Contents

List of Contributors xi
Preface xiii

1. Introduction

Lawrence J. Berliner

I. Electron Spin Resonance in Biology 1
II. Spin Labeling—A Reporter Group Technique 2
III. Prelude 3
    References 4

2. General Magnetic Resonance Theory

Pier Luigi Nordio

I. Introduction 5
II. Principles of Electron Spin Resonance 6
III. The Magnetic Interactions 10
IV. Analysis of the ESR Spectra 23
V. Line Shape Theory 36
    References 52

3. Theory of Slow Tumbling ESR Spectra for Nitroxides

Jack H. Freed

I. Introduction 53
II. Theory 55
III. Applications 71

Appendix A. General Solutions and Discussion of the Computer Program for Nitroxides 112
Appendix B. Computer Program for Slow Tumbling Nitroxides in Isotropic Liquids 121
    References 130
CONTENTS
9. Spin-Label-Induced Nuclear Magnetic Resonance Relaxation Studies of Enzymes
   Thomas R. Krugh
   I. Introduction 339
   II. Nuclear Spin Relaxation 341
   III. Measurement of Relaxation Times 357
   IV. Enzyme Studies 363
   V. Conclusions 369
   References 370

10. Anisotropic Motion in Liquid Crystalline Structures
    Joachim Seelig
    I. Classification of Liquid Crystals 373
    II. The Relevance of Spin Probes to the Investigation of Liquid Crystals 377
    III. Anisotropic Motion of Spin Probes in Liquid Crystals 378
    IV. Nitroxide Spin Probe Investigations of Liquid Crystalline Mesophases 398
    References 407

11. Oriented Lipid Systems as Model Membranes
    Ian C. P. Smith and Keith W. Butler
    I. Types of System 411
    II. Nature of the Spectra 415
    III. Applications 425
    IV. Conclusion 448
    References 448

12. Lipid Spin Labels in Biological Membranes
    O. Hayes Griffith and Patricia C. Jost
    I. Introduction 454
    II. Molecular Motion in Membranes 456
    III. Characterizing Macroscopic Order 484
    IV. Polarity Profile 495
    V. Measuring the Fraction of Membrane Lipids in the Fluid Bilayer 505
    VI. Lipid–Protein Interactions in Membranes 510
    References 519

13. Molecular Motion in Biological Membranes
    Harden M. McConnell
    I. Introduction 525
    II. “Fluid” Membranes 526
III. Lateral Phase Separations 328
IV. Lateral Diffusion 335
V. Membrane Function 338
VI. The Flexibility Gradient 341
VII. Membrane Fusion 351
Appendix A. Rotational Correlation Times 354
Appendix B. Direct Experimental Evidence for a Phospholipid Spin Label Tilt 357
References 358

Appendix I. Simulated X-Band Spectra—Isotropic Tumbling 362
Appendix II. Principal Values of the g and Hyperfine Tensors for Several Nitroxides Reported to Date 364
Appendix III. Commercial Sources of Spin Labeling Equipment and Supplies 366
Appendix IV. Practical Considerations for the Calculation of Order Parameters for Fatty Acid or Phospholipid Spin Labels in Membranes 367
Betty Jean Gaffney
Appendix V. Symbols and Abbreviations 372
Index 383

List of Contributors

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

Lawrence J. Berliner (1), Department of Chemistry, The Ohio State University, Columbus, Ohio
Keith W. Butler (411), Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada
Jack H. Freed (53), Department of Chemistry, Cornell University, Ithaca, New York
Betty Jean Gaffney (183, 567), Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland
O. Hayes Griffith (251, 453), Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon
Patricia Jost (251, 453), Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon
Thomas R. Krugh (339), Department of Chemistry, University of Rochester, Rochester, New York
J. Lajzerowicz-Bohneteau (239), Laboratory of Physical Spectroscopy, University of Science and Medicine of Grenoble, Grenoble, France
Geoffrey R. Luckhurst (133), Department of Chemistry, The University, Southampton, England
Haroon M. McConnell (525), Department of Chemistry, Stanford University, Stanford, California
Joel D. Morrisett (273), Department of Medicine, Baylor College of Medicine, Houston, Texas
Pier Luigi Nordio (5), Institute of Chemical Physics, University of Padua, Padua, Italy
Joachim Seelig (373), Department of Biophysical Chemistry, Biocenter of the University of Basel, Basel, Switzerland
Ian C. P. Smith (411), Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada
Preface

The spin label technique has received recognition to date in several specialized review journals.† In each case, either a broad review of the state of the art or a detailed account of a specific application was presented. However, nowhere has it been possible to treat comprehensively theory, techniques, and applications of the whole field of biomedical research, due either to the physical limitations of space or to the limits defined by the individual author.

We have attempted here to compile in one text most of the necessary background, theory, and applications of spin labeling. Our main intent has been to aim this book toward the level of the graduate student, that is, a level of understanding which can include readers from the medically oriented through the biochemically to the physically oriented backgrounds. Our aim is to elucidate for the reader the essential principles of spin labeling and to communicate not only its promises but also the limitations and pitfalls which occasionally arise. Topics are generally covered not necessarily as reviews of the current literature, but from a pedagogical point of view.

Much appreciation is due to the authors, all recognized experts in their fields, for their total and generous cooperation. I am also indebted to Ms. Barbara Cassity for her excellent secretarial and editorial assistance, and to the editorial staff of Academic Press for their continued help and cooperation.

Introduction

LAWRENCE J. BERLINER
DEPARTMENT OF CHEMISTRY
THE OHIO STATE UNIVERSITY
COLUMBUS, OHIO

I. Electron Spin Resonance in Biology  
II. Spin Labeling—A Reporter Group Technique  
III. Prelude  
References

I. ELECTRON SPIN RESONANCE IN BIOLOGY

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy has constantly found applications to biochemical and biomedical problems as a highly sensitive tool for the detection of free radical species. This applies to the detection (and identification) of free radical intermediates in metabolic reactions, to the observation of stable, naturally occurring paramagnetic species (e.g., transition metal ions), to the detection of free radicals produced by external radiation, and to the analysis of paramagnetic probes introduced into specific biological systems (spin labeling). A recent text on the general subject of ESR in biology is Swartz et al. (1972).

While there is great potential for the direct observation of paramagnetic free radical species associated with specific biochemical phenomena, the natural occurrence of paramagnetism in biological systems is relatively low. A great advantage is to be gained by the introduction of some versatile probe containing a free radical center.
II. SPIN LABELING—A REPORTER GROUP TECHNIQUE

The concept of labeling a biological system with some external probe molecule was termed the "reporter group" technique by Burr and Koshland (1964). The requirements of such a group are:

1. It must be some environmentally sensitive moiety that can be introduced into specific centers of the system of interest and it must subsequently "report" changes in its environment to an appropriate detector.
2. The physical properties of the reporter group that are detected must be either unique or distinct from the properties of the system under investigation. A pictorial representation of a reporter group in an enzyme is given in Fig. 1.

![Diagram of enzyme-substrate complex](image)

Fig. 1. A schematic representation of an enzyme-substrate complex in the native protein, the protein containing a reporter group (solid black area) adjacent to the substrate binding area, and the protein containing a reporter group distant from the substrate binding area. (Burr and Koshland, 1964.)

3. Last, it is obvious that an additional requirement of any reporter group must be that the biological system suffer little or no perturbation(s) in its structure and function as a result of the incorporation of the probe. ("One must ensure that the reporter group is reporting the news, not making the news.")

By virtue of the relatively low content of stable paramagnetic species in biological materials, an ESR-sensitive reporter group will usually be physically distinct from the rest of the system. An ideal spin label is a stable (organic) free radical of a structure and/or reactivity that facilitates its introduction to a specific target site in a biological system or macromolecule.

Although spin labeling generally refers to the use of nitroxide radicals, other examples, such as nitric oxide, Mn^{2+}, other paramagnetic transition metal ions, lanthanide ions, and a few other organic radicals, have been used as probes of specific environments. For example, the chlorpromazine radical cation was demonstrated to be an accurate reporter of DNA intercalation geometry (Ohnishi and McConnell, 1965). However, due to the great versatility, sensitivity, and wealth of information derived from nitroxide free radicals, this class of molecules has found the greatest use in spin label applications. A properly designed spin label may be sensitive to several aspects of its physieochemical environment: molecular motion, orientation, and local electric and magnetic fields. These features are developed and discussed throughout the text. The chapters to follow will illustrate the extreme power, potential, and future promise of this valuable technique.

III. PRELUDE

The text is arranged so that a cover to cover reading should smoothly develop the necessary theory and background preceding the various applications to follow. The next three chapters are primarily theoretical. They begin with the theory of magnetic resonance and then lead into detailed theoretical discussions of molecular motion and spectral simulations. Then the theoretical aspects of spin-spin interactions are covered in a separate chapter on nitroxide biradicals. These opening chapters are followed by discussions of the chemistry and molecular structure of nitroxide spin labels. Several syntheses are included, many of which either have never been published or have been modified since their original publication. Following these two chapters, a special chapter is included on instrumental methods, covering the operation of ESR spectrometers, the applications of computers, and other aspects of spin label methodology.
The text then divides its coverage into the two principal areas of biomedical research where spin labeling finds most application: proteins (and enzymes) and lipids (and membranes). Chapter 8 comprehensively covers techniques and applications to enzyme problems and Chapter 9 develops the theoretical and experimental background for the powerful complementary approach: nuclear magnetic resonance relaxation methods.

The last four chapters are devoted to an integrated coverage of spin labeling as applied to lipid and membrane systems: first, theory and applications to liquid crystals as model systems; second, applications to artificial lipid bilayers; and last, discussions of molecular motion, polarity, and fluidity in membranes, and of applications to phenomena at the level of membrane suprastructure.

Since the spin label literature contains a number of structural names for various nitroxide spin labels, we have attempted to interrelate the different structural nomenclatures. Each author has tended to use the nomenclature familiar to his own laboratory, while at the same time including several alternate structural descriptions that the reader may expect to find both in the literature and elsewhere within this text. Appendix V (p. 572) contains a list of symbols and abbreviations used in the text which the reader is strongly urged to make use of.

REFERENCES


I. INTRODUCTION

Electron spin resonance spectroscopy has developed at an outstanding pace since the late fifties, and now it can be safely stated that the technique is very well understood in its many aspects.
2. GENERAL MAGNETIC RESONANCE THEORY

momentum $L$. According to quantum theory, the magnetic moment of an atom in an electronic state specified by the quantum numbers $L$, $S$, and $J$ is

$$\mu = -g\beta_s J$$  \hspace{1cm} (1)

where $g$ is the spectroscopic splitting factor

$$g = 1 + \frac{J(J + 1) + S(S + 1) - L(L + 1)}{2J(J + 1)}$$  \hspace{1cm} (2)

and $\beta_s$ is the Bohr magneton, $|e|\hbar/2mc$. The interaction energy (Zeeman energy) of the magnetic moment with an external field $H$ of uniform intensity $H_0$ is calculated by the quantum mechanical operator

$$\mathcal{H} = -\mu \cdot H = g\beta_s H \cdot J = g\beta_s H_0 J_z$$  \hspace{1cm} (3)

where $J_z$ is the operator corresponding to the projection of the angular momentum along the field direction. The expectation values of $J_z$, which shall be denoted by $M_J$, range between $-J$ and $+J$ by integer steps, and therefore the effect of the magnetic field is to produce $2J + 1$ levels, each of which has an energy $E_M = g\beta_s H_0 M_J$, and a population given by the Boltzmann distribution law

$$P_{M_J} = \exp(-W_{M_J}/kT)$$

$$\sum_{M_J} \exp(-W_{M_J}/kT)$$  \hspace{1cm} (4)

Transitions can occur between the levels if the sample is irradiated with an electromagnetic field of proper frequency to match the energy difference. In the normal ESR experiments, this field is polarized in a plane perpendicular to the static field direction. The transitions are induced only between adjacent levels characterized by the quantum numbers $M_J$ and $M_J \pm 1$, hence the resonance condition leading to energy absorption by the sample from the field is met when

$$h\nu = \Delta W = g\beta_s H_0$$  \hspace{1cm} (5)

In the following, we shall treat only molecular systems in which the magnetic properties are principally due only to the spin angular momentum $S$. Actually, it can be shown that a magnetic moment caused by the orbital motion can result only in the cases of atoms, linear molecules, or systems with orbitally degenerate electronic states. Even in these cases, intermolecular interactions in the condensed phases or molecular distortions lifting the degeneracy may quench the orbital angular momentum. In any other nonlinear, nondegenerate molecule an effective quenching of the orbital angular momentum occurs, although small residual contributions still remain, due to a particular interaction between the spin and the orbital motion called spin-orbit coupling. As a result, the measured $g$ value deviates from the
value of two expected for free-spin systems, and the amount of this deviation can be a characterization of the molecule under investigation, as will be seen in the following section.

Electron spin resonance experiments are usually carried out at a fixed frequency, and the resonance condition is found by varying the intensity of the static magnetic field. Most ESR spectrometers operate with microwave fields at 9.5 GHz (X band) or 35 GHz (Q band), the resonant fields in these two cases being about 3,400 and 12,500 G, respectively. The magnetic energy is 0.3–1 cm⁻¹, some hundred times smaller than kT at ordinary temperatures, so that the difference in population of the magnetic levels, as given in Eq. (4), is very minute. However, this population difference is responsible for the detection of the ESR signal. In fact, when the sample is irradiated with the microwave field, it absorbs energy from the field and it is excited to higher energy levels. At the same time, the inverse transition occurs by stimulated emission, but since the rates of both processes are proportional to the population of the level from which the transition starts, a net energy absorption results. These circumstances, nevertheless, do not suffice to permit continuous detection of the signal. Since the prevailing absorption process would eventually equalize the population of the magnetic levels, causing the signal to disappear (saturation). Actually, there are relaxation mechanisms, which bring the system back to the Boltzmann equilibrium populations after it has been disturbed by the absorption of radiation. The equilibrium situation is restored by means of nonradiative transitions from the higher to the lower energy states and the consequent transfer to the environment of the magnetic energy, which is dissipated as thermal energy. The rate at which thermal equilibrium is restored is defined by a characteristic time called the spin–lattice or longitudinal relaxation time $T_1$, assuming the process to be represented by a single exponential decay:

$$\frac{d\mathcal{M}_z}{dt} = -\frac{\mathcal{M}_z - \mathcal{M}_{eq}}{T_1}$$

(6)

where $\mathcal{M}_z$ is the macroscopic magnetization of the sample and $\mathcal{M}_{eq}$ is given by the Curie law

$$\mathcal{M}_{eq} = N\xi g^2 \beta^2 S(S + 1)H_0/3kT$$

(7)

where $N$ is the number of spins, $\xi$ is the free-spin $g$ value, and $T$ is the absolute temperature.

The spin–lattice relaxation process shortens the lifetime of the magnetic levels, and therefore broadening of the spectral lines may occur because of the Heisenberg uncertainty principle. According to this principle, if a system maintains a particular state not longer than a time $\Delta t$, the uncertainty in the energy of the state cannot be less than $\Delta W = \hbar/\Delta t$. This means that the spectral linewidth, in frequency units, must be at least of the order of $1/T_1$.

However, under conditions of low microwave power, so as to avoid saturation effects, the linewidths are usually caused by other relaxation mechanisms, which produce modulation of the magnetic levels without causing transitions between them. These processes, which keep the total Zeeman energy constant in contrast with the spin–lattice relaxation mechanisms previously discussed, are characterized by a relaxation time $T_2$ called the transverse relaxation time.

Due to these processes, the perpendicular component of the magnetization, which in absence of relaxation would follow the oscillating microwave field with the same angular velocity $\omega = 2\pi v$, decays toward zero with the characteristic time $T_2$ according to the rate equation

$$\frac{d\mathcal{M}_x}{dt} = -\frac{\mathcal{M}_x}{T_2}$$

(8)

Equations (6) and (8) are known as the phenomenological Bloch equations for the macroscopic magnetization. As will be seen in some detail in Section V, the transverse relaxation processes produce an absorption curve that is described by a Lorentzian function. On an angular frequency scale, the normalized shape function for a resonance line centered at $\omega_0$ is in this case

$$f(\omega) = \frac{T_2}{\pi} \frac{1}{1 + T_2^2(\omega - \omega_0)^2}$$

(9)

Fig. 1. Plot of a Lorentzian function and of its first derivative.
2. GENERAL MAGNETIC RESONANCE THEORY

A. Zeeman Interaction

From the discussion of Section II, it follows that the Zeeman interaction energy of the magnetic moment associated to the electronic spin $S$ with an external field is expressed by the Hamiltonian

$$\mathcal{H}_Z = g_e \beta_e H \cdot S = g_e \beta_e H_0 S_z$$  \hspace{1cm} (11)

If the values of the components of the spin angular momentum $S$ along the magnetic field direction are denoted by $m_s$, then it follows that the energy of the permissible levels of the system (or, in quantum mechanical terms, the eigenstates of the operator $\mathcal{H}_Z$) are

$$W_{m_s} = g_e \beta_e H_0 m_s$$  \hspace{1cm} (12)

For a system with only one unpaired electron, $S = \frac{1}{2}$ and $m_s$ can assume only the values $\pm \frac{1}{2}$. The difference in energy between the two levels produced by the magnetic field is therefore (Fig. 2)

$$\Delta W = g_e \beta_e H_0$$  \hspace{1cm} (13)

[Diagram of Zeeman splitting of an $S = \frac{1}{2}$ state]

If the sample is irradiated with an electromagnetic radiation of frequency $\nu_0$, energy absorption will occur at a field value satisfying the resonance condition

$$H = \hbar \nu_0 / g_e \beta_e$$  \hspace{1cm} (14)

If the orbital angular momentum is completely quenched, $L = 0$, and so we expect from Eq. (2) the value of $g_e$ to be equal to 2, or more exactly to 2.00232 if quantum electrodynamic corrections are taken into account.

The reason for the quenching of the orbital angular momentum can be understood qualitatively from the following example.

Consider an electron in the atomic $p$ orbital. If the atom is isolated, three degenerate states, i.e., three states with the same energy, are possible. They
are represented by the three solutions \( p_1, p_0, \) and \( p_{-1} \) of the Schrödinger equation for the atomic system, and they have the complex form

\[
\left[ (x + iy)/\sqrt{2} \right] f(r), \quad z f(r), \quad \left[ (x - iy)/\sqrt{2} \right] f(r)
\]

where \( f(r) \) describes the radial part of the orbital and it is a spherically symmetric function. In the presence of an external field applied in the \( z \) direction, the three functions correspond to states in which the component of the angular momentum along this direction has a value of \( 1, 0, \) and \(-1\) (in \( \hbar/2\pi \) units), respectively. In other words, they are eigenfunctions of the projection operator

\[
L_z = -\hbar \left[ x (\partial/\partial y) - y (\partial/\partial x) \right]
\]

and \( 1, 0, \) and \(-1\) are the corresponding eigenvalues.

The system therefore possesses an orbital angular momentum, and hence an associated magnetic moment. This conclusion, however, is valid only for the isolated atom. If it is introduced into a molecule, the \( p \) electron is then subjected to the electrostatic interactions arising from other electrons and nuclei composing the molecule. If we fix a set of Cartesian axes \((x, y, z)\) to the atom under consideration, the interactions in the directions of these axes will be generally different, as a consequence of the fact that the local symmetry of the atom in the molecule is lowered with respect to the spherical symmetry of the isolated atom. As a result, the primitive degeneration of the three \( p \) states is lifted. The atomic functions that represent the new situation are the “symmetry-adapted” functions \( p_x, p_y, \) and \( p_z \):

\[
p_x = x f(r), \quad p_y = y f(r), \quad p_z = z f(r)
\]

These functions are real, and for all three the “expectation value” of the \( z \) component of the angular momentum, defined as

\[
\langle p_i | L_z | p_i \rangle = \int p_i L_z p_i \, dx \, dy \, dz, \quad i = x, y, z
\]

is zero, as found by direct integration on the angular part of the \( p \) functions. The same is true for the other angular momentum components, \( L_x \) and \( L_y \), being obtained from \( L_z \) of Eq. (15) by cyclic permutation of the variables (Slichter, 1963). Under these circumstances, it is said that the orbital angular momentum is “quenched.”

When the molecule is subjected to the action of a magnetic field, the combined effect of the field and of the spin–orbit coupling reinstates small contributions of orbital angular momentum into the ground state of the system. In this way the magnetic properties of the system are not due only to the spin angular momentum; nevertheless, it is convenient that we continue to regard the resulting magnetic moment as produced by the pure spin, defining consequently an effective \( g \) factor for the system. Deviations of the \( g \) factor from the free-spin value are small (\( \sim -1\%\)) in the case of aromatic free radicals, but they can be relatively large in molecules containing transition metal ions. Furthermore, the magnitude of such corrections depends on the orientation of the magnetic field with respect to the molecular axis system. This implies that the \( g \) factor of a molecule cannot be represented by a scalar, but must be represented by a second-rank tensor.

The detailed theoretical treatment leads to the following expression for the \( g \)-tensor components (Slichter, 1963):

\[
g_{ij} = g_e \delta_{ij} - 2\lambda \sum \frac{\langle \psi_0 | L_i | \psi_i \rangle \langle \psi_i | L_j | \psi_0 \rangle}{E_i - E_0}
\]

where \( \delta_{ij} \) is the Kronecker symbol, which is equal to one if \( i = j \) and zero otherwise; \( \lambda \) is the spin–orbit coupling; and \( \psi_0 \) and \( \psi_i \) are the wave functions that represent the ground and the excited states of the system, respectively, \( E_0 \) and \( E_i \) being the corresponding energies.

For those who are unfamiliar with tensor algebra, we note that a three-dimensional tensor of rank \( p \) is represented by \( 3^p \) components in the Cartesian space. Thus a scalar, which can be thought of as a zeroth-rank tensor, is defined by only one number, or component; a vector, or first-rank tensor, is defined by three components; and a second-rank tensor is defined by nine components.

The latter can be arranged for the sake of convenience to form a three-by-three matrix. Hence a general second-rank tensor \( T \) is represented by the matrix

\[
\begin{pmatrix}
T_{xx} & T_{xy} & T_{xz} \\
T_{yx} & T_{yy} & T_{yz} \\
T_{zx} & T_{zy} & T_{zz}
\end{pmatrix}
\]

If the tensor is symmetric (and we shall always deal with cases of this kind), \( T_{yx} = T_{xy}, \ T_{zx} = T_{xz}, \) and \( T_{yz} = T_{zy}, \) and then the number of independent components of the tensor is reduced to six.

The specification of the components of a tensor presupposes a definite choice of the coordinate axis system. If the axis system \((x, y, z)\) is rotated into the new set \((x', y', z')\), then the components of a vector (first-rank tensor) in the primed system are related to the components in the original system by the linear transformation

\[
T'_{i} = \sum_{j} R_{ij} T_{j}, \quad i, j = x, y, z
\]
The nine coefficients \( R_{ij} \) form a three-by-three matrix, known as the Euler matrix, which defines the relationship between the two coordinate sets. The rows of the matrix give the direction cosines of the new axes with respect to the original reference system, while the columns of the matrix are formed by the direction cosines of the unprimed axes in the rotated system. Analogous to Eq. (19), the transformation property of a second-rank tensor under rotation of the axis system is expressed as follows:

\[
T'_{ij} = \sum_{kl} R_{ik} R_{jl} T_{kl}
\]  

(20)

The result follows immediately from Eq. (19) if the nine components \( T_{kl} \) of the second-rank tensor are thought to be constructed from the products \( a_k b_l \) between the components of two vectors \( a \) and \( b \) (Brink and Satchler, 1968).

An important property, derived immediately from Eq. (20) by taking into account the orthogonality properties of the direction cosines, is the following: Any transformation of the type (20) will not affect the sum of the diagonal elements of a second-rank tensor. This sum is called the "trace" of the tensor, and it is indicated by the symbol \( \text{Tr} \):

\[
\text{Tr} \, T = \sum_i T_{ii}
\]

(21)

Furthermore, there exists a unique choice of the axis system in which a second-rank tensor takes on a particularly simple form, all the off-diagonal elements vanishing and only the diagonal elements being different from zero. The axis system that puts the tensor in diagonal form is called the principal axis system of the tensor, and the diagonal elements are known as its principal values. In the case of the \( g \) tensor, the principal axes always coincide with the axes of molecular symmetry, if these exist.

We conclude this digression on the tensor algebra by recalling some tensor multiplication rules. If \( T \) is a second-rank tensor and \( \mathbf{v} \) is a vector, the product \( T \cdot \mathbf{v} \) is still a vector, whose components are given by the matrix multiplication law:

\[
\begin{bmatrix}
T_{xx} & T_{xy} & T_{xz} \\
T_{yx} & T_{yy} & T_{yz} \\
T_{zx} & T_{zy} & T_{zz}
\end{bmatrix}
\begin{bmatrix}
v_x \\
v_y \\
v_z
\end{bmatrix}
= \begin{bmatrix}
T_{xv_x} + T_{yv_y} + T_{zv_z} \\
T_{zv_x} + T_{xv_y} + T_{yv_z} \\
T_{yv_x} + T_{zv_Y} + T_{xv_z}
\end{bmatrix}
\]

(22)

Inner multiplication of the resulting vector by another vector \( u \) gives a scalar, or zeroth-rank tensor. Thus the product

\[
\mathbf{u} \cdot T \cdot \mathbf{v}
\]

(23)

is a scalar, and it is invariant under rotation of the coordinate system.

2. GENERAL MAGNETIC RESONANCE THEORY

Following from the above considerations, we can write the Zeeman Hamiltonian for a paramagnetic molecule in the form

\[
\mathcal{H}_Z = \beta \mathbf{H} \cdot \mathbf{g} \cdot \mathbf{S}
\]

(24)

The physical observable corresponding to the Hamiltonian operator is the energy, and obviously it cannot depend on the orientation of the reference frame. It follows that the choice of the reference system is a completely arbitrary one. However, there are two particularly convenient possibilities to be considered:

(a) The laboratory axis system \((x, y, z)\) with the \( z \) axis coinciding with the direction of the magnetic field. In this system \( \mathbf{H} = k \mathbf{H}_0 \), and the spin operators are well defined, given that the direction of \( \mathbf{H} \) corresponds to the quantization axis. When expressed in the laboratory axis system, the Zeeman Hamiltonian becomes

\[
\mathcal{H}_Z = \beta_e H_0 (g_x S_x + g_y S_y + g_z S_z)
\]

(25)

(b) The molecular axis system \((p, q, r)\) where the \( g \) tensor is diagonal. In this case we obtain

\[
\mathcal{H}_Z = \beta_e (g_{pp} H_p S_p + g_{qq} H_q S_q + g_{rr} H_r S_r)
\]

(26)

where \( H_p, H_q, H_r \) are \( H \), the components of \( \mathbf{H} \) in the molecular axis system.

It is obviously possible to pass from one formulation to the other by making use of the relationships (19) and (20). Let us now consider as an example a case in which the molecule is oriented with one of the principal axes parallel to the field direction. The resonance condition will be met at field values

\[
\nu = \frac{g_{pp} \beta_e}{H_p}, \quad \nu = \frac{g_{qq} \beta_e}{H_q}, \quad \nu = \frac{g_{rr} \beta_e}{H_r}
\]

depending on which one of the principal axes is directed along the field. For any arbitrary orientation of the molecule, the resonant field is still given by the relation

\[
H = \frac{\nu}{g_{eff} \beta_e}
\]

(27)

where now the effective \( g \) factor is

\[
g_{eff} = \left( g_{pp}^2 + g_{qq} m^2 + g_{rr} n^2 \right)^{1/2}
\]

(28)

\((i, m, n)\) being the direction cosines of \( \mathbf{H} \) in the molecular axis system.

Let \((\alpha, \beta, \gamma)\) be the Euler angles that carry the laboratory axis system \((x, y, z)\) into coincidence with the molecular system \((p, q, r)\). These angles are defined by the three successive rotations (Rose, 1957; Edmonds, 1957): (i) rotation through \( \alpha \) about the original \( z \) axis; (ii) rotation through \( \beta \) about
the y’ direction obtained after the first step; (iii) rotation through y about the final r direction. According to this convention, one finds

\[ g_{rr}^2 = g_{pp}^2 \sin^2 \beta \cos^2 \gamma + g_{ee}^2 \sin^2 \beta \sin^2 \gamma + g_{rr}^2 \cos^2 \beta \] \hspace{2cm} (29)

This equation is further simplified if the tensor has an axial symmetry, i.e., if \( g_{pp} = g_{ee} \neq g_{rr} \). By denoting \( g_{pp} \) and \( g_{ee} \) by \( g_{1} \), and \( g_{rr} \), with \( g_{1} \), we obtain for this case

\[ g_{rr}^2 = g_{1}^2 \cos^2 \beta + g_{2}^2 \sin^2 \beta \] \hspace{2cm} (30)

The analysis of the angular dependence of the g factor for radical molecules trapped in a crystal lattice leads to the determination of the principal values of the \( g \) tensor and of the spatial disposition of the molecule relative to the crystal axes (Carrington and McLachlan, 1967).

B. Interaction with the Microwave Field

In addition to the effect of the static magnetic field, which determines the separation of the Zeeman levels, we have to consider the presence of the microwave field, which is responsible for the ESR transitions. The magnetic field associated with the radiation oscillates along the x axis perpendicular to the main field direction, and thus it has components

\[ H_{xx} = H_{1} \cos \omega t, \quad H_{yy} = H_{2} = 0 \] \hspace{2cm} (31)

The corresponding Hamiltonian for the interaction with the spin magnetic moment, neglecting for simplicity the g-factor anisotropy, is

\[ \mathcal{H} = g_{1} \beta_{e} H_{1} S_{z} \cos \omega t \] \hspace{2cm} (32)

Since \( H_{1} \) is much smaller than the main field \( H_{0} \), it is normally of the order of few milligauss to avoid saturation phenomena), \( \mathcal{H} \) can be treated as a time-dependent perturbation on the eigenstates of \( \mathcal{H}_{Z} \). From standard perturbation theory, it can be easily verified that the effect of \( \mathcal{H} \) is to induce transitions between the states \( | x \rangle \) and \( | x' \rangle \) of \( \mathcal{H}_{Z} \) of energies \( E_{x} \) and \( E_{x'} \) at the rate given by the transition probability (Hameka, 1965; Slichter, 1963)

\[ \omega_{m} = \frac{2\pi}{\hbar} g_{1} \beta_{e} H_{1}^{2} \langle \langle x | S_{z} | x' \rangle \rangle \delta(E_{x} - E_{x'} - \hbar \omega) \] \hspace{2cm} (33)

Here \( \delta \) is the Dirac delta function, which imposes the condition \( E_{x} - E_{x'} = \hbar \omega \). The condition for nonvanishing values of \( \langle x | S_{z} | x' \rangle \) is found by rewriting the spin operator \( S_{z} \) in terms of the "shift" operators \( S_{+} \) and \( S_{-} \):

\[ S_{z} = S_{x} \pm i S_{y}, \quad S_{x} = \frac{1}{2}(S_{+} + S_{-}) \]

2. General Magnetic Resonance Theory

The effect of these operators on the basis spin functions is

\[ S_{x} \left[ s, m_{s} \right] = (s(S + 1) - m_{s}(m_{s} + 1))^{1/2} \left[ s, m_{s} \pm 1 \right] \] \hspace{2cm} (34)

Therefore transitions will occur only between levels with energy separation \( \Delta E = \hbar \omega \), satisfying the selection rule \( \Delta m_{s} = \pm 1 \).

C. Hyperfine Couplings

From the results of Section IIIA one might think that the g values could be used to characterize individual radical species, but this is not always the case, because of the smallness of the g deviations from the free spin value, and the difficulty of an accurate theoretical interpretation in terms of molecular structure. By far more important for the identification of the paramagnetic molecules are the conspicuous multiplet structures, called hyperfine structures, that are very often present in the ESR spectra. The hyperfine structure is caused by the interaction of the electron spin magnetic moment with the magnetic moments of nuclei possessing nonvanishing nuclear spin angular momentum, such as hydrogen (\( I_{H} = \frac{1}{2} \)), nitrogen (\( I_{N} = 1 \)), and fluorine (\( I_{F} = \frac{1}{2} \)). Carbon and oxygen in their normal isotopic states, \( ^{12}C \) and \( ^{16}O \), have zero nuclear spin, but the rare isotopes \( ^{13}C \) (natural abundance 1.1%) and \( ^{17}O \) (natural abundance 0.04%) have nuclear spins of \( \frac{1}{2} \) and \( \frac{3}{2} \), respectively.

The multiplet structure of the ESR spectra arises from the fact that the electron spin magnetic moment interacting with the nucleus "feels" a different total field according to which of the \( 2I + 1 \) allowable orientations is assumed by the nuclear spin in the static magnetic field.

The magnetic interactions between electron and nuclear spins are represented by the Hamiltonian

\[ \mathcal{H} = -g_{e} \beta_{e} \gamma_{N} \frac{(1 \cdot S)^{2} - 3(1 \cdot r)(S \cdot r)}{r^{3}} - \frac{8\pi}{3} (1 \cdot S) \delta(r) \] \hspace{2cm} (35)

where \( r \) is the electron–nucleus distance vector and \( \delta(r) \) is the Dirac delta function. The first term appearing in Eq. (35) describes the electron–nucleus dipolar interaction, and it is readily derived by classical arguments (Hameka, 1965). The second term, which gives rise to the so-called Fermi contact coupling, can also be obtained by a simple classical treatment, although the derivation based on the relativistic Dirac equation is more advisable in this case (Slichter, 1963).

From the Hamiltonian (35) we can obtain a spin-Hamiltonian \( \mathcal{H}_{HF} \), acting on spin variables only, by integration over the electron spatial coordinates, i.e., by averaging over the electron probability distribution \( \psi^{2}(r) \) corresponding to the ground electronic state.
Let us examine separately the results obtained by the two terms of the Hamiltonian given in Eq. (35). The dipolar interaction takes the following form (Carrington and McLachlan, 1967):

$$\mathbf{H}_1 = \mathbf{I} \cdot \mathbf{A}' \cdot \mathbf{S}$$  \hspace{1cm} (36)

where $\mathbf{A}'$ is again a second-rank tensor, with elements given by the relationship

$$A_{ij} = -g_e \beta_e g_n \beta_n \langle r^2 \delta_{ij} - 3x_i x_j r^{-3} \rangle$$  \hspace{1cm} (37)

and the brackets denote the integration over the electron distribution.

The dipolar term is clearly symmetric, and its trace vanishes because $\Sigma x_i^2 = r^2$. Recalling what was previously said regarding the $g$ tensor, we can see that the magnitude of the dipolar interaction depends on the orientation of the molecule relative to the field direction.

For the contact interaction one obtains

$$\mathbf{H}_2 = a \mathbf{I} \cdot \mathbf{S}$$  \hspace{1cm} (38)

where the isotropic coupling constant $a$ is a scalar defined by

$$a = \frac{8\pi}{3} g_e \beta_e g_n \beta_n \psi^2(0)$$  \hspace{1cm} (39)

and $\psi(0)$ is the value assumed by the unpaired electron wave function at the nuclear position. Finally, the complete hyperfine Hamiltonian $\mathbf{H}_{hf}$, obtained by summing $\mathbf{H}_1$ and $\mathbf{H}_2$, can be written in the compact form

$$\mathbf{H}_{hf} = \mathbf{I} \cdot \mathbf{A} \cdot \mathbf{S}$$  \hspace{1cm} (40)

by defining a hyperfine tensor $\mathbf{A}$ with components

$$A_{ij} = A_{ij}' + \delta_{ij} a$$  \hspace{1cm} (41)

In the principal axis system of the hyperfine tensor, the Hamiltonian assumes the simpler form

$$\mathbf{H}_{hf} = A_{xx} \mathbf{I} \cdot \mathbf{S} + A_{yy} \mathbf{I} \cdot \mathbf{S} + A_{zz} \mathbf{I} \cdot \mathbf{S}$$  \hspace{1cm} (42)

It must be noted that if the electron distribution has a spherical symmetry, i.e., it is represented by an s-type function, then the integration of the dipole operator over the electron coordinates gives

$$\langle x^2/r^2 \rangle = \langle y^2/r^2 \rangle = \langle z^2/r^2 \rangle = \frac{1}{3} \langle 1/r^2 \rangle$$  \hspace{1cm} (43)

and so the dipolar terms vanish. Thus, only if the unpaired electron is in a p, d, f, … state will the dipolar interaction manifest itself. On the contrary, the contact term requires a finite electron density at the nuclear position, and only the s-type atomic wave functions have nonzero values at the origin. If the electron is described by a wave function that is a mixture of s and p states, both the dipolar and contact interactions are present simultaneously.

In most organic free radicals, the unpaired spin distribution is described by p-type orbitals. Among the very many examples are the planar $\cdot$CH$_3$ and NH$_2^-$ radicals, where the electron is concentrated on a 2p atomic orbital centered on the carbon or nitrogen atom, respectively; or the positive and negative ions of the aromatic hydrocarbons, where the electron is delocalized on $\pi$ molecular orbitals, which still can be constructed as linear combinations of 2p atomic orbitals. It is well known that the solution spectra of all of these systems exhibit a rich hyperfine structure due to contact interactions with the nuclei lying in the nodal plane of the $\pi$-electron distribution. This situation is not surprising, since it is well understood that the simple notion of electrons described by definite molecular shells, implied by the molecular orbital treatments, can only give an approximate picture of the electronic structures.

In fact, due to the presence of the unpaired electron, which interacts differently with electrons having opposite spin, a polarization of the doubly occupied $\sigma$ orbitals may occur, inducing a net spin density at the positions of the nuclei. This effect can be accounted for quantitatively in accurate calculations by refining the molecular functions with a technique known as configuration interaction. It consists in mixing to the first-approximation ground-state wave function, by a variational procedure, contributions from excited wave functions obtained by promotion of $\sigma$ electrons into the $\pi$ system. The adjustment of the electronic distribution produced in this way can be insignificant as far as the total electron density is concerned, although it is totally responsible for the observed isotropic couplings in $\pi$-electron systems. In fact it can be calculated that one unpaired electron in a hydrogen 1s atomic orbital or a nitrogen 2s orbital would produce a hyperfine interaction of 1420 or 1540 MHz, respectively. The observed coupling constants for the NH$_3$ radical ($a_H = -77$ MHz, $a_N = 55$ MHz) show that the induced unpaired electron density on each nucleus amounts to a small fraction of an electron. The negative sign of the hydrogen coupling constant means that on this nucleus there is an excess of electron with spin opposite to that of the unpaired electron on the 2PN orbital. The absolute sign of the coupling constants cannot be obtained directly from the observed spectra, but it is inferred from theoretical arguments. Experimental verification of the signs can be available only from the line shifts measured in the nuclear magnetic resonance spectra of paramagnetic molecules.

In contrast to the contact term, the dipolar interaction is a first-order effect that can be calculated approximately if an approximate $\pi$-electron distribution is known. Due to the $r^{-3}$ dependence, it is sensitive only to the local p-electron population.
Table I lists the principal values of the hyperfine dipolar tensor, together with the isotropic coupling constants, for the malonic acid radical $^{13}\text{CH}(\text{COOH})_2$ and $\text{NH}_3^+$ radical. These values are rather typical for any C-H or N-H fragment. The $p$ and $r$ molecular axes are parallel to the $X$-H bond direction and to the $2p$ orbital axis, respectively. The reader is referred to the book by Carrington and McLachlan (1967) for a discussion of the signs of the dipolar couplings.

### Table I

**Contributions to Hyperfine Splittings**

<table>
<thead>
<tr>
<th></th>
<th>$^{13}\text{C}$</th>
<th>$^{14}\text{N}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{pp}$</td>
<td>30</td>
<td>61</td>
</tr>
<tr>
<td>$\alpha_{mr}$</td>
<td>-50</td>
<td>-35</td>
</tr>
<tr>
<td>$\alpha_{mr}$</td>
<td>-30</td>
<td>-55</td>
</tr>
<tr>
<td>$\alpha_{rr}$</td>
<td>0</td>
<td>-6</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>-60</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

* Typical values (in MHz) of the anisotropic and isotropic contributions to the hyperfine splittings due to the magnetic nuclei present in the $^{13}\text{C}$-H and $^{14}\text{N}$-H molecular fragments.

If a C-H or N-H molecular fragment is inserted in a conjugated molecule where the unpaired electron is delocalized on several atoms, the isotropic coupling constant of a $^{13}\text{C}$ or $^{14}\text{N}$ nucleus is only roughly proportional to the unpaired $\pi$-electron density on that particular atom. On the contrary, the isotropic hyperfine coupling constants of hydrogen nuclei are strictly correlated to the unpaired $\pi$-electron density $\rho$ on the conjugated atoms to which they are bound, according to the well-known McConnell relationship (Carrington and McLachlan, 1967)

$$a_H = Q_{HH}^{H} \rho_X$$

where $Q_{HH}^{H}$ is a constant that depends exclusively on the nature of the X atom to which the hydrogen is attached. The value of the constant can be obtained from the experimental value of $a_H$ in molecules where the electron density on the atoms carrying the unpaired electron is known from symmetry considerations. From the examples given in Table I, where the unpaired electron density on the carbon and nitrogen atoms is equal to unity, it therefore follows that

$$Q_{HH}^{C} = -60 \text{ MHz}, \quad Q_{HH}^{N} = -77 \text{ MHz}$$

### 2. General Magnetic Resonance Theory

#### D. Interactions between Electron Spins

When a molecule possesses more than one unpaired electron, we need to consider the interactions between the electron spins in addition to the interaction of the total spin $S$ with the magnetic field and the hyperfine coupling with the nuclei. As examples of this type of system we refer to molecules in a triplet state, which have two unpaired electrons. In this case $S = 1$, and in the presence of an external field the allowed orientations of the magnetic moment correspond to the three values $+1, 0,$ and $-1$ of the spin projection quantum number.

