussed in Section IV. Note the intense resonance at 4.8 ppm that arises from the residual H_2O in the sample. The removal of solvent resonances is discussed in Section III.7.

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2. The Chemical Shift

There are electrons between the nucleus and the applied magnetic field B_0. Currents are set up in the electron cloud which produce a field opposing B_0. Such induced fields are directly proportional to B_0. The dependence of the resonance position on electronic environment depends on the various bonds an atom makes, as well as on the electron distribution. Table I gives the shift range observed for some nuclei. In certain cases a resonance can be assigned to a particular type of atom from its chemical shift position (see Section IV.3). The shifts are also sensitive to changes in the bond structure (e.g., those occurring on protonation). Following the ionization states of particular groups is one of the powerful applications of high resolution NMR (see Section V.2).

In addition to the primary effects listed previously, which depend on the electron distribution around the atom whose nuclear resonance is observed, secondary or through-space effects are very important in biological NMR. Two of these secondary effects can be quantified to some extent, thus giving valuable structural information. These are (*a*) the effect of nearby aromatic rings, and (*b*) the effect of paramagnetic ions.

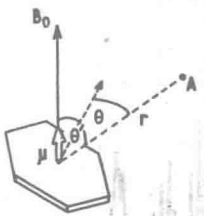

Figure 2. Illustration of ring current shift. The vector of length r joining the center of the aromatic ring and the nucleus A makes an angle θ with the perpendicular to the ring and an angle θ' with the B_0 field direction.

Consider a benzene ring: the π electrons are essentially delocalized, and if B_0 is applied perpendicular to the plane of the molecule, a field that opposes B_0 is produced by the circulating electrons. The field produced may be considered as a dipole $\boldsymbol{\mu}$, which produces a field at a nucleus A, a distance r away (Figure 2). The field at A is proportional to $(3\cos^2\theta' - 1)/r^3$, where θ' is the angle between the B_0 direction and the vector $\mathbf{r}$. In solution the molecule tumbles, and the field at A is averaged over all θ'; but since the induced dipole is very anisotropic (e.g., for a benzene ring $\mu_{//} = 0$), there is a net field experienced at A. This is of the form $(\mu_\perp - \mu_{//})\,(3\cos^2\theta - 1)/3r^3$, where θ is the angle between $\mathbf{r}$ and the perpendicular of the ring. Extensive tables and contour maps have now been constructed for the ring current shifts produced by amino acids and nucleotides (11–13).

The induced shift of a paramagnetic center has the same form as the ring current shifts, although the exact geometric dependence of the shift of the bound metal is less obvious and exchange of the paramagnetic center between different sites can cause complications. Paramagnetic ions especially the lanthanide ions, are a powerful means of determining structure, and these are discussed in Section V.4.

There are various other possible secondary shift effects, which are often very hard to quantify. These include electrostatic effects, the anisotropic shielding of carbonyl groups, and direct overlap of electrons from a paramagnetic center (this effect is often called a contact shift, see Section V.4). Chemical exchange effects are treated separately in Section II.6.

3. Spin-Spin Coupling

The resonances in a high resolution spectrum often exhibit multiplet structure that arises from a weak interaction (of magnitude J Hz) between magnetic nuclei. The interaction is conveyed between the nuclei by the electrons in a chemical bond. The nature of the resulting multiplet depends on the number of bonds between the coupled nuclei, the nature of the bond and on several other factors:

1. The *number* and *spin* of the bonded nuclei causing the fine structure of the resonance. For example, the spectrum of a nucleus coupled to one nucleus with $I = \frac{1}{2}$ is a doublet, whereas the spectrum when coupled to two equivalent nuclei with $I = \frac{1}{2}$ is a triplet with intensities in the ratio 1:2:1.

2. The *chemical shift* δ between the resonance of the observed nucleus and the resonances of the nuclei causing the splitting. When $J \ll \delta$, the spectra are called first order and the two components of a doublet have approximately equal intensity. When J is comparable to δ, the outer components of a pair of doublets are weaker than the inner ones and the spectra are, in general, complex. Computer programs are now available for simulating such spectra, and this aspect is not usually a problem in biological NMR, especially with the use of very high fields (δ increases with B_0, but J is constant).

3. *Bond angles.* The value of J for the ^1H–^1H coupling in a ^1H–X–Y–^1H fragment can be related to the dihedral angle using empirical curves. For example, the observed doublet splittings of the peptide NH resonances of rigid cyclic polypeptides have been related to bond angles (14,15). This method can also be extended to include ^{13}C and ^{15}N couplings.

4. The *populations* of conformers in solution. For example, when three rapidly interconverting rotamers exist in equilibrium, the observed J value is a weighted mean of each of the three possible J values. This is a very important consideration in structure determinations using coupling constants (see Section V.4).