The triplet state can be either the ground state of the molecule, as in the case of normal molecular oxygen, or an energetically excited state. Triplet ground states result whenever the unpaired electrons can occupy distinct atomic or molecular orbitals of the same energy. The interelectronic repulsions lead in this case to the stabilization of the state with the highest spin multiplicity, in agreement with the Hund rule for atoms. Well-known examples of excited triplet states are provided by the phosphorescent states resulting from photoexcitation of aromatic molecules. The decay to the ground singlet state ($S = 0$) is spin-forbidden, and this is the reason for the relatively long lifetime of the paramagnetic state.

An important interaction between the electron spins is the dipolar interaction, analogous to the electron spin–nuclear spin dipolar coupling. The dipolar Hamiltonian is in this case, neglecting $g$-factor anisotropy,

$$\mathcal{H} = g_s^2 \beta e^2 [(S_1 \cdot S_2) r^2 - 3(S_1 \cdot r)(S_2 \cdot r)]/r^8$$

(45)

where $r$ is the interelectronic distance. From this expression it is possible to derive a dipolar spin Hamiltonian $\mathcal{H}_D$, after averaging over the electron coordinates by a procedure similar to that used for the dipolar hyperfine coupling:

$$\mathcal{H}_D = S \cdot \mathbf{D} \cdot S$$

(46)

where $S = S_1 + S_2$, and

$$D_{ij} = \frac{1}{2} g_s^2 \beta e^2 \langle r^2 \delta_{ij} - 3x_i x_j \rangle/r^6$$

(47)

the brackets indicating the average over the electronic wave function.

The electron dipolar interaction lifts the degeneracy of a triplet state even in the absence of the field, giving rise to the so-called "zero-field splitting" (Fig. 3). As a result, in the presence of an applied field the allowed transitions $-1 \rightarrow 0$ and $0 \rightarrow 1$ do not have the same energy at a fixed field value. The spectrum will always consist of two lines, whose separation at sufficiently high field is independent of the field strength and dependent on
the principal values of $D$ and the orientation of the field direction with respect to the molecular axes. By introducing the new zero-field parameters

$$D = D_p - \frac{1}{2}(D_{pp} + D_{qq}), \quad E = \frac{1}{2}(D_{pp} - D_{qq})$$

(48)

we find that the separation between the line pair, called “fine splitting,” is $D - 3E, D + 3E,$ and $2D$ according to whether the field is directed along the $p$, $q$, or $r$ molecular axis, respectively.

In addition, a “half-field” transition of weaker intensity is usually observed. It is called a $\Delta m = \pm 2$ transition, but this definition is only conventional because the states involved are not pure states with $m = \pm 1$, due to the mixing produced by the zero-field interaction. When the magnetic field is directed along the principal axes of the $D$ tensor, the half-field transition is forbidden if the conventional equipment with the rf field polarized perpendicularly to the static field is adopted in the ESR spectrometer, but it can be observed with a parallel arrangement (van der Waals and De Groot, 1959).

For any other orientation of the magnetic field relative to the molecular axes, the transition is weakly allowed for both the parallel and perpendicular dispositions of the microwave field. Figure 4 shows the effect on the energy levels of the magnetic field when directed along the molecular $r$ axis. The heavy arrows indicate the “allowed” transitions and the dashed arrow the half-field transition.

In particular cases, especially when there is a pair of electrons on nearby sites with appreciably overlapping distributions, an isotropic coupling between the spins $S_1$ and $S_2$ must be introduced. This originates from the electrostatic interactions, which tend to couple the spins into a singlet and a triplet state. Following the Heitler–London description of the bonding, this interaction is termed exchange interaction, and it has the form

$$\mathcal{H}_e = J S_1 \cdot S_2 = \frac{1}{2}J(S^2 - S_1^2 - S_2^2) = \frac{1}{2}J(S^2 - \frac{3}{2})$$

with $S^2 = (S_1 + S_2)^2$ (49)

The eigenfunctions of $\mathcal{H}_e$ are therefore the familiar singlet and triplet spin functions, which are eigenfunctions of $S^2$ with eigenvalues $S(S + 1)$ equal to 0 and 2, respectively. The exchange energy $J$ corresponds to the singlet–triplet energy separation. Positive values of $J$ lead to a state of lowest energy with antiparallel disposition of the spins. Complex situations arise when the magnitude of $J$ is of the order of the hyperfine coupling $a$. The effects deriving from the presence of the exchange term is discussed in Chapter 4 on biradicals.

IV. ANALYSIS OF THE ESR SPECTRA

In the following sections we outline the methods of analysis of the ESR spectra for systems in the liquid phase, in single crystals, and in randomly oriented solids. In each of these cases the paramagnetic molecule is diluted in a diamagnetic host (the solvent, or a suitable crystal lattice) to avoid interactions between nearby spins, which would otherwise produce line broadening due to magnetic dipole interactions, or spin correlations due to exchange couplings.

We shall only treat systems with a single unpaired electron spin. The reader is referred to Chapter 4 for the discussion of the spectral features of systems with two or more interacting spins.
A. Solution Spectra

For a free radical in which the electron spin interacts via hyperfine coupling with \( N \) nuclei of individual nuclear spin quantum number \( I_k \), the magnetic Hamiltonian is

\[
\mathcal{H} = \beta_e \mathbf{H} \cdot \mathbf{g} \cdot \mathbf{S} + \sum_{k=1}^{N} S_i \cdot \mathbf{A}^{(k)} \cdot \mathbf{I}_k
\]  

(50)

If the paramagnetic species is dissolved in a low-viscosity solvent, then the very fast molecular reorientations due to thermal motions average out the anisotropic terms of the spin Hamiltonian. The position of the spectrum in the field and the magnitude of the hyperfine splitting are thus determined by the average values of the diagonal elements of the \( \mathbf{g} \) and \( \mathbf{A} \) tensors. The energy levels of the system can then be obtained in terms of the isotropic Hamiltonian

\[
\mathcal{H}_o = g \beta_e H_0 S_z + \sum_{k=1}^{N} a_k (S_i I_{kz} + S_y I_{ky} + S_z I_{kz})
\]

(51)

where

\[
g = \frac{1}{3} \text{Tr } \mathbf{g} \quad \text{and} \quad a_k = \frac{1}{3} \text{Tr } \mathbf{A}^{(k)}
\]

(52)

Since the hyperfine interactions are always much smaller than the Zeeman interaction energy, a perturbation procedure can be used to compute the energy levels, by treating the hyperfine term as a perturbation on the eigenstates of the Zeeman term. The eigenfunctions of the Zeeman Hamiltonian can be written as simple products of the usual electron spin and nuclear spin eigenfunctions of the spin operators \( S_i \) and \( I_{kz} \). These product functions are also eigenfunctions of the simplified Hamiltonian

\[
\mathcal{H}_o = g \beta_e H_0 S_z + \sum_{k} a_k S_i I_{kz}
\]

(53)

and the required first-order energies are the corresponding eigenvalues

\[
E = g \beta_e H_0 m_s + \sum_{k} a_k m_s M_k
\]

(54)

where \( m_s = \pm \frac{1}{2} \) and \( M_k = -I_k, -I_k + 1, \ldots , I_k \).

Due to the effect of the static field and of the hyperfine couplings, a number of sublevels are generated, with energy given by Eq. (54). Some of these levels will have the same energy, i.e., they will be degenerate, if the hyperfine interaction is the same for two or more nuclei. In this case the nuclei are said to be magnetically equivalent, and this property is immediately recognizable from the molecular geometry.

The rf field applied to the system in a normal ESR experiment induces transitions among the sublevels. However, not all transitions are allowed. First of all, the states involved in the transitions must satisfy the condition \( \Delta m_s = \pm 1 \), as already seen from Eq. (33). Moreover, the operator \( S_y \) acts on electron spin variables only, and so the disposition of the nuclear spins of the two states between which the transition takes place has to be the same. If this was not the case, the matrix element appearing in Eq. (33) would vanish, given the orthogonality of the nuclear spin functions.

In conclusion, the allowed ESR transition must satisfy the selection rules \( \Delta m_s = \pm 1, \Delta M_k = 0 \) for all nuclei.

Let us suppose for the sake of simplicity that all the \( N \) nuclei are equivalent, as in the methyl radical \( \cdot \text{CH}_3 \) or in the benzene radical ions \((\text{C}_6\text{H}_5)^\cdot\) and \((\text{C}_6\text{H}_5)^-.\). The energy of the hyperfine levels is then

\[
E(m_s, m) = g \beta_e H_0 m_s + a m_s M
\]

(55)

where \( M \) is the vectorial sum of the individual quantum numbers \( M_k \). The energy difference of the levels involved in the allowed transitions is

\[
\Delta E(M) = g \beta_e H_0 a M
\]

(56)

The spectrum will consist of a number of lines equal to the \( 2N + 1 \) permissible values of \( M \), with intensity proportional to the degeneracy of each nuclear \( M \) state, i.e., to the number of ways in which the individual spin components \( M_k \) can be combined to give the resultant \( M \). If \( I_k = \frac{1}{2} \), so that \( M_k \) can assume only the two values \( \pm \frac{1}{2} \), the intensities are proportional to the coefficients of the binomial expansion. The spectral lines are equally spaced, and the separation between adjacent lines is \( a/\hbar \) in frequency units.

In the ESR experiments, where the field is scanned at fixed frequency \( v_0 \) to meet the resonance conditions, the transitions will be symmetrically disposed about the center \( H_0 = h v_0 / g \beta_e \), and will occur at field values spaced by an amount \( a/\hbar \). For this reason, it is customary to give the coupling constants in frequency units or in equivalent gauss, instead of in energy units. From relation (5) we find that 1 G corresponds to 2.8 MHz for \( g = 2 \).

Figure 5 shows the energy diagrams for the case of an electron spin interacting with a nucleus of spin \( I = \frac{1}{2} \), with two equivalent nuclei of spin \( \frac{1}{2} \), and with a single nucleus of spin 1. The dependence on the magnetic field strength of the energy levels of the last two situations, and the corresponding simulated spectra, are displayed in Fig. 6.
2. General Magnetic Resonance Theory

1972). However, given that usually \(|A^{\text{iso}}| \ll g\beta, H_0, \) a first-order perturbation approach can again be adopted. We shall limit ourselves to the discussion of the results for the simple case of hyperfine interaction with a single nucleus of spin \(I\), with a further condition of coincidence for the principal axis systems of the \(g\) and hyperfine tensors. Under these circumstances the transition energies for any fixed orientation of the magnetic field relative to the molecular axes, specified by the Euler angles \((\beta, \gamma)\), are calculated by a relation analogous to Eq. (56):

\[
\Delta E(M) = g_{\text{eff}} \beta H_0 + A_{\text{eff}} M
\]

(57)

where \(g_{\text{eff}}\) has been already given in Eq. (29) and \(A_{\text{eff}}\) is similarly defined as

\[
A_{\text{eff}}^2 = A_{pp}^2 + A_{eq}^2 m^2 + A_{n}^2 n^2
\]

\[
= A_{pp}^2 \sin^2 \beta \cos^2 \gamma + A_{eq}^2 \sin^2 \beta \sin^2 \gamma + A_{n}^2 \cos^2 \beta
\]

(58)

When working at a fixed frequency, absorption will occur at field values determined by the resonance condition

\[
h\nu_0 = g_{\text{eff}}(\beta, \gamma)\beta H + A_{\text{eff}}(\beta, \gamma)M
\]

(59)

and thus the spectrum consists of the usual \(2I + 1\) lines, equally spaced with a separation in field units of \(A_{\text{eff}}(\beta, \gamma)\), and symmetrically disposed about a center determined by the value of \(g_{\text{eff}}\) according to Eq. (27).

If the hyperfine interaction tensor has axial symmetry, then the angular dependence of the splitting follows the relation

\[
A^2_{\text{eff}} = A_{||}^2 \cos^2 \beta + A_{\perp}^2 \sin^2 \beta
\]

(60)

\(\beta\) being the angle between the field direction and the symmetry axis of the magnetic tensor.

It is important to note that the experimental measurement of \(\Delta E\) in Eq. (57) can determine only the absolute values of the principal components of the hyperfine tensor. However, these are related to the isotropic coupling constant \(a\) by the relation (52):

\[
a = \frac{1}{2}(A_{pp} + A_{eq} + A_{n})
\]

The comparison between the value of \(a\) measured in solution and the values of the principal components of the tensor enables us to determine the relative sign of the isotropic and the purely anisotropic (or dipolar) part of the hyperfine interaction. On the other hand, the estimate of the absolute sign of the dipolar coupling is firmly established on theoretical grounds (Carrington and McLachlan, 1967; Atherton, 1973), so knowledge of the signs of the various terms of the hyperfine interaction can be safely assumed in most cases.

---

B. Spectra in Oriented Crystals

In order to find the angular dependence of the spectra for free radicals oriented in single crystals, we have to compute the energy levels of the complete Hamiltonian given in Eq. (50). Several procedures can be used to obtain the eigenstates of the Hamiltonian, with the degree of accuracy desired, for the complex case of many interacting nuclei (Poole and Farach,
C. Spectra of Nonoriented Systems

In polycrystalline or vitreous samples, the principal axes of the paramagnetic molecules assume all possible angles relative to the magnetic field direction. Under these conditions, the ESR spectrum is expected to be the superposition of the spectra corresponding to all the possible orientations, and to be spread over the entire field range determined by the $g$-factor and hyperfine anisotropy. The distribution of the absorption intensity, however, is not uniform in this range. This fact can be visualized if we consider a system with an axially symmetric $g$ factor, the symmetry axis being the $z$ axis. As a consequence of the random orientation, there will be many more molecules with the $x$ axis nearly perpendicular to the direction of the magnetic field than there are with the axis parallel to the field. Therefore there will be more paramagnets absorbing at the resonance field determined by $g_1$ than those absorbing in the field regions determined by $g_1$. At these two field values, which correspond to the extrema of the range in which transitions can occur, turning points with different intensity result in the absorption spectrum, and therefore maxima or minima appear in the first derivative presentation (Wertz and Bolton, 1972).

The shape of the spectral envelope arising from both $g$-factor and hyperfine anisotropy can be computed with reasonable accuracy if each component line is assumed to be described by a shape function $J$ independent of orientation. The amount of energy absorbed at a field $H_z$ is given by

$$A(H_z) = \int_{H_{\text{min}}}^{H_{\text{max}}} P(H) J(H_z - H) dH$$

(61)

where $P(H) dH$ represents the probability of finding molecules absorbing at the resonant field $H$, i.e., with orientation specified by the angles $(\beta, \gamma)$ satisfying the resonance condition according to Eq. (59). For randomly dispersed samples, this probability is proportional to the elementary solid angle $\sin \beta d\beta \, dy$. After the change to angular variables, numerical computation of the integral (61) is performed to obtain the overall absorption pattern. If the $g$-factor anisotropy is small and if the hyperfine component $A_r$ is larger than the other components, the outermost lines of the derivative spectrum have the appearance of absorption lines, centered at field values

$$\left(\hbar \nu \pm A_r I / g_r \beta_e \right)$$

A spectrum of this sort is shown in Fig. 7c.

Even in more complex situations the spectral parameters are obtainable from the analysis of the powder pattern, provided the spectrum is not too distorted by overlapping components (Weil and Hecht, 1963).

2. GENERAL MAGNETIC RESONANCE THEORY

D. Linewidths in Solution Spectra

We conclude this section concerning the analysis of the spectral features with some considerations on the linewidth effects very often observed in solution spectra.

Following from the results attained in the preceding discussion, if an unpaired electron of a radical molecule interacts with a nucleus of spin $I$, the observed spectrum will consist of $2I + 1$ hyperfine components, each of which is identified by the nuclear quantum number $M$ characterizing the sublevels involved in the transitions. These components are expected to have the same intensity, given that the energy separation between the magnetic levels is very small compared to the thermal energy $kT$, and so the populations of the levels, as seen from Eq. (4), are practically equal. In actual fact, we find that in most cases the heights of the various lines do not appear to be the same. Examination of the line shape reveals that this is due to the fact that the lines are not of the same width. For a Lorentzian curve as given in Eq. (9), the peak-to-peak height of the first derivative varies with the inverse square of the width, and therefore the relative height of two lines having the same intensity but different widths is given by

$$I_1/I_2 = [T_1(1)/T_2(2)]^2$$

(62)

The linewidth variations have to be ascribed in most cases to the fluctuations of the anisotropic terms of the magnetic Hamiltonian caused by the molecular motions (Freed and Fraenkel, 1963; Hudson and Luckhurst, 1969). Even if the anisotropic terms do not contribute to the magnetic parameters measured in solution, they do constitute an important source of line broadening. The Hamiltonian (50) can in fact be considered as the sum of two contributions: an isotropic, orientationally invariant part $H_0$, which has been given in Eq. (51), and a purely anisotropic, angular dependent part $H_1$, which can be written as

$$H_1 = \beta_e \cdot H \cdot g' \cdot S + \sum_{k=1}^{N} S \cdot A^{(k)} \cdot I$$

(63)

where $g'$ and $A^{(k)}$ are traceless tensors.

In liquid phase, the molecular tumbling makes $H_1$ a random function of time. As a consequence, there is a random modulation of the energy levels and of the transition frequencies. In spite of the vanishing average value of $H_1$, broadening of the absorption lines is expected to occur.

The frequency fluctuations can be characterized through their amplitude and coherence. The amplitude $\Delta$ is defined by the mean square value of the
2. GENERAL MAGNETIC RESONANCE THEORY

E. An Instructional Example: The Nitroxide Free Radicals

The nitroxide free radicals are molecules containing the paramagnetic moiety

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{R} \\
\text{H} & \quad \text{C} \\
\text{H} & \quad \text{N} \\
\text{H} & \quad \text{C} \\
\text{O} & \quad \text{CH}_3 \\
\text{H} & \quad \text{C} \\
\text{N} & \quad \text{C} \\
\text{R} & \quad \text{CH}_3
\end{align*}
\]

These species are remarkably stable and inert because of the protective effect exerted by the four methyl groups. The ESR spectra of these compounds comprise three sharp, well-resolved hyperfine lines resulting from the coupling of the unpaired electron spin with the nitrogen nuclear spin. Typical values of the magnetic parameters, obtained from single-crystal studies with the free radicals incorporated at low concentration into a diamagnetic host, are displayed in Table II (Griffith et al., 1965), the molecular axis system† being chosen as shown:

\[ N - O \rightarrow p \]

The value of the isotropic coupling constant (38 MHz compared with 55 MHz for the NH\(_2\) radical) and the angular dependence of the dipolar interaction confirm that the unpaired electron is largely localized on a \(2p_x\) orbital of the nitrogen atom.

| Magnetic Parameters for Nitroxide Free Radicals\(^*\) |
|-----------------------------|-----------------------------|
| \( g_{xx} \)                | 2.0089 \( A_{xx}(A_x) \)  |
| \( g_{yy} \)                | 2.0061 \( A_{yy}(A_y) \)  |
| \( g_{zz} \)                | 2.0027 \( A_{zz}(A_z) \)  |
| \( g \)                     | 2.0039 \( a \)            |

\* Editor’s note: A complete table of all nitroxide \( g \) and hyperfine tensors reported to date is compiled in Appendix I (p. 564).

† Editor’s note: This axis system is, of course, equivalent to the common Cartesian axis system for nitroxides, where \( p = x, q = y, r = z \).
Spectra (d) and (e) refer to free radicals dissolved in aqueous glycerol at 25°C and in a glassy matrix obtained by freezing a glycerol solution at −80°C.

Figure 8 shows the satellite lines revealed under high amplification due to the natural abundance of $^{13}$C and $^{15}$N nuclei. The three doublets come from the further subdivision of the nitrogen hyperfine levels in those molecules containing one $^{13}$C nucleus, the probability of finding two $^{13}$C on the same molecule being negligibly small. The two weakest lines come from the 0.365% of the molecules containing a $^{12}$N nucleus, which has spin 1/2. Note that the gyromagnetic ratio $g_N \beta_N$ for $^{12}$N is 1.40, and so the coupling constant for $^{12}$N is correspondingly 1.40 times the coupling constant for the normal isotope.

As far as the width of the three lines of the spectra in solution is concerned, we observe that the high-field component of the nitrogen hyperfine structure is always broader than the two other lines, which have approximately equal width. This fact can be rationalized if we refer to Eq. (64) and calculate the explicit expressions for the linewidth parameters $A$, $B$, and $C$ in...
terms of the magnetic anisotropies. The underlying theory will be developed in a following section, but here we shall anticipate some results.

Term A includes broadening contributions resulting from mechanisms that are different from the modulation of the anisotropic interactions considered so far. These broadening effects arise, for example, from the presence of oxygen in the solution or from instrumental factors, and they are all expected to be independent of line index \( M \). For the above reason, and also because it is easier to measure the relative heights of the lines instead of the breadth of any single line, it is convenient to rewrite expression (64) in the following way:

\[
(T_2(0)^{-1}[(T_2(0)/T_2(\pm 1)) - 1] = C + B \tag{64} \]

where \( T_2(0)^{-1} \) is the breadth of the central line, which corresponds to \( M = 0 \), as seen from Fig. 5c. Experimental values of \( T_2(0)/T_2(\pm 1) \) are obtained from the square root of the ratios of the experimental derivative curve peak heights, according to Eq. (62). Theoretical considerations lead to a positive value of the nitrogen isotropic coupling constant, and this permits the identification of the \( M = 1 \) nuclear spin state with the low-field hyperfine component.

In terms of the \( g \)- and hyperfine-tensor anisotropies, one obtains for the \( B \) and \( C \) parameters

\[
B = \frac{1}{2} b (\Delta \gamma) \mu_0, \quad C = \frac{1}{2} b^2 \tau \tag{66} \]

where

\[
b = 2\pi \mathcal{A}_v - 2\pi (\mathcal{A}_o - \mathcal{A}) = \frac{3}{2} \mathcal{P} (\mathcal{A}_H - \mathcal{A}_V) \tag{67} \]

all the \( \mathcal{A} \) values being expressed in MHz units, and

\[
\Delta \gamma = \beta e \hbar^{-1} [\mathcal{A}_e - \frac{1}{2} (\mathcal{A}_p + \mathcal{A}_d)] \tag{68} \]

By introducing the values listed in Table II, one finds that \( C \approx -B \) for field values of about 3,400 G. In conclusion, one can see from Eq. (64) that, due to the negative sign of \( B \), only the high-field line (having \( M = -1 \)) will appear to be broader than the central one in the X-band spectra of nitroxide free radicals. In the case where field intensity was increased to 12,500 G (Q band), the \( B \) term would become accordingly more negative, and the spectrum of Fig. 9b would be the result.

The very simple expressions for \( B \) and \( C \) given above are correct if (i) the molecular motion is isotropic, and (ii) the correlation time falls in a range satisfying the conditions \( \omega_0^2 \tau > 1 \), and \( \Delta \tau < 1 \), \( \omega_0 \) being the resonance frequency and \( \Delta \) being practically the largest of the magnetic anisotropies.

The first of the two conditions is discussed in Section V, where general expressions for the linewidth parameters, which are valid also for the case of anisotropic motion, are presented.

In an X-band experiment the resonance frequency is 9.5 GHz, which is equivalent to \( 6 \times 10^{10} \) sec\(^{-1} \) in angular frequency units. On the other hand, \( \Delta \approx \omega \) is calculated from Eq. (67) to be \( 3 \times 10^{8} \) sec\(^{-1} \). Thus the above treatment is applicable to X-band spectra of nitroxide free radicals if the correlation times fall in the range \( 5 \times 10^{-11} - 10^{-9} \) sec.

The paramagnetic resonance spectra of the nitroxide free radicals are sensitive not only to molecular motions, but also to the nature of the medium in which they are dissolved. This arises from the fact that the magnetic parameters of any radical species are very sensitive functions of the electronic distribution in the molecule, and therefore are influenced by perturbations due to the environment. Thus the isotropic coupling constant of the nitrogen atom in the nitroxide group increases by more than \( 10\% \) when we go from hydrophobic to hydrophilic solvents. A noticeable decrease of the isotropic \( g \) factor is correspondingly observed. These effects can be understood qualitatively in terms of specific interactions of the polar solvents with the lone pairs of electrons on the oxygen atom. These interactions are expected to lower the energy of the nonbonded electrons and increase the electron affinity of the oxygen atom. In this way the three-electron distribution of the N-O group is shifted towards a situation which is well represented by the limiting structure corresponding to a maximum unpaired electron density on the nitrogen atom:

\[
\frac{\mathcal{N}}{1} \left( \begin{array}{c} \mathcal{N} \mathcal{O} \mathcal{O} \end{array} \right) \tag{69} \]

On the other hand, a rather crude estimate of the \( g \) factor based on Eq. (17) of Section IIIA shows that the deviation from the free-spin value arises essentially from the term

\[
\lambda_{0}\rho_{e}/\Delta E_{\text{iso}} \tag{69} \]
where \( \lambda_0 \) is the spin-orbit coupling of the oxygen atom, \( \rho_0^g \) is the spin density on the oxygen, and \( \Delta E_{\pi \pi} \) is the \( n \to \pi \) excitation energy (Brière et al., 1965). It is evident that the factors that cause the increase of \( a_0 \) lead to a parallel reduction of the observed \( g \) value. The relation between the \( g \) value and the \( n \to \pi \) energy difference is well demonstrated by the experimentally observed blue shift of the \( n \to \pi \) transition in the UV spectrum on passing from aprotic to polar solvents.†

## Y. LINE SHAPE THEORY

As already mentioned in the previous sections, line shape analysis is very important in biological applications of ESR spectroscopy because it represents the only way to obtain information on molecular motions. Unfortunately, the theoretical approach to this matter remains undoubtedly the most difficult problem of magnetic resonance theory. For this reason, we find it useful to give an outline of the quantum mechanical description of the problem, with the aim of providing a guide to readers who desire to go deeper into this area. They will then find exhaustive answers to the many questions that this concise account will certainly raise in the textbooks by Abragam (1961), Slichter (1963), and Atherton (1973), in the basic papers of Freed (1964), Freed and Fraenkel (1963), and Kivelson (1972), and in an excellent review by Hudson and Luckhurst (1969).

In the following we shall briefly introduce the density matrix formalism that plays an important role in relaxation theory. In addition, the spherical coordinate representation of the magnetic interaction tensor shall be discussed. Tensor components expressed in this way correspond to definite projection quantum numbers, and this makes their use particularly convenient when the averages of the anisotropic terms of the Hamiltonian are calculated. The basic definitions of the statistical distribution functions that are needed to characterize the random motion in liquids shall also be given.

The solution of the line shape problem derived here is valid only in the fast motional region, with an upper limit for correlation times of about \( 10^{-3} \) sec. Beyond this limit, the methods presented in Chapter 3 have to be adopted.

### A. Density Matrix

As known from the basic principles of quantum theory, any physical observable is associated with a quantum mechanical operator. Thus, if a system is described by a wave function \( \psi_k \), the expectation value of the physical property \( \mathcal{A} \) will be

\[
A_k = \int \psi_k^* \mathcal{A} \psi_k \; dv
\]

If \( \psi_k \) is expanded in a complete set of orthonormal time-independent functions \( u_m \), then we have

\[
\psi_k = \sum_n c_n^k u_n
\]

\[
A_k = \sum_{n,m} c_n^k c_m^* \int u_n^* \mathcal{A} u_m \; dv = \sum_{n,m} c_n^k c_m^* \langle n \mid \mathcal{A} \mid m \rangle
\]

This is the case in which maximal information on the property \( \mathcal{A} \) can be achieved. In many instances, however, our knowledge of the system is not so complete. Consider, for example, a system composed of a large number of spins, \( 10^{23} \) say, interacting among themselves or with the environment in which they are embedded, in the presence of a static magnetic field. The angular momentum and spin component of an individual spin are no longer defined, and if we wish to compute the value of the macroscopic magnetic moment induced by the field, we must take into account all possible spin orientations and add up the expectation values of \( \mathcal{S} \) for each of these situations weighted with some probability factors.

Going back to Eq. (71), we see that in a composite system the products \( c_n^k c_m^* \) can be different for the various components of the system because they are represented by different functions \( \psi_i \), but the matrix elements of the operator \( \mathcal{A} \) on the basis set \( u_m \) are always the same.

We define therefore the ensemble average of \( \mathcal{A} \) by the relation

\[
\langle \mathcal{A} \rangle = \sum_k p_k A_k = \sum_{k,m} p_k c_n^k c_m^* \langle n \mid \mathcal{A} \mid m \rangle
\]

where \( p_k \) is the statistical weight of \( \psi_k \) in the specification of the system. It is convenient to define a density operator \( \rho \) through its matrix elements:

\[
\langle m \mid \rho \mid n \rangle = \sum_{k,m} p_k c_m^k c_n^k
\]

so that a more compact form for \( \langle \mathcal{A} \rangle \) results:

\[
\langle \mathcal{A} \rangle = \sum_{n,m} \langle m \mid \rho \mid n \rangle \langle n \mid \mathcal{A} \mid m \rangle = \sum_m \langle m \mid \rho A \mid m \rangle = \text{Tr} \rho A
\]

Here \( \text{Tr} \) stands for the trace, and its value can be shown to be independent of the choice of the basis set functions, as expected on physical grounds.
If the system is at equilibrium, the explicit form for the density matrix operator $\rho$ is
\[
\rho_{eq} = e^{-\mathcal{H}_{eq}/kT}/\text{Tr} \ e^{-\mathcal{H}_{eq}/kT}
\]
its diagonal matrix elements being the Boltzmann factors given in Eq. (4).
If the state of the system develops in time, the wave functions $\psi_t$, describing the system will then be time dependent. Since the basis functions $\psi_n$ are time independent, the coefficients $c_n$ must carry the time dependence. As a consequence, the density matrix will also vary with time, and its time evolution is expressed in terms of the Hamiltonian $\mathcal{H}$ of the system by the equation of motion (Slichter, 1963)
\[
d\rho(t)/dt = (i/\hbar)[\rho(t), \mathcal{H}] = (i/\hbar)[\rho(t)\mathcal{H} - \mathcal{H}\rho(t)]
\]
Accordingly, the time variation of the macroscopic observable $A$ is determined by:
\[
\langle A(t) \rangle = \text{Tr} \ \rho(t)A
\]
In the development of the theory of spin relaxation, we are interested in the calculation of the time dependence of the macroscopic magnetization $\mathcal{M}$. According to Eqs. (1) and (77), the components of the macroscopic magnetization are related to the quantum mechanical spin operators $S_x$, $S_y$, and $S_z$ by the expression
\[
\mathcal{M}(t) = -Ng\beta_S \langle S_i \rangle = -Ng\beta_S \text{Tr} \ \rho(t)S_i, \quad i = x, y, z
\]
where $N$ is the number of spins.
We have seen that the Hamiltonian for paramagnetic species in liquids always consists of a time-independent part $\mathcal{H}_0$ and a part $\mathcal{H}_1(t)$ that is a random function of time. Therefore the equation for the density matrix of the spin system becomes
\[
d\rho(t)/dt = (i/\hbar)[\rho(t), \mathcal{H}_0 + \mathcal{H}_1(t)]
\]
It is not possible to obtain exact solutions to this equation, and thus we must make use of time-dependent perturbation expansions. According to a method proposed by Redfield, the procedure to solve this equation consists in an iterative integration by successive approximations, followed by the statistical averaging over the ensemble defined by the probability distribution characteristic of $\mathcal{H}_1(t)$. To second order of approximation, we end up with the following system of linear differential equations (Slichter, 1963; Redfield, 1966):
\[
d\rho_{sa}/dt = (i/\hbar)[\rho, \mathcal{H}_0]_{sa} + \sum_{\beta \beta'} R_{sa\beta\beta'} \rho_{\beta\beta'}
\]
\[
= (i/\hbar)(E_a - E_s)\rho_{sa} + \sum_{\beta \beta'} R_{sa\beta\beta'} \rho_{\beta\beta'}
\]
The matrix elements of the spin density operator $\rho$ are calculated on the basis of the eigenfunctions of $\mathcal{H}_0$, and the $R_{sa\beta\beta'}$ are constant coefficients. The summation is restricted to states that satisfy the condition among the energies $E_a - E_s = E_b - E_p$. The coefficients $R_{sa\beta\beta'}$ form a matrix which is known as relaxation matrix. The derivation of Eq. (80) is rather lengthy and the reader is referred to the texts of Slichter and Atherton for the discussion of the various approximations employed. Furthermore, it has to be understood that Eq. (80) only gives an approximate time dependence for $\rho(t)$ and therefore cannot be used if the perturbation expressed by $\mathcal{H}_1(t)$ is too strong, or if it acts for too long a time. In fact, the applicability of Eq. (80) is restricted to the region of fast frequency modulation.
Equation (80) does not consider the effect of the applied alternating field, which induces the transitions between the levels. In order to include this effect, we must add to $\mathcal{H}_0 + \mathcal{H}_1(t)$ of Eq. (79) an extra term $\mathcal{H}_2(t)$ in the form given by Eq. (32) of Section III.B. To first order, the differential equation for the density matrix is modified in the following way (Slichter, 1963, p. 156):
\[
d\rho_{sa}/dt = (i/\hbar)[\rho, \mathcal{H}_0 + \mathcal{H}_2(t)]_{sa} + \sum_{\beta \beta'} R_{sa\beta\beta'} \rho_{\beta\beta'}
\]
\[
= (i/\hbar)(E_a - E_s)\rho_{sa} + (i/\hbar) \sum_{\alpha} [\rho_{sa} \langle \alpha' | \mathcal{H}_2(t) | \alpha' \rangle - \langle \alpha | \mathcal{H}_2(t) | \alpha'' \rangle \rho_{sa\beta\beta'}] + \sum_{\beta \beta'} R_{sa\beta\beta'} \rho_{\beta\beta'}
\]
Explicit expressions for the coefficients $R_{sa\beta\beta'}$ will be given in the next section. We will limit ourselves for the moment to examine, with a specific example, the effect of the relaxation terms in the density matrix equation.
Let us consider a system of spin $S = 1/2$, for which there are only two levels $a$ and $b$, corresponding to the eigenvalues $+1/2$ and $-1/2$ for $S_z$, respectively. In this case, complete knowledge of the system is provided by the four elements $\rho_{aa}$, $\rho_{bb}$, $\rho_{ab}$, and $\rho_{ba}$ of the density matrix.
We want now to calculate the value of the $\mathcal{M}_z$ component of the magnetization with the aid of Eq. (78). Keeping in mind that the only nonvanishing matrix elements of $S_z$ are $\langle a | S_z | b \rangle = \langle b | S_z | a \rangle = 0$, both having a value of $1/2$, and that $\rho$ is Hermitian, so that $\rho_{aa} = \rho_{bb}$, we obtain
\[
\langle S_z \rangle = \text{Tr} \ \rho S_z = \rho_{aa} \langle a | S_z | a \rangle + \rho_{bb} \langle b | S_z | b \rangle = \frac{1}{2}(\rho_{aa} + \rho_{bb}) = \text{Re} \ \rho_{aa}
\]
where $\text{Re} \ \rho_{aa}$ means the real part of $\rho_{aa}$, which in general will be a complex function.
The magnetic moment $\mathcal{M}_z$ is induced by the oscillating field applied in the direction perpendicular to the static field:
\[
H_z(t) = H_1 \cos \omega t
\]
If this field is thought of as being removed at time \( t = 0 \), the time development of the transverse magnetization is obtained by solving the equation for \( \rho_{px} \) in the absence of the rf field, i.e., in the form given by Eq. (80) (Kivelson, 1972):
\[
\frac{d\rho_{px}}{dt} = (i\omega_0 + R_{\rho px})\rho_{px}
\]
where \( \omega_0 = (E_e - E_p)/\hbar \).

This equation is solved immediately, and by setting
\[
-R_{\rho px} = 1/T_2
\]
we find for \( \mathcal{M}_x(t) \)
\[
\mathcal{M}_x(t) \propto \cos \omega_0 t \exp(-t/T_2)
\]
We see in this way that the macroscopic magnetization component \( \mathcal{M}_x(t) \) after the rf field is turned off, executes a damped precession and relaxes finally to zero with a decay time constant \( T_2 \). Equation (84) gives theoretical support to the physical intuitions leading to the phenomenological equation (8), and permits us to relate the experimentally observable relaxation time \( T_2 \) to microscopic properties of the system through the calculation of the relaxation matrix element \( \rho_{\rho px} \).

B. Relaxation and Line Shapes

In a magnetic resonance experiment the shape of the resonance absorption curve is determined by the profile of the intensity of the energy absorbed as a function of the frequency \( \omega \) of the exciting rf field (the experiment is thought of as being performed by keeping the main magnetic field at a fixed value \( H_0 \) and by varying the frequency of the oscillating field). As previously noted, the oscillating field \( H_x(t) \) induces a magnetic moment \( \mathcal{M}_x(t) \) in the macroscopic sample, and the power absorbed by the sample from the field is
\[
P = -\mathbf{\hat{H}} \cdot \mathbf{dH}/dt = -\mathcal{M}_x(dH_x/dt)
\]
The actual spectrum is determined by the average power absorbed per cycle (Abragam, 1961; Pake, 1962)
\[
\overline{P(\omega)} = -(\omega/2\pi) \int_0^{2\pi/\omega} \mathcal{M}_x(dH_x/dt) \, dt
\]
\( \mathcal{M}_x(t) \) can still be obtained by solving the differential equation for the density matrix, but this time the equation must be written in the complete form given in (81), to account for the presence of the rf field. The details of the calculation are given by Atherton (1973) and Slichter (1963); we shall only give here the final result. In the limit of small \( H_1 \) (that is, in the absence of saturation), \( \mathcal{M}_x(t) \) is found to be proportional to \( H_1 \) and to be composed of a part in phase with the oscillating field and another part out of phase with it:
\[
\mathcal{M}_x(t) = H_1 [\chi'(\omega) \cos \omega t + \chi''(\omega) \sin \omega t]
\]
(87)

By substituting this expression into Eq. (86), we find after the integration that only \( \chi'(\omega) \) actually determines the spectral line shape:
\[
\overline{P(\omega)} = \frac{\chi_0}{4\pi} \omega \chi'(\omega) |H_1|^2
\]
(88)

When the lines are narrow, as is usually observed in ESR, \( \omega \) varies very little within the linewidth and so the spectral shape is well represented by \( \chi'(\omega) \).

In the case of the two-level system discussed above, one finds
\[
\chi'(\omega) = \frac{\chi_0}{4\pi} \omega \chi'(\omega) f(\omega)
\]
(89)
where \( \chi_0 \) is the static susceptibility and \( f(\omega) \) is the Lorentzian function described by Eq. (9) and illustrated in Fig. 1.

It will be noticed that the shape function \( f(\omega) \) is nothing else than the Fourier transform of the relaxation function \( \mathcal{M}_x(t) \) given in Eq. (84), describing the decay of the \( x \) component of the magnetization after the turning off of the rf field. These results are quite general in the framework of linear response theory (Abragam, 1961, p. 94). Relations like that given in Eq. (87) are expected to be obtained for the response of any system to monochromatic excitations, the exciting being in this case the rf field and the response the induced moment \( \mathcal{M}_x \). Furthermore, the Fourier transform is the general relation between the decay of the response measured in the time domain after the removal of the excitation, and the frequency dependence of the same physical property determined in the frequency domain under continuous excitation.

If the relaxation behavior of the system cannot be described by a single exponential decay, the absorption curve will be the superposition of Lorentzians of different widths (Freed and Fraenkel, 1963). The density matrix calculation relates the line-shape function to the microscopic structure of the spin system.

C. Relaxation Matrix and Correlation Functions

From the former discussion it is evident that the crucial point for the theoretical interpretation of the spectral line shapes is the computation of the relaxation matrix. We recall that Eq. (80) results from an approximate procedure that enable us to obtain the solutions for the density matrix of a system where a time-dependent perturbation \( \mathcal{W}(t) \) is acting. Given the random nature of the time dependence of \( \mathcal{W}(t) \), the effect of the perturbation on the system must be treated in a statistical way.
The general expression for $R_{\alpha \beta \gamma \delta}$ is found to be (Slichter, 1963)

$$R_{\alpha \beta \gamma \delta} = 2J_{\beta \gamma \delta \alpha}(\omega_{\eta \delta} - \delta_{\alpha \beta}) \sum_{\gamma} J_{\alpha \gamma \delta \eta}(\omega_{\eta \delta})$$

$$- \delta_{\alpha \beta} \sum_{\gamma} J_{\alpha \gamma \delta \eta}(\omega_{\eta \delta})$$

$$J_{\alpha \gamma \delta \eta}(\omega) = \frac{1}{2} \int_{-\infty}^{+\infty} G_{\alpha \gamma \delta \eta}(t) e^{-i\omega t} dt$$

$$G_{\alpha \gamma \delta \eta}(t) = h^{-2} \langle \mathcal{H}_1(0)_{\alpha \gamma} \mathcal{H}_1(t)_{\delta \eta} \rangle$$

(90)

Here $\mathcal{H}_1(t)_{\alpha \gamma}$ is a shorthand notation for the $\alpha \beta$ matrix element of $\mathcal{H}_1(t)$, the brackets mean the average over the statistical ensemble, and $\omega_{\eta \delta} = (E_{\eta} - E_{\delta})/\hbar$. $G(t)$ is called the correlation function of $\mathcal{H}_1(t)$. Even if the statistical average of $\mathcal{H}_1(t)$ is zero, the mean square interaction $\langle |\mathcal{H}_1(t)|^2 \rangle$ is not, and so $G(t)$ tells how the value of $\mathcal{H}_1$ at any time $t_0$ is correlated with values at later times $t_0 + t$. This function is independent of the time origin, due to the stationary nature of the random processes that cause the fluctuations of $\mathcal{H}_1$, as discussed later. The detailed time dependence of the correlation function depends on the physical model that is assumed to account for the main mechanism of modulation of $\mathcal{H}_1(t)$, e.g., anisotropic interactions modulated by rotational diffusion, but in general any statistical model leads to an exponential decay of the form

$$G(t) = h^{-2} \langle |\mathcal{H}_1|^2 \rangle e^{-\tau_\omega t}$$

(91)

where $\tau_\omega$ is the correlation time for the random motion.

We recall that $\mathcal{H}_1$ is a function of the Euler angles, and these are random functions of the time. Thus we need the definition of suitable probability distributions for the molecular orientations in order to be able to calculate the correlation function of the random function $\mathcal{H}_1(t)$.

The random processes that we shall encounter can always be described in terms of two probability distributions (Pedersen, 1972):

- $P(x, t) \, dx$, which is the probability of finding $x$ in the range $(x, x + dx)$ at time $t$;
- $P(x_1, t_1, x_2, t_2) \, dx_1 \, dx_2$, which is the joint probability of finding $x$ in the range $(x_1, x_1 + dx_1)$ at time $t_1$ and in the range $(x_2, x_2 + dx_2)$ at time $t_2$.

It is useful to define a conditional probability $P(x_1, t_1 \mid x_2, t_2)$ as the probability that, given $x_2$ at time $t_2$, one finds $x$ in the range $(x_2, x_2 + dx_2)$ at time $t_1$. The conditional probability is obviously related to the joint probability:

$$P(x_1, t_1, x_2, t_2) = P(x_1, t_1 \mid x_2, t_2) P(x_2, t_2)$$

(92)

2. GENERAL MAGNETIC RESONANCE THEORY

For any function $f(x)$ of the random variable $x$ we can now define the average value $\langle f(t) \rangle$ and the correlation function $\langle f(t_1) f^*(t_2) \rangle$:

$$\langle f(t) \rangle = \int f(x) P(x, t) \, dx$$

$$\langle f(t_1) f^*(t_2) \rangle = \int f(x_1) f^*(x_2) P(x_1, t_1, x_2, t_2) \, dx_1 \, dx_2$$

(93)

The random processes that we are interested in are stationary in character, which means that they are invariant under translation of the time axis. It follows that the distribution probability $P(x, t)$ is independent of time, and that the joint or conditional probability depends on the times $t_1$ and $t_2$ only through their difference $t_2 - t_1 = t$. Under these circumstances we have

$$\langle f \rangle = \int f(x) P(x) \, dx$$

$$\langle f(0) f^*(t) \rangle = \int f(x_1) f^*(x_2) P(x_1, 0, x_2, t) \, dx_1 \, dx_2$$

$$= \int dx_1 f(x_1) P(x_1) \int dx_2 f^*(x_2) P(x_1 \mid x_2, t)$$

$$\langle f(0) f^*(t) \rangle = \langle f(-t) f^*(0) \rangle = \langle f(0) f^*(-t) \rangle$$

(94)

The correlation time $\tau_\omega$ is defined by

$$\tau_\omega = \int_0^\infty \langle f(0) f^*(t) \rangle \, dt / \langle f^2 \rangle$$

(95)

The probability distribution functions are normalized to unity:

$$\int P(x) \, dx = 1, \quad \int P(x_1 \mid x_2, t) \, dx_2 = 1$$

(96)

The following equalities result from the properties of $P(x)$ and $P(x_1 \mid x_2, t)$:

$$\int P(x_1) P(x_1 \mid x_2, t) \, dx_1 = P(x_2)$$

$$\lim_{t \to \infty} P(x_1 \mid x_2, t) = P(x_2)$$

(97)

Therefore the correlation function goes to $\langle f \rangle^2$ for $t \to \infty$ and reduces to $\langle f^2 \rangle$ at $t = 0$. 

...
D. Hamiltonian in Spherical Basis

The Hamiltonian given in Eq. (63) is a function of the Euler angles, which are in turn random functions of time. The dependence on the angles can be made explicit by transforming the Cartesian tensors from space to molecular axes. The expression so obtained is rather cumbersome, and for our purposes it would be desirable to have the functional dependence in a more tractable form. This can be achieved if the tensors are expressed in a spherical basis instead of in the usual Cartesian set.

A spherical tensor of rank \( l \) is a quantity represented by \( 2l + 1 \) components that transform under rotation according to the law (Rose, 1957; Brink and Satchler, 1968)

\[
T^{(l, m)} = \sum_{m'} D^{m'}_{m}(\alpha \beta \gamma) T^{(l, m')}
\]

(100)

This transformation is analogous to the relation that gives the eigenfunctions of the angular momentum projection operator \( L_{z} \) in terms of the original eigenfunctions \( | l, m \rangle \) of \( L_{z} \) after a rotation of the quantization axis.

Since \( m, m' \) can only assume the values \( l, l - 1, \ldots, -l \), the coefficients \( D^{m'}_{m}(\alpha \beta \gamma) \) form, for a given value of \( l \), a matrix of dimension \( 2l + 1 \), which is known as a Wigner rotation matrix. For the inverse rotation one has

\[
T^{(l, m)} = \sum_{m'} D^{m'}_{m}(\alpha \beta \gamma) T^{(l, m')}
\]

\[
= \sum_{m'} (-1)^{m-m'} D^{m}_{m'}(\alpha \beta \gamma) T^{(l, m')}
\]

(101)

The Wigner rotation matrix elements \( D^{m'}_{m}(\alpha \beta \gamma) \) are found to have the following functional dependence on the Euler angles:

\[
D^{m'}_{m}(\alpha \beta \gamma) = e^{-im\phi} d_{m}(\beta) e^{-im\phi}
\]

(102)

The reduced rotation matrices are real functions (Table III) and obey the symmetry relations

\[
d_{m, m'}^{l} = d_{-m, -m'}^{l} = (-1)^{m-m'} d_{m, m'}^{l}
\]

The Wigner functions reduce in some special cases to spherical harmonics or to Legendre polynomials

\[
D^{l}_{m=0}(\alpha, \beta, 0) = \left[ \frac{4\pi}{2l + 1} \right]^{1/2} Y_{m}^{l}(\beta, \alpha)
\]

\[
D^{l}_{m=0}(0, \beta, \gamma) = (-1)^{m} \left[ \frac{4\pi}{2l + 1} \right]^{1/2} Y_{m}^{l}(\beta, \gamma)
\]

(103)

\[
D^{l}_{m=0}(0, \beta, 0) = P_{l}(\cos \beta)
\]

2. GENERAL MAGNETIC RESONANCE THEORY

TABLE III

<table>
<thead>
<tr>
<th>REDUCED WIGNER ROTATION MATRIX ELEMENTS ( d_{m, m'}^{l}(\beta) ) FOR ( l = 0, 1, \text{AND} 2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( d_{0,0}^{0} )</td>
</tr>
<tr>
<td>( d_{1,0}^{0} )</td>
</tr>
<tr>
<td>( d_{1,1}^{1} )</td>
</tr>
<tr>
<td>( d_{1,-1}^{1} )</td>
</tr>
<tr>
<td>( d_{0,0}^{0} )</td>
</tr>
<tr>
<td>( d_{2,0}^{0} )</td>
</tr>
<tr>
<td>( d_{2,1}^{1} )</td>
</tr>
<tr>
<td>( d_{2,-1}^{1} )</td>
</tr>
<tr>
<td>( d_{2,2}^{2} )</td>
</tr>
<tr>
<td>( d_{2,-2}^{2} )</td>
</tr>
<tr>
<td>( d_{2,0}^{0} )</td>
</tr>
<tr>
<td>( d_{2,2}^{2} )</td>
</tr>
<tr>
<td>( d_{2,-2}^{2} )</td>
</tr>
</tbody>
</table>

They form a complete orthogonal set and the following orthogonality relation holds:

\[
\int D_{m}^{l}(\alpha \beta \gamma) D_{m'}^{l}(\alpha \beta \gamma) \sin \beta \ d\beta \ d\gamma = \frac{8\pi}{2l + 1} \delta_{m,m'} \delta_{l,l'}.
\]

Finally, it should be remembered that the Wigner functions are the solutions of the Schrödinger equation for the symmetric rotator.

The relation between spherical components and Cartesian components of any tensor is easily found, beginning with the definition of the spherical components of a first-rank tensor and then by using the rule of the product of spherical tensors.

The spherical components of a first-rank tensor with Cartesian components \( T_{x}, T_{y}, \text{and} T_{z} \) are

\[
T^{(l, 1)} = -(1/\sqrt{2})(T_{x} + iT_{y})
\]

(105)

\[
T^{(l, 0)} = T_{z}
\]

\[
T^{(l, -1)} = (1/\sqrt{2})(T_{x} - iT_{y})
\]

Now a spherical tensor of rank \( l \) can be constructed from two tensors of ranks \( l_{1} \) and \( l_{2} \) according to the multiplication rule

\[
T^{(l, m)}(A, B) = \sum_{m_{1}} C(l_{1}, l_{2}; l; m_{1}, m - m_{1}) A^{(l_{1}, m_{1})} B^{(l_{2}, m_{2})}
\]

(106)

where \( C(l_{1}, l_{2}; l; m_{1}, m_{2}) \) is a Clebsch–Gordan coefficient (Rose, 1957), which vanishes unless \( |l_{1} - l_{2}| \leq l \leq |l_{1} + l_{2}| \). Note again that this relation is
simply a generalization of the product of two angular momentum eigenfunctions $|l_1 m_1\rangle$ and $|l_2 m_2\rangle$ to give the eigenfunctions of the total angular momentum operator $\vec{J}$. Numerical tables of the Clebsch–Gordan coefficients are found in the book by Heine (1964). By using Eq. (106) and the tabulated values of the $C$ coefficients, we can build up successively, from the first rank components $T^{(1,0)}$, the spherical components of any rank. In this way we have obtained the irreducible spherical components of rank zero, one, and two corresponding to a general second-rank Cartesian tensor of components $A_{ij}B_j$, and presented in the second column of Table IV.

Spherical tensors of the same rank can be contracted into an invariant according to the equation

$$A : B = \sum_{m} (-1)^{m} A^{(i,m)} B^{(i,-m)}$$  \hspace{1cm} (107)

This corresponds to the scalar product of Cartesian tensors, which for second-rank tensors assumes the form

$$A : B = \sum_{ij} A_{ij} B_{ij}$$  \hspace{1cm} (108)

The definitions used so far for Cartesian and spherical tensors can be extended directly to quantum mechanical operators. For example, the components $S_z$, $S_x$, and $S_y$ can be regarded as the Cartesian components of a first-rank tensor, and the corresponding spherical components, written in terms of the “shift” operators $S_\pm$, are

$$S^{(1,1)}_\pm = -(1/\sqrt{2})(S_x \pm iS_y) = -(1/\sqrt{2}) S_\pm$$  \hspace{1cm} (109)

Higher rank tensor operators are obtained in the same manner as just described. The third column of Table IV lists the components of rank zero, one, and two derived from the product of two first-rank spherical tensor operators. The two sets of expressions presented in Table IV are actually identical, but the spherical tensor operators are written for convenience in terms of the shift operators $A_z$ and $B_z$.

As already stated, the Hamiltonian must be invariant under rotation of the reference system and therefore it can be expressed as a sum of scalar products of tensors. In fact, the various terms of the magnetic Hamiltonian,

$$\mathbf{H} \cdot \mathbf{g} \cdot \mathbf{S} = \sum_{ij} H_{ij} g_{ij} S_j \quad \mathbf{I} \cdot \mathbf{A} \cdot \mathbf{S} = \sum_{ij} I_{ij} A_{ij} S_j$$

are the invariants obtained by the contraction of second-rank tensors with the irreducible spherical components corresponding to the second-rank Cartesian tensor $A_{ij}B_j$.

<table>
<thead>
<tr>
<th>$i, m$</th>
<th>$T^{(i,m)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 0</td>
<td>[ (1/\sqrt{3}) (A_{11} B_1 + A_{12} B_2 + A_{13} B_3) - (1/\sqrt{3}) (A_{31} B_1 + A_{32} B_2 + A_{33} B_3) ]</td>
</tr>
<tr>
<td>1, 1</td>
<td>[ -\frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) - \frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) ]</td>
</tr>
<tr>
<td>1, 0</td>
<td>[ -\frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) - \frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) ]</td>
</tr>
<tr>
<td>1, -1</td>
<td>[ \frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) - \frac{1}{2} (A_{11} B_1 - A_{31} B_3 + (A_{12} B_2 - A_{32} B_3)) ]</td>
</tr>
</tbody>
</table>

are the invariants obtained by the contraction of second-rank tensors with components $g_{ij}$ and $H_{ij}$, of $A_{ij}$ and $I_{ij}$, and of $D_{ij}$ and $S_j$, respectively.

In the spherical basis, according to Eq. (107), the total Hamiltonian is expressible in the form

$$\mathcal{H} = \sum_{i,m} (-1)^{m} T^{(i,-m)} A^{(i,m)}$$  \hspace{1cm} (110)

where $\mu$ specifies any particular interaction (Zeeman, hyperfine, etc.). $T^{(i,m)}$ are the irreducible spherical components related to the Cartesian tensor $\mu$, and $A^{(i,m)}$ are the corresponding spin operators.

It is logical to have the spin operators expressed in the laboratory coordinate system; thus the spherical tensors in Eq. (110) will be specified in this system. However, the magnetic interaction tensors are naturally defined in the molecular axis system, and so we shall express the $T^{(i,m)}$ components in terms of the components $F^{(i,m)}$ in the molecular system, according to the transformation law given in Eq. (101).

The magnetic Hamiltonian then takes the form

$$\mathcal{H} = \sum_{\mu} \sum_{i,m} (-1)^{m} F^{(i,-m)} D^{(i,m)} A^{(i,m)}$$  \hspace{1cm} (111)

The interactions we have considered are represented by second-rank Cartesian tensors; hence the index $i$ can only assume the values 0, 1, and 2. The explicit expressions for $F^{(i,m)}$ corresponding to a particular interaction $\mu$ can be derived from the second column of Table IV by setting $\mu = A_{ij}B_j$, whereas the most suitable choice for the operators $A^{(i,m)}$ are given by the third column, as already pointed out. From the table we see that all the $F^{(i,m)}$
terms vanish for symmetric tensors, which is our case. In addition, if the molecular axis system in which the magnetic interactions are expressed coincides with the principal axis system of the tensor \( \mu \), the off-diagonal elements \( \mu_{ij} \) are zero and a simpler expression for \( F^{(0)} \) results. A further simplification occurs if the tensor is axially symmetric because in this case only the \( F^{(0)} \) and \( F^{(3)} \) terms are left.

In solution, due to the molecular tumbling, the Wigner functions must be averaged over the probability distribution \( P(a|\beta) \) to obtain the probability of finding the molecular orientation defined within a volume element in the Euler space. For isotropic liquids this distribution is uniform and so all terms with \( l \neq 0 \) vanish because of the orthogonality properties of the rotation matrix components.

**E. Rotational Diffusion Model**

The functional form of the distribution functions needed to calculate the correlation function \( G(t) \) in Eq. (90) can be obtained starting from a reasonable model for the random process under investigation. Thus the random orientations of a molecule subjected to Brownian motion are usually described in terms of the rotational diffusion equation introduced by Debye (1945). If we denote the Euler angles that specify the orientation of the molecule relative to a fixed coordinate system by \( \Omega \), then the solution of the diffusion equation gives the probability \( P(\Omega, t) \) of finding the molecule within the solid angle \( d\Omega \) at time \( t \).

In the case of axially symmetric molecules, the diffusion equation is formally analogous to the Schrödinger equation for the symmetric rotator, where the rotational constants are replaced by the principal values \( D_{xx} \) and \( D_{yy} \) of the diffusion tensor (Freed, 1964). As already mentioned, the eigenfunctions of the symmetric rotator are the Wigner functions \( D_{lm}(\Omega) \), and the corresponding eigenvalues are

\[
\lambda_{lm} = -\left[ (j + 1)D_{xx} + (D_{yy} - D_{zz})m^2 \right] \tag{112}
\]

The general solution of the diffusion equation is therefore

\[
P(\Omega, t) = \sum_{l,m} c_{lm}(0)D_{lm}(\Omega) \exp(\lambda_{lm}t) \tag{113}
\]

The conditional probability is obtained by imposing the initial condition \( P(\Omega, 0) = \delta(\Omega - \Omega_0) \) and by using the expansion of the Dirac delta function on the complete orthogonal set of the \( D_{lm} \) functions:

\[
\delta(\Omega - \Omega_0) = \sum_{l,m} \frac{2j + 1}{8\pi^2} D_{lm}^*(\Omega_0)D_{lm}(\Omega) \tag{114}
\]

**2. General Magnetic Resonance Theory**

We obtain therefore for \( P(\Omega_0 | \Omega_t) \)

\[
P(\Omega_0 | \Omega_t) = \sum_{j,m} \frac{2j + 1}{8\pi^2} D_{lm}^*(\Omega_0)D_{lm}(\Omega) \exp(\lambda_{lm}t) \tag{115}
\]

According to Eq. (99), the distribution probability \( P(\Omega) \) is uniform and equal to \( 1/8\pi^2 \), as expected. As we shall see in next subsection, the relevant quantities to be evaluated are the correlation functions of the Wigner matrices, which in Eq. (114) carry out the transformation of the laboratory-fixed to the molecule-fixed coordinate system. We have then, using the orthogonality properties of the Wigner functions,

\[
\langle D_{lm}^*(\Omega)D_{lm}(\Omega) \rangle = \int d\Omega_0 D_{lm}^*(\Omega_0)P(\Omega_0 | \Omega_t) \prod \int d\Omega_0 P(\Omega_0 | \Omega_t) = \langle D_{lm}^*(\Omega_0)D_{lm}(\Omega_0) \rangle \exp(\lambda_{lm}t) \tag{116}
\]

The correlation function decays exponentially with a characteristic time \( \tau_{lm} = -1/\lambda_{lm} \). The absolute sign for \( t \) is taken to satisfy the conditions expressed by Eq. (95). We can note that for an isotropic molecular diffusion, \( D_{ij} = D \) and the correlation functions of all the Wigner rotation matrix components corresponding to the same value of \( j \) decay with the same characteristic time \( \tau_{lm} = [j(j + 1)D]^{-1} \), where \( D \) is the diffusion constant. The values of the principal components of the diffusion tensor for the case of nonspherical molecules can be calculated from the molecular dimensions according to formulas reported by Freed (1964).

**F. Linewidth Parameters**

In this subsection we derive the linewidth parameters \( A, B, \) and \( C \) for a free radical in a liquid medium. We must calculate those elements \( R_{x,y,z} \) of the relaxation matrix that are involved in the determination of the \( x \) component of the magnetization. Equations (74) and (78) show that the indices \( a, a' \) and \( \beta, \beta' \) must correspond to states involved in allowed ESR transitions, the only ones that are connected by the operator \( S_x \). It follows therefore from the condition \( E_a - E_{a'} = E_{\beta} - E_{\beta'} \) that the relaxation matrix will be diagonal if there is no degeneracy in the ESR transitions. The simplest case for which this condition is satisfied is the case of an electron spin interacting with a single nuclear spin, and this will be treated in detail. We shall further assume that the paramagnetic molecules have an axially symmetric diffusion tensor, and the \( g \) factor and hyperfine anisotropies are the only (or the major) causes of spin relaxation. In this way we neglect fluctuating solvent interactions or molecular geometry variations, which can modify...
the instantaneous values of the magnetic parameters. Under these assumptions we have for the perturbing Hamiltonian \( \mathcal{H}_1(t) \)

\[
\mathcal{H}_1(t) = \sum_{\mu} \sum_{m,m'} (-1)^m F_{\mu}^{m-m'} D_{m,m'}^2(t) A^{(m)}_{\mu} 
\]  

(117)

The correlation function of \( \mathcal{H}_1(t) \) is then

\[
G_{\alpha \beta}(t) = \hbar^{-2} \sum_{\mu} \sum_{m,m'} (-1)^m F_{\mu}^{m-m'} D_{m,m'}^2(t) A^{(m)}_{\mu} 
\]

\[
\times \langle \alpha | A^{(m)}_{\alpha} | \alpha' \rangle \langle \beta | A^{(-m')}_{\beta} | \beta \rangle 
\]  

(118)

All the relevant interactions are described by second-rank tensors, and so the index 2 has been omitted for simplicity. In the above expression \( g_{\alpha \beta}(t) \) stands for the correlation function of the rotation matrix component \( D_{2\alpha \beta}(\alpha \beta) \). Cross-correlation terms of the type \( \langle D_{2\alpha \beta}(\alpha \beta) D_{2\alpha \beta}(\alpha \beta') \rangle \) vanish according to Eq. (116).

Let \( j_{\alpha \beta}(\omega) \) be the Fourier transform of the correlation function \( g_{\alpha \beta}(t) \). From Eq. (116) we have

\[
j_{\alpha \beta}(\omega) = \frac{1}{2} \int_{-\infty}^{\infty} \frac{1}{\tau_m} \exp(-|t|/\tau_m) \exp(-i \omega t) dt = \frac{1}{2} \tau_m/(1 + \omega^2 \tau_m^2)
\]  

(119)

Finally we write for \( J(\omega) \) of Eq. (90)

\[
J_{\omega \alpha \beta}(\omega) = \hbar^{-2} \sum_{\mu} (-1)^m F_{\mu}^{m-m'} F_{\mu}^{-(m')} j_{\alpha \beta}(\omega)
\]

\[
\times \langle \alpha | A^{(m)}_{\alpha} | \alpha' \rangle \langle \beta | A^{(-m')}_{\beta} | \beta \rangle 
\]  

(120)

The matrix elements of the spin operators on the basis of eigenfunctions of \( \mathcal{H}_0 \) are promptly calculated by standard methods. We note that the contributions to the relaxation matrix \( R_{\omega \alpha \beta} \) can be conveniently divided into three groups of terms, which are called secular, pseudosecular, and nonsecular terms. The first come from those parts of \( \mathcal{H}_1(t) \) that commute with the unperturbed Hamiltonian \( \mathcal{H}_0 \), and so have only diagonal matrix elements on the basis of the eigenfunctions of \( \mathcal{H}_0 \). Spin operators of the type \( H_S, S^2 \) and \( I^2 \) satisfy this requirement, and for these terms \( \omega_{\alpha \beta} = 0 \). Pseudosecular terms result from spin operators, such as \( I_z S_z \), that connect spin states whose energy difference is the hyperfine coupling constant \( a \), or \( h\omega_d \) if \( \omega_d \) is the hyperfine splitting in angular frequency units. The nonsecular part of \( \mathcal{H}_1(t) \) contains the spin operators \( S_k \) that have matrix elements between states with energy difference \( h\omega_m \), where \( \omega_m \) is the resonance frequency. This distinction is very important, because \( a \) is typically two orders of magnitude less than \( g\beta H_0 = h\omega_m \), and so, except for very rapid motions, it may happen that \( a \gg 1 \) and \( \omega_m \gg 1 \). In this case we see from Eq. (119) that secular and pseudosecular terms give comparable contributions, and the nonsecular terms can be neglected.

After calculation for all the contributions to the relaxation matrix, the width of the spectral line corresponding to the value \( M \) of the nuclear projection quantum number is found to be

\[
[T_2(M)]^{-1} = \frac{1}{2} \sum_{\alpha \beta} \sum_{m} (-1)^m F_{\alpha}^{m}(F_{\beta}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2
\]

(121)

\[
A = \frac{1}{2} \hbar^2 \sum_{\mu} (-1)^m F_{\mu}^{m}(F_{\mu}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2
\]

+ \frac{1}{2} \hbar I(t+1) \sum_{\mu} (-1)^m F_{\mu}^{m}(F_{\mu}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2

B = \frac{1}{2} \hbar^2 \sum_{\mu} (-1)^m F_{\mu}^{m}(F_{\mu}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2

C = \frac{1}{2} \hbar^2 \sum_{\mu} (-1)^m F_{\mu}^{m}(F_{\mu}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2

- \frac{1}{2} \hbar I(t+1) \sum_{\mu} (-1)^m F_{\mu}^{m}(F_{\mu}^{-m}) |j_{\alpha \beta}(0) + 3 j_{m}(\omega_0)|^2

(122)

The presence of a linear and a quadratic term in the expression for \( [T_2(M)]^{-1} \) implies an asymmetric linewidth variation about the center of the spectrum. Examples of this behavior have been seen in the nitroxide free-radical spectra.

For moderately fast correlation time the nonsecular terms can be neglected, and \( j_{\alpha \beta}(\omega_\alpha) \approx j_{\alpha \beta}(0) \). In this case, if an approximate spherical symmetry of the diffusion tensor can be assumed, the following simplified expressions result for the linewidth parameters \( A, B, \) and \( C \):

\[
A/\tau_c = \frac{2}{15} \beta^2 h^{-2} H_0^2 (g': g')
\]

+ \frac{1}{20} \hbar I(t+1) \langle A': A' \rangle

B/\tau_c = \frac{4}{15} \beta, h^{-2} H_0^2 (g': A')

C/\tau_c = \frac{1}{12} \hbar^{-2} \langle A': A' \rangle

\tau_c = \frac{[6D]}{[123]}

where the scalar product of second-rank spherical tensors has been written in terms of the Cartesian tensors according to Eqs. (107) and (108). The expression for \( B \) and \( C \) used in Section IV.E are promptly derived from Eq. (123) after some simple manipulation.

The complete form of the relaxation matrix for the more general case of an electron spin interacting with several nonequivalent nuclei has been derived by Freed and Fraenkel (1963).
Theory of Slow Tumbling ESR Spectra for Nitroxides

JACK H. FREED
DEPARTMENT OF CHEMISTRY
CORNELL UNIVERSITY
ITHACA, NEW YORK

I. Introduction
53
II. Theory
55
A. General Method
55
B. Rotational Modulation in Isotropic Liquids
60
C. Anisotropic Liquids
63
D. Exchange and Slow Tumbling
67
E. Nitroxides
69
III. Applications
71
A. Isotropic Liquids: Experiments
71
B. Simplified Methods of Estimating \( \tau_0 \)
83
C. Very Anisotropic Rotational Reorientation
91
D. Anisotropic Liquids: Simulations
96
E. Anisotropic Liquids: Experiments
104
F. Saturation and Nonlinear Effects
109
Appendix A. General Solutions and Discussion of the Computer Program for Nitroxides
112
Appendix B. Computer Program for Slow Tumbling Nitroxides in Isotropic Liquids
121
References
130

I. INTRODUCTION

In this chapter we develop the theory for slow tumbling in ESR spectroscopy, with specific application to nitroxide free radical spectra. The slow tumbling region is that range of rotational reorientation times for which the
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

II. THEORY

A. General Method

A quantum mechanical wave function $\Psi$ can be expanded in a complete set of orthonormal functions $U_n$ as

$$\Psi(t) = \sum_n c_n(t) U_n$$

(1)

The density matrix is defined to be

$$\rho_{mn}(t) = c_m(t) c_n^*(t)$$

(2)

where the bar indicates an average over a statistical ensemble. A useful property of the density matrix is the calculation of the expectation value of an operator $O$ for a system described by the wave function $\Psi$. Thus

$$\langle \Psi | O | \Psi \rangle = \sum_{m,n} \langle U_m | O | U_n \rangle \langle U_n | \Psi \rangle = \sum_{m,n} c_n(t) c_m^*(t) O_{mn}$$

(3a)

and for an average expectation value of an ensemble of such systems we have

$$\langle \langle \Psi | O | \Psi \rangle \rangle = \sum_{m,n} \rho_{mn} O_{mn} = \text{Tr} \rho O$$

(3b)

where Tr is the trace. The trace is invariant to the choice of the complete orthonormal basis set. Thus all the information for calculating observable quantities is contained in the density matrix.

Since the wave function $\Psi$ will vary with time, the coefficients $c_n(t)$ will be functions of time, and this time dependence can be obtained from the time-dependent Schrödinger equation. Then the density matrix equation of motion, assuming the same Hamiltonian $\mathcal{H}(t)$ for all members of the ensemble, is given by the quantum mechanical Liouville equation

$$\frac{\partial \rho}{\partial t} = -i [\mathcal{H}(t), \rho]$$

(4)

Now assume that the time dependence of the spin Hamiltonian $\mathcal{H}(t)$ for a free radical arises from interactions with its environment such that $\mathcal{H}(t)$ is fully determined by a complete set of random variables $\Omega$. Also assume that this time dependence of $\Omega$ is described by a stationary Markov process, so that the probability of being in a state $\Omega_1$ at time $t$, if in state $\Omega_2$ at time $t - \Delta t$, is (a) independent of the value of $\Omega$ at any time earlier than $t - \Delta t$ and (b) depends only on $\Delta t$ and not on $t$. A stationary Markov process can be described by a differential equation

$$\frac{\partial P(\Omega, t)}{\partial t} = -\Gamma \rho P(\Omega, t)$$

(5)

where $P(\Omega, t)$ is the probability of the free radical being in a state $\Omega$ at time $t$. 

---

ESR spectrum can no longer be described as a simple superposition of Lorentzian lines, characteristic of the fast motional or motional narrowing region, for which the theory developed in the previous chapter applies. In this chapter we further take it to be the region for which the ESR spectrum still shows effects of the motion; i.e., the motion is not so slow as to yield a proper rigid-limit spectrum. For nitroxide free radicals, this usually means we are considering the range of rotational correlation times $10^{-9}$ sec $\leq t_{\tau} \leq 10^{-6}$ sec. This is an important range for nitroxide probes in viscous media or for nitroxide spin labels attached to large macromolecules.

In this region, the spectra are affected in a complicated way by both the motions and the magnetic spin interactions. As a result, a theory which can deal rigorously with describing this region must be both powerful and general. It is our objective to set forth this theory, which has been developed in the last few years, and to emphasize its general foundations. The general theory is presented in Section II.A. It is a characteristic of the slow motional region that it requires that we ask more intricate questions about the detailed nature of the molecular motions in order to properly analyze the spectra than is true for studies in the motional narrowing region. Thus in much of the remainder of Section II the dynamics of molecular motions is developed to an extent consonant with the need to explain actual slow tumbling experiments.

It has also been our objective to demonstrate how the general theory can be applied to actual cases. Thus in Section III we give a detailed discussion of comparisons between the theoretical predictions and recent experimental studies. Wherever it appears reasonable, we have tried to indicate simplified approaches in the analysis of slow tumbling spectra short of running detailed computer simulations. Nevertheless, there are many cases where the researcher will need detailed simulations tailored to his specific needs. For that reason, we have, in Appendix B, supplied the computer program that is applicable to isotropic liquids.

The development of the theory and the specific examples given here draw most heavily on the recent work of the Cornell group, with which the author is most familiar. Detailed references to other work can be found in a recent review article (Freed, 1972b). Recently, Polnánek (1975b) has reviewed our methods and compared them with the other approaches. It should be emphasized that the slow tumbling theory is in many ways based on a generalization of stochastic theories of jump models for magnetic resonance (Abragam, 1962; Kubo, 1962, 1969; Johnson, 1965) and we sometimes make use of the analogies when appropriate. A familiarity with motional narrowing theory, such as is discussed in Chapter 2, would be useful preparation for this chapter, and a familiarity with quantum mechanics and some statistical mechanics is assumed in Section II.
Since the process is assumed stationary, \( \Gamma_0 \) is independent of time. The evolution operator \( \Gamma_0 \) is an operator only on the random variables \( \Omega \) and is independent of spin space. \( \Gamma_0 \) includes such general Markov operators as the diffusion operators given by the Fokker–Planck equations and transition rate matrices among discrete states \( \Omega_1, \Omega_2, \ldots, \Omega_n \). In this discussion, \( \Omega = \alpha, \beta, \gamma \) will represent Euler angles specifying orientation and \( \Gamma_0 \) will be a rotational diffusion operator.

It is also assumed that the stochastic process has a unique equilibrium distribution \( P_0(\Omega) \) characterized by

\[
\Gamma_0 P_0(\Omega) = 0
\]  

We can show (Kubo, 1969; Freed et al., 1971; Freed, 1972a) that Eqs. (4)–(6) lead to the stochastic Liouville equation of motion

\[
\frac{\partial \rho(\Omega, t)}{\partial t} = -i[H(\Omega), \rho(\Omega, t)] - \Gamma_0 \rho(\Omega, t)
\]  

where \( \rho(\Omega, t) \) is now understood to be the value of \( \rho \) associated with a particular value of \( \Omega \), hence of \( \hat{H}(\Omega) \). Thus, instead of looking at the explicit time dependence of the spin Hamiltonian \( \hat{H}(t) \) involving the interaction with its environment, the spin Hamiltonian is written in terms of random angle variables \( \Omega_1, \Omega_2, \ldots, \Omega_n \) and its modulation (due to rotational motions) is expressed by the time dependence of \( \Omega \).

The steady-state spectrum in the presence of a single rotating microwave frequency field is determined by the power absorbed from this field. We find for the 4th hyperfine line at an orientation specified by \( \Omega \)

\[
P_4(\Omega) = 2N\hbar d_4 Z_4^{(1p)}(\Omega)
\]  

where \( P_4(\Omega) \) is the power absorbed, \( N \) is the concentration of electron spins, \( d_4 \) is a "transition moment" given by

\[
d_4 = \frac{1}{2} \gamma_1 H_1 \langle \lambda^+ | S^- | \lambda^+ \rangle
\]  

(\( S^- \) is the electron spin-lowering operator and \( \lambda^\pm \) are the \( M_S = \pm \frac{1}{2} \) states of the electron spin for the \( \lambda \)th transition), and \( Z_4^{(1p)} \) is defined by the series of expressions

\[
Z_4^{(1p)} = \sum_{n=-\infty}^{\infty} \text{exp}(in\omega t) Z_4^{(1p)}
\]

and

\[
Z_4^{(1p)} = Z_4^{(1p)} + i Z_4^{(1p)}
\]  

(Actually, the experimentally observed signal is proportional to \( Z_4^{(1p)} \) and not to \( d_4 Z_4^{(1p)} \)).

3. Theory of slow tumbling ESR spectra for nitroxides

In Eq. (5), \( \rho_0(\Omega) \) is the equilibrium spin density matrix. Equation (8) displays the fact that it is the \( n = 1 \) harmonic, i.e., the component rotating with the microwave field, that is directly observed. In the case of simple lines we can identify \( Z_4^{(1p)} \) and \( Z_4^{(1p)} \) with the magnetization components \( M_x \) and \( M_y \) for the 4th line in the rotating frame.

The notation for a matrix element of an operator \( O \) is

\[
O_{ab} = \langle a | O | b \rangle
\]  

\[
O_1 = \langle \lambda^- | O | \lambda^+ \rangle
\]  

\[
O_{\lambda 2} = \langle \lambda^+ | O | \lambda^- \rangle
\]

where \( a, b \) are eigenstates and \( \lambda^+, \lambda^- \) are, respectively, the upper and lower electron spin states between which the \( \lambda \)th ESR transition occurs. For a nitroxide there are three allowed ESR transitions.

The total absorption is then obtained as the equilibrium average of Eq. (8) over \( \Omega \). Thus averages are introduced such as

\[
\langle Z_{\lambda 4}^{(1p)} \rangle = \int d\Omega Z_{\lambda 4}^{(1p)}(\Omega) P_4(\Omega)
\]

so that

\[
P_{\lambda} = 2N\hbar d_4 \langle Z_{\lambda 4}^{(1p)} \rangle
\]

where \( d_4 \) has been taken to be independent of orientation. The total spin Hamiltonian \( \hat{H}(t) \), expressed in angular frequency units, is now separated into three components,

\[
\hat{H}(t) = \hat{H}_0 + \hat{H}_1(\Omega) + \hat{H}_2(t)
\]

In the high-field approximation

\[
\hbar \hat{H}_0 = g e H_0 \hat{S}_z - \hbar \sum_i \gamma_i \hat{I}_i H_0 - \hbar \sum_i a_i \hat{S}_i \hat{I}_i
\]

yields the zeroth-order energy levels and transition frequencies (cf. Chapter 2). \( \hat{H}_1(\Omega) \) is the perturbation depending on the orientation angles \( \Omega \), and, being a scalar, can be expressed as the scalar product of two tensors. That is, in general, we write \( \hat{H}_1(\Omega) \) as [in the notation of Freed and Fraenkel (1963)]

\[
\hat{H}_1(\Omega) = \sum_{l, n, m, n', m'} \hat{S}_{n, m, n', m'}(\Omega) F_{\mu, \nu}^{(l, m)} A_{\mu, \nu}^{(l, m')}
\]

where the \( F_{\mu, \nu}^{(l, m)} \) and \( A_{\mu, \nu}^{(l, m')} \) are irreducible tensor components of rank \( L \) and component \( m \), with the \( F \) being spatial functions in molecule-fixed coordinates, while \( A \) consists only of spin operators quantized in the laboratory axis system. The subscripts \( \mu \) and \( \nu \) refer to the type of perturbation and
to the different nuclei, respectively. The Wigner rotation matrix elements 
$R_{m,n}^{m,n}(\Omega)$ include the transformation from the molecule-fixed axis system 
$(x', y', z')$ into the laboratory axis system $(x, y, z)$. We shall be concerned 
with the $A$ and $g$ tensors, for which $L = 2$. [It has been found that effects of 
the $^{14}N$ quadrupole tensor is negligible (Goldman et al., 1972a; Goldman, 
1973).] In addition,

$$\hat{t}(t) = \begin{pmatrix} 1 + \hat{S}_+ \exp(-i\omega t) + \hat{S}_- \exp(i\omega t) \end{pmatrix}$$

is the interaction of the electron spin with the oscillating magnetic radiation field. [When more than one oscillating field, e.g. ELDOR or ENDOR, is 
present and/or when field modulation effects are to be explicitly incorporated 
then Eq. (18) may be appropriately modified to include these effects.]

When we take the $\langle \xi^- | \xi^+ \rangle$ matrix elements of Eq. (7) and utilize 
Eqs. (9)–(11), we find the steady-state equation for $Z^{\xi^0}(\Omega)$ to be

$$\omega - \omega_0)Z^{\xi^0}(\Omega) + \left[ \hat{\mathcal{P}}(\Omega), Z^{\xi^0}(\Omega) \right]_{\xi^0} - \left[ \mathbf{G}_m(\omega) Z^{\xi^0}(\Omega) + d_{\xi^0} \frac{\chi_{\xi^0}}{\chi_{\xi^0}} - \frac{\chi_{\xi^0}}{\chi_{\xi^0}} \right] = \frac{\omega_0}{\omega} d_{\xi^0}$$

(19)

[The superscripts to $\chi_{\xi^0}$ refer to harmonics in the sense of Eq. (10). The 
harmonic components of any other oscillating fields present may be 
introduced in a similar manner.] For reasonable temperatures and typical 
ESR field strengths we may write $\rho_0 = N'^{ -1} - qA\rho_0$, where $N'$ is the 
number of spin eigenstates of $\mathcal{H}_0$ and $q = \hbar/NkT$. Also, $\delta_{\xi^0} = E_{\xi^0} - E_{\xi^-}$ 
and the $Z^{\xi^0}(\Omega)$ are spin matrices defined by Eqs. (9)–(11), and the $E_{\xi^\pm}$ are 
the eigenenergies of $\mathcal{H}_0$ for the $\xi^\pm$ states.

It is convenient at this point to introduce a "symmetrizing" transformation 
for the evolution operator. It is not needed for isotropic liquids but 
becomes useful for anisotropic liquids. Thus

$$\mathbf{G}(p) = P_0^{1/2} \mathbf{G}(p) P_0^{-1/2}$$

(20)

and similarly

$$Z^{\xi^0}(\Omega) = P_0^{-1/2} \left[ Z^{\xi^0}(\Omega) \right]_{\xi^0}$$

(21)

This transformation usually renders $\mathbf{G}(p)$ Hermitian. Now, Eqs. (6) and (20) 
combine to give

$$\mathbf{G}(p) P_0^{1/2} = 0$$

(22)

Equations (21) and (13) yield

$$Z^{\xi^0}_l \left( \Omega \right) = \int d\Omega P_0^{1/2} Z^{\xi^0}_l(\Omega)$$

(23)

and Eq. (19) with Eqs. (20)–(21) becomes

$$(\omega - \omega_0) \left[ Z^{\xi^0}_l(\Omega) + \left[ \hat{\mathcal{P}}_l(\Omega), Z^{\xi^0}_l(\Omega) \right]_{\xi^0} - \left[ \mathbf{G}_m(\omega) Z^{\xi^0}_l(\Omega) + d_{\xi^0} \frac{\chi_{\xi^0}}{\chi_{\xi^0}} - \frac{\chi_{\xi^0}}{\chi_{\xi^0}} \right] = \frac{\omega_0}{\omega} d_{\xi^0} P_0^{1/2}$$

(24)

In order to solve for the absorption, Eq. (23), we first solve the diffusion 
equation (5). As in quantum mechanics, the solution of such a partial differential 
equation can be expressed in terms of a complete orthonormal set of 
eigenfunctions, call them $G_m(\omega)$, such that

$$\mathbf{G}(\omega) = P_0^{1/2}$$

(25)

where $\tau_m^{-1}$ is the $m$th "eigenvalue." We generally find

$$G_m(\omega) = P_0^{1/2}$$

(26)

Then we expand matrix elements of $Z^{\xi^0}(\Omega)$ in the complete orthonormal set 
$G_m(\omega)$:

$$Z^{\xi^0}_l(\Omega) = \sum_m \left[ C_m(\omega) \right]_{\xi^0} G_m(\omega)$$

(26)

where the coefficient $C_m(\omega)$ is an operator in spin space and is a function of 
$\omega$, but is independent of $\Omega$.

Substituting Eq. (26) into Eq. (24), premultiplying the resulting equation 
by $G_m(\omega)$, and then integrating over $\Omega$ and taking advantage of the orthonormal 
properties of $G_m(\omega)$, we obtain:

$$[(\omega - \omega_0) - i\tau_m^{-1} \left( C^{\xi^0}_m(\omega) \right)]_{\xi^0} + \left[ \int d\Omega G_m(\omega) \left[ \hat{\mathcal{P}}_l(\Omega), C_m(\omega) \right] \right]_{\xi^0} + d_{\xi^0} \frac{\chi_{\xi^0}}{\chi_{\xi^0}} - \frac{\chi_{\xi^0}}{\chi_{\xi^0}} = \omega_0 d_{\xi^0}$$

(27)

Since the absorption, Eq. (14), depends only on $Z^{\xi^0}_l$, then solving Eq. (27) 
for $[C^{\xi^0}_m(\omega)]$ for all allowed transitions will give the spectral line shapes.

In the absence of saturation we can set $d_{\xi^0} = 0$ on the left-hand side of 
Eq. (27) and then let $n = 1$, to obtain the needed expression.