5. The *lifetime* of the nucleus-nucleus configuration within the bond. This period can be short if one of the nuclei has a fast relaxation rate (see Section II.4.B) or if it undergoes rapid chemical exchange. For example, in peptides, the ^{14}N spin-spin coupling is removed because of the fast relaxation rate of the ^{14}N nucleus. Intermediate effects can also be observed when $1/T_1$ the relaxation rate is of the order of $\mathcal{J}$.

6. *Double resonance.* In a manner somewhat analogous to the lifetime effects just described, collapse of multiplicity can be brought about by "stirring" one nuclear spin state with an applied radiofrequency field. This is known as decoupling a resonance (see Section III.6).

The reader is referred to refs. 9 and 10 for further discussion of the appearance of the high resolution spectra of various spin systems.

4. Magnetization and Relaxation

A. MAGNETIZATION

In an applied magnetic field, an assembly of nuclear magnetic moment vectors $\boldsymbol{\mu}$ distribute themselves among the nuclear energy levels according to the Boltzmann distribution law. Because of the small energy separation between the levels, the populations of the states are nearly equal and a linear expansion of the Boltzmann exponential is nearly always valid, as is the application of classical mechanics. In a two-level system, the equilibrium magnetization along the applied magnetic field direction (z) is $M_z^0 = \gamma\hbar(N_2^0 - N_1^0) = \gamma\hbar n_0$, where N_2^0 and N_1^0 are the populations of the levels. We shall see later that M_z^0 is proportional to the maximum observable signal in the NMR experiment, and this means that resonance intensity can be related directly to the number of spins in the sample.

B. SPIN-LATTICE OR LONGITUDINAL RELAXATION

In NMR all the transitions between energy levels are induced by fluctuating fields at appropriate frequencies because other transitions, such as spontaneous emission, have negligible probability. How then is a Boltzmann distribution set up? The answer is that the local field fluctuations are caused by thermal motions of the "lattice" (nuclei or electrons around the nucleus of interest), and since the lattice energy has a Boltzmann distribution, the probabilities of inducing upward and downward NMR transitions are not equal but differ by a factor determined by the Boltzmann law. This can be simply expressed, for a system of two energy levels, as is observed for spin $\frac{1}{2}$ nuclei; if W_{12} and W_{21} are the transition probabilities defined in Figure 3.

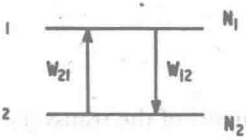

Figure 3. Two energy levels (1 and 2) are shown with populations N_1 and N_2. The probability for the transition $2 \rightarrow 1$ is W_{21} and for $1 \rightarrow 2$ is W_{12}.

The rate equation is

$$\frac{dN_1}{dt} = N_2 W_{21} - N_1 W_{12} \qquad [2]$$

The signal observed is proportional to $M_z = \gamma\hbar n$, where $n = N_2 - N_1$, $n_0 = N_2^0 - N_1^0$. At equilibrium $N_2^0/N_1^0 = W_{12}/W_{21}$, and using the relationship $N_1 + N_2 = N_1^0 + N_2^0$, the rate equation can be rewritten in terms of n and n_0

$$\frac{dn}{dt} = -(n - n_0)(W_{12} + W_{21}) \qquad [3]$$

This is a simple first-order rate process with rate constant $W_{12} + W_{21} = 1/T_1$, where T_1 is defined to be the relaxation time of the magnetization along the field direction. It is also known as the spin-lattice or longitudinal relaxation time (because it arises from interactions with the surrounding "lattice" and it takes place along the field direction). The solution of [3] is $n = n_0[1 + A\exp(-t/T_1)]$, where A is an integration constant. For example, if at $t = 0$, $n = 0$, $A = -1$, and n recovers from zero to the equilibrium value n_0 at a rate of $1/T_1$. Typical values of T_1 are of the order of 1 sec.

Transitions between energy levels can also be induced by an oscillating radiofrequency field, but now the upward and downward transition probabilities are equal. As a result of this, n tends to zero under the influence of an applied field, a phenomenon known as *saturation* (see Section III.2).

C. THE BLOCH EQUATIONS AND TRANSVERSE MAGNETIZATION

Thus far the discussion has considered only the populations of the energy levels. Since the spin has angular momentum, however, each nuclear moment $\boldsymbol{\mu}$ behaves more like a gyroscope than a bar magnet. In an applied magnetic field, the moments experience a twisting force that makes them precess at a constant angle to the field direction. This means that there may be components of the magnetization at right angles to the field direction. This transverse magnetization is very important, since nearly all the transitions between levels are induced and detected by oscillating fields in this plane. The net magnetization from all the individual nuclei, the bulk magnetization, obeys classical mechanics, and the rate equations for the components of this magnetization in the

x,y-plane are of the form

$$\frac{dM_{x,y}}{dt} = -\frac{M_{x,y}}{T_2}$$

where T_2 defines the decay rate of the x- and y-components of the transverse magnetization.