A rotationally invariant Lorentzian linewidth $T^{-1}_2$ can be included in 
Eq. (19) or Eq. (27) by letting

$$\omega_0 \rightarrow \omega_0 + iT^{-1}_2$$

(28)

In the near-rigid limit, this linewidth corresponds to the linewidth in a single 
crystal, or more precisely to a residual linewidth in a powder spectrum.

More generally, an angular variation of the width can be introduced. For 
simplicity, this variation is allowed to take the form

$$T^{-1}_2(\theta) = \alpha + \beta \cos^2 \theta$$

(29)
where $\theta$ is the angle between the magnetic field axis and the molecular $z$ axis.

The above equations yield coupled complex algebraic equations for the coefficients $[C_{0}^{2n}]_{n}$, and we attempt to solve for these equations utilizing only a finite number of coefficients. (The complete orthonormal set includes an infinite number of such eigenfunctions.) The convergence depends essentially on the ratio $|E_{n}^{(i)}|/\alpha_{n}^{-1}$. The larger the value of this ratio of off-diagonal to diagonal terms, the more terms $C_{0}^{2n}$ that are needed. The results obtained by relaxation theory valid in the fast motional limit (cf. Chapter 2) are recovered when only one order beyond $[C_{0}^{2}]_{n}$ is included.

**B. Rotational Modulation in Isotropic Liquids**

When the general method of the previous section is applied to rotational modulation, $\Omega$ refers to the Euler angles for a tumbling molecular axis with respect to a fixed laboratory axis system. For a molecule undergoing many collisions, causing small random angular reorientations, the resulting isotropic Brownian rotational motion is a Markov process, which can be described by the rotational diffusion equation (Freed et al., 1971; Freed, 1972a,b).

$$\partial P(\Omega, t)/\partial t = R V_{\Omega}^{2} P(\Omega, t) \tag{30}$$

where $V_{\Omega}^{2}$ is the Laplacian operator on the surface of a unit sphere and $R$ is the rotational diffusion coefficient. If the molecule is approximated by a rigid sphere of radius $a$ rotating in a medium of viscosity $\eta$, then a rotational Stokes–Einstein relationship yields

$$R = kT/8\pi a^{3}\eta \tag{31}$$

In an isotropic liquid, the equilibrium probability $P \Omega(\Omega)$ of Eq. (6) will be equal for all orientations, so that $P \Omega(\Omega) = 1/[8\pi^{2}]$. Here the Markov operator $\Gamma_{\Omega}$, Eq. (5), for isotropic Brownian rotation, Eq. (30), is $-RV_{\Omega}^{2}$, which is formally the Hamiltonian for a spherical top whose orthonormal eigenfunctions are the normalized Wigner rotation matrices or generalized spherical harmonics:

$$G_{n} \rightarrow \phi_{n,n}(\Omega) = [(2L + 1)/8\pi^{2}]^{1/2} Y_{n,m}(\Omega) \tag{32}$$

with eigenvalues $R(L + 1)$ (Freed, 1964, 1972a). Note that for $K = 0$, $Y_{n,m}(\Omega, \theta, \phi) = [4\pi/(2L + 1)]^{1/2} Y_{n,m}(\theta, \phi)$, where $Y_{n,m}$ is the well-known spherical harmonic (Rose, 1954; Edmonds, 1957).

Similarly, the Markov operator for axially symmetric Brownian rotation about a molecule-fixed $z$ axis is formally the Hamiltonian for a symmetric top whose symmetry axis is the $z$ axis. The orthonormal eigenfunctions are again the normalized Wigner rotation matrices with eigenvalues given by

$$\Gamma_{\Omega} \phi_{n,n}(\Omega) = [R_{z} L(L + 1) + (R_{x} - R_{y})K^{2}] \phi_{n,n}(\Omega) \tag{33}$$

where $R_{z}$ and $R_{y}$ are the rotational diffusion constants about the $x$, $y$ axes and $z$ axis, respectively (Freed, 1964; Favro, 1965). The "quantum numbers" $K$ and $M$ of the Wigner rotation matrices refer to projections along the body-fixed symmetry axis and along a space-fixed axis, respectively.

For completely asymmetric Brownian rotation (Freed, 1964, 1972a; Favro, 1965) $R_{x} \neq R_{y} \neq R_{z}$, where $R_{x}$, $R_{y}$, and $R_{z}$ are the respective rotational diffusion constants about the $x$, $y$, and $z$ axes, the Markov operator $\Gamma_{\Omega}$ has more complex solutions (see below). Also, for fast rotation about one axis (e.g., the $z$ axis, so that $R_{x} \gg R_{y}$, $R_{z}$), the completely asymmetric rotation can be treated as axially symmetric with eigenvalues $R_{z} L(L + 1) + (R_{x} - R_{y})K^{2}$, where $R_{x} = 1/(R_{x} + R_{y})$.

In the fast rotational motion region, all rotational reorientation processes yield the same ESR line shapes, which, as predicted by earlier relaxation theories, yields Lorentzian lines (Abragam, 1961; Freed and Fraenkel, 1963). Naturally, in the very slow rotational motion region all models should tend to the rigid-limit powder line shape of the equilibrium distribution. However, in the intermediate slow rotational region $|E_{n}^{(i)}|/\alpha_{n} \geq 1$, the ESR line shapes are found to be sensitive to the details of the molecular reorientation process.

A number of different models for rotational reorientation can be proposed. Some useful ones are: (a) Brownian rotational diffusion; (b) free diffusion in which a molecule rotates freely for time $\tau$ (i.e., inertial motion with $\tau = I/B$, with $I$ the moment of inertia and $B$ the friction coefficient) and then reorients instantaneously; and (c) jump diffusion in which a molecule has a fixed orientation for time $\tau$ and then "jumps" instantaneously to a new orientation (Goldman et al., 1972; Egelstaff, 1970). For isotropic reorientation, we can summarize the results for these models as

(a) $\tau_{L}^{-1} = L(L + 1)R \tag{34}$

(b) $\tau_{L}^{-1} = L(L + 1)R/(1 + L(L + 1)\tau_{L})^{1/2} \tag{35}$

and

(c) $\tau_{L}^{-1} = \tau^{-1} \left[ 1 + (2L + 1)^{-1} \int_{0}^{1} dW(\alpha) \right]^{1/2} \tag{36}$

where $W(\alpha)$ is the distribution function for diffusive steps by angle $\alpha$ and is normalized so that

$$\int_{0}^{1} W(\alpha) d\alpha = 1 \tag{37}$$
One convenient form for $W(e)$ is

$$W(e) = A \sin(e) \exp(-e/\theta)$$  \hspace{1cm} (38)

where $A$ is a normalization constant. For $\theta < \pi$, we obtain

$$\tau_2^{-1} = L(L + 1)R/L[1 + R_t(L + 1)]$$  \hspace{1cm} (39)

and

$$(e^2)_{\text{avg}} = 6\theta^2$$  \hspace{1cm} (40)

where $R_t$ is proportional to the size of a mean diffusive step when the diffusion coefficient is defined as

$$R = (e^2)_{\text{avg}}/6$$  \hspace{1cm} (41)

Other possible choices for $W(e)$ are summarized elsewhere (Goldman et al., 1972). A good formal theoretical discussion of jump diffusion as has been employed here is given by Cukier and Lakatos-Lindenberger (1972); also the basic work of Ivanov on jump diffusion has recently been reviewed by Valiev and Ivanov (1973).

It should be noted that Eq. (35) is only an approximate expression for free diffusion. Since free diffusion includes inertial effects, the orientation of the molecule is not properly described as a simple Markovian process. A more accurate treatment of free diffusion must include angular momentum as well as orientational degrees of freedom, but recent work by Bruno and Freed (1974b) shows that the results for the more complete formulation of free diffusion are similar to those obtained using the simple model.

A comparison of Eqs. (34)–(36) shows that the $L$ dependence of $\tau_2$ depends on the choice of reorientational model. Thus in the slow motional region, where the line shape is simulated in terms of an expansion in $\delta_{k,m}^L$ with eigenvalues $\tau_{2,k}^{-1}$, the ESR spectra will be model sensitive. We can summarize these equations with the simple expression

$$\tau_2^{-1} = B_2 L(L + 1)R$$  \hspace{1cm} (42)

with the \textit{model parameter} $B_2 = 1$ for Brownian motion; $B_2 = [1 + L(L + 1)]^{-1}$ for strong jump diffusion with $R_t = 1$; and $B_2 = [1 + L(L + 1)]^{-1/2}$ for free diffusion and $R_t = 1$. For purposes of comparison, the definition of $\tau_2$ is generalized to

$$\tau_k = (6B_k R)^{-1}$$  \hspace{1cm} (43)

where $B_k$ is the appropriate model parameter for $L = 2$. Thus in the motionally narrowed region, where only the $L = 2$ term is important, it follows from Eqs. (42) and (43) that all models yield the same Lorentzian width for the same value of $\tau_k$.

3. Theory of slow tumbling ESR spectra for nitroxides

For anisotropic rotational diffusion, there are no convenient solutions for the jump and free diffusion models. The most straightforward course then is to generalize the equations for spherically symmetric rotation. Thus we let

$$\tau_2^{-1} = (B_2/R_t)[R_t L(L + 1) + (R_t - R_t)K^2]$$  \hspace{1cm} (44)

where $B_2$ is the same model parameter as in Eq. (42). In effect, it is assumed in Eq. (44) that although the $L$ dependence of $\tau_2^{-1}$ is model dependent, the \textit{quantum number} $K$ plays the same role in all models (Goldman et al., 1972). Other interpretations of the model dependence in terms of more fundamental analyses of microscopic molecular dynamics have been discussed by Hwang et al. (1975).

C. Anisotropic Liquids

Suppose now that the liquid has a preferred axis of orientation, i.e., the director axis (cf. Chapter 8). We now write the perturbing Hamiltonian (17) as (Polnaszek et al., 1973) [with the $(-1)^k$ which was included into the $F_{\mu,1}^{(L,K)}$ of Eq. (17) now explicitly displayed]:

$$\mathcal{H}_1(\Omega, \Psi) = \sum_{L,M,N,M',k_1} (-1)^k \delta_{k,M,N}^{L,M}(\Omega)\delta_{k,M',N}(\Psi)^{\mu,1}F_{\mu,1}^{(L,K)}$$  \hspace{1cm} (45)

Equation (45) is based on two sets of rotations of the coordinate systems: first from the molecular axis system $(x', y', z')$ into the director axis system $(x'', y'', z'')$ with Euler angles $\Omega = (\alpha, \beta)$; and then into the laboratory axis system $(x, y, z)$ with Euler angles $\Psi$. The orientation of the director relative to the laboratory frame can be specified by the two polar angles $\theta'$ and $\phi'$ such that $\Psi = (0, \theta', \phi')$. More precisely, one means by the molecular coordinate system $(x', y', z')$ the principal axis system for the orientation of the molecule in the mesophase. It may also be necessary to transform from the principal axis system of the magnetic interactions $(x'', y'', z'')$ to the $(x', y', z')$ system with Euler angles $\Theta$, according to

$$F_{\mu,1}^{(L,K)} = \sum_k \delta_{k,L}^{L,M}(\Theta)F_{\mu,1}^{(L,K)}$$  \hspace{1cm} (46)

where $\Theta = (\alpha, \beta', \gamma)$.

The diffusion equation for a particle undergoing Brownian rotational diffusion in the presence of a potential $V$ is given by (Pavlík, 1965; Polnaszek and Freed, 1975; Polnaszek, 1975a)

$$\frac{\partial P(\Omega, t)}{\partial t} = -\mathbf{\dot{N}} \cdot \left[ \mathbf{R} \cdot \frac{\mathcal{H}(\Omega)}{kT} + \mathbf{R} \cdot \mathbf{\dot{N}} \right] P(\Omega, t) \equiv -\Gamma_{\alpha} P(\Omega, t)$$  \hspace{1cm} (47)
where $V(\Omega)$ can be taken to be the orienting pseudopotential for a liquid crystal, $\mathcal{M}$ is the vector operator, which generates an infinitesimal rotation, and is identified with the quantum mechanical angular momentum operator for a rigid rotator, and $\mathbf{R}$ is the diffusion tensor of the molecule. Both $\mathcal{M}$ and $\mathcal{S}$ are defined in the $(x', y', z')$ molecular coordinate system. The angular momentum operator $\mathcal{M}$ is defined by

$$\mathcal{M}_z \phi_{K\Omega}(\Omega) = K \phi_{K\Omega}(\Omega)$$

$$\mathcal{M}\phi_{K\Omega}(\Omega) = [(L \pm K)(L \pm K + 1)]^{1/2} \phi_{K+1, \Omega}(\Omega)$$

Where the $\phi_{K\Omega}(\Omega)$ are the eigenfunctions of $\mathcal{S}$ and $\mathcal{M}$, given by Eq. (32), and

$$\mathcal{M}_x = \mathcal{M} \pm i \mathcal{M}_y$$

When $V = 0$, Eq. (47) is simply the equation for (asymmetric) Brownian rotational diffusion in isotropic liquids. [Simpler expressions have been given for special cases by Nordio and Busolin (1971), Nordio et al. (1972), and Poliaszek et al. (1973).] Equation (47) is based on the assumption that the external torque $\mathcal{T}$ is derived from the potential $V(\Omega)$:

$$\mathcal{T} = -i \mathcal{M} V(\Omega)$$

[Cf. Eq. (30), where we have set $\mathcal{V} = -\mathcal{M}^2$.] The equilibrium solution to Eq. (47) is given by

$$P_0(\Omega) = \frac{\exp[-V(\Omega)/kT]}{\int d\Omega \exp[-V(\Omega)/kT]}$$

When the symmetrized forms of Eqs. (20)–(22) are used, we obtain the diffusion equation

$$\frac{\partial P(\Omega, t)}{\partial t} = -\Gamma \frac{\partial P(\Omega, t)}{\partial t}$$

where

$$\Gamma = \mathcal{M} \cdot R \cdot \mathcal{M} + \frac{(\mathcal{M} \cdot R \cdot V)}{2kT} + \frac{\mathcal{S} \cdot R \cdot \mathcal{S}}{(2kT)^2}$$

The restoring potential for liquid crystals can be written in its most general form as

$$V(\Omega) = \sum_{L, K, M} e^{iK} M \mathcal{D}_{L}^{M}(\alpha, \beta, \gamma)$$

The assumption of cylindrical symmetry about the director axis $n$ implies that all averages taken over the angle $\gamma$ vanish unless $M = 0$ (Glarum and}

3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

Marshall, 1966, 1967). The uniaxial property of nematic liquid crystals (i.e., $n = -n$) implies that $L$ must be even. It is useful to use the linear combinations of the $\mathcal{D}_{L}^{M}$ that are of definite parity, i.e., the real linear combinations:

$$V(\Omega) = \sum_{L = 0}^{L} \sum_{K > 0}^{L} e^{iK} M \left[ \mathcal{D}_{L}^{M}(\Omega) + \mathcal{D}_{L}^{M}(\Omega) \right]$$

These have simpler properties for molecular symmetries less than cylindrical.

Usually we consider only the leading term $e^{i}\mathcal{D}_{L}^{M}(\Omega)$, i.e., the Meier-Saupe (1958) potential. The cylindrically symmetric case when $e^{i}\neq 0$ has also been considered (Poliaszek et al., 1973) and it was shown that typical ESR spectral predictions are not very sensitive to having $e^{i}\neq 0$. In general, however, we expect the terms for $L > 2$ to be less important than those for $L = 2$, and we can approximate

$$V(\Omega) \approx e^{i} \mathcal{D}_{L}^{M}(\Omega) + \frac{2}{kT} \sum_{K > 0}^{L} e^{iK} M \left[ \mathcal{D}_{L}^{M}(\Omega) + \mathcal{D}_{L}^{M}(\Omega) \right]$$

The $e^{i}$ and $e^{i}$ are $e^{i} \pm e^{i}$ (with the upper sign for $K > 0$, and the lower sign for $K < 0$) are themselves second-rank irreducible tensor components, so that, in the principal axis of molecular orientation system ($x', y', z'$) their Cartesian components $e^{i}$ are diagonalized, with $Tr e^{i} = 0$, and complete specification is given by just $e^{i}$ and $e^{i}$. [Equation (56) can be thought of as the scalar product of second-rank irreducible tensors.] The ordering tensor is defined by

$$\langle \mathcal{D}_{L}^{M}(\Omega) \rangle = \int d\Omega P_{0}(\Omega) \mathcal{D}_{L}^{M}(\Omega)$$

where $L = 2$ and $M = 0$. It is also a second-rank irreducible tensor whose symmetry properties are related to those of the $e^{i}$. Thus from Eqs. (51) and (56) and the orthogonality of the $\mathcal{D}_{L}^{M}(\Omega)$ terms it follows that in the $(x', y', z')$ system only $\langle \mathcal{D}_{L}^{M}(\Omega) \rangle$ and $\langle \mathcal{D}_{L}^{M} + \mathcal{D}_{L}^{M} \rangle$ are nonzero, i.e., $\langle \mathcal{D}_{L}^{M}(\Omega) \rangle$ is also diagonalized. Thus the “diagonalized” potential (retaining only $L = 2$ terms) becomes

$$V(\Omega) = e^{i} \mathcal{D}_{L}^{M}(\Omega) + e^{i} \left[ \mathcal{D}_{L}^{M}(\Omega) + \mathcal{D}_{L}^{M}(\Omega) \right]$$

or equivalently

$$V(\alpha, \beta, \gamma) = \gamma \cos^2 \beta + \alpha \sin^2 \beta \cos 2\alpha$$

where $e^{i} = 2\gamma/3$ and $e^{i} = 2\epsilon(6)^{-1/2}$. For molecules in which the molecular $x'$ and $y'$ axes are aligned to different extents, $\epsilon$ is nonzero. If we choose the orientation coordinate system such that the $x'$ axis tends to align to a
greater degree either parallel or perpendicular to the director than does the $x'$ axis or the $y'$ axis, we have $|y_2| > |x_2|$. The case $\epsilon < 0$ corresponds to the $y'$ axis being ordered preferential to the $x'$ axis along the direction of $\epsilon$ and/or to the $x'$ axis being ordered to a greater degree perpendicular to the $x'$ than is the $y'$ axis.

We can utilize Eqs. (50), (48b) and (48c) to obtain $F$ from the potential in Eq. (58) in terms of its components in the $(x', y', z')$ coordinate system:

$$ F_{x'} \equiv \pm (\sin 2\beta)(\epsilon e^{-i\epsilon} - y_2 e^{i\epsilon}) $$

$$ F_{y'} = -2\alpha \sin^2 \beta \sin 2\alpha $$

We assume axially symmetric rotation about $x'$ such that $R_{x'y'} = R_{\perp}$ and $R_{x'y'} = R_{\parallel}$. Further, we introduce the definitions

$$ \lambda \equiv -y_2/kT $$

and

$$ \rho \equiv -\epsilon/kT $$

Then the symmetrized Markov operator defined in Eq. (52) becomes (Polnaszek and Freed, 1975; Polnaszek, 1975a)

$$ \Gamma = N \cdot R \cdot N = f(R_{\perp}, R_{\parallel}, \lambda, \rho, \Omega) $$

where

$$ f(R_{\perp}, R_{\parallel}, \lambda, \rho, \Omega) = \sum_{L=0, 2, 4} X_{00}^L \mathcal{O}_{00}^L + \sum_{0 < k < L} X_{2k}^L \mathcal{O}_{2k}^L + X_{L}^L \mathcal{O}_{L}^L $$

with

$$ X_{00}^L = -2(15^L \lambda^2 + 3\rho^2) $$

$$ X_{20}^L = 2(\lambda - (\lambda^2 + 3\rho^2)/21) $$

$$ X_{40}^L = 4(\lambda^2 + \rho^2) $$

$$ X_{2k}^L = 6^{1/2} \rho [k(1 + (2\lambda/7))] $$

$$ X_{4k}^L = 4(10^{1/2} \rho^2/35 $$

and $N \cdot R \cdot N$ is just the $\Gamma_0$ of Eq. (33) with its associated eigenvalues.

We can also write the diffusion equation in terms of the general angular momentum operator $N$ referred to the director frame (Favro, 1965). This is appropriate when $R$ is diagonal in this frame, i.e., one has anisotropic viscosity. We then generate an analogous set of expressions (Polnaszek, 1975a; Polnaszek and Freed, 1975). It is now assumed that $R_{xx} = R_{yy} = R_{\perp}$, $R_{zz} = R_{\parallel}$. The result is

$$ \Gamma_{\parallel} = N \cdot R_{\parallel} \cdot N - \tilde{R}_{\perp} \tilde{f}(\lambda, \rho, \Omega) $$

where

$$ \tilde{f}(\lambda, \rho, \Omega) = \sum_{L=0, 2, 4} \left[ X_{00}^L \mathcal{O}_{00}^L + \sum_{0 < k < L} X_{2k}^L \mathcal{O}_{2k}^L + X_{L}^L \mathcal{O}_{L}^L \right] $$

with

$$ \tilde{X}_{00}^{2k} = -2(15^L \lambda^2 + 3\rho^2)/15 $$

$$ \tilde{X}_{20}^{2k} = 2(\lambda - (\lambda^2 + 3\rho^2)/21) $$

$$ \tilde{X}_{40}^{2k} = 4(\lambda^2 + \rho^2)/35 $$

and

$$ N \cdot R \cdot N \cdot \Phi_{LM} = [\tilde{R}_{\perp} L(L + 1) + \tilde{R}_{\parallel} M^2] \Phi_{LM} $$

It is shown in Appendix A how the slow tumbling equations for isotropic liquids can be simply modified to deal with anisotropic liquids in the simple case of a Meier-Saue potential and with $\Psi = (0, 0, 0)$ and $\Theta = (0, 0, 0)$ (except that permutation of the labeling of the molecular axis system is permitted). The more complex expressions for the general cases are given by Polnaszek (1975a).

Finally, note that when $|\lambda| > 1$, and the eigenfunction expansion in $\Phi_{LM}(\Omega)$ is only slowly convergent for solutions of the diffusion equation, then there are other types of eigenfunction expansions, specifically tailored to these limiting cases, which become very useful (Polnaszek et al., 1973).

D. Exchange and Slow Tumbling

In cases where the concentration of nitroxide spin probes is high, we also have to consider the effects of Heisenberg spin exchange. This phenomenon involves bimolecular collisions of radicals during which an exchange integral $J'$ is turned on, and, because of the Pauli principle, can be written as an added term in the spin Hamiltonian:

$$ H_{\text{ex}} = J'(S_1 \cdot S_2) $$

(67)
where \( J = 2J' \) and is time dependent due to the relative motion of the radical pairs. Its effect (viewed from an ESR point of view) is to cause the electron spins to exchange their nuclear environments.

A rigorous analysis of exchange (cf. Freed, 1967; Eastman et al., 1969) shows that we can add to the left side of Eq. (19) for the allowed transitions \( \lambda \), the terms:

\[
-\omega_{SS}(1 - \frac{2D_{\lambda}}{N})Z_\lambda + \omega_{SS} \sum_{\eta \neq \lambda} \frac{2D_{\eta}}{N} Z_\eta
\]  

where \( D_{\lambda} \) and \( D_{\eta} \) are the degeneracies of the \( \lambda \)th and \( \eta \)th allowed ESR transitions, respectively. That is, for nitroxides \( D_3 = D_1 = 1 \) and \( N' = 6 \). The sum in Eq. (68) is over all allowed transitions \( \eta \) not equal to \( \lambda \). Also, the effective exchange frequency \( \omega_{SS} \) obeys

\[
\omega_{SS} = \tau_2^{-1} J^2 \tau_1^2 / (1 + J^2 \tau_1^2)
\]

where \( \tau_2 \) is the mean time between successive new bimolecular encounters of radicals, and \( \tau_1 \) is the lifetime of the interacting pair. In the case of simple Brownian diffusion of uncharged radicals in solution we can write (Eastman et al., 1969; Pedersen and Freed, 1973a,b):

\[
\tau_2^{-1} = 4\pi dDN
\]

\[
\tau_1^{-1} = D/d\Delta r
\]

where \( N \) is the density of radicals, \( d \) is the “interaction distance” for \( J(r) \) which is nonzero (and equal to \( J \)) only in the range of \( d < r < d + \Delta r \), and the diffusion coefficient in a Stokes–Einstein model is

\[
D = kT/6\pi nm
\]

Expression (68) is only appropriate for the allowed transitions, which then couple together by this mechanism. For each forbidden ESR transition (see below), we have instead of (68) to add the term to the left side of Eq. (19):

\[
-\omega_{SS} Z_\lambda
\]

If we make the simplifying assumption that \( \omega_{SS} \) is independent of any orientational effects [i.e., \( J(r) \) is taken as a function only of the relative internuclear separation of the radical pair], then (68) and (73) can be added to the LHS of Eq. (27), where we let \( Z_\lambda(\Omega) \rightarrow [C_{0\lambda}^2]_\lambda \), etc.

Expressions (69) and (70) are based on a simple contact-exchange model (Eastman et al., 1969). More complex models of the motional modulation of \( J(r_1, r_2) \) can also be dealt with (Pedersen and Freed, 1973a,b). Effects of radical charge and/or electrolyte concentration on Eqs. (70) and (71) must also be considered (Eastman et al., 1970). Expressions (68) and (72) are also useful for two-dimensional motions, but Eqs. (69)–(72) would have to be modified. Also, in general, effects of intermolecular electron–spin dipolar interactions become important at higher concentrations as the motions slow down (Eastman et al., 1969).

E. Nitroxides

For nitroxides, there are three allowed ESR transitions and six forbidden transitions which must be considered in a rigorous solution of Eq. (27). They are illustrated in Fig. 1a. The asymmetric \( g \) tensor \( g \) and the hyperfine tensor \( A \) yield an \( \mathcal{H}_1(\Omega) \) given by

\[
\mathcal{H}_1(\Omega) = \mathcal{D}_{2-1}(\Omega) \left[ F_+ + D^{(1)} \right] S_z + \left[ \mathcal{D}_{2-1}(\Omega) \mathcal{I}_z - \mathcal{D}_{2-1}(\Omega) \mathcal{I}_- \right] \mathcal{D}_z
\]

\[
\times \left( \mathcal{D}_{2-1}(\Omega) \mathcal{I}_+ + \mathcal{D}_{2-1}(\Omega) \mathcal{I}_- \right)
\]

\[
-\left[ \mathcal{D}_{2-1}(\Omega) + \mathcal{D}_{2-1}(\Omega) \right] \mathcal{D}(\mathcal{I}_- \mathcal{S}_z)
\]

where

\[
F_+ = \sqrt{3} g_{00} h^0 \beta_x H_0
\]

\[
g_{00} = g - \frac{1}{2}(2d_D - (g_x + g_y))
\]

\[
g_{22} = \frac{3}{2}(g_x - g_y)
\]

\[
D = \sqrt{3} \gamma_x (A_z + A_y - 2A_x)
\]

\[
D^{(2)} = \frac{1}{2} \gamma_x (A_y - A_x)
\]

with \( D' = -(8/3)^{1/2} D \) and \( D^{(2)} = -(8/3)^{1/2} D^{(2)} \). The forbidden transitions 4–9 of Fig. 1a are coupled into the expressions for the allowed transitions because of the pseudoscalar terms in Eq. (74), i.e., the terms involving \( \mathcal{I}_z \) \( \mathcal{S}_z \) (where \( \mathcal{I}_z \) are the nuclear spin raising and lowering operators). The Euler angles \( \Omega = \phi, \theta, \gamma \) define the rotation between the laboratory coordinate system \( (x, y, z) \) and the principal axis system in the molecular frame in which the \( g \) and hyperfine tensors are diagonal. We assume they are diagonal in the same axis system [which is rigorously the \( x''', y'''', z''' \) system, but
Eqs. (75a)–(75c) and (76a) and (76b) are written as though they correspond to the $x', y', z'$ system.

It can be shown that the nuclear Zeeman term (which appears in the resonance frequencies of the forbidden transitions) makes a negligible contribution, so one can neglect it. Then it is only necessary to consider forbidden transitions 4 and 5, 6 and 7, and 8 and 9 in pairs, and this simplifies the expressions (see Appendix A).

We can now evaluate Eq. (27) (for $n = 1$) and the resulting expressions, neglecting saturation [i.e., set $d = 0$ on the LHS of Eq. (27)], are given in Appendix A. They define an infinite set of coupled algebraic equations coupling the allowed and forbidden transitions. Only even values of the "quantum number" $L$ appear for the allowed transitions for which $M = 0$. Also, one has the general restriction

$$0 \leq K \leq L \quad \text{with } K \text{ even}$$  \hspace{1cm} (77)

while $M = 1$ for the coefficients $C_{LM}$ representing the "single forbidden" transition pairs (4, 5) and (6, 7) and $M = 2$ for the "doubly forbidden" pair (8, 9).

Approximations to the complete solution can be obtained by terminating the coupled equations at some finite limit by letting $C_{L0}(l) = 0$ for all $L > n_L$. While the number of equations needed to obtain a satisfactory convergent solution depends on the value of $\tau_0$ (the larger is $\tau_0$, the greater is the value of $n_L$ needed) the convergence also depends on the rotational reorientational model. The model that yields eigenvalues with the greatest dependence on $L$ value in Eqs. (34)–(40) will have the fastest convergence. Therefore, in general, the convergence becomes poorer as one proceeds from Brownian to simple free and intermediate jump, to strong collisional jump diffusion. For Brownian rotational diffusion with $\tau_0 \approx 2 \times 10^{-8}$ sec, $n_L = 5$ is sufficient; with $\tau_0 \approx 2 \times 10^{-7}$ sec, $n_L = 12$ is sufficient; and with $\tau_0 \approx 2 \times 10^{-6}$ sec, $n_L = 24$ is sufficient. However, for simple free diffusion and $\tau_0 \approx 2 \times 10^{-8}$ sec, $n_L = 10$ is needed and for a strong jump model $n_L = 16$ is needed. It is also often useful to terminate $K$ at some value considerably less than $n_L$ (i.e., for $n_k < n_L$). This would be especially applicable for isotropic or for axially symmetric reorientation about an axis parallel to the 2p $\pi$ orbital of nitrogen, because in such a principal axis system, the $A$ tensor is almost axially symmetric. Typical values for the nitroxide radical in this coordinate frame (cf. Table 1) are $|F_0| \approx 4.8$ G and $|D| \approx 10.4$ G, while the asymmetric values are $|F_2| \approx 1.6$ G and $|D^{(2)}| = 0.4$ G. These smaller terms are the coefficients for coupling the variable $C_{L0}(l)$ with $K \neq 0$ into the problem, so their smallness guarantees faster convergence (cf. Appendix A). Also, if $R_0 \gg R_L$ or $R_\perp$, then this will greatly improve the convergence in $K$ relative to that in $L$.

3. Theory of Slow Tumbling ESR Spectra for Nitroxides

The computer program in Appendix B has provision for separate terminating values for $K$ and $L$. The number of coupled equations is then found to be

$$r = 3 + \frac{2}{3}(n_k - \frac{1}{3}n_L + 1) + \frac{1}{3}n_L, \quad n_k \geq n_L, \quad n_k \geq 2$$  \hspace{1cm} (78a)

or

$$r = 9 + 3(n_k - 2) \quad \text{if } n_k = 0 \quad \text{and } n_L \geq 2$$  \hspace{1cm} (78b)

A further reduction in the number of equations can be made by distinguishing between the allowed and forbidden transition coefficients. That is, if the coefficients for the allowed transitions $C_{k0}(l)$ are terminated at $L = n_k$, then the coefficients for the singly forbidden transitions $C_{k1}(j, k)$ are terminated at $L = n'^*$ and the doubly forbidden $C_{k2}(8, 9)$ at $L = n''$, where $n', n'' \leq n_L$. We have found that for convergence usually $n' \approx n_L$ or $n_L - 2$, but $n''$ may be truncated at values significantly below $n_L$ (e.g., when $n_L = n' = 16$ was needed, $n'' = 8$ was sufficient). Also the terms of odd $L$, which exist only for the forbidden transitions, may be truncated at values of $L \leq n_L$. The computer program in Appendix B also provides for these truncations.

III. Applications

A. Isotropic Liquids: Experiments

The validity of the slow tumbling theory has now been carefully confirmed in studies on model systems of PADS (peroxylamine disulfonate) and PD-TEMPONE (perdeuterated 2,2,6,6-tetramethyl-4-piperidone-1-oxyl) (cf. Fig. 1b) in viscous media (Goldman et al., 1972a; Goldman, 1973; Hwang et al., 1975).

One of the most important requirements in analyzing a slow tumbling spectrum is to have accurate values for the magnetic tensors $A$ and $g$. These are best obtained from viscous solutions in the same solvent as is the slow tumbling spectrum. This is because, in general, nitroxides will exhibit magnetic parameters which are rather solvent dependent. Figure 2 shows one
TABLE I

<table>
<thead>
<tr>
<th>MAGNETIC PARAMETERS</th>
<th>PADS in frozen D$_2$O</th>
<th>PADS in 85% glycerol-H$_2$O</th>
<th>DTBN'</th>
<th>PADS in (KSO$_4$)$_2$NOH'</th>
<th>$^{17}$O PADS in 85% glycerol-H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_x'$</td>
<td>2.0081 ± 0.0002</td>
<td>2.00815 ± 0.0002</td>
<td>2.00872 ± 0.00005</td>
<td>2.0094 ± 0.0004</td>
<td>--</td>
</tr>
<tr>
<td>$a_y'$</td>
<td>2.0057 ± 0.0002</td>
<td>2.00590 ± 0.0002</td>
<td>2.00616 ± 0.00005</td>
<td>2.0055 ± 0.0004</td>
<td>--</td>
</tr>
<tr>
<td>$a_z'$</td>
<td>2.0025 ± 0.0001</td>
<td>2.00265 ± 0.0001</td>
<td>2.00270 ± 0.00003</td>
<td>2.0025 ± 0.0004</td>
<td>--</td>
</tr>
<tr>
<td>$a_{1/2}^y$</td>
<td>2.00545 ± 0.000017</td>
<td>2.00547 ± 0.00017</td>
<td>2.00586 ± 0.00005</td>
<td>2.0058 ± 0.0004</td>
<td>--</td>
</tr>
<tr>
<td>$a_{2/2}^y$</td>
<td>2.00545 ± 0.000002</td>
<td>2.00548 ± 0.00001</td>
<td>2.00548 ± 0.00000</td>
<td>2.0054 ± 0.0004</td>
<td>--</td>
</tr>
<tr>
<td>$a(0)^{1/2}$</td>
<td>-3.61 ± 0.15 x 10^{-8}</td>
<td>-3.47 ± 0.13 x 10^{-8}</td>
<td>-3.54 ± 0.12 x 10^{-8}</td>
<td>-3.50 ± 0.11 x 10^{-8}</td>
<td>--</td>
</tr>
<tr>
<td>$a(2)^{1/2}$</td>
<td>3.1 ± 0.1 x 10^{-4}</td>
<td>5.3 ± 0.2 x 10^{-4}</td>
<td>5.2 ± 0.1 x 10^{-4}</td>
<td>5.1 ± 0.2 x 10^{-4}</td>
<td>--</td>
</tr>
<tr>
<td>$A_x(G)$</td>
<td>7.7 ± 0.5</td>
<td>5.5 ± 0.5</td>
<td>7.59 ± 0.05</td>
<td>7.7 ± 0.2</td>
<td>-8.7</td>
</tr>
<tr>
<td>$A_y(G)$</td>
<td>5.4 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td>5.95 ± 0.05</td>
<td>5.5 ± 0.2</td>
<td>-8.7</td>
</tr>
<tr>
<td>$A_z(G)$</td>
<td>3.0 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>80.2 ± 0.8</td>
</tr>
<tr>
<td>$A_{1/2}^y(G)$</td>
<td>3.1 ± 0.4</td>
<td>13.1 ± 0.4</td>
<td>13.1 ± 0.4</td>
<td>13.1 ± 0.4</td>
<td>--</td>
</tr>
<tr>
<td>$e_{1/2}^z$</td>
<td>11.1 ± 0.03</td>
<td>13.0 ± 0.03</td>
<td>13.0 ± 0.03</td>
<td>13.0 ± 0.03</td>
<td>--</td>
</tr>
<tr>
<td>$-D_{1/2}^z$</td>
<td>28.5 ± 0.5</td>
<td>28.5 ± 0.5</td>
<td>28.5 ± 0.5</td>
<td>28.5 ± 0.5</td>
<td>--</td>
</tr>
<tr>
<td>$-D_{1/2}^y$</td>
<td>-2.0 ± 0.7</td>
<td>-2.0 ± 0.7</td>
<td>-2.0 ± 0.7</td>
<td>-2.0 ± 0.7</td>
<td>--</td>
</tr>
<tr>
<td>$-D_{1/2}^x$</td>
<td>17.0 ± 0.6</td>
<td>16.2 ± 0.6</td>
<td>16.2 ± 0.6</td>
<td>16.2 ± 0.6</td>
<td>--</td>
</tr>
</tbody>
</table>
### TABLE I (cont.)

<table>
<thead>
<tr>
<th>PD-TMPONE(^a) in</th>
<th>PD-TMPONE(^a) in</th>
<th>PD-TMPONE(^a) in</th>
<th>PD-TMPONE(^a) in</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene-(d_{8})</td>
<td>85% glycerol-(d_{1})-(D_{2}O)</td>
<td>Acetonitrile-(d_{3})</td>
<td>Ethanol-(d_{6})</td>
</tr>
<tr>
<td>(\theta_{1})</td>
<td>2.0096 ± 0.0002</td>
<td>2.0064 ± 0.0002</td>
<td>2.0059 ± 0.0003</td>
</tr>
<tr>
<td>(\theta_{2})</td>
<td>2.0063 ± 0.0002</td>
<td>2.0064 ± 0.0002</td>
<td>2.0062 ± 0.0003</td>
</tr>
<tr>
<td>(\theta_{3})</td>
<td>2.0022 ± 0.0001</td>
<td>2.0022 ± 0.0001</td>
<td>2.0022 ± 0.0002</td>
</tr>
<tr>
<td>(\phi_{1})</td>
<td>2.0060 ± 0.00017</td>
<td>2.0065 ± 0.00017</td>
<td>2.0060 ± 0.00027</td>
</tr>
<tr>
<td>(\phi_{2})</td>
<td>2.00602 ± 0.00095</td>
<td>2.00630 ± 0.00005</td>
<td>2.00596 ± 0.00050</td>
</tr>
</tbody>
</table>

### Table: Tempone\(^a\) \(\theta_{1}\), \(\theta_{2}\), \(\phi_{1}\), \(\phi_{2}\) in \(\phi_{1}\) \(\times 10^{-3}\) vs \(\phi_{2}\) \(\times 10^{-3}\)

<table>
<thead>
<tr>
<th>(\phi_{1})</th>
<th>(\phi_{2})</th>
<th>(\text{Tempone}^a)</th>
<th>(\text{Tempone}^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\theta_{1})</td>
<td>2.0096 ± 0.0002</td>
<td>2.0064 ± 0.0002</td>
<td>2.0084 ± 0.0002</td>
</tr>
<tr>
<td>(\theta_{2})</td>
<td>2.0063 ± 0.0002</td>
<td>2.0064 ± 0.0002</td>
<td>2.0059 ± 0.0003</td>
</tr>
<tr>
<td>(\theta_{3})</td>
<td>2.0022 ± 0.0001</td>
<td>2.0022 ± 0.0001</td>
<td>2.0022 ± 0.0002</td>
</tr>
<tr>
<td>(\phi_{1})</td>
<td>2.0060 ± 0.0017</td>
<td>2.0065 ± 0.0017</td>
<td>2.0060 ± 0.0027</td>
</tr>
<tr>
<td>(\phi_{2})</td>
<td>2.00602 ± 0.00095</td>
<td>2.00630 ± 0.00005</td>
<td>2.00596 ± 0.00050</td>
</tr>
</tbody>
</table>

* The magnetic parameters are given in their principal axis system \((x', y', z')\), but the triple primes have been dropped for convenience. The \((x', y', z')\) axes are the principal axes for the rotational diffusion.

* From Goldman et al. (1972a), PADS = paraxyamine disulfonate; cf. Fig. 1b.


* From Hamrick et al. (1972).

* From Goldman et al. (1972b); entries give \(^{1}O\) hyperfine entries. Note that it is assumed here that \(A_{x} = A_{y}\).

* Measured in motionally narrowed region. Error limits reflect total range of observed values.
axis perpendicular to the other two. [These axes should more rigorously be written as $x''$, $y''$, $z''$, cf. Eq. (46), but the primes have been dropped for convenience, since there should be no confusion with the laboratory axes.] The $x'$, $y'$, $z'$ axes are the principal axes of the diffusion tensor $\mathbf{R}$ and they are assumed in Table I to be either the same as the $x$, $y$, $z$ axes or else to be a cyclic permutation of them. It should be clear from this tabulation that (1) nitroxides do exhibit significant solvent dependencies in their magnetic parameters and (2) the different nitroxides will exhibit some difference in their magnetic parameters. One interesting observation in this context is the result for PD-TEMPONE (cf. Fig. 1b) in ethanol-$d_6$. It exhibits two distinct values of $A_z$, the larger one characteristic of the values in hydrogen-bonding solvents, while the smaller one is characteristic of the values in non-hydrogen-bonding solvents. In general, one must expect some variation in the magnetic parameters from site to site in a given solvent, and this will be an important source of the [orientation-dependent, cf. Eq. (29)] rigid-limit intrinsic width. These matters are discussed elsewhere (Hwang et al., 1975).

Another source of valuable information for the analysis of the slow tumbling spectrum is the relaxation results from the fast motional spectrum in less viscous media, if it is at all available.

Figure 3 shows the results for PADS (cf. Fig. 1b) in D$_2$O (Goldman et al., 1972a) where the derivative width $\delta(M)$ is plotted as

$$\delta(M) = A + B\bar{M} + CK^2$$  \hspace{1cm} (79)

The analysis of motional narrowing spectra is discussed by Nordio in Chapter 2. Suffice it to say here, that the motional narrowing theory, coupled with experimental values of $B$ and $C$ and accurate values of the magnetic parameters, is sufficient to determine $R_1$ and $R_2$ at each temperature. It is important to note that in Fig. 3 the curves for $B$ and $C$ are very nearly parallel. This fact and the temperature insensitivity of $a_0$ and $g_0$ provide strong evidence against competing relaxation mechanisms affecting the interpretation. Figures 4a and 4b are plots of $C$ versus $B$ for PADS in glycerol-H$_2$O and for PD-TEMPONE in toluene-$d_8$, respectively. These results can be analyzed to yield

$$\tau_R \equiv (6R)^{-1}$$  \hspace{1cm} (80)

and

$$R \equiv (R_1R_2)^{1/2}$$  \hspace{1cm} (80')

$$N \equiv R_1/R_2$$  \hspace{1cm} (81)

It is found that the PADS system exhibits anisotropic rotational diffusion ($N \approx 4.7$ in aqueous glycerol solvents) where the $z$ axis for the rotational diffusion tensor is parallel to the line through the two sulfur atoms, while the PD-TEMPONE system rotates isotropically (within experimental error). An interesting sidelight to this result is that, if we were to interpret the PD-TEMPONE motional narrowing results in glycerol solvent in terms of the magnetic parameters from toluene solvent, we would obtain $N \approx 3.6$, with fastest rotation about the molecular $y$ axis, but when we use the correct magnetic parameters for glycerol, we again obtain $N = 1$.

The value of these motional narrowing results for the slow tumbling studies is that one can extrapolate the information obtained on $\tau_R$ and $N$ into the slow motional region. That is, $\log \tau_R$ is found to have a nearly linear dependence on $1/T$, as expected for an activation process. More precisely, for PD-TEMPONE in toluene, it is linear in $\eta/T$, as shown in Fig. 5. This is expected from Eq. (31) for Stokes–Einstein-type behavior. Also, $N$ is found to be temperature independent. Figure 6 shows a such comparison of an experimental spectrum with computer simulated results utilizing the values of $\tau_R$ and $N$ extrapolated from the motional narrowing region. This is the case of PADS in D$_2$O, where $N = 3$, and is one of incipient slow tumbling.
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

Fig. 5. $\tau_2$ versus $1/T$ for PD-TEMPONE in toluene-$d_8$. Motional narrowing results are designated by $\Delta$ ($\tau_2$ from $B$ values) and $\bigcirc$ ($\tau_2$ from $C$ values). They are extrapolated to the slow tumbling region (---) and are compared to best fits for the different models: (I) free diffusion, (A) Brownian diffusion, (■) strong jump diffusion. (---) $N = 1$, $\epsilon = 5.4$. (Δ-Δ) $N = 1$, $\epsilon = 1$.

Fig. 4. Comparisons of experimental and calculated values of $B$ vs. $C$. The curves show the results and the fit for $N = 1$, $\epsilon = 10^{-8}$ sec. The solid line is the best fit. [Reprinted with permission from Hwang et al., J. Phys. Chem. 79, 489-511 (1975). Copyright by the American Chemical Society, 1975.]

($\tau_R = 4 \times 10^{-9}$ sec). A comparison is given for different values of $N$, and it clearly shows that the best fit is for $N = 3$. These simulations were performed for a Brownian diffusion model, with the model parameter $B_L = 1$. When slower motional spectra were obtained ($\tau_R \geq 10^{-8}$ sec), it was found that a Brownian motion model was not yielding good simulations for the small spin probes PADS and PD-TEMPONE. Therefore other models were tried, i.e., the strong jump diffusion of Eq. (39) with $R_T = 1$ and the free diffusion of Eq. (35) also with $R_T = 1$. A typical comparison for the three models is shown in Fig. 7, from which it is clear that the best experimental fit is obtained with the free diffusion result. In this context, it is important to recognize that the values of $B_L$ for this free diffusion model are not unique. For $\tau_R \leq 10^{-8}$ sec the simulated spectrum is determined mainly by coefficients of $L = 2, 4, 6,$ and $8$. Over this range of values of $L$, it is possible to reproduce the values of $B_L$ of the free diffusion case reasonably well by the jump diffusion models given by Eq. (36) [and the special case of Eq. (39) with $R_T \approx 0.13$ corresponding to an rms jump angle of 50°]. In fact,
Fig. 6. A comparison of simulated and experimental spectra for PADS in frozen D₂O at T = -50°C: (---) experimental spectrum, (--) calculated for Brownian diffusion with τ₈ = 4 × 10⁻⁸ sec, A' = 0.2 G, and A, N = 1; B, N = 3; and C, N = 6. [From Goldman et al. (1972a).]

Computer simulations of such jump models do yield virtually identical results. Figure 8 shows the results for PADS in D₂O in the model-sensitive region of τ₈ for free diffusion and a range of values of N. This is also a case where free diffusion fits best.

Another way of testing the model dependence of the results is to extrapolate the τ₈ values obtained in the motional narrowing region and to compare them with the “best” τ₈ values obtained for each model. (This is associated with the S parameter discussed in Section III.B.) It is seen in Fig. 5 that the free diffusion model gives good agreement, but the others do not, especially for the slower values of τ₈ > 10⁻⁸ sec.

The parameters τ₈ and N are not the only ones that can be extrapolated from the motional narrowing region. Another is the parameter A' in G (cf. Fig. 7), which is that part of A in Eq. (79) that is not attributable to g- or A-tensor sources. (It is given as $T_{T1,2} = \frac{1}{3} |g_e| A'$ in sec⁻¹ in the computer programs.) The best A' for free diffusion again falls closest to the values extrapolated from the motional narrowing region, although the distinction here is not so clear (cf. Mason et al., 1974).

An important point to emphasize at this stage is that the model-dependent studies summarized above were greatly aided by the very well-resolved spectra obtained from PADS and PD-TEMPONE in deuterated solvents. In general, the added intrinsic widths of typical spin labels due to the unresolved proton superhyperfine splitting will tend to obscure many of the spectral details in the slow motional and rigid-limit spectra. The analysis of the motional narrowing spectra would be particularly seriously affected.
B. Simplified Methods of Estimating $\tau_R$

An important characteristic of a typical nitroxide slow motional spectrum is that it has two well-separated outer hyperfine extrema with an overlapped central region. It has been found that a useful parameter for describing these spectra is $S = A'_s / A_s$, where $A'_s$ has already been defined in Fig. 2 as one-half the separation of the outer hyperfine extrema, and $A_s$ is the slow tumbling value for the same spectral feature (Goldman et al., 1972b). McCalley et al. (1972) have discussed the separate deviations of high-field and low-field positions from their rigid-limit values. Figure 9 compares the quantities $A_s$ and $A'_s$. Note that $A'_s$ decreases monotonically from its rigid-limit value of $A_s$ as the motion becomes more rapid. Thus $S$ is a sensitive, monotonically increasing function of $\tau_R$. Furthermore, simulations performed for axial and asymmetric $A$ and $g$ tensors show that for a given value of $A_s$, the value of $S$ is insensitive to changes in $A_x$, $A_y$, and the $g$-tensor components. Changes in the magnitude of $A_s$, however, do affect the value of $S$. This is expected, of course, since, as can be seen from Fig. 2, $A_x$, $A_y$, and $g$ only contribute to the central regions of the rigid limit spectrum. This

---

Fig. 8. A comparison of simulated (---) and experimental (----) spectra for PADS in frozen D$_2$O at $T = -60^\circ$C. The simulated spectra are calculated for free diffusion with $\tau_R = 2 \times 10^{-8}$ sec, $A' = 0.6$ G, and $A, N = 1$; $B, N = 3$; and $C, N = 6$. [From Goldman et al. (1972a).]

It is interesting to note, however, that the ESR spectra obtained by McCalley et al. (1972)† from spin-labeled oxyhemoglobin in H$_2$O at $\tau_R \cong 2.6 \times 10^{-8}$ sec show many of the features that are characteristic of Brownian diffusion (cf. Fig. 7). This is an important confirmation of the theory, because one would expect that a macromolecule (unlike the small spin probes) would obey simple Brownian motion.

† Editor's note: Appendix I (p. 562) contains simulated spectra calculated by this model for a broad range of $\tau_R$ values.

Fig. 9. Superposition of computed rigid-limit nitroxide spectrum with a computed slow-tumbling spectrum at $\tau_R = 5.0 \times 10^{-8}$ sec, demonstrating the measurements required for the parameters $S = A'_s / A_s$, $W'_s = \Delta_s / \Delta_l$, and $W_s = \Delta_s / \Delta_l$. In an actual experiment, it is often necessary to estimate the $\Delta'_s$ in place of the $\Delta_l$ as described in the text. The magnetic parameters utilized are $\delta = 3.0$ G, $g_s = g_t = 2.0075$, $g_x = 2.007$, $A_x = A_y = 6.0$ G, $A_s = 32.0$ G, and $B_0 = 3.300$ G. [Reprinted with permission from Mason and Freed, J. Phys. Chem. 78, 1321–1323 (1974). Copyright by the American Chemical Society.]
dependence can be approximately expressed in the functional form 
\[ S = S(\tau_r, A_s) \]
where \( S \) is simply dependent on the product \( \tau_r A_s \). This functional dependence permits the scaling of results for one value of \( A_s \) to the range of values of \( A_s \) typical for nitroxides (27–40 G) with an error of less than 3%. Thus, if we know how \( S \) is affected by changes in the linewidth and rotational diffusion model, then it is possible to estimate \( \tau_r \) without the necessity of making detailed line-shape calculations and comparisons. This is particularly useful for nitroxides that are broadened by inhomogeneous intramolecular or intermolecular (solvent) hyperfine and dipolar interactions. As already noted, this line broadening decreases the spectral resolution and obscures other \( \tau_r \)-dependent line-shape changes.

The variation of \( S \) with \( \tau_r \) is shown in Fig. 10 for Brownian, free, and strong jump diffusion models and isotropic diffusion, with \( A_s = 32 \) G and peak-to-peak derivative Lorentzian linewidths \( S = (2/\sqrt{3}) |\gamma|^{-1}T_e^{-1} \) of 0.3 and 3.0 G. It can be seen that \( S \) is model sensitive, and for an equivalent value of \( \tau_r \), \( S \) increases from a Brownian to a free to a jump reorientational model. (This is consistent with the analysis we have already given for the best \( \tau_r \) fit as a function of model; cf. Fig. 7.) These curves can be fit to the expression

\[ \tau_r = a(1 - S)^b \]

(82)

to within 2, 3, or 5% in the value of \( \tau_r \) for a given \( S \) for jump, Brownian, or free diffusion, respectively, with the values of \( a \) and \( b \) given in Table II.

### Table II

<table>
<thead>
<tr>
<th>Diffusion model</th>
<th>Linewidth (G)</th>
<th>( a )</th>
<th>( b )</th>
<th>( \tau_r(S = 0.99)^b ) (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brownian diffusion</td>
<td>0.3</td>
<td>( 2.57 \times 10^{-10} )</td>
<td>-1.78</td>
<td>( 9 \times 10^{-7} )</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>( 5.4 \times 10^{-10} )</td>
<td>-1.36</td>
<td>( 3 \times 10^{-7} )</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>( 8.52 \times 10^{-10} )</td>
<td>-1.16</td>
<td>( 2 \times 10^{-7} )</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>( 1.09 \times 10^{-9} )</td>
<td>-1.05</td>
<td>( 2 \times 10^{-7} )</td>
</tr>
<tr>
<td>Free diffusion</td>
<td>0.3</td>
<td>( 6.99 \times 10^{-10} )</td>
<td>-1.20</td>
<td>( 3 \times 10^{-7} )</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>( 1.10 \times 10^{-9} )</td>
<td>-1.01</td>
<td>( 1 \times 10^{-7} )</td>
</tr>
<tr>
<td>Strong diffusion</td>
<td>0.3</td>
<td>( 2.46 \times 10^{-9} )</td>
<td>-0.589</td>
<td>( 4 \times 10^{-8} )</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>( 2.53 \times 10^{-9} )</td>
<td>-0.613</td>
<td>( 3 \times 10^{-8} )</td>
</tr>
</tbody>
</table>

* These values are calculated for an axial nitroxide with \( A_\perp = 32 \) G, \( A_\parallel = 6 \) G, \( \theta_\perp - \theta_\parallel = 0.004 \), and isotropic reorientation. (From Goldman et al., 1972b.)

* These models are discussed in detail in the text.

* Peak-to-peak derivative Lorentzian width: \( \delta \). For this \( \tau_r \) value, \( 1 - S = 0.01 \).

3. Theory of Slow Tumbling ESR Spectra for Nitroxides (Goldman et al., 1972b; Goldman, 1973). The parameters for Brownian diffusion with linewidths of 5 and 8 G are also given. It should be noted that for \( \tau_r < 7 \times 10^{-9} \) sec, \( S \) is undefined since the outer lines begin to converge to the motionally narrowed spectrum. For longer \( \tau_r \)'s than shown in Fig. 10, the spectrum approaches the rigid limit, and the value of \( 1 - S \) becomes comparable to experimental uncertainties. The value of \( \tau_r \) for which \( 1 - S = 0.01 \) is given in Table II. The least squares fit to Eq. (82) was calculated for \( 7 \times 10^{-9} \) sec \( \leq \tau_r \leq \tau_r(S = 0.99) \).

The effect of linewidth on the value of \( S \) is also shown in Fig. 10. For Brownian and free diffusion models, \( S \) increases with increasing linewidth, while for jump diffusion a decrease in \( S \) is observed. The uncertainty in estimating \( \tau_r \) due to an uncertainty in intrinsic linewidth, for a given value of \( S \), increases for longer \( \tau_r \). Thus for a Brownian diffusion model and a 1.5 G uncertainty in the intrinsic width, the uncertainty in calculating \( \tau_r \) for a given value of \( S \) increases from about 5% for \( \tau_r \approx 1 \times 10^{-8} \) sec to about 50% for \( \tau_r \approx 1 \times 10^{-6} \) sec to an order of magnitude for \( \tau_r \approx 1 \times 10^{-6} \) sec. Linear interpolations along the vertical line between the curves A and B (or
C and D, or E and F) give the correct results for intermediate linewidth values.

The curves in Fig. 10 were calculated for isotropic rotational reorientation. For anisotropic diffusion about the z axis, with \( R_z > R_x \), the results are relatively straightforward. This type of rotation preserves the approximate axial symmetry of the spin parameters, and the observed value of \( S \) is the value expected for isotropic diffusion and \( \tau_R = (6R_z)^{-1} \). For relatively more rapid diffusion about the \( x \) or \( y \) axis, the results are more complicated. For small anisotropies about these axes, i.e., \( R_y = 3R_z \), the value of \( S \) is very slightly changed from the value calculated for isotropic diffusion and \( \tau_R = (6R_z)^{-1} = 4(R_x R_y)^{-1/2} \). This corresponds to a decrease of about 8% in the apparent value of \( \tau_R \) obtained from Fig. 10. For larger anisotropies, a decrease in the value of \( S \) is observed (e.g., for \( R_y = 3 \times 10^{-8} \) sec, Brownian diffusion, and an \( A' = 0.3 \) G, \( S \) decreases from 0.931 for \( R_y / R_x = 1 \) to 0.897 for \( R_y / R_x = 20 \), or an apparent decrease in \( \tau_R \) obtained from Fig. 10 by a factor of two). The magnitude of this decrease is independent of whether the \( x \) or \( y \) axis is the symmetry axis. However, in general, if the axis of rotation is unknown or does not correspond to a molecular coordinate axis, or if the rotation is completely asymmetric, then estimates of the components of the rotational diffusion tensor can only be obtained from detailed spectral simulations.

Two criticisms of the general applicability of the method based on measuring \( S \) are (1) it becomes very insensitive when \( \tau_R > 10^{-8} \) sec and (2) in the region \( \tau_R > 10^{-8} \) sec the results are sensitive to the choice of residual width. A related simple technique has been proposed which may prove helpful in getting around these difficulties. Before we present it, it is useful to attempt a qualitative explanation of the observed behavior of the \( S \) parameter.

In the rigid limit (for an isotropic distribution of nitroxide spin labels), the outer hyperfine extrema arise from those nitroxide radicals for which the 2p \( \pi \) orbital of the nitrogen atom is nearly parallel to the applied field direction and for which the component of nuclear spin along the electron spin direction is +1 or -1. The Stanford group (McConnell and McFarland, 1970; Hubbell and McConnell, 1971) have shown that the derivative patterns of these outer hyperfine extrema are reasonably approximated as absorption curves with a shape function characteristic of the inhomogeneous broadening. Thus, when there are incipient motional effects in the near-rigid-limit spectrum, one can try to use the well-known magnetic resonance analogy of exchange occurring between distinct (and separated) resonance lines (Abragam, 1961; Johnson, 1965). In this well-known case, as the exchange rate increases, the lines are first observed to broaden and then to shift closer together. It is line shifts of just this type that cause \( S < 1 \). In fact, in the simple exchange case of two jump sites with equal probability, for which very simple equations exist (Johnson, 1965), we find that the separation of the lines decreases by the factor \( [1 - 2(\tau_s)^{-2}]^{1/2} \approx 1 - (\tau_s)^{-2} \) when the line shifts are small, where \( \tau_s \) is the lifetime in one state and the separation between peaks, respectively. If we now draw the analogy between the rotational motion (carrying the nitroxide radical between different orientations corresponding to substantially different ESR frequencies) at a rate of the order of \( \tau_R^{-1} \) and the \( \tau^{-1} \) of the two jump model, and we use the further analogy between \( A_\tau \) and \( s \), then the result noted above, that \( S = S(\tau_R A_\tau) \), is seen to follow. If we employ the two-jump expression for small shifts to the present case, then we would predict the form of Eq. (82) with \( a = 1.75 \times 10^{-4} \) sec (for \( A_H = 32 \) G) and \( b = -0.5 \). We see from Table II that these results are of the correct order for strong jump diffusion, where the analogy is probably the best, but are substantially different than the Brownian diffusion results.

However, the analogy between \( \tau_R^{-1} \) and \( \tau^{-1} \) suggests that we examine the adhesive line broadening of the outer hyperfine extrema, which should (1) roughly correspond to \( \tau_R^{-1} \) and (2) be a more sensitive function of the motion than the shifts in position. Indeed, accurate computer simulations have confirmed these suggestions as being theoretically correct (Mason and Freed, 1974). In fact, the residual width is found to be given by \( \tau_R^{-1} \) within a factor of \( \sim 2 \) (or \( \frac{1}{2} \)) over most of the range of interest. This is about as good an agreement as we might hope for, when we recognize that the analogy is incomplete, because in the slow tumbling case, the rotational motion (a) modulates the ESR frequency over a continuous range, and (b) induces nuclear spin flips as well because the quantization axis of the nuclear spins is orientation dependent; this is known as a nonadiabatic effect (Freed, 1972a). However, a recent approximate treatment, for small pseudosecular terms in Eq. (74), has shown that this latter effect is roughly proportional to \( \tau_R^{-1} \) (Freed, 1974).

We give now a more quantitative discussion of this width effect (Mason and Freed, 1974). The average of the measured half-widths at half-heights, \( \Delta \), for the outer extremum of a rigid-limit spectrum is found from simulations to be equal, to a very good approximation, to \( \sqrt{\frac{1}{2}} \Delta \delta = |\gamma_\tau|^{-1} \tau_\tau^{-1} \). The heights of the hyperfine extrema are measured from the true baseline (cf. Fig. 9). More precisely, we have

\[ 2\Delta_i = 1.59\delta \quad (83a) \]
\[ 2\Delta_H = 1.81\delta \quad (83b) \]

where the subscripts \( i \) and \( H \) refer to the low- and high-field lines, respectively, and the superscript \( \tau \) refers to the rigid-limit value. This result is found to be independent of \( \delta \) over the range \( 1.0 \leq \delta \leq 4.0 \) G and virtually
independent of \( A_r \) over the range \( 27 \leq A_r \leq 40 \) G. It is, of course, essentially independent of variations in the other nitroxide rigid-limit parameters. Equations (83a) and (83b) are valid for the assumption of Lorentzian inhomogeneous broadening. (No calculations for non-Lorentzian broadening have yet been performed for the method discussed.)

In the slow motional region, near the rigid limit, the linewidth \( \Delta \) for Lorentzian line shapes can be decomposed into two contributions (cf. Abragam, 1961): (1) the Lorentzian inhomogeneous component given by Eqs. (83a) and (83b) and (2) the excess motional width (of order of magnitude \( \tau_e^{-1} \)). (It is convenient to think in terms of this decomposition even though it is not necessary for the method.) A useful dimensionless parameter for describing these spectra is then

\[
W_i \equiv \Delta_i/\Delta_1, \quad W_i - 1 = (\Delta_i - \Delta_1)/\Delta_1
\]

(84)

where \( i = \ell, h \). In general, \( W_i - 1 \) is about an order of magnitude larger than 1 - \( S \) for a particular value of \( \tau_e \) (cf. Fig. 11), and furthermore, it can be measured to at least comparable accuracy (~ 1%; cf. Fig. 9). The results in Fig. 11 were calculated utilizing the computer program in Appendix B. A study of how \( W_i \) is affected by changes in (1) the spin parameters, (2) linewidth, and (3) rotational diffusion model has been made. It was found that \( W_i \), like \( S \), is insensitive to deviations from axial \( A \) and \( g \) tensors, as well as to variations in \( A_2 \) and \( g \) typical of a nitroxide. However, in contrast to \( S \), which is dependent on the product \( \tau_e A \), \( W_i \) is virtually independent of \( A_r \) over the range \( 27 \leq A_r \leq 40 \) G; (we have used \( A_r = 32 \) G in obtaining the results in Fig. 11), as expected from our simple analogy. However, \( W_i \) is found to depend on \( \delta \). Generally, a smaller \( \delta \) implies a larger \( \Delta_i - \Delta_1 \) for a given \( \tau_e \). In particular, \( \delta = 1 \) G yields values of \( \Delta_i - \Delta_1 \) ranging from 1.3 to 2.5 times greater than those for \( \delta = 3 \) G. We can try to explain this observation qualitatively. The rigid-limit extrema of finite width \( \Delta_i \) arise from those nitroxide radicals whose \( 2 \pi \) N-atom orbitals lie within a cone of angle \( \Omega \) about the applied field direction, and the size of the cone increases rapidly with an increase in the rigid-limit \( \delta \) (McConnell and McFarland, 1970). If we roughly identify the excess width \( \Delta_i - \Delta_1 \) with the rate at which radicals reorient out of the cone, then extrema from the larger cones (which result from larger values of \( \delta \)) will be less broadened, since it takes longer for the radicals to leave the cone. The observation that \( \Delta_i - \Delta_1 \) is always significantly larger than \( \Delta_i - \Delta_1 \) at a given \( \tau_e \) could be explained in a similar manner. It is known that the high-field resonance for a single-crystal spectrum changes with angle more rapidly than the low-field resonance; thus the range of \( \Omega \) contained in the observed cone (from a polycrystalline sample) must be smaller for the high-field line. Reorientations out of the high-field cone thus occur at a more rapid rate, and, in general, \( W_i \) is a more sensitive function of \( \tau_e \) than is \( W_1 \), as can be seen from Fig. 11.

The choice of a proper \( \delta \) is clearly at the heart of the method. Near the rigid limit, an appropriate estimate of \( \delta \) can be deduced from the \( \Delta_i \). The narrowest rigid-limit \( \delta \) found in our laboratory is 1.5 G, which corresponds to \( 2 \Delta_i = 2.4 \) G. Hubbell and McConnell (1971) reported values of 4.6 and 5.5 G for \( 2 \Delta_i \) for pseudoaxial "rigid-limit" spectra, corresponding to \( \delta \) of 2.9 and 3.5 G, respectively. The rigid limit spectrum of the \( N_{-polyl-4',4'-dimethyl-azoxide} \) derivative of \( 6x-\text{androstane-3-one} \) appears to have \( 2 \Delta_i \approx 5.0 \) G, which corresponds to a \( \delta \) of 3.1 G (McConnell and McFarland, 1970). The motional broadening can easily double the widths of the outer extrema when the separation of the hyperfine extrema is not much different from the rigid-limit value (i.e., \( S > 0.95 \)). Thus, very near the rigid limit, where \( \delta \) can be determined from the rigid-limit extrema widths, two independent determinations of \( \tau_e \) can be made using Fig. 11.
As has been noted, the major contributions to the $\Delta f$ are electron nuclear hyperfine interactions between the electron and the protons of the spin label and host, while heterogeneity of the environment also contributes to $\Delta f$. These interactions will be quickly averaged with the onset of molecular motion, resulting in a decrease in the appropriate $\delta$. When this is the case, it becomes necessary to estimate a single $\delta$ such that the rotational correlation times obtained from Fig. 11 for both the low- and high-field extrema are equal within experimental error. For this process we can define "effective inhomogeneous widths" $\Delta f^*$, which obey Eqs. (83a) and (83b) and which generally obey the relation $\Delta f^* \leq \Delta f$. Then we should rewrite Eq. (84) as

$$W_i = \Delta / \Delta f^*$$

(84')

with the $W_i$ given by Fig. 11. This procedure should then yield both $\tau_0$ and $\delta$. As noted, the uncertainty in $\delta$ can result in serious errors when $\tau_0$'s of $> 3 \times 10^{-8}$ sec are determined from $S$. Once $\delta$ has been determined from the $W_i$, another estimate of $\tau_0$ may (when feasible) be obtained from a measurement of $S$. In other words, $\tau_0$ and $\delta$ could be obtained as a function of three experimental parameters, $S$ and the $W_i$.

The model-dependent results shown in Fig. 11 were obtained for Brownian diffusion and free diffusion, as before. The free diffusion model results in a more nearly linear dependence (in a log-log plot) of $W_i - 1$ versus $\tau_0$ in Fig. 11 than the Brownian motion model. The plots in Fig. 11 have been fitted to the form

$$\tau_0 = a'(W_i - 1)^{-b'}$$

(85)

for the region $W_i - 1 > 0.01$, and the coefficients are given in Table III. The maximum variation between the curves and the results predicted from Eq. (85) is also given. It is clear that the use of Eq. (85) is a less accurate means of estimating $\tau_0$ than the curves. However, the fact that $b' \approx 1$ (except for the anomalous curve F, which is presumably affected by overlap) is consistent with the interpretation of $\Delta f - \Delta f^*$ as a lifetime broadening.

When there is axially symmetric rotational diffusion about the molecular $z$ axis with $R_2 > R_1$, it should mean that $\tau_0$ is again obtained. Also, the introduction of an angle-dependent rigid-lid width given by Eq. (29) should have no effect on the outer hyperfine extrema, except that now

$$\delta = (2/\sqrt{3}) |\gamma_e|^{-1/2}(T_{2,1}^{-1} + T_{2,1}^{-1}) = (2/\sqrt{3}) |\gamma_e|^{-1}(\alpha + \beta)$$

(86)

It is very important in the experimental application of this method to avoid distortion of the true linewidth by overmodulation of the magnetic field and/or power saturation. Experimental applications of this method have not yet been reported.

### Table III

<table>
<thead>
<tr>
<th>Curve</th>
<th>$a \times 10^3$ sec</th>
<th>$b'$</th>
<th>Maximum deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.29</td>
<td>1.033</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>1.96</td>
<td>1.062</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>5.32</td>
<td>1.076</td>
<td>18</td>
</tr>
<tr>
<td>D</td>
<td>7.97</td>
<td>1.123</td>
<td>18</td>
</tr>
<tr>
<td>E</td>
<td>1.15</td>
<td>0.943</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>2.12</td>
<td>0.776</td>
<td>18</td>
</tr>
<tr>
<td>G</td>
<td>5.45</td>
<td>0.999</td>
<td>30</td>
</tr>
<tr>
<td>H</td>
<td>9.95</td>
<td>1.014</td>
<td>55</td>
</tr>
</tbody>
</table>

*Table is based on approximate fit of Fig. 11 data to Eq. (85) for $W_i - 1 > 0.01$. See Fig. 11 for explanation of the different curves.

[Reprinted with permission from Mason and Freed, J. Phys. Chem. 78, 1321-1323 (1974). Copyright by the American Chemical Society.]

* Based on comparing values in Fig. 11 with Eq. (85).

The simplified methods discussed above are based on the relatively simple to analyze behavior of the outer extrema. While it is true that the central region of the nitroxide spectrum is very sensitive to motional effects, it is also very sensitive to the deviations of the nitroxide magnetic parameters from cylindrical symmetry and this can vary considerably (cf. Table I). Thus it would be difficult to develop general methods based on the central region; computer simulation with accurate magnetic parameters is probably required.

### C. Very Anisotropic Rotational Reorientation

The simplified methods discussed in the previous section have an important failing in that they are not really applicable to spectra arising from highly anisotropic motion, especially when the molecular $z$ axis is not itself a principal axis. The phenomenon of spin labels undergoing very rapid anisotropic rotational reorientation is a common one. In fact, the Stanford group (Hubbell and McConnell, 1969a,b, 1971; McConnell and McFarland, 1970; McFarland and McConnell, 1971) have developed a simple analysis in terms of an effective time-independent spin Hamiltonian $\mathbf{H}_{\text{eff}}$ to account for rapid anisotropic motion.

Suppose that there is very rapid motion about some molecular axis $\mathbf{v}$, motion perpendicular to that axis is very slow. This is the case, for example, if the nitroxide spin label rotates about a single bond while the overall motion is that of the macromolecule to which it is attached. Then we
can introduce effective $g'$ and $A'$ tensors that are axially symmetric about $v$, and this yields an effective rigid-limit Hamiltonian to predict the spectrum. The use of such an effective Hamiltonian implies (1) a large enough $R_i$ (representing rotational reorientation about $v$) that residual time-dependent effects of the averaging process, which could lead to line broadening, are negligible; and (2) motion about axes perpendicular to $v$, described by an effective $R_i$, is so slow that its effects on the spectrum are negligible.

Suppose that either of these conditions is not fulfilled, and that motional effects assert themselves in the spectrum. If condition 1 is fulfilled, but $R_i$ becomes fast enough to affect the spectrum, then one can simulate spectra using the program in Appendix B but with the effective axial tensors $A'$ and $g'$. The tensor $A'$ is given in terms of the true $A$ and the direction cosines $x_i$ ([1] $z''$, $y''$, or $z''$, but we drop the triple primes for convenience in this section) of $v$, in the molecular principal axis system as

$$A_i' = \sum_j x_i' x_j A_j$$  \hspace{1cm} (87)

$$A_i' = \frac{1}{2} \sum_j (1 - x_i' x_j) A_j$$  \hspace{1cm} (88)

The simplified methods of the previous section would only apply in modified form if $A_i'$ and $A_i''$ are not much different from typical nitroxide values (see below).

If, however, condition 1 is relaxed somewhat, then a motional narrowing theory can be applied to consider how the motion represented by $R_i$ yields line broadening, etc., from the deviations between $x_i''$ and the true $x_i$. However, using our example above, the relaxation effects are a function of the orientation of the macromolecule, and we would have to compute such effects from $R_i$ for each orientation.

However, the general theory given here can be rigorously applied to this example, including effects from both types of motion simultaneously. A series of simulations in which $R_1$ is just large enough to show incipient slow motional effects, namely $\tau_{R_1} = 5 \times 10^{-8}$ sec, were performed where $\tau_{R_1}$ is allowed to vary from $5 \times 10^{-8}$ to $6 \times 10^{-11}$ sec (Mason et al., 1974). This series of simulations, shown in Fig. 12b, was motivated by experimental results (cf. Fig. 12a) of Wee and Miller (1973) on a spin-labeled polybenzyl glutamate in DMF solution (cf. Fig. 13). These simulations required that the principal axes of $R_i$ (i.e., $x_i', y_i'$, and $z_i'$) be tilted relative to the principal axes of the magnetic tensors ($x', y', z'$). In this modified computer program, $A$ and $g$ are expressed in the $x', y', z'$ coordinate system (cf. Polnaszek, 1973). The spectra were calculated for a spatially isotropic distribution of spin labels with a tilt angle between respective $z$ axes of 41.7°. The simulated spectra of

Fig. 12b for $\tau_{R_1}$ of the same order of magnitude as $\tau_{R_1}$ appear similar to the isotropic and near-isotropic motional spectra already shown, and for which the simplified approaches already apply. But for $\tau_{R_1} \ll \tau_{R_1}$, there are marked qualitative differences. (Note that this is an ideal case for $K$ truncation, i.e., $\eta_K < \eta_L$.) The general progression of spectra in Fig. 12b from A to I, where $\tau_{R_1}$ is increasing bears considerable resemblance to the progression of experimental spectra in Fig. 12a from A to G, where the polymer concentration, and hence the solution viscosity, is increasing. Rather close agreement is found between pairs 12b-I and 12a-G (the near-rigid limit), 12b-B and 12a-B (where $\tau_{R_1}$ is very fast), and 12b-F and 12a-F (an intermediate case). These results provide evidence that there is fast motion of the pipericine ring about the NH-CH bond, and no observable motion of the overall polymer (i.e., $\tau_p > 10^{-7}$ sec).

The question now remains of the range of validity of the effective time-independent Hamiltonian in terms of $g'$ and $A'$. Its use by Hubbell and
where the bar implies a time average and the second equality is based on the fact that $A_s = A_i$ as is the case in many solids. $S_s$ is a measure of the extent of the motion leading to $2\text{e}^a$. Then we have

$$A' = \frac{1}{3} A^2 = A_s - A_i$$

$$A_s = \frac{1}{2} A^2 - A_i = A'$$

with permission from Wee and Miller, J. Phys. Chem. 71, 280 (1967). Copyright by the American Chemical Society.

**TABLE IV**

<table>
<thead>
<tr>
<th>Spectrum index</th>
<th>$r_{s_n}$ (sec)</th>
<th>$A'_s$ (G)</th>
<th>$A_i$ (G)</th>
<th>$\Delta A_s$ (G)</th>
<th>$\Delta A_i$ (deg)</th>
<th>$S_s$</th>
<th>$\delta_s$</th>
<th>$\delta_i$</th>
<th>$\cos^{-1}(\alpha)$</th>
<th>$\cos^{-1}(\beta)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.06</td>
<td>18.9</td>
<td>11.5</td>
<td>7.4</td>
<td>4.0</td>
<td>-0.008</td>
<td>0.299</td>
<td>2.0035</td>
<td>2.0059</td>
<td>2.0056</td>
</tr>
<tr>
<td>B</td>
<td>0.10</td>
<td>18.9</td>
<td>11.3</td>
<td>7.6</td>
<td>4.2</td>
<td>-0.008</td>
<td>0.304</td>
<td>2.0035</td>
<td>2.0059</td>
<td>2.0056</td>
</tr>
<tr>
<td>C</td>
<td>0.20</td>
<td>19.2</td>
<td>11.6</td>
<td>7.6</td>
<td>4.2</td>
<td>-0.010</td>
<td>0.324</td>
<td>2.0050</td>
<td>2.0060</td>
<td>2.0057</td>
</tr>
<tr>
<td>D</td>
<td>0.40</td>
<td>20.2</td>
<td>10.7</td>
<td>9.5</td>
<td>4.0</td>
<td>-0.014</td>
<td>0.380</td>
<td>2.0047</td>
<td>2.0051</td>
<td>2.0056</td>
</tr>
<tr>
<td>E</td>
<td>0.67</td>
<td>22.6</td>
<td>10.3</td>
<td>12.3</td>
<td>4.4</td>
<td>-0.025</td>
<td>0.490</td>
<td>2.0037</td>
<td>2.0042</td>
<td>2.0054</td>
</tr>
<tr>
<td>F</td>
<td>1.00</td>
<td>24.1</td>
<td>10.9</td>
<td>13.2</td>
<td>4.1</td>
<td>-0.017</td>
<td>0.528</td>
<td>2.0033</td>
<td>2.0058</td>
<td>2.0050</td>
</tr>
<tr>
<td>G</td>
<td>1.60</td>
<td>27.3</td>
<td>11.6</td>
<td>15.7</td>
<td>4.2</td>
<td>-0.016</td>
<td>0.547</td>
<td>2.0031</td>
<td>2.0056</td>
<td>2.0050</td>
</tr>
<tr>
<td>H</td>
<td>10.0</td>
<td>27.7</td>
<td>11.7</td>
<td>16.0</td>
<td>4.4</td>
<td>-0.025</td>
<td>0.573</td>
<td>2.0030</td>
<td>2.0056</td>
<td>2.0050</td>
</tr>
<tr>
<td>I</td>
<td>500</td>
<td>29.7</td>
<td>11.7</td>
<td>18.0</td>
<td>4.5</td>
<td>-0.025</td>
<td>0.587</td>
<td>2.0030</td>
<td>2.0056</td>
<td>2.0050</td>
</tr>
<tr>
<td>J</td>
<td>0.00</td>
<td>30.4</td>
<td>11.8</td>
<td>18.6</td>
<td>4.5</td>
<td>-0.025</td>
<td>0.587</td>
<td>2.0030</td>
<td>2.0056</td>
<td>2.0050</td>
</tr>
</tbody>
</table>


1. Peak-to-peak residual derivative width $\delta$ of 5.0 G was used.
2. Peak-to-peak residual derivative width $\delta$ of 2.0 G was used.
3. The inner hyperfine extrema are not resolved.
4. $R_s$ and $R_i$ are zero.
5. $S_s$ and $\sigma_s$ are defined by Eq. (89).
6. $A_s$ is defined by Eq. (91).
7. $g'_s$ is defined as $\frac{1}{3}(g'_i + g'_s + g'_i)$.
8. From construction, $\cos^{-1}(\alpha) = \frac{\pi}{2}$.
9. If $S_s = 0.336$ is used, $\cos^{-1}(\alpha) = \frac{\pi}{2}$ equals 50°; see text.
10. If correction is made for the finite $r_{s_n} = 5 \times 10^{-3}$ see, then $S_s = 0.338$ (cf. Mason et al., 1974).
A check on the validity of using $\mathcal{K}_{\text{eff}}$ for interpreting a spectrum in terms of the "pseudoaxial" rigid limit is that one must have $\text{Tr} A = \text{Tr} A'$, which follows directly from the rotational invariance of the trace of a tensor. The simulated spectra of Fig. 12b have been analyzed just as though they were experimental results from anisotropically immobilized spin labels, and the results are summarized in Table IV (Mason et al., 1974). These results are in fact very similar to those obtained by Wee and Miller (1973) and in spin label studies in membrane models and membranes (Hubbell and McConnell, 1969a,b, 1971; McConnell and McFarland, 1970; McFarland and McConnell, 1971). Note that (1) $A'_x$ does vary slightly from $A_x = 14.13$ G but (2) the apparent $S_a$ varies considerably even though the calculated spectra were from a tilt angle of $41.7^\circ$, corresponding to a constant true value of $S_a = 0.336$. The results for $A$ and $B$ in Fig. 12b, where $\tau_{R_1}$ is very fast, are reasonable; the only discrepancy with the true results probably arises from the residual motional effects on the spectrum of having $\tau_{R_1} = 5 \times 10^{-8}$ sec. However, for spectra such as E and F, where $\tau_{R_1} \approx 5 \times 10^{-10}$ sec, the motional effects for the fast motion about a single bond are already slow enough for us to utilize the simple time-independent $\mathcal{K}_{\text{eff}}$. Thus the invariance of $S_a$ is a necessary but not really sufficient condition for the simple approach.

Thus we note that a change in $S_a$ can arise from a real change in the angle between $v$ and $z$ or a change in the rotational rate about $v$, as is the predominant phenomenon in the spectra of Fig. 12a (the spectrum A, however, has a shorter $\tau_R$ than the other spectra) (Mason et al., 1974). In general, these two phenomena cannot be distinguished unless the rotational rate about $v$ slows to where $A'_x$ is clearly anomalous and/or the slowed motion manifests itself in the other spectral characteristics shown in Fig. 12a.

Note that the analysis in terms of a single $R_1$ and $R_2$ represents a considerable simplification of the complex dynamics of polymer motion including localized bond motions and internal rotations. However, (1) as long as the internal rotation is much faster than the overall motion, it can be treated as uncoupled from the latter, and (2) if the overall motion is only showing marginal spectral effects, it would be difficult to obtain anything more precise than an effective $\tau_{R_1}$.

D. Anisotropic Liquids: Simulations

Some examples will now be given for nitroxides oriented in nematic liquid crystals (Polanszuk et al., 1973; Polanszuk, 1975a). For convenience, the Meier-Saue potential, coincidence of the magnetic and orientation principal axes, and axial magnetic parameters were used, unless otherwise noted. In all cases the director was assumed parallel to the static magnetic field.

3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

First, the effect of keeping the rotational correlation time $\tau_R = (6\tau)^{-1}$ constant but varying the ordering by changing the potential parameter $\lambda$ is considered. Figure 14 shows such a case for an oblate nitroxide, i.e., one that tends to orient with its $z$ axis perpendicular to the magnetic field. The rotational correlation time was held constant at a value of $1.84 \times 10^{-9}$ sec, which is in the incipient slow motional region for nitroxides. One sees that the effect of increasing the absolute magnitude of the orienting potential is (1) to decrease linewidths considerably, and (2) to introduce larger shifts in the positions of the lines. The first effect is due to the fact that the effective $[\mathcal{K}_{\text{eff}}(t) - \langle \mathcal{K}_{\text{eff}} \rangle]$ is reduced as the sample is being ordered, while $\langle \mathcal{K}_{\text{eff}} \rangle$, the average part of the perturbation, which causes the line shifts, departs more from its isotropic value of zero. It appears that the slow tumbling spectra begin to resemble motional narrowing results as $|\lambda|$ is increased at a constant $\tau_R$, although the shift of line positions is characteristic of liquid crystals in the nematic range. However, the observed line shifts are not predicted correctly by expressions appropriate for the motional narrowing region (cf. Fig. 14. First derivative line shapes for a nitroxide as a function of $\lambda$ for Brownian diffusion. The different $\lambda$ values are $(- - -) 0, (- - -) -2.0, (- - -) -3.5, (- - -) -7.5$. All correspond to $\tau_R = 1.84 \times 10^{-9}$, $\sigma = 2.002^\circ$, $\delta = 2.007^\circ$. $A_1 = 33.4$ G, $A_2 = 3.42$ G, and $\delta = 0.1$ G. [From Polanszuk et al. (1973)].
Chapter 10. We can use these shifts as an indication of slow motion in the mesophase. For $\lambda > 0$ a prolate top (a nitroxide that tends to orient with its $z$ axis parallel to the field) similar linewidth behavior is observed, except that the spectrum spreads out and shifts to higher fields. In Fig. 15, $\lambda$ is held constant at a value of $-0.975$, corresponding to a low degree of ordering typical of small nitroxides. Comparison with figures for isotropic liquids (cf. Figs. 6–8) shows that the trends are quite similar in both cases, but that there are distinct quantitative differences in the details of the line shapes at comparable $\tau_k$'s.