It is, however, convenient to include in the equation of the transverse magnetization the fact that there is precession at the frequency $\omega_0 = \gamma B_0$ (from [1]) and the effects of an applied rotating field of magnitude B_1 and frequency ω_1. The simplest way of treating this is to view the motion of the bulk magnetization in a new coordinate system rotating at frequency ω_1. The magnetization then behaves as if it were in an effective field $B_0 - \omega_1/\gamma$. When $\omega_1 = \omega_0$, the effective field vanishes. The equations of motion for the components of the magnetization in the transverse plane are now

$$\begin{aligned} \frac{du}{dt} &= (\omega_0 - \omega_1)v - \frac{u}{T_2} \\ \frac{dv}{dt} &= -(\omega_0 - \omega_1)u - \frac{v}{T_2} + \gamma B_1 M_z \end{aligned} \qquad [4]$$

where u and v are the components, in the x'- and y'-directions, of the new coordinate system, and B_1 is applied along the rotating x'-axis. These equations simply mean that the effective precession frequency is $\omega_0 - \omega_1$ and that a rotating field B_1 along the x'-direction induces components of M_z into the y'-direction of the rotating frame (see also Section III.2).

Equation [4] together with a version of [3]

$$\frac{dM_z}{dt} = -\frac{M_z - M_0}{T_1} - \gamma B_1 v \qquad [5]$$

are collectively known as the Bloch equations, and these equations, with some simple modifications, can be used in nearly all cases to interpret the transient behavior of the nuclear magnetization.

Note that the transverse relaxation time T_2 depends on different processes from T_1 and the two of them are often not equal.

D. PHASE

The directions of the components in the x,y-plane are experimentally observable in NMR. A way of defining the direction of these components is to use a phase angle ϕ (Figure 4*a*). For example, the u and v components are 90° out of phase with respect to each other; components along y' and $-y'$ are 180° out of phase, and so on.

Viewed another way, a solution of [4] is $u = \sin[(\omega_0 - \omega_1)t] \exp(-t/T_2)$, which is an oscillation at frequency $\omega_0 - \omega_1$, which decays with a time

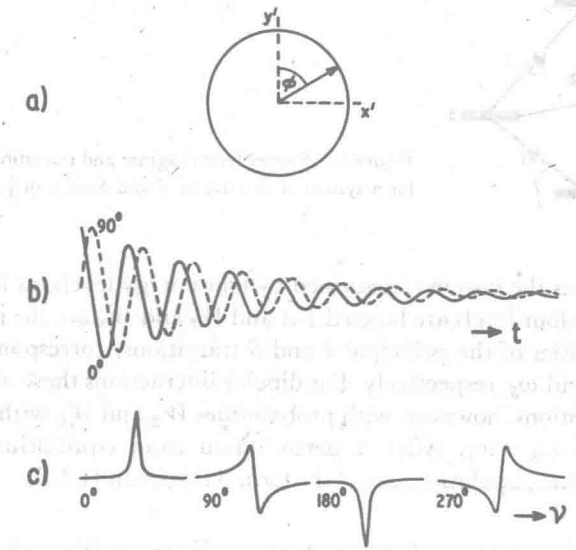

Figure 4. Three representations of the idea of phase in NMR. (*a*) The rotating x', y'-plane, with a magnetization vector that makes a phase angle ϕ with the y'-axis at $t = 0$. (*b*) The signal that would be detected in the x,y-plane as the magnetization vector rotates around the z-axis, for two different values of ϕ. (*c*) The Fourier transformation of signals like those in (*b*) for four different values of ϕ (see also Figures 11, 12, 17 and Section III.5).

constant T_2 (Figure 4). The solution for v, in the absence of B_1, is $v = \cos[(\omega_0 - \omega_1)t] \exp(-t/T_2)$, which is a signal 90° out of phase with respect to the u component.

This concept of *phase* is referred to extensively in Sections III and IV.

5. Dipolar Relaxation

The most important mechanism causing relaxation in solutions of macromolecules is the thermal motion of the molecule, which modulates the local fields produced by the dipole-dipole interactions. The field exerted by one dipole on another has a component proportional to $(3\cos^2\theta - 1)/r^3$, and since molecular motion causes θ to vary, this component fluctuates. Some of the resulting field fluctuations are effective in inducing transitions between the energy levels of the system.

A. THE RATE EQUATIONS AND T_1

Consider two spin $\frac{1}{2}$ nuclei I and S, coupled by dipolar interactions. In general the energy states of I will be influenced by the interaction of S, and

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