In Figs. 16a and b, $\lambda$ is held constant (at $-7.5$ and $8.5$, respectively) for large ordering parameters, with the correlation time varying over several orders of magnitude. They correspond respectively to large disklike and rodlike nitroxide molecules, which tend to be very well ordered. They show that spectra with correlation times $< 3 \times 10^{-8}$ sec will be fairly insensitive to changes in $\tau_k$. In fact the observed ordering parameter for the oblate top nitroxide is nearly equal to the theoretical value for $\tau_k \leq 10^{-9}$ sec, and it is found that the rigid limit is not approached until $\tau_k \approx 10^{-6}$ (cf. Fig. 16c), compared to $\tau_k \approx 3 \times 10^{-7}$ for the low ordering case in Fig. 15. Thus for a very highly ordered nitroxide, we can extend the upper limit of rotational correlation times obtainable from the unsaturated slow motional line shapes.

For values of the potential parameter $\lambda$ that lead to intermediate ordering (e.g., $\lambda = -3.5$, corresponding to $\langle Q_0^2 \rangle = -0.30$), it has been found that the deviations from symmetric lines and from the theoretical ordering parameter begin to occur at somewhat longer values of $\tau_k$ in the incipient slow tumbling region than for isotropic liquids, but that the linewidth asymmetry starts to be appreciable at somewhat shorter $\tau_k$'s than those for which the apparent ordering parameter deviates significantly from the correct value. Therefore the linewidth asymmetry can be used as an indication of slow tumbling in nematic phases. This can also be seen from Fig. 16 for highly ordered nitroxides.

The effect of using different models of rotational reorientation has also been studied. For weakly ordered systems, one again sees the same qualitative behavior of spectral changes as the rotational model is changed, but there are qualitative differences between the spectral changes for the isotropic and nematic phases. As has been usually done for isotropic liquids, the correlation times for the non-Brownian models were determined to be those that gave the same values for the separation of the outer hyperfine extrema (i.e., $S$) as observed in the Brownian diffusion case. For liquid crystals $S$ is expected to be a function of $\lambda$ as well as of $\tau_k$ and $A$, thus complicating any attempts to use it as a quantitative measure of $\tau_k$'s in nematic phases. This was found to be true for weakly ordered systems. For the strongly ordered cases, the parameter $S$ is meaningless since no outer extrema are observed.

Figure 17 gives an example in which the principal axis of orientation is permuted among the three principal axes of the nitroxide magnetic tensors. The hyperfine tensor is taken as axially symmetric, but the $g$ tensor is asymmetric, as is typical for nitroxides. The rotational correlation time is $10^{-8}$ sec and $\lambda$ was adjusted to make the $S$ values nearly equal. The $x$- and $y$-axis spectra are cases where the molecule tends to align parallel to those axes, respectively, while the $z$ axis tends to be perpendicular to the field. One sees significant changes in the line shapes as the principal axis of orientation is changed, even for this weakly ordered system. There are also "apparent" shifts in $\Delta g_y$. The $g$ shifts persist in the motional narrowing region for this case of three different orientations. Thus, especially when using any cylindrically symmetric potential, one must be careful to choose the principal axis of orientation correctly. However, when an asymmetric potential such as that given in Eq. (58) is used, the potential is invariant to permutation of the principal axes (i.e., the relabeling of $x''$, $y''$, $z''$ to obtain $x'$, $y'$, $z'$). Since the coefficients of the potential transform as the components of an irreducible tensor, we can transform the potential parameters for a principal axis system
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

Fig. 16. First derivative line shapes for nitroxides in highly ordered cases as a function of $\tau_k$ for Brownian diffusion: (---) $\tau_k = 3 \times 10^{-9}$ sec, $IF = 779$; (-----) $\tau_k = 3 \times 10^{-9}$ sec, $IF = 529$; (-----) $\tau_k = 3 \times 10^{-9}$ sec, $IF = 163$. (a) $\lambda = -1.2$; (b) $\lambda = 8.5$. (c) $\lambda = -7.5$ and $\tau_k = 3 \times 10^{-7}$ sec, $IF = 298$; (-----) $\tau_k = 1 \times 10^{-6}$ sec, $IF = 41.0$. All other parameters as in Fig. 15. [From Polnanskii et al. (1973) and Polnanskii (1975a)]

in the molecule into those for another molecular axis system. For the potential given by Eq. (58), one has for a permutation of axes such that the $y$ axis $\rightarrow z'$ axis

$$\lambda_y = -\left(\lambda_z - 3\rho_x\right)/2$$  \hspace{1cm} (92a)

and

$$\rho_y = -\left(\lambda_z + \rho_x\right)/2$$  \hspace{1cm} (92b)

where the subscripts refer to the principal axis of orientation of the molecule. We can also determine $\lambda_x$ and $\rho_x$ from the relation

$$\sum_{i} \lambda_i = \sum_{i} \rho_i = 0$$  \hspace{1cm} (93)

which follows from the fact that the ordering tensor is traceless.
It is well known that liquid crystals exhibit an anisotropic viscosity when oriented in a magnetic field (Miesowicz, 1946). The effects of anisotropic viscosity on nitroxide slow tumbling spectra are shown in Fig. 18. In this figure, \( \tau_{R_1} = (6R_1)^{-1} \) is kept constant at \( 1 \times 10^{-8} \) sec, the potential parameter \( \lambda \) is \(-0.975\), and \( \tau_{R_2} \) is varied. The effect of keeping \( R_1 \) constant is to keep the value of \( S \) virtually constant. However, there are gross changes in the central region of the spectrum as \( R_2 \) is increased relative to \( R_1 \). Note from Eq. (66) that the terms that contain the effect of anisotropic viscosity have \( M \neq 0 \). It is seen from Eq. (74) that such terms are the pseudosecular terms in \( H_{\tau}(\Omega) \). Thus we must include them in the Hamiltonian in order to see the effects of anisotropic viscosity. The effects of anisotropic viscosity on the slow motional line shapes are negligible when the ordering parameter is large or if the molecule tends to be aligned with its \( z \) axis, the axis of cylindrical symmetry of the hyperfine tensor, parallel to the field. Note that the effects of anisotropic molecular reorientation on slow tumbling spectra from nitroxides are not as dramatic as the effects of anisotropic reorientation with respect to the director axis. Birrell et al. (1973) observed a system that can be thought of in terms of a highly anisotropic viscosity. Nitroxide free radicals were oriented in tubular cavities in inclusion crystals in which the molecule is free to rotate about the long axis but with its rotation hindered about the other two axes because of the cavity geometry. The system behaves as a highly ordered liquid crystal, which, as has already been noted, is fairly insensitive to the dynamics of the motion.

In all the preceding discussion, it has been assumed that \( n \) is fixed along the laboratory \( z \) axis, so that \( \Psi = (0, 0, 0) \). When \( n \) is tilted relative to the \( z \) axis, then Eq. (45) must be used to expand \( \Psi_{\phi}(\Omega, \Psi) \), otherwise the same diffusion equations in terms of \( \Omega \) are applicable. If there are random static distributions of directors, then, in principle, one must solve the problem for each value of \( \Psi \) and then integrate over the correct static distribution to predict the spectrum. When there is residual motion of the director, then
the stochastic Liouville equation can be augmented to deal effectively with
the simultaneous motions of $\Psi$ and $\Omega$ (Polnaszek, 1975a; Polnaszek and
Freed, 1975).†

E. Anisotropic Liquids: Experiments

Experiments have now been carried out to test the applicability of the
slow tumbling theory to anisotropic liquids using the same general approach
as has already been described for studies in isotropic liquids (Polnaszek,
1975a; Polnaszek and Freed, 1975). In particular, the PD-TEMPO probe
has been studied in several viscous nematic solvents. The study was limited
by the fact that before very large viscosities could be reached, the nematons
would freeze. However, in the case of phase V solvent, it was possible to
reach the slow motional region (cf. Fig. 19). Note in Fig. 20 how the apparent
$\langle \mathcal{B}_0^2 \rangle$ changes markedly when the slow motional region is approached.

In the isotropic studies, the motional narrowing line shapes were care-
fully corrected for the residual inhomogenous broadening effects of the
deuteron splittings given in Table I. In the nematic phase, the splittings were
found to vary with temperature as a result of the increase of $\langle \mathcal{B}_0^2 \rangle$ with
decreasing temperature. This added factor had to be corrected for in order to
adequately deal with the line shapes.

Furthermore, careful measurements of the $\delta N$ and $g$ shifts clearly
demonstrated that the radical ordering required the use of the two-
parameter potential of Eq. (58). For most cases (including phase V solvent),
if the choice $x'' = x'$, $y'' = y'$, and $z'' = z'$ is made, then $|\lambda| > |\rho|$, with
typical values for phase V being $\lambda = -0.8$ and $\rho = 0.3$. The higher
temperature spectra from the isotropic phase again showed $N = 1$ for the aniso-
tropic diffusion parameter, corresponding to isotropic rotation. However, in
the nematic phase, only if the correct ordering potential involving both $\lambda$
and $\rho$ were used could the linewidth results be fit to isotropic rotational
diffusion.

The appropriate values of $\tau_k$, $N = 1$, $\lambda$, and $\rho$ were then extrapolated to
the slow motional region to obtain parameters to predict the slow motional
spectrum. A typical comparison is shown in Fig. 21a, where
$\tau_k = 3.6 \times 10^{-5}$ sec at $-25^\circ$. There are serious discrepancies between
experiment and prediction, unlike the good agreement (cf. Fig. 16) for
isotropic liquids. This incipient slow tumbling region has been found to be
rather model insensitive. However, careful analysis of the motional narrowing
region for $\tau_k > 2 \times 10^{-10}$ sec showed that the discrepancy was developing

† Editor's note: Further and more expanded discussions of order parameters and the
anisotropic motion of spin labels in liquid crystals and bilayer model membrane systems are
found in Appendix IV and Chapters 10-12, respectively.

Fig. 19. ESR spectra of PD-TEMPO in liquid crystal: phase V, at different temperatures. The scan range is 40 G. [From Polnaszek (1975a).]

Fig. 20. Ordering parameter $\langle \mathcal{B}_0^2 \rangle$ vs. reduced temperature $T^* = T/T_c$ for PD-
TEMPO in several liquid crystals; O, phase V solvent. [Reprinted with permission from
Chemical Society.]
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

Formula would be expected to arise if the rotational reorientation is significantly coupled to other degrees of freedom of the molecule or its surroundings (e.g., the angular momentum of the molecule or internal rotational degrees of freedom). A detailed statistical mechanical theory in terms of fluctuating torques experienced by the spin probe in its liquid environment has been proposed by Hwang et al. (1975).

The pseudosecular terms reflect the nuclear spin-flip transitions \( \omega_\pm \approx a_\pi / 2 \) for isotropic liquids and \( \langle a_\pi \rangle / 2 \) for nematics. Thus one might try

\[
J(\omega_\pm) = \tau_\pi / \left(1 + \varepsilon \omega_\pm^2 \tau_\pi^2 \right)
\]

It was found that a large value of \( \varepsilon \) could account reasonably for the motional narrowing and slow tumbling spectra in the nematic (cf. Fig. 21b).

Actually, the analysis of this effect is fairly complex, involving inceptive slow tumbling corrections to which transpires that the secular spectral densities are also characterized for nonzero frequencies and the pseudosecular spectral densities are modified. The proper analysis, which is complex, is discussed by Polnaszek and Freed (1975). It is found that the pseudosecular spectral densities require a correction of \( \varepsilon \pi \tau \sim 15-20 \) while for the secular spectral densities \( \varepsilon \pi \tau \sim 1-2 \). This yields satisfactory agreement with experiment (cf. Fig. 21b). Another approach to this problem, in terms of slowly fluctuating torques, has been introduced by Polnaszek and Freed (1975). It is based upon the concept of a local structure or ordering which is relaxing more slowly than the probe molecule. This structure is expected to result from the surrounding rodlike nematic solvent molecules, which reorient more slowly than the smaller spin probe. [Such effects are not included in the Meier-Saupe (1958) mean field analysis that leads to the effective pseudopotential.] A simple analysis of the spin relaxation effects of such a mechanism shows that it has many of the proper trends. We may let \( \Psi \) be the slowly relaxing set of Euler angles between the local structure and the lab frame, then for an axially symmetric potential of the spin probe relative to the local structure, rough estimates of \( S_0^2 = \langle \Omega_0^2 \rangle \sim 0.1 \) and \( \tau_\pi / \tau_\pi \sim 10 \) are obtained from the simple model, where \( \tau_\pi \) is the local structure relaxation time. If such a hypothesis were correct, further careful studies including larger spin probes of different shapes could shed further light on this phenomenon.

Some preliminary experimental spectra of the rodlike cholesteric spin label 3-doxyl-5a-cholesterol in the viscous nematic phase of phase V are shown in Fig. 22 (Polnaszek, 1975a). The system is highly ordered and the apparent splitting constants do not change appreciably with temperature. The observed \( \langle \Omega_0 \rangle \) values calculated from a motional narrowing theory analysis (cf. Chapter 8) are \(-0.35, -0.39, \) and \(-0.46 \) for \( T = 26, 3, \) and \(-26^\circ\text{C} \), respectively. The two splittings are not equal at the lowest tempera-

\[
\text{Fig. 21. Comparison of theoretical (-----) and experimental (- - -) spectra for PDE-TEMPONE in phase V liquid crystal at (a) \( \varepsilon = 1 \) with } \tau_\pi = 2.5 \times 10^{-9} \text{ sec at } -20^\circ\text{C and } \tau_\pi = 3.6 \times 10^{-9} \text{ sec at } -25^\circ\text{C (A' = 0 G); (b)} \quad \varepsilon_\pi = 1.2, \varepsilon'_\pi = 20. \text{ The values at } -6^\circ, -14^\circ, -20^\circ, \text{ and } -25^\circ \text{C are for } \tau_\pi: 0.9, 1.6, 2.5, \text{ and } 3.6 \times 10^{-9} \text{ sec, respectively, and for } A': 0.55, 1.0, 1.45, \text{ and } 1.75 \text{ G. The magnetic parameters are given in Table IV. [Reprinted with permission from Polnaszek and Freed, } J. \text{ Phys. Chem. } 79 \text{ (in press) (1975). Copyright by the American Chemical Society.}]}
\]

there as well [i.e., the \( \tau_\pi \) obtained from the \( B \) and \( C \) terms in Eq. (79) were no longer the same]. It was found possible to largely remove this discrepancy by the physically unreasonable model that anisotropic viscosity was developing such that while \( \tau_\pi \) increased with \( \eta / T \) as is normal for a liquid, \( \tau_\pi \) remained virtually constant at \(-2 \times 10^{-13} \text{ sec. However, some alternative explanations have been proposed. To appreciate them, one must first examine Figs. 3 and 4 for isotropic liquids. There it is found that the nonsupercritical spectral densities for the rotational motion, which are expected to obey a Debye-type expression (cf. Chapter 2)

\[
J(\omega) = \tau_\pi / (1 + \omega_\pi^2 \tau_\pi^2)
\]

were better fitted instead by the expression

\[
J(\omega) = \tau_\pi / (1 + \omega_\pi^2 \tau_\pi^2)
\]

with \( \varepsilon \approx 5 \). A similar correction was found to be the case in the work with nematic solvents both above and below the isotropic-nematic transition with a smaller one below the transition. Deviations from the simple Debye
3. Theory of Slow Tumbling ESR Spectra for Nitroxides

F. Saturation and Nonlinear Effects

The general slow tumbling theory presented in Section I can also be applied to saturation phenomena (Freed et al., 1971; Goldman et al., 1973; Goldman, 1973; Bruno, 1973). A careful study of saturation and slow tumbling for PADS in viscous media has shown that the stochastic Liouville approach can be effectively employed to predict slow tumbling spectra (Goldman et al., 1973). This is illustrated in Fig. 23. Note that the simulations were performed for axial nitroxide parameters, so the agreement should not be expected to be perfect.

In the discussion of Section II.B on simplified methods of estimating $\tau_R$ from features of the outer hyperfine extrema, it was found that in the region where $\tau_R > 10^{-8}$ sec they are quite insensitive to all parameters other than $\tau_{R_{\perp}} A_z$, and $\mathcal{A}$ (the intrinsic width), while the central region is affected by the other magnetic tensor components. It is therefore reasonable to expect that simulations based on axial parameters should agree quite well with the saturation behavior of the outer extrema but not necessarily with the central extrema. Such a comparison is shown in Fig. 24, which shows the ratios of the $d_{\max}(M) = \frac{1}{2} \gamma_e H_{\max}(M)$ (where $H_{\max}(M)$ is the microwave field strength at which the $M = -1, 0, +1$ regions of the spectra maximize, where these regions correspond, respectively, to the low field, central, and high-field extrema). In particular, for $\tau_R > 10^{-8}$ sec, there is rather good agreement between experiment and prediction for $d_{\max}(+1)/d_{\max}(-1)$. It is possible to use such a ratio (which depends only on relative values of $B_i$ and not on its absolute magnitude) as a means of estimating $\tau_R$, but this has not yet been studied in detail. In particular, we are adding the new parameter $W_e$, the electron-spin relaxation rate, into the analysis. Also note the rather small changes in the ratio for large changes in $\tau_R$.

At the heart of all saturation and nonlinear phenomena is the fact that the eigenfunction expansion coefficients for the density matrix elements representing population differences (cf. Eq. (27), the terms $|C_m^\perp|^2 - |C_m^\parallel|^2$, where $n = 0$) are relaxed at rates that depend on

$$2W_e + B_L R L (L + 1)$$

(96)

when (1) an orientation-independent $W_e$ is used and (2) isotropic reorientation is assumed. There is, of course, coupling among the different ESR transitions due to the nuclear spin-flip transitions induced by the pseudoscalar terms in $\mathcal{A}^\perp(\Omega)$. Thus while the expansion coefficients for $L = 0$, representing the population differences for the proper isotropic average over orientations, is relaxed by $T_{L=0} = (2W_e)^{-1}$, the coefficients for $L > 0$ are relaxed by the rotational motion as well. Furthermore, when $R \gg W_e$ (typical values of $W_e$ are $\sim 10^{-3}$ sec), then (1) the rotational motion is very...
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

![Graph showing variation of $d_{\text{eff}}(+1)/d_{\text{eff}}(0)$ and $d_{\text{eff}}(+1)/d_{\text{eff}}(-1)$ with $\tau_c$. Solid lines are drawn through the experimental points for the system PADS in 85% glycerol-$\text{H}_2\text{O}$. [From Goldman et al. (1973).]]

Effective in spreading the saturation throughout the spectrum, and (2) only the $L = 0$ component is effectively saturated. These ideas and the implications they have for pulsed saturation recovery studies are explored elsewhere (Freed, 1974). It is found in that work that the saturation recovery times (or at least the slowest relaxing mode) are often well approximated by $T_{1,\text{eff}} \approx (2W_c)^{-1}$ over a wide range of conditions, including the slow tumbling region.

Such considerations suggest that slow tumbling ELDOR experiments could be very interesting (Bruno, 1973; Bruno and Freed, 1974a; Smigel et al., 1974). In such experiments, we saturate with a pump microwave field at a particular resonant frequency corresponding to a particular orientation (and nuclear spin) of the nitroxide and then observe at another position corresponding to a different orientation. Then the indirect saturation at the observing position will depend on the rotational motion of the molecule. We therefore expect that the ratio $R/W_c$ will determine the relative importance of transmission of saturation to different environments (a form of "spin diffusion") versus simple ESR spin relaxation. The application of the stochastic Liouville theory to such experiments is discussed in detail elsewhere (Bruno, 1973; Bruno and Freed, 1974a; Smigel et al., 1974).

Finally, we note the existence of a great variety of nonlinear phenomena, such as the modulation-frequency dependence of adiabatic rapid-passage effects explored by Hyde and Dalton (1972). This latter, more complex phenomenon can also be dealt with by stochastic Liouville methods and is
discussed elsewhere (Dalton, 1973; Leniart, 1972; Thomas and McConnell, 1974). The rather striking spectral effects observed have been calibrated with typical samples by the use of the $S$ parameter and extrapolation of $\tau_R$ with known values of $\eta/T$ to yield estimates of $\tau_R \gtrsim 10^{-6}$ sec (Hyde and Dalton, 1972).

In the discussion of saturation and nonlinear effects there is an important distinction to be made with respect to intrinsic widths, which was not important in the theory for unsaturated spectra. Note that in Eqs. (28) and (29) intrinsic widths were introduced by replacing $\omega_2$ by $\omega_2 + iT_{2z}^{-1}$ with no distinction made as to whether these widths are due to homogeneous or inhomogeneous broadening. It is possible to show, from the general form of the solutions in terms of superpositions of "complex Lorentzians" (cf. Appendix A), that this is entirely adequate for the case of a Lorentzian distribution of inhomogeneous broadening and no saturation (Abragam, 1961; Goldman et al., 1973). If the inhomogeneous widths may not be approximated as Lorentzian, then we must convolute the line shapes obtained from the slow tumbling theory given here with a more appropriate inhomogeneous line-shape function (e.g., a Gaussian). This can become a real problem when unresolved proton extra hyperfine structure is a dominant source of broadening (in which case rigorous line-shape simulations require adequate knowledge of the proton splittings and their orientation dependence). It is well known, however, that in the case of saturation, homogeneous and Lorentzian inhomogeneous lines are no longer formally equivalent (Abragam, 1961). Then Eqs. (28) and (29) are only appropriate for the homogeneous broadening. Again, inhomogeneous broadening could be accounted for by convolution methods, but for Lorentzian inhomogeneous broadening there is a simpler method. The solutions for saturated cases require both the coefficients $C_{k\mu}(t)$ of Eq. (27) and their complex conjugates $C_{k\mu}(t)^*$, to which they are coupled by the saturating terms. For homogenous broadening we replace $\omega_2 \rightarrow \omega_2 + iT_{2z}^{-1}$ for the former, and $\omega_1 - iT_{2z}^{-1}$ for the latter. But for Lorentzian inhomogeneous broadening, we can merely let $\omega_2 \rightarrow \omega_2 + iT_{2z}^{-1}$ for both types of terms (Goldman et al., 1973). The computer simulations of Fig. 23b were made for the case of homogenous broadening.

### APPENDIX A. GENERAL SOLUTIONS AND DISCUSSION OF THE COMPUTER PROGRAM FOR NITROXIDES

The equations upon which the computer program given in Appendix B for the ESR spectrum of a nitroxide in an isotropic fluid are based are (Bruno, 1973)

3. Theory of Slow Tumbling ESR Spectra for Nitroxides

$$[(\omega - \omega_s + 2b) - i(T_{2z}^{-1} + \tau_{2z}^{-1})]C_{k\mu}(t)$$

$$- (F_0 + D + iT_{2z}^{-1}) \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ -K \\ 0 \\ K \end{array} \right] \left[ \begin{array}{c} L \\ 2 \\ L' \\ L' \end{array} \right] C_{k\mu}(t)$$

$$- (F_2 + D) \sum_{L'} N(L, L') \left[ \begin{array}{c} L \\ 2 \\ L' \\ 0 \\ 0 \end{array} \right]$$

$$\times \left[ \begin{array}{c} L \\ -K \\ 2 \\ K + 2 \end{array} \right] C_{k+2, \mu}(t)$$

$$+ \left[ \begin{array}{c} L \\ -K \\ 2 \\ K - 2 \end{array} \right] C_{k-2, \mu}(t)$$

$$+ D \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ -K \\ 0 \\ K \end{array} \right] \delta(L, 0)$$

$$\times C_{k, 1}(4, 5) + D \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ 2 \\ L' \\ 0 \\ 0 \end{array} \right]$$

$$\times \left[ \begin{array}{c} L \\ -K \\ 2 \\ K + 2 \end{array} \right] C_{k+2, 1}(4, 5)$$

$$+ \left[ \begin{array}{c} L \\ -K \\ 2 \\ K - 2 \end{array} \right] C_{k-2, 1}(4, 5)$$

$$= 2^{1/2} \omega_s d_1 \delta(L, 0) \delta(K, 0)$$

(A.1)

$$[(\omega - \omega_s) - i(T_{2z}^{-1} + \tau_{2z}^{-1})]C_{k\mu}(t)$$

$$- (F_0 + iT_{2z}^{-1}) \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ -K \\ 0 \\ K \end{array} \right] \left[ \begin{array}{c} L \\ 2 \\ L' \\ L' \end{array} \right] C_{k\mu}(t)$$

$$- F_2 \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ 2 \\ L' \\ 0 \\ 0 \end{array} \right] \left[ \begin{array}{c} L \\ 2 \\ L \\ L' \end{array} \right] C_{k+2\mu}(t)$$

$$+ \left[ \begin{array}{c} L \\ -K \\ 2 \\ K + 2 \end{array} \right] C_{k+2\mu}(t)$$

$$+ D \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ 2 \\ L' \\ 0 \\ 0 \end{array} \right]$$

$$\times \left[ \begin{array}{c} L \\ -K \\ 0 \\ K \end{array} \right] C_{k, 1}(4, 5) + C_{k, 1}(6, 7)$$

$$+ D \sum_{L'} N(L, L') \left[ \begin{array}{c} L' \\ 2 \\ L' \\ 0 \\ 0 \end{array} \right]$$

$$\times \left[ \begin{array}{c} L \\ -K \\ 0 \\ K \end{array} \right] C_{k, 1}(4, 5) + C_{k, 1}(6, 7)$$

$$= 2^{1/2} \omega_s d_2 \delta(L, 0) \delta(K, 0)$$

(A.2)
\[
[(\omega - \omega_e - 2b) - i(T_{\lambda,1} + r_{\lambda,1})]C_{K,0}(3)
- (F_0 - D' + iT_{\lambda,1}') \sum L N(L, L) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
0
\end{array} \right) C_{K,0}(3)
- (F_2 - D'^{(2)}) \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
\times \left[ \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K+2,0}(3) + \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K-2,0}(3) \right]
\times D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
- \frac{1}{2} \frac{\omega_0}{\omega} \frac{\alpha_0}{\delta}(L, L') \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
\]

3. Theory of Slow Tumbling ESR Spectra for Nitroxides

\[
\times \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K+2,0}(3) + \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K-2,0}(3)
\times D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
- \frac{1}{2} \frac{\omega_0}{\omega} \frac{\alpha_0}{\delta}(L, L') \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
\]

\[
[(\omega - \omega_e - b) - i(T_{\lambda,1} + r_{\lambda,1})]C_{K,1}(4, 5)
+ (F_0 - \frac{1}{2} D' + iT_{\lambda,1}') \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,1}(4, 5)
+ (F_2 + \frac{1}{2} D'^{(2)}) \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,1}(4, 5)
+ D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left[ \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,1}(4, 5) + \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K-2,1}(4, 5) \right]
\times D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
- \frac{1}{2} \frac{\omega_0}{\omega} \frac{\alpha_0}{\delta}(L, L') \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
\]

\[
[(\omega - \omega_e - b) - i(T_{\lambda,1} + r_{\lambda,1})]C_{K,2}(8, 9)
+ (F_0 - \frac{1}{2} D' + iT_{\lambda,1}') \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,2}(8, 9)
+ (F_2 + \frac{1}{2} D'^{(2)}) \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,2}(8, 9)
+ D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right)
\times \left[ \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K,2}(8, 9) + \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) C_{K-2,2}(8, 9) \right]
\times D \sum L N(L, L) \left( \begin{array}{c}
L \ 2
\hline
-K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
- \frac{1}{2} \frac{\omega_0}{\omega} \frac{\alpha_0}{\delta}(L, L') \delta(K, 0)
\left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) \left( \begin{array}{c}
L \ 0
\hline
K
\end{array} \right) C_{K,0}(3)
\]
where the plus sign is used for even \( L \) and the minus sign for odd \( L \). For even \( L \), \( i \) refers to all transitions; for odd \( L \), \( i \) refers only to transitions 4, 5, 6, 7, 8, and 9;

\[
C_{K,i}^\pm(8, 9) = 2^{-i/2}[C_{K,i}^+(8, 9) \mp C_{K,i}^-(8, 9)]
\]

where \((i, j)\) are either \((4, 5)\) or \((6, 7)\), and the minus (plus) sign is for even (odd) \( L \); and

\[
C_{K,8}^\pm(8, 9) = 2^{-i/2}[C_{K,8}^+(8, 9) \mp C_{K,8}^-(9)]
\]

where the (minus) sign is for even (odd) \( L \). Also,

\[
N(L, L) = [(2L + 1)(2L + 1)]^{1/2}
\]

and

\[
T_{1/2}^\pm = \frac{\alpha + \frac{1}{2}\beta}{\sqrt{2}},
\]

\[
T_{3/2}^\pm = \frac{\alpha + \frac{3}{2}\beta}{\sqrt{2}}.
\]

The quantity \((\frac{\alpha}{\beta} \frac{\beta}{\gamma})\) is a \( 3j \) symbol, whose values are tabulated or given by formulas (Rotenberg et al., 1959; Edmonds, 1957). These are used to evaluate the integrals on the LHS of Eq. (27), utilizing

\[
\int d\Omega \mathcal{P}_{m_1, m_2} (\Omega) \mathcal{P}_{l_1, m_2} (l_1) \mathcal{P}_{l_2, m_3} (l_2)
\]

with the relationship

\[
\mathcal{P}_{m_1, m} (\Omega) = (-)^{m - m_1} \mathcal{P}_{-m_1, -m} (\Omega)
\]

There are a number of symmetry relations (Edmonds, 1957) in a \( 3j \) symbol \((\frac{\alpha}{\beta} \frac{\beta}{\gamma})\) Among the more useful ones are: (1) The sum of the \( m \) values must be zero. (2) Naturally, \( L \) must be positive and the absolute value of \( m \) must not be greater than the corresponding \( L \) in a given column. (3) The columns can be permuted without changing the value of the \( 3j \) symbol if the sum of \( L \) values is even. If the sum of \( L \) values is odd, then a permutation of the columns results in a change in sign for the value of the \( 3j \) symbol. (4) If the sum of \( L \) values is even, then all the \( m \) values can change sign without changing the value of the \( 3j \) symbol. If the sum of \( L \) values is odd, then a change in sign for all \( m \) values results in a change in sign for the value of the \( 3j \) symbol. (5) The triangular property holds whereby the sum of any two \( L \) values must be equal to or greater than the third \( L \) value. Properties (4) and (5) result in the equations in \( L \) being coupled only to the equations in \( L \) and \( L \pm 2 \), for the three allowed transitions; but the forbidden transitions (only
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

\[
Y = \left[ 2^{-1/2} q \sigma_0 d_e \right]^{-1}
\]

(A.19b)

where we use the fact that \( \alpha_1 \approx \alpha_2 \approx \alpha_3 \approx \alpha_4 \) in high fields and \( d_1 = d_2 = d_3 = d_4 \). It follows from Eqs. (A.17) and (A.18) that

\[
\mathbf{Z}'' = \text{Im}[(\mathbf{O})^\dagger (\sigma'_d + k_1)^{-1} (\mathbf{O}^\ast U)]
\]

(A.20)

which can be written for the \( r \) dimensions as

\[
\mathbf{Z}'' = Y \text{Im} \left[ \sum_{i=1}^T \left( (\mathbf{O}^\ast U)_i \right)^2 \right]
\]

(A.21)

and for the first derivative of an absorption field-swept spectrum

\[
\frac{d\mathbf{Z}''}{d\alpha} = Y \text{Im} \left[ \sum_{i=1}^T \left( (\mathbf{O}^\ast U)_i \right)^2 \right]
\]

(A.22)

Thus only a single diagonalization is required to calculate an absorption line shape or the \( n \)th derivative of the absorption. Another advantage is that \( \sigma'_d \) and \( \mathbf{O} \) are not functions of \( T^{-1}_{2,1} \), so that spectral line shapes can be calculated for different values of \( T^{-1}_{2,1} \) without performing additional diagonalizations.

The diagonalization subroutine used for the slow tumbling computer program in Appendix B is due to Gordon and Messenger (1972). This subroutine had the fastest execution time of all diagonalization subroutines tried. Besides its speed in diagonalizing a matrix, it has characteristics that make it especially useful in solving the slow motions equations. First, the subroutine takes advantage of the symmetry of \( \sigma'_d \), so that only the elements to one side of the diagonal are stored. Second, the subroutine retains the banded nature of the equations, so that only the subdiagonals containing nonzero elements are stored. Third, the subroutine performs the operation \( (\mathbf{O}^\ast U) \) "instantaneously" for each step of the diagonalization, so that only a single column vector \( \mathbf{O}^\ast U \) need be stored rather than the construction and storage of the entire \( r \times r \) \( \mathbf{O}^\ast \) matrix. A modified version of this subroutine, which can be used to obtain all the eigenvectors, is given by Bruno (1973).

Anisotropic Liquids

Because \( \Gamma_{2,1} \) for anisotropic liquids is composed of a simple sum of terms in \( \sigma'_d(\mathbf{O}) \) plus the isotropic liquid \( \Gamma_{2,1} \), there is much similarity between the equations for anisotropic liquids and those for isotropic liquids. In fact, the resulting equations for anisotropic liquids can be obtained by simple modifications of the isotropic liquid equations (A.1)-(A.6). These modifications can be specified by the following definitions for the simple...
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES

for $n$ even. Appropriate expressions for the more complicated cases are given by Polnaszek (1975a).

Convergence for isotropic spectra has been discussed in Section II.E in terms of an $n_e$. The effect of having $\lambda \neq 0$ means that the ordering affects the convergence of the solutions. For example, in the motionally narrowed region, when $n_e = 2$ is sufficient for isotropic liquids, then for $\lambda = -0.9$ (weak ordering) one needs $n_e = 4$ and for $\lambda = -3.5$ (moderately strong ordering) $n_e = 6$. It appears safe, for the slow motional region, to use as $n_e$ the sum of the value required for isotropic liquids and $n_e - 2$, where $n_e$ is required for convergence for that value of $\lambda$ in the motional narrowing region, although usually smaller values of $n_e$ may be used.

Computer programs are given by Polnaszek (1975a) for calculating nitroxide line shapes when (1) the asymmetric potential defined by Eq. (58) describes the orientation of a nitroxide radical for which the principal magnetic ($x'$, $y'$, $z'$) and orientation ($x$, $y$, $z$) axes are coincident, and (a) Eqs. (61a), (61b), and (62a)–(62f) and Eqs. (63), (64), and (65a)–(65f) apply or (b) Eqs. (64) and (65a)–(65f) apply; (2) a Meier–Saupe potential is used; but the $x'$ and $z'$ axes are tilted by angle $\beta$; (3) different reorientational models are used for a Meier–Saupe potential. All these programs contain the correction terms for nonsecular contributions to the resonant frequency shifts.

All the programs, including that given in Appendix B, have been written in FORTRAN IV language for an IBM 360/65 computer.

APPENDIX B. COMPUTER PROGRAM FOR SLOW TUMBLING NITROXIDES IN ISOTROPIC LIQUIDS

The following program was written in FORTRAN IV and is listed with 72 print positions per line. One of the subroutines in this program has been taken from Gordon and Messenger (1972), pp. 376–381, and has been reproduced with 60 print positions per line. The Gordon and Messenger subroutine is used with permission of Plenum Press.
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES
3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITRONIDES

C. Transition.

D. Phases.

E. Phase transitions phase transitions phase transitions.

F. Phase transitions phase transitions.

G. Phase transitions phase transitions.

H. Phase transitions phase transitions.
ACKNOWLEDGMENTS

Support of this work by grants from the National Science Foundation, the Petroleum Research Fund (Grant No. 6818-AC6) administered by the American Chemical Society, and the Cornell University Materials Science center gratefully acknowledged. The efforts of the past and present members of the Cornell Chemistry Department ESR group have been the basis for this chapter and their critical comments are greatly appreciated. Special thanks are due Dr. G. V. Bruno for developing the computer program given here and Dr. C. F. Polnarek for his considerable help with it. This chapter was completed while the author was a guest professor at the Department of Physical Chemistry of Aarhus University (Spring semester, 1974), and he greatly appreciates the facilities made available to him.

REFERENCES


3. THEORY OF SLOW TUMBLING ESR SPECTRA FOR NITROXIDES


Biradicals as Spin Probes

GEOFFREY R. LUCKHURST
DEPARTMENT OF CHEMISTRY
THE UNIVERSITY, SOUTHAMPTON, ENGLAND

I. Introduction

The obvious success of the spin probe technique stems from the ability of the environment to influence the appearance of the probe’s electron resonance spectrum. It is important therefore to understand the parameters that determine the form of this spectrum and how these can be modified by the interaction of the probe with its surroundings. For the purpose of the discussion it is convenient, but not essential, to assume that any molecular motion is sufficiently fast to give a motionally narrowed spectrum. The positions of
the spectral lines will then be determined by the scalar magnetic interactions provided the system is isotropic. If, however, some space-fixed orientations are preferred, then the line positions will depend on the anisotropic as well as the scalar couplings. In general the linewidths are affected by a variety of spin relaxation processes, but for organic free radicals the dominant process is invariably associated with the anisotropic interactions coupled to the molecular rotation. There are then four variables that determine the spectral shape and each can be influenced by the interaction between the probe and its environment. Such interactions have been described in earlier chapters and so we shall mention just three examples encountered with nitroxide monoradicals. The formation of a hydrogen bond with the nitroxide group increases the nitrogen coupling constant and this change has been employed to probe the polarity of the environment. The spacing between the hyperfine lines is altered by the partial alignment of the spin probe because of the anisotropy in the nitroxide hyperfine interaction. This behaviour has been employed to considerable advantage in the study of membranes and other liquid crystals. Finally, the rate of molecular reorientation is profoundly influenced by the macroscopic and the local viscosity of the environment; changes in these viscosities are reflected in the linewidths because of the anisotropy in the \( g \) and nitrogen hyperfine tensors.

Nitroxide monoradicals would therefore appear to satisfy the particular requirements for a spin probe and so it is proper to ask what advantages, if any, are provided by biradical probes. Of course any magnetic interaction present in the monoradical will also occur in the corresponding biradical. But, in addition, there is a new interaction, which is the coupling between the two electron spins; this, like all magnetic interactions, has an isotropic and an anisotropic part. The scalar component or exchange interaction is found to depend critically on the conformation of the biradical, whereas the anisotropic part is determined by the electron-electron separation. For flexible biradicals both of these quantities will be influenced by their surroundings and so biradical probes provide a way of examining another facet of the environment. Further, the rate at which the environment modifies the conformation of the probe can also be inferred from the widths of the spectral lines. The anisotropy in the electron-electron interaction provides an alternative method for studying those features already investigated with the aid of the anisotropy in the \( g \) and hyperfine interactions.

In the following sections we shall consider, in some detail, how the introduction of these electron-electron interactions influences the appearance of the electron resonance spectrum. We shall confine our attention to biradicals, largely because no new principles are encountered for polyradicals but also because only biradicals appear to have been employed as spin probes. At various stages in the development of the theory we shall pause to consider those specific applications of biradicals as spin probes that illustrate this theory. We shall not pay particular attention to systems in which the molecular motion is slow, although we shall comment on the difficulties encountered in such systems. Finally, we shall return to the problem of the relative merits of monoradical and biradical spin probes in Section V.

II. THE TRIPLET STATE

A. Isotropic Solutions

We shall be predominantly concerned in this chapter with those biradicals formed by joining two nitroxides together with saturated linkages. Consequently, the spin Hamiltonian will contain contributions from the hyperfine interactions between the electron and nuclear spins; it is, however, convenient to begin our discussion by ignoring such terms. The orientation-independent scalar Hamiltonian therefore contains the electron Zeeman interaction and the electron exchange coupling:

\[
\mathcal{H}^0 = g\beta B(S_1^1 + S_2^1) + J S_1^{(1)} \cdot S_2^{(1)}
\]

where \( J \) is the exchange integral.† The biradical is taken to be symmetric and so the \( g \) factors for the two electrons have been set equal. The three triplet spin functions

\[
|1\rangle = |xz\rangle, \quad |0\rangle = \frac{1}{\sqrt{2}}(|xz\rangle + |yz\rangle), \quad |-1\rangle = |yz\rangle
\]

and the singlet

\[
|0\rangle = \frac{1}{\sqrt{2}}(|xy\rangle - |yx\rangle)
\]

are eigenfunctions of the spin Hamiltonian with energies

\[
E_1 = g\beta B + J/4, \quad E_0 = J/4, \quad E_{-1} = -g\beta B + J/4
\]

and

\[
E_0 = -3J/4
\]

The subscripts \( a \) and \( e \) on the spin states denote symmetric and antisymmetric functions, respectively. The allowed spin transitions are within the triplet manifold and satisfy the selection rule

\[
\Delta M_s = \pm 1
\]

† Editor's note: Note that here the symbol \( B \) instead of \( H \) is used for magnetic field. Both symbols are common in the literature.
where $M_\pi$ is the total spin quantum number. The observed transitions are then

$$| -1 \rangle \leftrightarrow | 0, \rangle \quad (\equiv | - \rangle) \quad (7)$$

and

$$| 0, \rangle \leftrightarrow | 1 \rangle \quad (\equiv | + \rangle) \quad (8)$$

These are degenerate and occur when

$$B = \hbar \nu_0 / g \beta$$

where $\nu_0$ is the operating frequency of the spectrometer. The position of this single line is independent of the exchange integral, although it can influence the intensity $J$ of the spectrum. This intensity is proportional to the population of the triplet state and, provided the sample is not saturated, is given by

$$J \propto \frac{1}{T} \frac{3e^{jRT}}{1 + 3e^{jRT}} \quad (10)$$

When $J$ is comparable to the thermal energy the spectral intensity will increase with increasing temperature if the exchange integral is positive, corresponding to a singlet ground state, but decrease if it is negative. Under favorable conditions the sign and often the magnitude of $J$ can be obtained from the temperature dependence of the signal intensity.

The width of the single line in the solution spectrum of a triplet is often found to be rather large and in certain cases the line is undetectably broad. The linewidths are considerable because the anisotropic electron-electron interaction coupled to the molecular reorientation modulates the energies of the spin levels and induces transitions between them (Weissman, 1958). The calculation of the linewidth resulting from this powerful spin relaxation process can be accomplished with the aid of Redfield's relaxation theory, which is outlined in Chapter 2. The first step in the calculation is the formulation of the dynamic spin Hamiltonian, which is that for the anisotropic electron-electron coupling:

$$\mathcal{H} = 2S_x^1 D_{xx} S_x^2 + S_y^1 D_{yy} S_y^2 + S_z^1 D_{zz} S_z^2 + \frac{1}{2} D_{xy} S_x^1 S_y^2 + \frac{1}{2} D_{yx} S_y^1 S_x^2 + \frac{1}{2} D_{yz} S_y^1 S_z^2 + \frac{1}{2} D_{zy} S_z^1 S_y^2$$

where $D$ is the zero-field splitting tensor and $\mathcal{H}$ is written in a molecular-fixed coordinate system XYZ. It is important to note that the zero-field splitting spin Hamiltonian takes the same form when written in terms of the total spin operator

$$\mathcal{H} = D_{xx} S_x^2 + D_{yy} S_y^2 + D_{zz} S_z^2$$

but now the coefficients are just one-half the value required for the Hamiltonian involving individual spin operators (Carrington and McLachlan, 1967). The tensor $D$ is referred to as the zero-field splitting because this interaction removes the degeneracy of the three triplet spin levels even in the absence of a magnetic field.

As the triplet rotates, the spin operators fluctuate in time and this time dependence is conveniently handled using the irreducible operator techniques developed in Chapter 2. The dynamic perturbation is therefore written as

$$\mathcal{H}'(t) = \sum_{\rho, \sigma} \langle - \rangle \langle - \rangle D_{\rho, \sigma}^{(2)} T_{\rho, \sigma}(t)$$

where $T_{\rho, \sigma}(t)$ denotes the time-dependent spin operators. This time dependence can be removed by transforming to a laboratory coordinate system where the operators are independent of time. This transformation is accomplished with a Wigner rotation matrix and gives:

$$\mathcal{H}'(t) = \sum_{\rho, \sigma} \langle - \rangle \langle - \rangle D_{\rho, \sigma}^{(2)} \tilde{S}_6 \tilde{S}_6(t) T_{\rho, \sigma}(t)$$

Redfield's relaxation matrix contains four elements because the transitions given in Eqs. (7)-(8) are doubly degenerate; these four elements are readily calculated, from Eq. (10) of Chapter 2, with the result

$$\begin{pmatrix}
| + \rangle & | - \rangle \\
\langle + | & A \\
\langle - | & B
\end{pmatrix}$$

where

$$A = -\frac{1}{3} \sum_{\rho} \langle - \rangle \langle - \rangle D_{\rho, \sigma}^{(2)} S_{\rho, \sigma}(j_0 + 3j_1 + 2j_3) \quad (15)$$

and

$$B = -\frac{1}{3} \sum_{\rho} \langle - \rangle \langle - \rangle D_{\rho, \sigma}^{(2)} S_{\rho, \sigma}(j_0 + 3j_1 + 2j_3) \quad (16)$$

The spectral densities $j_\pi$ are defined by

$$j_\pi = \tau / (1 + 4\pi^2 n_j \sigma^2)$$

where $\tau$ is the correlation time characterizing the isotropic reorientation process. The linewidths are simply minus one times the eigenvalues of the relaxation matrix and for this problem both transitions are found to have the same width

$$T_{j_\pi}^{-1} = \tau / \sum_{\rho} \langle - \rangle \langle - \rangle D_{\rho, \sigma}^{(2)} T_{\rho, \sigma}(j_0 + 3j_1 + 2j_3)$$

and so the line shape is Lorentzian (Carrington and Luckhurst, 1964).
Consequently, if rotational modulation of the zero-field splitting constitutes the dominant relaxation process, the width of the single line can be used to estimate the correlation time (Michon and Rassat, 1974). However, since there is only one line, it may prove difficult to make an unambiguous assignment of the relaxation process. There has been one attempt to assign the relaxation process for the biradical I dissolved in ethyl alcohol (Rassat, 1972). The peak-to-peak linewidth $\Delta B_{pp}$ was measured as a function of temperature, with the results given in Fig. 1, where $\Delta B_{pp}$ is plotted against $\eta/T$. The linearity of the plot can be understood in the following way. For a

$$\eta/T \times 10^{-3} \text{ cP/}^{\circ} \text{C}$$

![Graph showing $\Delta B_{pp}$ vs. $\eta/T$](image)

Fig. 1. The temperature dependence of the peak-to-peak linewidth $\Delta B_{pp}$ for the biradical I dissolved in ethyl alcohol. (Rassat, 1972.)

The Lorentzian line shape the linewidth $T^{-1}$ is directly proportional to $\Delta B_{pp}$ and for moderately viscous systems the spectral densities $j_1$ and $j_2$ may be ignored in comparison with $j_0$, since $n^2v_0^2\tau^2 > 1$. The peak-to-peak separation is then directly proportional to $\tau$, which, according to the Debye model of rotation, is related to the viscosity and size of the molecule by

$$\tau = \frac{3}{4} \rho \eta r^3/kT$$

(20)

Although the Debye model is admittedly crude, it has been found to work rather well for molecules as large as biradical I. The only necessary modification is the introduction of an anisotropic interaction parameter $\kappa$, which reflects the anisotropy in the solute-solvent intermolecular potential and is apparently independent of temperature (Hwang et al., 1973); the modified Debye equation is

$$\tau = \frac{3}{4} \rho \eta r^3/kT$$

(21)

Accordingly, the peak-to-peak separation should be proportional to $\eta/T$, in agreement with experiment; the deviation from linearity at high viscosities is probably associated with the failure of Redfield's theory. It is important to note that the linewidth will also be proportional to the correlation time, and hence $\eta/T$, if the molecular reorientation is fast in the sense $n^2v_0^2\tau^2 < 1$, for now all of the spectral densities are equal to $\tau$.

The validity of the expression for the linewidth of a triplet state rotating rapidly in fluid solution can be tested in quite a different way. This involves the measurement of the linewidth, at constant temperature, but for different microwave frequencies $v_0$. In practice such experiments are restricted to three readily available frequencies: 3 GHz (S band), 9.5 GHz (X band), and 35 GHz (Q band); although these are usually sufficient. The frequency dependence of $T^{-1}$ given in Eq. (19) is caused by the occurrence of $v_0$ in the spectral densities $j_1$ and $j_2$, as we can see from Eq. (18). Consequently, provided $n^2v_0^2\tau^2$ is comparable to unity, changing the frequency will modify the spectral densities and hence $T^{-1}$; in general the linewidth should decrease on increasing the microwave frequency. Clearly, if the zero-field splitting $D$ is known, then measurement of the linewidth at different frequencies will permit independent estimates of the correlation time; these should, of course, be equal if the theory is correct. Alternatively, if $D$ is not known, then such variable-frequency experiments should enable us to determine the correlation time together with the inner product $\sum (\cdots pD^{12} \cdot n)$. This procedure has yet to be applied to the triplet state, although it has been used with some success in studies of spin relaxation in states of higher multiplicity, such as iron(III) and manganese(II) (Levanon et al., 1970). However, it must be realized that such experiments are essentially tests of one aspect of the theory, namely the assumption of an exponential decay of the correlation function for the rotation matrices.

The expression for the linewidth assumes that all of the components of the diffusion tensor $R$ are the same and in fact equal to $C_0^2$. Although this assumption may be reasonable for biradical I it can hardly be realistic for II,
where the diffusion tensor should possess cylindrical symmetry about the long molecular axis. The linewidth predicted (Luckhurst et al., 1975) for such a biradical is

\[ T_J^{-1} = \frac{1}{2} \sum_p (-1)^p D^{(2),p} D^{(2),-p}(3j_{0p} + 5j_{1p} + 2j_{2p}) \]  
(22)

where the spectral densities are

\[ J_p = \tau_p / (1 + 4\pi^2 n^2 \tau_p^2 \nu_0^2) \]  
(23)

Now the correlation times depend on \( p \) as well as on the components of the diffusion tensor parallel \( (R_{||}) \) and perpendicular \( (R_{\perp}) \) to the symmetry axis:

\[ \tau_p = \left[ 6R_{\perp} + (R_{||} - R_{\perp})p^2 \right]^{-1} \]  
(24)

When applying Eq. (22) for the linewidth, it is important to remember that the coordinate system employed to calculate the irreducible components of the zero-field splitting must also diagonalize the diffusion tensor. Since the observed linewidth depends on two components of the diffusion tensor, it will never be possible to determine them from this single width and so triplet spin probes are not of value in studying rapid anisotropic rotational diffusion.

B. Anisotropic Environments

Although, as we have seen, the zero-field splitting has a pronounced effect on the linewidth when the triplet is tumbling rapidly in an isotropic solvent, it does not influence the line position. This is not the case when the spin probe is dissolved in a macroscopically anisotropic system such as a liquid crystal (Falle et al., 1966); the properties of such systems are described more fully in Chapter 10. Since all orientations are no longer equivalent, D is not averaged to zero by reorientation and its retention in the static spin Hamiltonian removes the degeneracy of the two spin transitions. The spectrum will therefore contain two lines and we shall now seek to calculate their separation. Our starting point is the spin Hamiltonian for a particular orientation of the spin probe; this is the sum of the scalar Hamiltonian given in Eq. (1) and the anisotropic Hamiltonian in Eq. (13):

\[ \mathcal{H}(t) = \mathcal{H}^0 + \sum_p (-1)^p D^{(2),p} T^{(2),-p} \]  
(25)

As we have seen, the spin operators \( T^{(2),-p} \) fluctuate in time as the molecule rotates, but this time dependence can be removed by transforming to a laboratory coordinate system:

\[ \mathcal{H}(t) = \mathcal{H}^0 + \sum_{p,q} (-1)^p D^{(2),p} \mathcal{G}^{(2),-p}_q T^{(2),q} \]  
(26)

4. BIRADICALS AS SPIN PROBES

The static Hamiltonian is obtained from this expression by taking a time or ensemble average of the Wigner rotation matrix. In an isotropic environment the ensemble average of any rotation matrix \( \mathcal{G}^{(2)}_{p,q} \) is just

\[ \mathcal{G}^{(2)}_{p,q} = \delta_{p,0} \delta_{q,0} \]  
(27)

and so only the scalar terms in Eq. (26) contribute to the static spin Hamiltonian since \( \mathcal{G}^{(2)}_{p,q} \) is zero. This is not the case for an anisotropic environment; however, to evaluate the ensemble average \( \mathcal{G}^{(2)}_{p,q} \) and hence the form of the spectrum it is necessary to know the symmetry of both the spin probe and the potential that is responsible for its partial alignment. The optical properties of nematic and smectic A liquid crystals show that this ordering potential is cylindrically symmetric and it is convenient and reasonable to adopt the same symmetry for other anisotropic systems. With this assumption \( \mathcal{G}^{(2)}_{p,q} \), and indeed the averages of all even rotation matrices, vanish unless \( q = 0 \); the static spin Hamiltonian then reduces to

\[ \mathcal{H} = \mathcal{H}^0 + \sum_p (-1)^p D^{(2),p} \mathcal{G}^{(2),-p}_0 T^{(2),q} \]  
(28)

in a coordinate system containing the direction of the ordering potential as one of the axes. This is entirely equivalent to the spin Hamiltonian for a triplet state with a cylindrically symmetric zero-field splitting tensor with the symmetry axis parallel to the ordering potential. The components of the effective or partially averaged zero-field splitting are denoted by \( D_i \) and \( D_j \). The electron resonance spectra expected for such a Hamiltonian are well understood as a result of numerous studies involving transition metal ions (Low, 1960). Indeed the energy levels for a comparable spin Hamiltonian are shown in Fig. 4 of Chapter 2 together with the allowed spin transitions. Thus the spectrum is found to contain two lines whose separation \( d \) changes as the orientation of the sample with respect to the magnetic field is varied. The angular dependence for our problem is

\[ d = 3(\tilde{D}_i q) / (3 \cos^2 \gamma - 1) / 2 \]  
(29)

provided \( \tilde{D} \) is sufficiently small compared with the Zeeman splitting that the nonsecular terms may be ignored. Here \( \gamma \) is the angle between the magnetic field and the symmetry axis of the environment. The result in Eq. (29) can be obtained by extending the following argument. When the magnetic field is parallel to the symmetry axis of \( \tilde{D} \) the static spin Hamiltonian in Eq. (28) reduces to

\[ \mathcal{H} = g\beta BS_+ + \frac{1}{2} J(S^2 - \frac{1}{2}) + \frac{1}{2} \tilde{D}_i (3S_i^2 - S^2) \]  
(30)

where \( S \) is the total spin operator. The triplet functions given in Eq. (2) are
still eigenfunctions of $\mathcal{A}$ but with eigenvalues
\[ E_1 = g\beta B + \frac{1}{2} J + \frac{1}{2} \tilde{D}_\parallel, \quad E_0 = \frac{1}{2} J - \tilde{D}_\parallel \]
\[ E_{-1} = -g\beta B + \frac{1}{2} J + \frac{1}{2} \tilde{D}_\parallel \]
(31)

The two allowed transitions are again $|+\rangle$ and $|-\rangle$ but they are no longer degenerate:
\[ B_+ = (h\nu_0/g\beta) - (3\tilde{D}_\parallel/2g\beta) \]
(32)
\[ B_- = (h\nu_0/g\beta) + (3\tilde{D}_\parallel/2g\beta) \]
(33)

and the separation between the two spectral lines is $3\tilde{D}_\parallel/g\beta$, in agreement with Eq. (29).

We shall now consider two examples that illustrate these ideas. The first is for the biradical dispiro((dimethyl-4,4'-oxazolidine-3-oyl)-2,1'-cyclohexane-4,2'-((dimethyl-4''4''-oxazolidine-3''-oyl))) III dissolved in a

nematogen. The spectrum found for the isotropic phase is shown in Fig. 2a and contains the expected single broad line; the three sharp lines originate from a monoradical impurity. In the nematic mesophase this single line is split into two, as the spectrum in Fig. 2b shows, because the liquid crystal is aligned with its symmetry axis parallel to the magnetic field (cf. Chapter 8).

These observations confirm the theoretical analysis and also provide a dramatic confirmation of the anisotropic environment provided by a liquid crystal. The magnitude of the line separation in this experiment is just $3\tilde{D}_\parallel$, which is related to the total zero-field splitting tensor by
\[ \tilde{D}_\parallel = \frac{3}{2} \sum_p (-1)^p D_{\pi}^{(2),p} \overline{\mathcal{D}_{0,-p}^{(0)}} \]
(34)

where the averages $\overline{\mathcal{D}_{0,-p}^{(0)}}$ are a measure of the partial alignment of the spin probe.

These order parameters can also be described in terms of the direction cosines of the environmental symmetry axis in a molecular coordinate system, as we shall see in Chapter 10. This description follows naturally when the various magnetic interactions are expressed as Cartesian rather than spherical tensors and leads directly to the concept of the ordering matrix which was originally introduced by Sauer (1964). The relationship between this ordering matrix and the order parameters $\mathcal{D}_{0,-p}^{(1)}$ has been

Fig. 2. The electron resonance spectra of the biradical III dissolved in (a) the isotropic and (b) the nematic mesophase of a liquid crystal. The three lines marked with an asterisk come from a monoradical impurity.

shown to be relatively straightforward, as we might have expected (Faile and Luckhurst, 1970). The determination of these parameters is of some importance because they provide a valuable insight into the anisotropy of the solute–solvent intermolecular potential (Humphries et al., 1971). In general there are five independent order parameters and it is clearly not possible to determine all of them using a biradical spin probe if the anisotropic interactions are restricted to the electron–electron coupling. However, when the biradical is cylindrically symmetric only one order parameter $\mathcal{D}_{0,0}^{(0)}$ is required and such uncommon biradicals could prove useful in studying the extent of solute alignment.

In the second example (Keana and Dinerstein, 1971) the cholestane biradical II was used to study a dipalmitoyl lecithin bilayer whose structure is described in Chapters 10 and 11. An attempt was made to obtain a homogeneous sample by spreading the spin-doped membrane onto a glass plate since the symmetry axis of the bilayer has been found to be oriented perpendicular to the surface. The electron resonance spectra are shown in
Fig. 3 for three orientations of the glass plate with respect to the magnetic field; the central line should be ignored since it originates from a monoradical impurity. Provided the rotational motion of the biradical is fast, the spectrum should contain just two lines with a separation given by Eq. (29). Two lines are observed when the magnetic field is either parallel or perpendicular to the glass surface, although the line shape is not symmetric. This asymmetry is probably caused by the imperfect alignment of the membrane but it could result if the molecular motion was slow, as we discovered in Chapter 3 (Brooks et al., 1971; Norris and Weissmann, 1969). Both interpretations are consistent with the observation of four peaks in the spectra for intermediate orientations. Similar, although less pronounced, asymmetry in the line shape is also found for the liquid crystal spectrum shown in Fig. 2b. Such observations present rather a problem in electron resonance studies of liquid crystals because it is extremely difficult to devise ways of distinguishing unambiguously between the two possible origins of the asymmetric line shapes. The separation $d$ between the lines is difficult to estimate because of the asymmetry; however, $d$ is about 450 G when $\gamma$, the angle between the field and the symmetry axis, is 0° and decreases to 250 G when $\gamma$ is 90°. These numbers are in reasonable agreement with Eq. (29), which predicts the modulus of the ratio to be 2:1. Finally, the observed value for $\tilde{D}_z$ of 420 MHz is extremely close to the largest component of the total zero-field splitting, which suggests that the alignment of the long axis of the spin probe in the membrane is virtually complete.

The dominant spin relaxation process for a triplet reorienting in an anisotropic environment almost certainly results from the anisotropy in the electron–electron interaction coupled to this rotation. There are, however, two essential differences between the operation of this relaxation mechanism in isotropic and in anisotropic solvents (Luckhurst et al., 1975). The first of these results from the partial alignment of the spin probe, which means that not all of the zero-field splitting contributes to the dynamic spin Hamiltonian:

$$\mathcal{H}(t) = \sum \left\{ -1 \right\}^p D_z^2 \langle \mathcal{S}^2 \rangle - p(t) - \langle \mathcal{S}^2 \rangle \delta_{q0} \rangle T^{(2,0)} \tag{35}$$

Second, the widths of the two lines, like their positions, now depend on the orientation of the sample with respect to the magnetic field. The angular dependence of the linewidths is found to be

$$T_2^{-1}(\gamma) = X_0 + X_2 P_2(\cos \gamma) + X_4 P_4(\cos \gamma) \tag{36}$$

where $P_n(\cos \gamma)$ is the $n$th Legendre polynomial. The magnitudes of the angular linewidth coefficients $X_n$ depend on the model adopted for the

---

Fig. 3. The spectral angular dependence for the spin probe II dissolved in a dipalmitoyl lecithin bilayer. The central peak comes from a monoradical impurity. [Reprinted with permission from Keana and Dinerstein, J. Amer. Chem. Soc. 93, 2808-2810 (1971). Copyright by the American Chemical Society.]
reorientation process. The strong collision model is mathematically attractive, for according to this model reorientation proceeds via collisions with no correlation between the molecular orientation before and after a collision. This model can be modified to allow for molecular anisotropy by the introduction of several correlation times; for example, if the molecule is axially symmetric, there would be two such times (Luckhurst and Sanson, 1972). One, \( \tau_0 \), would be associated with collisions resulting in reorientation of the symmetry axis, whereas the other, \( \tau_2 \), would relate to reorientation about this axis. The angular coefficients for such an axially symmetric spin probe are found to be

\[
X_0 = \frac{3}{10}(D^{(2, 0)})^2(1 - P_2^2)\tau_0 + 2D^{(2, 2)}P_2^2 \tau_2 \\
X_2 = \frac{3}{10}(D^{(2, 0)})^2(P_2 - P_2^3)\tau_0 - 2D^{(2, 2)}P_2^2 \tau_2 \\
X_4 = \frac{9}{35}(3D^{(2, 0)})^2(P_2 - P_2^3)\tau_0 + D^{(2, 2)}P_2^2 \tau_2
\]

(37) (38) (39)

provided the nonsecular terms in \( \mathcal{K}(t) \) are negligible. These coefficients depend on the order parameters \( P_2 \) and \( P_4 \), where \( P_2 \) is the equivalent of the order parameter \( \Theta^{(2)} \) encountered for molecules with less than axial symmetry; it can, of course, be determined from the line positions. There are few techniques capable of yielding the other parameter \( P_4 \), and so such linewidth studies may prove to be invaluable in the investigation of anisotropically systems.

The three linewidth coefficients depend on three unknowns \( P_2, \tau_0, \) and \( \tau_2 \); consequently each of these can be determined if the angular dependence of the linewidth is known. This contrasts with the situation for isotropic systems, where, as we have seen, it is impossible to obtain both

Quantitative application of the ideas developed in this section clearly demands a knowledge of the zero-field splitting tensor for the biradical employed as the spin probe. The most satisfactory determination of \( D \) involves the study of the biradical incorporated into a diamagnetic single crystal, for then the spectrum can be obtained for particular orientations of the biradical with respect to the magnetic field (Cieciarska-Tworek et al., 1973). For example, if the magnetic field is parallel to a principal axis of the zero-field splitting tensor, then the spacing between the observed doublet is three times the component of \( D \) for that axis. Determination of the complete angular dependence of this separation provides the most accurate method for estimating the zero-field splitting tensor. Alternatively, the principal components of \( D \) can often be determined from the spectrum of a sample in which the biradical adopts all orientations with respect to the magnetic field. The spectra of such polycrystalline samples are found to contain three pairs of lines with separations equal to three times the principal components of the zero-field splitting tensor. An example of a polycrystalline spectrum is

4. BIRADICALS AS SPIN PROBES

shown in Fig. 4 for biradical III dissolved in frozen toluene. Nitrogen hyperfine structure is only observed on one of the pairs of lines because the components of the hyperfine tensor associated with the other two directions are so small. The trace of the zero-field splitting is zero and so only two quantities are required to define the principal components of the tensor; it is conventional to choose these as

\[
D = \frac{3}{2}D_{xx}
\]

(40)

and

\[
E = \frac{1}{2}(D_{xx} - D_{yy})
\]

(41)

where the \( Z \) direction corresponds to the largest component of \( D \). These parameters are directly related to the irreducible components of \( D \):

\[
D^{(2, 0)} = (2/3)^{1/2}D
\]

(42)

and

\[
D^{(2, \pm 2)} = \pm E
\]

(43)

The values of \( D \) and \( E \) for the nitroxide biradicals I-III and for biradicals IV and V are listed in Table I.
C. The Zero-Field Splitting

We must now consider what factors determine the magnitude of the zero-field splitting parameters $D$ and $E$. In general the calculation of the zero-field splitting tensor is a difficult task in quantum mechanics because it demands a precise knowledge of the spatial electronic wave functions for the molecule. Given these functions, $D$ is then obtained from the matrix elements

$$D = \frac{1}{2} g^2 \beta z^2 \left< -|D_\varphi| - \right>$$

where $| - \rangle$ is the antisymmetric wave function

$$| - \rangle = (1/\sqrt{2})(|a(1)b(2)> - |a(2)b(1)>$$

where $|a\rangle$ and $|b\rangle$ are the molecular orbitals containing the unpaired electrons. The spatial operators are defined in terms of the coordinates of these two electrons as

$$D_{ab} = \left< (r^2 \delta_{ab} - 3x_1x_2) / r^5 \right>$$

in which $|a\rangle$ and $|b\rangle$ are the molecular orbitals containing the unpaired electrons. The spatial operators are defined in terms of the coordinates of these two electrons as

where $r$ is the electron-electron separation and $x_12$ is the difference in the $z$ coordinates of the electrons. Unless the principal axes of $D$ are determined by the molecular symmetry, the total tensor must be calculated in some arbitrary coordinate system and the principal components obtained by diagonalization.

This entire procedure is considerably simpler when the size of the molecular orbitals $|a\rangle$ and $|b\rangle$ is small in comparison with their separation, for then the electrons may be assumed to be localized. When the electrons are confined to specific locations, the matrix elements in Eq. (44) simply reduce to $D_{ab}$ with the localized coordinates of electrons in place of the spatial operators. The obvious choice of coordinate system for this system is with the $Z$ axis parallel to the interelectron vector, although the assignment of the $X$ and $Y$ axes is immaterial. If one electron is found at the origin of this system then the coordinates of the other electron are

$$Z_2 = r, \quad X_2 = 0, \quad Y_2 = 0$$

Calculation of the appropriate differences from these coordinates then gives the components of the zero-field splitting tensor as

$$D_{zz} = -g^2 \beta z^2 / r^5, \quad D_{xx} = D_{yy} = -g^4 \beta^2 / 2r^3, \quad D_{xy} = D_{xz} = D_{yz} = 0$$

The tensor is therefore diagonal and cylindrically symmetric within this coordinate system; of course this result follows immediately from the symmetry of two localized electrons. The zero-field splitting parameters are then

$$D = 3g^2 \beta z^2 / 2r^3 = -1.949 \times 10^4 g^2 \beta^2 / r^3 \text{ MHz}$$

where $r$ is expressed in angstroms and $E = 0$. Consequently, a knowledge of $D$ should enable the electron-electron separation $r$ to be determined and occasionally the molecular configuration to be assigned to the biradical. Strictly a necessary but not sufficient condition for these calculations is that $E$ is zero or at least small in comparison with $D$ and this is the case for the biradicals listed in Table I. Consider, as an example, the biradical III, which may exist in either a cis or a trans configuration. However, the electron-electron separation estimated from $D$ is about 6 Å and this separation is only compatible with the trans configuration, where the separation is found, from molecular models, to be 6.2 Å (Michon, 1970; Rassat, 1971). Similarly, the zero-field splittings for the biradicals I, II, and IV correspond to an...
electron–electron separation of about 5 Å, which is in accord with a twistboat configuration.

Of course the assumption of localized electrons cannot explain the non-zero values of $E$ observed for certain biradicals and is not strictly valid for nitrooxide biradicals where the electrons are delocalized over their respective nitrooxide groups. Consequently it is necessary to extend the theory if a more precise analysis of the experimental data is required and a more rigorous theory is available (Pullman and Kochanski, 1967). This theory starts with Eq. (44) but now the molecular orbitals are written as a sum of atomic $2p$ orbitals, for example,

$$|a\rangle = \sum_i C_i^a |i\rangle$$  \hspace{1cm} (50)

where $|i\rangle$ denotes an orbital on atom $i$ with a coefficient $C_i^a$, which could be calculated from Hückel molecular orbital theory. With this approximation, which would be valid for most organic biradicals, the zero-field splitting tensor is found to be

$$D = \frac{1}{2} g^2 \beta^2 \sum_{i<j} (C_i^a C_j^a - C_j^a C_i^a)^2 \langle ii | D^{ij} | jj \rangle$$  \hspace{1cm} (51)

where $\langle ii | D^{ij} | jj \rangle$ is a two-center integral for the two electrons in orbitals on atoms $i$ and $j$. The problem is therefore reduced to the evaluation of these integrals; this calculation is particularly simple, and yet accurate, if the $2p$ orbitals are represented as two half charges (McWeeny, 1961). The calculation proceeds in analogous manner to that for localized electrons which was described previously; however, there is now an arbitrary parameter corresponding to the separation between the two half charges. The most appropriate value of this separation is found to be 1.4 Å by comparing experimental and theoretical values of the zero-field splitting (Pullman and Kochanski, 1967). The complete evaluation of $D$ is now reduced, in essence, to a geometrical calculation and has been applied with some success to a conjugated nitrooxide biradical (Calder et al., 1969). The calculation is still simpler for aliphatic nitrooxide biradicals because the electrons are localized on their respective nitrooxide groups and so the coefficients in Eq. (51) only involve the spin densities on the nitrogen and oxygen atoms.

III. BIRADICALS WITH HYPERFINE INTERACTIONS

A. Spectral Analysis

The previous sections contained examples of nitrooxide biradicals and in each of these there must be a coupling between the electron and nuclear spins. It was possible to ignore such hyperfine interactions because their effect on the spectra was obscured by the large linewidths. In this section we shall be concerned with biradicals for which the hyperfine structure can be resolved; however, before considering biradicals that exhibit such structure we shall find it helpful to deal with a biradical where the electron spins are associated with different $g$ factors. The scalar spin Hamiltonian is then

$$\mathcal{H} = g^{(1)} \beta S_z^{(1)} + g^{(2)} \beta S_z^{(2)} + J S^{(1)} \cdot S^{(2)}$$  \hspace{1cm} (52)

which is equivalent to that for an AB system in nuclear magnetic resonance spectroscopy (cf. Carrington and McLachlan, 1967). When the $g$ factors are identical the eigenfunctions of $\mathcal{H}$ are just the triplet and singlet spin states. However, the difference in the $g$ factors is responsible for a competition for the two spins and the result of this competition depends on the ratio $\Delta g \beta B / J$, where

$$\Delta g = g^{(1)} - g^{(2)}$$  \hspace{1cm} (53)

At one extreme, when this ratio is infinite, the two electrons are unaware of each other and the spectrum contains two lines at resonance fields $h v_0 / (g^{(1)} \beta)$ and $h v_0 / (g^{(2)} \beta)$. The other extreme corresponds to a strong coupling between the electron spins and so the ratio is zero; as a consequence they share a common resonance field, which is $2 h v_0 / (g^{(1)} + g^{(2)} \beta)$. These two extreme situations are frequently described as the strong ($\Delta g \beta B / J < 1$) and weak ($\Delta g \beta B / J > 1$) exchange limits. It is not uncommon to see them referred to as the fast and slow exchange limits, but this terminology is quite misleading, for no physical motion is involved. For intermediate values of $\Delta g \beta B / J$ the spectrum is more complex and therefore more informative; we shall now determine the form of such spectra. The choice of the basis set clearly depends on the value of the ratio and in our calculation we shall find it convenient to employ the triplet and singlet spin functions. With this basis the Hamiltonian matrix is

$$\begin{pmatrix}
|1\rangle & |0_+\rangle & |0_0\rangle & |1\rangle \\
\frac{g^{(1)} + g^{(2)}}{2} \beta B + \frac{J}{4} & 0 & 0 & 0 \\
0 & \frac{J}{4} & \Delta g \beta B & 0 \\
0 & \Delta g \beta B & -3J & 0 \\
0 & 0 & 0 & -\frac{g^{(1)} + g^{(2)}}{2} \beta B + \frac{J}{4}
\end{pmatrix}$$  \hspace{1cm} (54)
where only states $|0_+\rangle$ and $|0_\pm\rangle$ are mixed by the $g$-factor difference. The matrix can therefore be cast in diagonal form by taking linear combinations of these two states; the appropriate combinations are

$$|0_+\rangle = \cos \phi |0_+\rangle + \sin \phi |0_\pm\rangle$$

and

$$|0_\pm\rangle = \cos \phi |0_\pm\rangle - \sin \phi |0_+\rangle$$

provided

$$\tan 2\phi = \Delta g\beta B/J$$

The eigenvalues for the two new levels are then

$$E_{0_+} = \frac{1}{2}J + \frac{1}{2}\Delta g\beta B \tan \phi$$

and

$$E_{0_-} = -\frac{3}{2}J - \frac{1}{2}\Delta g\beta B \tan \phi$$

The allowed transitions are now

$$|0_+\rangle \leftrightarrow |1\rangle \quad \text{for} \quad h = 1 + \cos 2\phi$$

$$|0_\pm\rangle \leftrightarrow |1\rangle \quad \text{for} \quad h = 1 - \cos 2\phi$$

$$|1\rangle \leftrightarrow |0_+\rangle \quad \text{for} \quad h = 1 + \cos 2\phi$$

$$|1\rangle \leftrightarrow |0_-\rangle \quad \text{for} \quad h = 1 - \cos 2\phi$$

where $h$ is the transition probability obtained from the square of the matrix element of $(S_{z}^{(1)} + S_{z}^{(2)})$ between the relevant states. The resonance fields for the two transitions involving $|0_+\rangle$ are determined by

$$h_{0_+}(T) = \frac{1}{2}(g^{(1)} + g^{(2)})\beta B \pm \frac{1}{2}\Delta g\beta B \tan \phi$$

and those involving $|0_-\rangle$ are

$$h_{0_-}(S) = \frac{1}{2}(g^{(1)} + g^{(2)})\beta B \pm J \pm \frac{1}{2}\Delta g\beta B \tan \phi$$

In general the spectrum will contain two pairs of lines, each centered on the mean resonance field. When the ratio $\Delta g\beta B/J$ is comparable to or smaller than one, it is possible to label these transitions and spectral lines in a particularly convenient manner. For this region the value of $\cos \phi$ is approximately one and so the state $|0_+\rangle$ is largely composed of the triplet function $|0_\pm\rangle$, whereas $|0_-\rangle$ closely resembles the singlet function. The transitions involving $|0_+\rangle$ may therefore be thought of as triplet (T) transitions while those involving $|0_-\rangle$ are essentially singlet (S) transitions. The spacing between the triplet lines is $\Delta g\beta B \tan \phi$ and that between the singlet pair is $2J + \Delta g\beta B \tan \phi$. The spectra calculated for such a biradical are shown in Fig. 5 for a range of values for the ratio $\Delta g\beta B/J$.

We are now in a position to develop the relevant theory for a biradical composed of two essentially independent halves, which could, for example, be joined by a saturated linkage. The spin Hamiltonian for the system is usually taken to be the sum of the Hamiltonians for the isolated fragments together with the familiar exchange interaction:

$$\mathcal{H} = g^{(1)}\beta S_{z}^{(1)} + \sum_i a_i^{(1)}(\hat{S}^{(1)}_i \cdot \hat{S}^{(1)}) + g^{(2)}\beta S_{z}^{(2)} + \sum_j a_j^{(2)}(\hat{S}^{(2)}_j \cdot \hat{S}^{(2)}) + J\sum_i \hat{S}^{(1)}_i \cdot \hat{S}^{(2)}$$

Here $a_i^{(1)}$ is the coupling constant for the $i$th nucleus in fragment 1 (Slichter, 1955). The conditions under which the hyperfine contribution to the spin Hamiltonian takes this form were derived by Reitz and Weissman (1960); their derivation is outlined in Appendix A. When the hyperfine interactions are small compared with the electron Zeeman splitting, the nonsecular
hyperfine terms may be ignored and $\mathcal{H}$ reduces to
\[
\mathcal{H} = g^{(1)} \beta BS_z^{(1)} + \sum_i d_i^{(1)} I_z^{(1)} S_z^{(i)} + g^{(2)} \beta BS_z^{(2)} + \sum_i d_i^{(2)} I_z^{(2)} S_z^{(i)} + JS^{(1)} \cdot S^{(2)}
\]
(64)

The basis set must now be extended to include the nuclear spin states and the simplest way to achieve this is to employ product spin functions of the form $| M_r, \sum_i m_i^{(1)}, \sum_i m_i^{(2)} \rangle$, where $m_i^{(1)}$ is the quantum number for nucleus $i$ in fragment 1. Because the nuclear components of the spin states are not mixed by any term in $\mathcal{H}$, we can obtain an effective spin Hamiltonian that operates solely on the electron spin states. The effective operator is found by evaluating the matrix elements of the nuclear spin operators between the product spin states, which gives
\[
\mathcal{H}_{\text{eff}} = \left[ g^{(1)} \beta B + \sum_i d_i^{(1)} m_i^{(1)} \right] S_z^{(1)} + \left[ g^{(2)} \beta B + \sum_i d_i^{(2)} m_i^{(2)} \right] S_z^{(2)} + JS^{(1)} \cdot S^{(2)}
\]
(65)

This has exactly the same form as the spin Hamiltonian in Eq. (52) for a biradical without hyperfine interactions and we can therefore define effective $g$ factors by
\[
g_i^{(1)} = g^{(1)} + (1/\beta B) \sum_i d_i^{(1)} m_i^{(1)}
\]
(66)

with a similar expression for fragment 2. It is possible therefore to determine the transition fields and probabilities for even the most complex biradical simply by substituting the effective $g$ factors into Eqs. (60)–(62).

However, our objectives are far less ambitious, for we shall consider a symmetric nitroxide biradical in which the only important hyperfine interaction is with the nitrogen nuclei. The effective $g$ factors are
\[
g_i^{(1)} = g + am^{(1)}/\beta B
\]
(67)

and
\[
g_i^{(2)} = g + am^{(2)}/\beta B
\]
(68)

where the subscript on the nuclear quantum number is omitted because there is only one nucleus in each fragment; the labels on the coupling constant have been dropped for similar reasons. The spectral behavior is, of course, determined by $\Delta g_{\text{eff}} \beta B/J$, which is $a(m^{(1)} - m^{(2)})/J$. The line positions are readily calculated from the expressions for the effective $g$ factors and the results embodied in the single equation
\[
\hbar \omega = g \beta B + \frac{a}{2} M_z + \left( \frac{\pm 1}{\mp 1} \right) \frac{a \Delta m}{2} \tan \phi + \left( \frac{0}{\pm 1} \right) J
\]
(69)

4. BIRADICALS AS SPIN PROBES

where $M_z$ is the total nuclear quantum number and $\Delta m$ is the difference $m^{(1)} - m^{(2)}$. In the strong exchange limit $\tan \phi$ vanishes and so the triplet transitions occur when
\[
h \omega_0(T) = g \beta B + \frac{a}{2} M_z
\]
(70)

The singlet transitions are, of course, forbidden in this limit. Since the spin of the common nitrogen isotope is 1, the total spin quantum number takes all integer values from 2 to −2. The spectrum therefore contains five lines and, as we can see from Table II, they have intensities $1:2:3:2:1$ with a spacing of $a/2 \beta B$, which is just one-half the coupling constant for the corresponding monoradical. The biradical bis[2,2,6,6-tetramethyl piperidin-4-one-1-oxylazide] (V) exhibits strong exchange, as we can see from the spectrum shown in Fig. 6b. There are five hyperfine lines, with the expected relative intensities, each separated by 7.5 G; this gives a value for the nitrogen coupling constant $a$ of 42 MHz, which is equal to that found for the relevant monoradical 2,2,6,6-tetramethyl piperidin-4-one-1-oxyl (Nakajima et al., 1972). Of course it is not possible to determine the magnitude of $J$ from the electron resonance spectrum in the strong exchange limit. However, as the exchange interaction is reduced the degeneracy of the spin transitions is removed and it is possible to estimate $J$. The magnitude of the splitting of the triplet transitions can be obtained from Eq. (69) by expanding $\tan \phi$ for small $\phi$ and this gives
\[
h \omega_0(T) = g \beta B + \frac{a}{2} M_z \pm \left( a^2 \Delta m^2/4J \right)
\]
(71)

Accordingly, those transitions for which $\Delta m$ is zero are unshifted but all other lines do move. Their new positions are given in Table II, and, as we
can see, the central peak is split into three lines with a spacing between adjacent lines of $a^2/2Jg\beta$. The degeneracy of the peaks with $M_I = \pm 1$ is also removed but here the splitting between the two lines is only $a^2/4Jg\beta$. When these component lines can be resolved it is then possible to estimate the exchange integral $J$, although the accuracy of such measurements is not high. An example of this situation is provided by bis(2,2,6,6-tetramethyl piperidin-4-yl-1-oxyl) sulfite (VI), whose spectrum is shown in Fig. 6a,

![Figure 6](image_url)

where only the splitting of the central peak is large enough to be observed; in this case $a/J$ was found to be 0.33 (Nakajima et al., 1972). When the splitting is insufficient to allow the various components to be resolved the removal of the degeneracy will produce an inhomogeneous broadening of the central peak. In addition there will be a smaller broadening of the two lines with $M_I = \pm 1$. However, the use of the widths of the spectral lines to gauge the magnitude of $J$ is fraught with difficulties, for, as we shall see in Section IV.B, the widths of just these lines are strongly influenced by spin relaxation processes. Consequently when the spectrum of a nitroxide biradical contains just five lines the only sound conclusion which may be drawn is that the strong exchange limit obtains. It is unfortunate that several pioneering studies with biradicals as spin probes failed to appreciate this point, thus invalidating their conclusions (Calvin et al., 1969; Ferruti et al., 1969; Hsia et al., 1969).

**B. The Exchange Integral**

Now that we know how to determine the magnitude of the exchange interaction in biradicals we shall require a theoretical basis with which to rationalize these results. A variety of mechanisms may contribute to the
exchange integral and a quantitative prediction of their magnitudes is often difficult. However, in the case of unconjugated nitrooxide biradicals there are just two contributions to \( J \) and the qualitative behavior of these is readily appreciated. Let us consider a particularly simple system containing two electrons, which may occupy the two molecular orbitals \(|a\rangle\) and \(|b\rangle\). The spatial Hamiltonian for the system will be composed of the Hamiltonians for the individual components together with the term \( \epsilon^2/r \), which allows for electron-electron repulsion. It is straightforward to show that \( J \), which is equal to the energy separation between the bonding and antibonding combinations of \(|a\rangle\) and \(|b\rangle\), is given by

\[
J = 2\epsilon^2\langle a(1)b(2) | r^{-1} | a(2)b(1) \rangle
\]  

(72)

The symbol \(|a(1)\rangle\) implies that electron 1 occupies orbital \(|a\rangle\); these orbitals are discussed in greater detail in Appendix A. The magnitude of the matrix element clearly depends on the direct overlap between \(|a\rangle\) and \(|b\rangle\); indeed it is suggested that this mechanism makes a negligible contribution to \( J \) if the molecular orbitals are separated by more than 14 Å. In contrast if the separation is less than 10 Å the contribution is said to be large, although the basis of these statements is obscure (Calvin et al., 1969). This direct contribution to \( J \) is positive and clearly sensitive to the configuration of the biradical. It is, of course, just this configurational dependence which makes flexible biradicals potentially useful as environmental spin probes.

The second mechanism by which the spins are made aware of each other is not immediately obvious from the form of Eq. (72), for it involves the spin polarization of the \( \sigma \) core in the biradical. An entirely analogous mechanism was described in Chapter 2 to account for the scalar hyperfine interaction when the unpaired electron did not appear to occupy an orbital with some \( s \) character. McConnell (1960) has employed perturbation theory to estimate the magnitude of \( J \) caused by this mechanism; although we shall not be concerned with the details, the final result is of interest. When the orbitals containing the unpaired electrons are linked by \( n \sigma \) bonds the spin polarization contribution to \( J \) is approximately

\[
J = (-1)^n \times 10^5 - n \text{ MHz}
\]  

(73)

As expected, \( J \) decreases rapidly with the number of \( \sigma \) bonds separating \(|a\rangle\) and \(|b\rangle\); in addition, the calculation makes the interesting prediction that the sign of the exchange integral should alternate.

We shall now see how these ideas can be used to help us understand the temperature dependence of \( J \) for the biradical bis(2,5,6-tetramethyl piperidin-4-ol-1-oxyl) oxalate (VII) dissolved in carbon disulfide (Glarum and Marshail, 1967). This temperature dependence is shown in Fig. 8 and is typical of that found for structurally related biradicals. At low temperatures the exchange integral reaches a limiting value of about 14 MHz. The fluctuations in molecular geometry are presumably quenched at these temperatures and the biradical then adopts a configuration in which the direct contribution to \( J \) is negligible. The observed limiting value is then attributable to the indirect mechanism and, according to Eq. (73), should only be about

![Fig. 8. The temperature dependence of the exchange integral for biradical VII dissolved in carbon disulfide. (Glarum and Marshall, 1967.)](image)
$3 \times 10^{-2}$ MHz. This enormous discrepancy between theory and experiment probably results from relatively minor errors in the integrals involved in McConnell's calculation. Indeed, the agreement is considerably improved if the exchange integral is attenuated by a factor of $\frac{1}{4}$ rather than $\frac{1}{3}$ by each $\sigma$ bond as suggested in Eq. (73). As the temperature is increased there is a slight decrease in $J$, followed by an exponential increase, which may be attributed to the population of high-energy configurations with the nitrooxide groups in close proximity. The slope of the log $J^{-1}/T$ plot corresponds to an activation energy of about 12 kJ mole$^{-1}$ but it is not possible to use this value to identify the nature of the process responsible for the configurational changes. These changes might be controlled simply by the barriers to rotation about single bonds in the biradical or they could be determined by positional changes in the solvent sheath. Indeed, the exchange integral does exhibit a solvent dependence; for example, the low-temperature limiting values of $J$ for bis(2,2,6,6-tetramethyl-piperidin-4-ol-1-oxyl) carbonate (VIII) range from 125 MHz in chloroform through 95 MHz in carbon disulfide to 74 MHz in $n$-hexane. There is an apparent correlation with the anisotropy of the solvent molecules which may force the spin probe to adopt less favorable configurations for the direct contribution to $J$ even at low temperatures.

The sensitivity of the exchange integral to the biradical's environment has been employed in a study of micelle formation in aqueous solutions of sodium dodecyl sulfate (Ohnishi et al., 1970). The biradical used was $N,N'$-bis[4-(1-oxyl-2,2,6,6-tetramethyl piperidyl)] urea (IX), which gives the spectrum shown in Fig. 9a when dissolved in water. The singlet transitions are observed, the spectrum is therefore readily analyzed, and $J$ is found to be 65 MHz. Addition of sodium dodecyl sulfate had no effect on the spectrum until the critical micelle concentration of $8 \times 10^{-2} M$ was reached. At this point additional lines were observed in the spectrum and these are indicated by arrows in Fig. 9b. Further increase in the concentration of the surfactant yields the single spectrum shown in Fig. 9c; the singlet lines can still be discerned and give a value for the exchange integral of 56 MHz. This is close to the value found when the biradical is dissolved in hydrocarbon solvents. The interpretation of these results is quite straightforward. Below the critical micelle concentration the spin probe experiences an aqueous environment. Far above this concentration the probe is associated entirely with the hydrocarbon region of the micelles. The relatively sharp spectral lines observed for the spin probe in this environment imply a highly fluid state for the interior of the micelle. At intermediate concentrations the biradical is partitioned between the micelles and the surrounding aqueous phase. The observation of spectra from both regions shows that the rate of exchange between the two environments is slow. Similar conclusions have also been reached with the aid of monoradical spin probes (Atherton and Strach, 1972; Oakes, 1972).

In these experiments the molecular configuration and hence the exchange integral were modified by the weak van der Waals interactions with the solvent. However, in an idealized application of biradicals as spin probes there should be a much stronger interaction with the environment (Calvin et al., 1969). For example, we might hope to attach the spin probe via covalent or ionic links to the system under investigation, which could be a membrane or a nucleic acid. Then changes in the conformation of the system, induced by an external stimulus, would induce corresponding structural variations in
the biradical and these could be detected by changes in the exchange integral. The success of such experiments clearly demands a rather special spin probe, for not only must it bind specifically to the system, but, in addition, the exchange integral must be comparable to the hyperfine interaction in order for the magnitude of \( J \) to be determined from the spectrum (Ferruti et al., 1970).

C. Anisotropic Environments

In this section we shall consider the spectral analysis for a biradical dissolved in a macroscopically anisotropic environment with particular reference to nematic liquid crystals. As we saw in Section II.B, the difference between isotropic and anisotropic systems is the retention of angle-dependent contributions in the static spin Hamiltonian \( \mathcal{H} \). A straightforward extension of Eq. (28) to a biradical for which there are several angle-dependent terms shows that the formal expression for \( \mathcal{H} \) is

\[
\mathcal{H} = \mathcal{H}_0 + \sum_{\nu \lambda \rho} (-1)^\nu F^{\nu(2)} \mathcal{S}^{(2)}_{\nu \lambda \rho} \mathcal{T}^{(2)}_{\nu \lambda \rho}
\]

(74)

when the symmetry axis of the system is parallel to the magnetic field. These various magnetic interactions are denoted by \( \mu \) and the interaction tensors \( F^{(2)} \) are expressed in a molecular coordinate system. If the contributions are restricted to the Zeeman, hyperfine, and electron-electron interactions, then this spin Hamiltonian is

\[
\mathcal{H} = g_1^{(1)} \beta S_1^{(1)} + g_1^{(2)} \beta S_2^{(2)} + A_1^{(1)} j_1^{(1)} S_1^{(1)} + A_1^{(2)} j_1^{(2)} S_2^{(2)} + (J - B) S_1^{(2)} S_2^{(2)} + 3 D_1 S_1^{(2)} S_2^{(2)}
\]

(75)

provided the nonsecular hyperfine terms are negligible. The parallel components of the partially averaged \( g \) and hyperfine tensors are defined in an analogous manner to that for the zero-field splitting tensor \( D \). The two \( g \) tensors \( g_1^{(1)} \) and \( g_1^{(2)} \) are not equal even in a symmetric biradical unless the principal axes of the total \( g \) tensors are parallel; when this condition is satisfied the two electrons are said to be completely equivalent. Similarly the partially averaged hyperfine tensors for the two nitrogen nuclei will only be identical if the nitrogens are completely equivalent. The contribution of the zero-field splitting to \( \mathcal{H} \) is identical to that given in Eq. (30) but has been rewritten in terms of the individual electron spin operators.

According to Eq. (75), the partial alignment of the biradical shifts both the \( g \) factor and the coupling constant from their isotropic values; however, the major spectral change results from the contribution of the anisotropic electron-electron coupling (Falle et al., 1966). In the strong exchange limit this coupling removes the degeneracy of the electron spin transitions, as we saw in Section II.B. Consequently the number of spectral lines is doubled and the spacing between these doublets is \( 3B \). The splitting of the spectral lines is illustrated in Fig. 10 for bis(2,2,6,6-tetramethyl piperidin-4-ol-1-oxyl) glutarate (X) dissolved in the nematogen 4,4'-dimethoxyazoxybenzene. The spectrum in the isotropic phase (142°C) contains five lines indicative of strong exchange, although the lines do not have their expected relative heights. This deviation is a result of symmetric line broadening, which is described in Section IV.B. In the liquid crystal phase each of the lines is indeed split into a doublet, although only nine of the ten lines are observed, because of overlap in the center of the spectrum. Determination of this additional splitting together with a knowledge of the total zero-field splitting tensor would then provide a value for the order parameter of the spin probe.
The spectral changes produced by partial alignment are much more complex for intermediate values of spin exchange (Lemaire, 1967; Lemaire et al., 1968; Corvaja et al., 1970). The problem is further complicated by the contribution of the zero-field splitting to $\mathcal{H}$, which has the same form as the scalar electron–electron coupling and as a consequence influences the extent of triplet–singlet mixing. The effect of this mixing can best be appreciated by returning to an effective spin Hamiltonian, for the Hamiltonian matrix is readily found to be

$$
\begin{bmatrix}
|1\rangle & |0\rangle & |0\rangle & |-1\rangle \\
\frac{3}{4}g_{||}^2 + \frac{5}{4}g_{\perp}^2 & 0 & 0 & 0 \\
\frac{1}{4}J' + \frac{3}{4}D_1 & \frac{1}{4}J' - \frac{3}{4}D_1 & \frac{1}{4}J' - \frac{3}{4}D_1 & 0 \\
0 & \frac{1}{4}D_1 & -\frac{1}{2}J' + \frac{3}{4}D_1 & 0 \\
0 & 0 & -\frac{1}{4}g_{\parallel}^2 + \frac{5}{4}g_{\perp}^2 & \frac{1}{4}J' + \frac{3}{4}D_1
\end{bmatrix}
$$

(76)

where

$$J' = J - D_1$$

(77)

This matrix is identical in form to Eq. (54) and so the eigenfunctions are still given by Eqs. (55) and (56), but now

$$\tan 2\phi = \frac{A_0 g_{\parallel} \beta B}{J'}$$

(78)

Evaluation of the eigenvalues shows that the four allowed spin transitions occur when

$$h\nu_0(T) = (g_{||}^{(1)} + g_{||}^{(2)})(\beta B/2) \pm \Delta g_{\parallel}(\beta B/2) \tan \phi \mp \frac{1}{2}D_1$$

(79)

and

$$h\nu_0(S) = (g_{||}^{(1)} + g_{||}^{(2)})(\beta B/2) \pm J' \pm \Delta g_{\parallel}(\beta B/2) \tan \phi \pm \frac{1}{2}D_1$$

(80)

The partially averaged zero-field splitting makes the anticipated contribution to the spacing between the triplet lines, but in addition there is also a contribution to the separation between the singlet lines. Since these two separations depend in different ways on $J$ and $D_1$, it is possible to determine both from the spectrum of the biradical in the ordered phase. Such determinations are potentially important because the molecular configurations should change on passing from a disordered to an ordered system and these changes will be reflected in $J$. However, environmental changes in the exchange integral should be negligible for the rigid biradical azoxy-5,5'-tetraethyl-1,1,3,3 isindoline oxyl-2 (XI) synthesized by Giroud

\[\textit{et al.} (1974).\] This biradical could be a particularly valuable spin probe because its structure closely resembles that of many liquid crystals containing the azoxybenzene nucleus. In addition, the determination of the partially averaged $g$ factor, nitrogen hyperfine splitting, and zero-field splitting from the spectrum in the liquid crystal mesophase should permit all of the order parameters $D_\mu^{(1)}$ to be obtained.

IV. SPIN RELAXATION

An appreciation of the various factors which may influence the linewidths in a spectrum is important not only as an aid to a correct analysis but also because the linewidths can often afford a valuable insight into the molecular dynamics. There are two distinct relaxation processes which contribute to the linewidths for a biradical exhibiting hyperfine structure. One results in an asymmetric variation in the widths of the spectral lines and in the other case the symmetric appearance of the spectrum is preserved.

A. Asymmetric Line Broadening

When the solvent viscosity is sufficiently high to impede molecular rotation the modulation of the anisotropic interactions by this reorientation provides the dominant spin relaxation process (Lemaire, 1967; Luckhurst and Pedulli, 1971). Except in a few rare cases, these interactions are second rank and so the dynamic perturbation required in the linewidth calculation can be written formally as

$$\mathcal{H}'(t) = \sum_{\mu,\nu,\rho} \langle \mu,\nu | \rho,\nu \rangle T_{\mu,\rho}(t)$$

(81)

where the Wigner rotation matrix connects the molecular and space-fixed coordinate systems. For nitroxide biradicals the subscript $\mu$ denotes the anisotropic Zeeman coupling, nitrogen hyperfine interaction, and electron-electron coupling. The calculation of the Redfield relaxation matrix for these three interactions is tedious; however, the bookkeeping can be reduced by assuming that the two electrons and two nuclei are completely equivalent as
well as by ignoring the nonsecular terms in the dynamic perturbation. This can then be written as

\[ \Gamma(t) = \sum_p (-1)^p \left( g^2 \tau^2 \mu B \mathcal{D}_{2,1} \tau(t) \left( \frac{2}{3} (S_z^1 + S_z^2) \right) 
+ g^2 \tau^2 \mu B \mathcal{D}_{2,1} \tau(t) \left( \frac{2}{3} (I_z^1 S_z^1 + I_z^2 S_z^2) \right) 
+ \mathcal{D}_{2,1} \tau(t) \left( \frac{1}{2} (I_z^1 S_z^1 + I_z^2 S_z^2) \right) \right) 
+ \mathcal{D}_{2,1} \tau(t) \left( \frac{1}{2} (I_z^1 S_z^1 + I_z^2 S_z^2) \right) \right) \]

Neglect of the nonsecular terms will, of course, only be valid when the rotational correlation time \( \tau \) satisfies the inequality \( 4 \pi \mu B \tau^2 \gg 1 \).

The size of the relaxation matrix is determined by the degeneracy of the transition and this, for a biradical, is a sensitive function of the ratio \( \alpha/J \). For example, in the strong exchange limit the central component of the five-line multiplet observed from a nitroxide biradical is sixfold degenerate because of the triple degeneracy of the nuclear spin states and the double degeneracy of the electron spin transitions. In contrast, when \( \alpha \) is comparable to \( J \) each transition is only doubly degenerate. For those transitions between states with \( m^{(1)} \) equal to \( m^{(2)} \) this degeneracy is associated with the electron spin. However, for the other transitions the electron spin degeneracy is removed and the double degeneracy comes from the nuclear spin states. The detailed calculation of the elements of the relaxation matrix, whatever its size, is somewhat involved and since no new principles are introduced, we shall not be concerned with these details. In addition, the results of the calculation are complicated and so have been relegated to Appendix B. It is difficult to draw qualitative conclusions simply by inspection of these results, especially since the widths of the component lines for a doubly degenerate transition may differ and so cause the overall line shape to deviate from a Lorentzian.

In view of these difficulties we shall illustrate the theory by simulating spectra for the typical biradical VIII with the configuration shown, for various correlation times. The other parameters required by the linewidth calculations are the \( g \) and hyperfine tensors, which have been determined from single-crystal studies of the relevant monoradicals. The zero-field splitting tensor has not been measured for this biradical but can be estimated with the aid of the point-dipole approximation. The calculations are shown in Fig. 11 for three values of the rotational correlation time; when the motion is fast the spectrum exhibits a slight broadening of the spectral lines which is asymmetric with respect to the center of the spectrum. As the correlation time is increased the asymmetric linewidth variation becomes more pronounced. Indeed the sharp features of the triplet spectrum rapidly disappear, although in contrast the singlet transitions become more important because their widths are less affected by this relaxation process. Figure 11 also includes, for comparison, the electron resonance spectrum of biradical VIII dissolved in toluene at three different temperatures. The general agreement between theory and experiment is good and clearly demonstrates the value of the theory in analyzing the spectra of biradical spin probes in viscous environments.

When the spin probe is rigid, the only unknown in the calculation is the rotational correlation time and so a comparison between theory and experiment simply yields \( \tau \). A more interesting situation obtains when the spin probe is nonrigid, for then the configuration and, hence, the zero-field splitting may be influenced by the environment and so are not known. Consequently analysis of the asymmetric line broadening should enable both \( D \) and \( \tau \) to be determined even though the spin probe is in fluid solution. The sensitivity of the spectral appearance to the zero-field splitting is demonstra-
ted by the following calculation. Biradical VIII is assumed to adopt a hypo-
thetical configuration in which the piperidine rings are parallel and with the
largest component of D parallel to that of the nitrogen hyperfine tensor. In
other words the principal components of the zero-field splitting are un-
changed although the orientation of the principal coordinate system with
respect to that for the hyperfine tensor has been rotated through 90°. The
spectrum, calculated for a correlation time of \( 7.23 \times 10^{-16} \) sec, is shown in
Fig. 12; it is quite unlike any of those obtained for the real configuration and
so confirms the dramatic dependence of the spectrum on the zero-field
splitting.

![Graph](image)

Fig. 12. A simulated spectrum for a hypothetical configuration of VIII. (Luckhurst and
Pedulli, 1971.)

**B. Symmetric Linewidth Variations**

According to Eq. (72), the exchange integral is sensitive to the separation
between the orbitals containing the unpaired electrons and so in a flexible
biradical \( J \) will vary with the molecular configuration. This modulation of \( J \)
by the intramolecular motion constitutes the dominant spin relaxation
process for a nonrigid biradical dissolved in a low-viscosity solvent (Luck-
hurst, 1966). This behavior is not entirely unexpected, for as we saw in
Section III.A, the positions of the spectral lines are dependent on \( J \). Further,
according to the theoretical spectra in Fig. 7, the singlet lines are extremely
sensitive to the value of the exchange integral, whereas the triplet lines are
less sensitive and indeed certain of these lines are independent of \( J \)
altogether. When the modulation of the exchange integral is an important

**4. BIRADICALS AS SPIN PROBES**

relaxation process we must therefore expect the singlet lines to be con-
siderably broader than the triplet lines, while certain of these will remain
unbroadened. We shall now seek to quantify these predictions.

It will again prove advantageous to consider a biradical described by an
effective spin Hamiltonian in which the hyperfine interactions have been
incorporated into an effective \( g \) factor. This approach is valid because the
dynamic perturbation involves just the electron–electron interaction and so
does not mix the nuclear spin states. The rate of \( J \) modulation is assumed to
be fast in the sense that the static spin Hamiltonian, which determines the
line positions, can be obtained by taking an average of the exchange integral
over all molecular configurations. This time-averaged spin Hamiltonian is

\[
\bar{\mathcal{H}} = \mathcal{H}^{(1)} + \mathcal{H}^{(2)} + J S^{(1)} \cdot S^{(2)}
\]  

(83)

where the bar indicates a time or ensemble average. The dynamic pertur-
bation is obtained by subtracting the static from the instantaneous spin Hamil-
tonian; this gives

\[
\mathcal{H}^{(e)}(t) = (J(t) - \bar{J}) S^{(1)} \cdot S^{(2)}
\]  

(84)

which has a zero time average. In general there are four allowed spin
transitions for our effective biradical and they are listed in Eq. (60). These
transitions are nondegenerate and so the relevant relaxation matrices contain a
single element for which the expression given in Chapter 2 reduces to

\[
R_{\alpha \sigma', \sigma} = 2J_{\alpha \sigma', \sigma}(0) - \sum_{\gamma} \left( J_{\alpha \sigma', \gamma} + J_{\alpha \gamma, \sigma} \right)
\]  

(85)

for the transition between states \( | \alpha \rangle \) and \( | \alpha' \rangle \). In this problem the spectral
density \( J_{\alpha \beta}(\omega) \) is

\[
J_{\alpha \beta}(\omega) = \frac{1}{2} \int_{-\infty}^{\infty} \left( J(0) - J(t) \right) e^{i\omega t} dt
\]  

(87)

This calculation gives the widths of the two triplet transitions as

\[
T_{2}^{-1}(T) = j(0) \sin^{4} \phi + j(\Delta \omega)^{2} \sin^{2} 2\phi
\]  

(88)

where

\[
\Delta \omega = J + \Delta g \beta \tan \phi
\]  

(89)
which is the frequency separating states $|0_+\rangle$ and $|0_-\rangle$. There is an analogous expression for the widths of the singlet lines:

$$T_2^{-1}(S) = j(0) \cos^2 \phi + j(\Delta \omega) \sin^2 2\phi$$  \hfill (90)

It is now possible to see why the singlet satellite lines may be difficult to detect for flexible biradicals (Glarum and Marshall, 1967). Consider the situation when $\Delta g \beta B/J$ is one-half; then the widths of the triplet and singlet lines are calculated to be

$$T_2^{-1}(T) = 0.0022j(0) + 0.038j(\Delta \omega)$$  \hfill (91)

and

$$T_2^{-1}(S) = 0.62j(0) + 0.038j(\Delta \omega)$$  \hfill (92)

Since $j(\Delta \omega)$ can never be greater than $j(0)$, the widths of the singlet lines could well be orders of magnitude greater than those of the triplet lines. The absolute linewidths will depend on the magnitude of the spectral density $j(0)$, which will, in turn, be a function of the rate at which the exchange integral is modulated as well as the values which it adopts. It is, however, difficult to calculate $j(\omega)$ in any rigorous fashion because the exact dependence of $J$ on the molecular configuration is not available and in addition the nature of the motion between configurations is unknown. The qualitative features of $j(\omega)$ can be obtained by appealing to a mathematically tractable but possibly naive model in which the biradical is assumed to exist in just two configurations, each with its own lifetime and exchange integral. An entirely similar problem was considered in Chapter 2 and we can use these results to write the spectral density as

$$j(\omega) = (J_1 - J_2)^2 \frac{\tau^3}{(\tau_1 + \tau_2)^2} \frac{1}{1 + \omega^2 \tau^2}$$  \hfill (93)

where $\tau$ is the geometric mean of the individual lifetimes. The magnitude of the spectral density therefore depends on the difference in the exchange integral for the two configurations and is essentially proportional to the lifetime. The model could, in principle, be made more realistic by increasing the number of configurations, although this would increase the number of adjustable parameters in the theory. From a pragmatic point of view such extensions are not warranted, for, at best, the spectrum in the limit of rapid modulation only provides us with the two spectral densities $j(0)$ and $j(\Delta \omega)$. Finally, we consider the width of the triplet line in the strong exchange limit when $\Delta g \beta B/J$ is small. This limiting width is of considerable interest, for many flexible biradicals are found to exhibit strong exchange. By taking the limit of Eqs. (88) and (89) for small $\Delta g \beta B/J$ the width of the triplet line is found to be

$$T_2^{-1} = (\Delta g \beta^2 B^2/4J^2)j(J)$$  \hfill (94)

We can now proceed to consider the linewidths for nitroxide biradicals, which exhibit hyperfine structure, simply by replacing the $g$-factor difference in Eqs. (88), (90), and (94) by the difference in the effective $g$ factors. As an example of this procedure, we consider a symmetric nitroxide biradical exhibiting strong spin exchange with intramolecular motions modulating the exchange integral. The spectrum therefore contains five lines with intensities $1:2:3:2:1$ and their widths are obtained by substituting the effective $g$ factors defined by Eqs. (67) and (68) into Eq. (94), as

$$T_2^{-1}(m^{(1)}, m^{(2)}) = [a^2(m^{(1)} - m^{(2)})^2/4J^2] j(J)$$  \hfill (95)

The linewidths calculated from this expression for the nine transitions corresponding to the nine combinations of $m^{(1)}$ and $m^{(2)}$ are listed in Table III. Since the linewidth depends on the square of $(m^{(1)} - m^{(2)})$, lines whose total quantum numbers are related by a reversal of sign will have the same width and consequently this broadening mechanism preserves the symmetry about the center of the spectrum; this is readily seen from the linewidths listed in Table III. The three lines for which $m^{(1)}$ is equal to $m^{(2)}$ are not broadened by

<table>
<thead>
<tr>
<th>$M_t$</th>
<th>$m^{(1)}$</th>
<th>$m^{(2)}$</th>
<th>$T_2^{-1}[a^2(J)/4J^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>-1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>-2</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
</tr>
</tbody>
</table>

modulation of the exchange integral. However, although one component of the central line is unBroadened, the widths of the other two are increased considerably and we can now see why the width of this line may not provide a reliable method for determining the exchange integral. The lines with $M_t = \pm 1$ are broadened by the relaxation process and so the widths of the spectral lines will alternate. An example of such linewidth alternation is
4. BIRADICALS AS SPIN PROBES

unbroadened, while the peaks with \( M_I = \pm 1 \) are rather broad. As the temperature is reduced the widths of the \( M_I = \pm 1 \) lines increase still further, although those of the other peaks remain unchanged. At \(-22^\circ C\) only the three lines with \( m^{(1)} \) equal to \( m^{(2)} \) are observed and this spectrum is identical to that expected for a biradical exhibiting weak exchange. Clearly, considerable care must be exercised when interpreting a biradical spectrum that is identical to that for the corresponding monoradical (Lemaire et al., 1968). According to Eq. (95), these spectral changes reflect the temperature dependence of the ratio \( j(J)/J^2 \) and it is not possible to associate them with either variations in \( j(J) \) or \( J \) alone.

The preceding linewidth analysis is based on Redfield's relaxation theory and so will only be valid when the exchange interaction is modulated rapidly by the intramolecular motion. If this motion is not fast, then theoretical techniques analogous to those described in Chapter 3 must be employed to calculate not only the widths of the spectral lines but also their positions. However, a rigorous calculation is not possible with a flexible biradical, for exactly the same reasons that the spectral density \( j(\omega) \), defined by Eq. (87), cannot be evaluated. We are therefore forced to perform calculations based on the simple two-site model of intramolecular motion. The line-shape theory is relatively straightforward (Parmon and Zhdanov, 1974) and can be simplified by the use of an effective spin Hamiltonian. The details of the theory is outside the scope of this chapter and we must be content to quote some of the more pertinent results. These can be illustrated by reference to the calculation for a nitroxide biradical interconverting between two conformations with equal lifetimes; in one configuration the exchange integral is zero and for the other it is 30 times the nitrogen hyperfine interaction. The spectra shown in Fig. 14 were calculated for a range of lifetimes \( \tau \), which are listed in the figure as the dimensionless parameter \( \tau r_1 \). The average exchange integral \( J \) is 15\( a \) and so when the intramolecular motion is fast the spectrum corresponds to the strong exchange limit; this situation can be seen in Fig. 14g. As the lifetime is increased certain lines in the simulated spectra are observed to broaden, in complete accord with the predictions of Eq. (95). It is also of interest to note the similarity between the theoretical spectra and the experimental spectra shown in Fig. 13. The spectrum given in Fig. 14c contains essentially three lines of equal intensity because of the extreme widths of the other peaks; however, as the lifetime is increased still further new lines appear in the spectra, as we can see from Figs. 14b and 14a. The origin of these new peaks is readily understood, for in the limit of slow conversion the observed spectrum will be the sum of the spectra from the two configurations. For one of these the exchange integral is zero and it therefore has a three-line spectrum, whereas the other configuration corresponds to strong spin exchange and has a five-line spectrum. It is the peaks

Fig. 13. Symmetric linewidth variations for the nitroxide biradical X. [Reprinted with permission from Luckhurst and Pedulli, J. Amer. Chem. Soc. 92, 4738–4739 (1970). Copyright by the American Chemical Society.]

provided by the spectra, shown in Fig. 13, for the nitroxide biradical X dissolved in toluene (Luckhurst and Pedulli, 1970). Even at 46°C the relative line heights are not in the ratio of 1:2:3:2:1 expected when the linewidths are equal; indeed only one component of the central line can be observed. The extreme spectral lines corresponding to \( M_I = \pm 2 \) are also
4. BIRADICALS AS SPIN PROBES

From linewidth measurements. Since the correlation time that can be determined by such techniques depends on the anisotropy in the magnetic interactions, nitroxide biradicals are more useful than the corresponding monoradical. This advantage accrues because the zero-field splitting in a biradical can be varied simply by changing the separation between the two nitroxide groups, whereas the $g$ and hyperfine tensors for a nitroxide are essentially independent of the molecular structure.

The other applications as spin probes would seem to be unique to biradicals and so there is no direct conflict with the use of monoradicals. The ability to investigate the molecular configuration of the biradical from a knowledge of the exchange integral is important. Of course the detailed molecular geometry cannot be inferred from the magnitude of $J$ but changes in the exchange integral will reflect conformational variations induced by an external stimulus. In addition the rate and nature of the intramolecular motion can often be deduced from the linewidths; consequently changes in the dynamics caused by a specific perturbation to the system can also be examined. Finally, the ability to determine the zero-field splitting tensor from the line broadening in moderately viscous systems is important because the interelectron separation can be determined and so the effect of the environment on the molecular geometry investigated.

Nitroxide biradicals would therefore appear to possess unique properties which make them valuable as environmental spin probes and yet their applications to real problems are rare. It is not too difficult to see why they should be so unpopular in comparison with their monoradical counterparts. In the first instance the additional magnetic interactions in the biradical often make its electron resonance spectrum complex and the lines poorly resolved. Further, the analysis of such a spectrum need not be straightforward or even unambiguous. The lack of suitable biradicals for which the exchange integrals can be determined from their spectra may also be a reason for their unpopularity. Nonetheless, it is to be expected that as the theory demanded for the spectral analysis becomes more appreciated and as the range of biradicals is extended their obvious merits as spin probes will be exploited.

APPENDIX A. THE SPIN HAMILTONIAN

Here we seek to justify the form of the spin Hamiltonian given in Eq. (63) for a biradical exhibiting hyperfine structure. The scalar coupling between the electron and nuclear spins is represented in the Hamiltonian by the Fermi contact interaction, which was described in Chapter 2. For a biradical, this takes the form

$$\mathcal{H}_e = \frac{(8\pi/3)g\beta S}{\beta} \sum_i \left[ \delta(r_1 - r_i)S^{(1)} + \delta(r_2 - r_i)S^{(2)} \right] \cdot J^0 \quad (A.1)$$
where the summation is restricted to nuclei in one-half of the biradical; there is a comparable summation, which has been omitted, for the other half. Here \( g_n \) is the nuclear \( g \) factor, \( \beta_n \) is the nuclear magneton, and \( \delta(r_1 - r_i) \) vanishes unless the coordinates \( r \) of electron 1 and nucleus \( i \) coincide. This Hamiltonian contains both spatial and spin operators; since we require a spin Hamiltonian, the spatial operators must be removed by evaluating their matrix elements. The molecular orbitals \( |a\rangle \) and \( |b\rangle \) containing the unpaired electrons can be combined to form symmetric,

\[
|+\rangle = (1/\sqrt{2})(|a(1)b(2)\rangle + |a(2)b(1)\rangle)
\]

and antisymmetric,

\[
|-\rangle = (1/\sqrt{2})(|a(1)b(2)\rangle - |a(2)b(1)\rangle)
\]

functions. The total wave function is obtained by combining these spatial functions with spin functions in such a way that the total function is antisymmetric with respect to the interchange of the electron labels. Consequently the symmetric state \( |+\rangle \) can only combine with the antisymmetric singlet spin function and the antisymmetric state \(|-\rangle\) must combine with the triplet spin functions. To evaluate the matrix element of the hyperfine spin Hamiltonian between the triplet spin functions we have first to obtain the matrix element of the spatial operators between the \(|-\rangle\) states:

\[
\langle - | \mathcal{H}_s | - \rangle = (8\pi/3)g \beta g_n \beta_n \sum_i \frac{1}{2} [\langle a | \delta(r_i) | a \rangle + \langle b | \delta(r_i) | b \rangle - 2\langle a | \delta(r_i) | b \rangle \langle b | a \rangle] J(0). (S^{(1)} + S^{(2)})
\]

(A.4)

together with a corresponding term for the other half of the biradical. Here the symbol \( \langle a | \delta(r_i) | a \rangle \) implies that the square of the wave function is evaluated at nucleus \( i \). Before considering the matrix elements for the other states we shall simplify the expression in Eq. (A.4). If the orbitals containing the unpaired electrons are orthogonal, then the last term in the square brackets vanishes. In addition, if these orbitals are spatially separated, then when the summation is over nuclei in the fragment associated with orbital \( |a\rangle \) the value of the wave function \( |b\rangle \) at nucleus \( i \) will be negligibly small. The matrix element reduces to

\[
\langle - | \mathcal{H}_s | - \rangle = \sum_i \frac{1}{2} a^{(0)}_i J(0). (S^{(1)} + S^{(2)})
\]

(A.5)

where

\[
a^{(0)} = (8\pi/3)g \beta g_n \beta_n \langle a | \delta(r_i) | a \rangle
\]

(A.6)

and is identical to the coupling constant for the \( i \)th nucleus in the corresponding monoradical.

4. BIRADICALS AS SPIN PROBES

Similar arguments show that the operator required for evaluating the matrix element for the singlet state is also given by Eq. (A.5). In contrast the spin operator employed in calculating the matrix element between the triplet and singlet spin functions is

\[
\langle + | \mathcal{H}_s | - \rangle = \sum_i \frac{1}{2} a^{(0)}_i J(0). (S^{(1)} - S^{(2)})
\]

(A.7)

Since the electron spin operator \( (S^{(1)} - S^{(2)}) \) only connects triplet with singlet spin levels, we can obtain an equivalent hyperfine Hamiltonian to operate on any spin function simply by adding Eqs. (A.5) and (A.7). This gives

\[
\mathcal{H}_s = \sum_i a^{(0)}_i J(0). S^{(1)}
\]

(A.8)

with a corresponding term for the nuclei in the fragment associated with orbital \( |b\rangle \). We have achieved our objective, for this expression is identical to the hyperfine operator given in Eq. (63).

In conclusion we note that when the conditions concerning the overlap and extent of the molecular orbitals are relaxed, the coefficients in Eqs. (A.5) and (A.7) are different and not equal to the coupling constant for the appropriate monoradical.

APPENDIX B. THE LINEWIDTHS FOR A NITROXIDE BIRADICAL

We list here the equations for the linewidths of a nitroxide biradical when the dominant spin relaxation process results from the molecular rotation coupled to the anisotropy in the magnetic interactions.

In the strong exchange limit the linewidth for the transition

\[
| M_s ; m^{(1)}_s m^{(2)}_s \rangle \leftrightarrow | 0_s ; m^{(1)}_s m^{(2)}_s \rangle
\]

(B.1)

where \( M_s \) takes the values \( \pm 1_1 \), is

\[
T_2^{-1} = \frac{\tau_2}{3} [\frac{1}{3} \beta^2 B^2 (g : g) + \frac{M_s \beta B}{h} (g : D) + \frac{3}{8} (D : D) + \frac{2 M_s \beta B}{3 h} (g : A) + \frac{M_s M_s}{2} (A : D) + \frac{M_s^2}{6} (A : A)] + \frac{(A : A) \Delta m^2}{10} \left( \frac{1}{3} J(J) + \frac{2}{3} (J + 1 - |m^{(1)}_s + m^{(2)}_s|) \left[ \frac{J(J)}{2} + J(J) \right] \right)
\]

(B.2)
The symbol \((X : Y)\) denotes the inner product of the two second-rank tensors \(X\) and \(Y\):
\[
(X : Y) = \sum \chi_{i2}^* \alpha_{j2} \chi_{i2} \alpha_{j2}^*
\]  
(B.3)

The spectral density \(j(\omega)\) is defined by
\[
j(\omega) = \tau/(1 + \omega^2 \tau^2)
\]  
(B.4)

where \(\tau\) is the rotational correlation time; when applying Eq. (B.2) the argument \(\omega\) is usually assumed to be small compared with \(\tau\) and the spectral densities are set equal to this correlation time.

For intermediate values of the exchange integral the contribution of the secular terms in the dynamic spin Hamiltonian to the widths of the triplet and singlet lines is
\[
T_2^{-1(\text{sec})} = \frac{\tau}{5} \left[ \frac{2 \beta B^2}{3 \hbar^2} (g : g) + \frac{\beta BM}{3 \hbar} (2 + \cos 2\phi)(g : D) \right.
\]
\[
+ \left. \frac{(2 + \cos 2\phi)^2}{(2 - \cos 2\phi)^2} \frac{(D : D)}{24} + \frac{2 \beta B}{3 \hbar} \left( M_x M_y - M_z \Delta m \sin 2\phi \right) (g : A) \right.
\]
\[
+ \left. \frac{M_y}{M_z} \left( 2 + \cos 2\phi \right) - \Delta m \sin 2\phi \left( 2 + \cos 2\phi \right) \right] (A : D)
\]
\[
+ \left. \frac{M_x}{M_z} \left( 2 - \cos 2\phi \right) + \Delta m \sin 2\phi \left( 2 - \cos 2\phi \right) \right] (A : A) \right]
\]
\[
+ \frac{1}{6} \left( (M_x - M_z \Delta m \sin 2\phi)^2 / (A : A) \right)
\]
\[
+ \left. \frac{M_y}{M_z} \left( 2 + \cos 2\phi \right) - \Delta m \sin 2\phi \left( 2 + \cos 2\phi \right) \right] (A : A) \right]
\]
\[
+ \frac{1}{6} \left( (M_x - M_z \Delta m \sin 2\phi)^2 / (A : A) \right)
\]
\[
+ \Delta m \sin \frac{4}{12} \left( (A : D) + \Delta m^2 \cos^2 2\phi \left( (A : A) / 6 \right) \right)
\]  
(B.5)

The pseudosecular terms in \(\mathcal{H}'(t)\) make the same contribution to the widths of both triplet and singlet lines:
\[
T_2^{-1(\text{pse-sec})} = \frac{\tau}{3} (2m^2(1 + m^2)) \times \left[ \frac{1}{2} \left( (A : A) \right) \right]
\]
\[
+ \sin^2(\phi + \phi')(o_{12}) - \frac{1}{2} M_x [\sin^2(\phi + \phi') + o_{12}]
\]
\[
- \sin^2(\phi + \phi') [o_{12} + \cos^2(\phi + \phi') + o_{12}]
\]
\[
- \cos^2(\phi + \phi') [o_{12} + \cos^2(\phi + \phi') + o_{12}]
\]  
(B.6)

4. BIRADICALS AS SPIN PROBES

The new symbols in this equation are
\[
\tan 2\phi = a(\Delta m + 1)/J
\]  
(B.7)

\[
\tan 2\phi' = a(\Delta m - 1)/J
\]  
(B.8)

\[
\omega' = \frac{1}{2} \left( \sec 2\phi' \pm \sec 2\phi \right)
\]  
(B.9)

with analogous expressions for \(\omega'\). For nitroxide biradicals both \(a\) and \(J\) are usually small compared with the inverse of the rotational correlation time and so the expression in Eq. (B.6) can be reduced to
\[
T_2^{-1(\text{pse-sec})} = \frac{\tau}{3} \left( 2m^2(1 + m^2) \right) \left( (A : A) / 6 \right)
\]  
(B.10)

The total linewidth is obtained by adding Eq. (B.10) to Eq. (B.5) after replacing \((J \sec 2\phi)\) by \(\tau\).

REFERENCES


4. BIRADICALS AS SPIN PROBES


The Chemistry of Spin Labels

BETTY JEAN GAFFNEY

DEPARTMENT OF CHEMISTRY
THE JOHNS HOPKINS UNIVERSITY
BALTIMORE, MARYLAND

I. The Stability of the Paramagnetic Nitroso Group in Spin Labels
II. The Synthesis of Spin Labels
   A. Isotopically Labeled Nitrosoxides
   B. Synthetic Precursors
   C. Spin-Labeled Protein Modification Reagents
   D. Lipid Spin Labels
   E. Spin-Labeled Nucleotides
   F. Spin-Labeled Co-factors and Prosthetic Groups
   G. Spin-Labeled Sugars
   H. Spin-Label Probes of Binding-Site Structure
   I. Spin Labels in Immunochemistry
III. Experimental Procedures for Preparation of Spin Labels

References

184
187
189
190
192
196
200
201
203
204
206
209
232

Much of the organic chemistry of nitrosoxides as stable free radicals has been covered in several books and articles (Buchachenko, 1965; Forrester et al., 1968; Nelson, 1973; Rozantsev, 1970; Rozantsev and Scholle, 1971). The stability of the free radical portion of nitrosoxides under synthetic conditions that are aimed at the non-free radical portion of the molecule and also in biological samples is probably the most important aspect of nitrosoxide chemistry for spin-label studies. This subject is covered in Section I of this chapter. The synthesis of the spin labels often occupies a major part of the time devoted to a spin-label investigation of a problem of biological or biophysical interest. Section II of this chapter summarizes the types (i.e.,
spin-labeled protein modification reagents, spin-labeled nucleotides, carbohydrate spin labels, etc.) of spin labels that have been synthesized and pertinent considerations for the syntheses. Section III presents the experimental procedures for preparation of a representative sampling of spin labels. Many of these procedures have not been published previously.

1. THE STABILITY OF THE PARAMAGNETIC NITROXIDE GROUP IN SPIN LABELS

Although nitroxides are extraordinarily stable free radicals, they may be destroyed, with loss of paramagnetism, by components of some biological systems and under some of the experimental conditions employed in synthetic steps aimed at the nonparamagnetic portions of a spin-label molecule. The reactions, which involve the unpaired electron of nitroxides, have been discussed in detail (Buchachenko, 1965; Forrester et al., 1968; Nelson, 1973; Rozantsev, 1970; and Rozantsev and Scholle, 1971; Coker et al., 1966). In summary, nitroxides have been observed to undergo the following types of reactions:

1. Disproportionation (Dumyere and Rassat, 1966)
2. Free-radical reaction at oxygen (Keana et al., 1971)
3. Carbon-nitrogen bond cleavage (Keana and Baitis, 1968)
4. One-electron oxidation (Golubev et al., 1965)
5. One-electron reduction (Golubev et al., 1965; Neimann et al., 1964)
6. Reaction with strong acids (Neimann et al., 1964).

\[
\begin{align*}
2 \cdot \cdot \cdot & \leftrightarrow \cdot \cdot + \cdot \cdot \cdot \\
\text{Disproportionation} & \\
\cdot \cdot & + R \rightarrow \cdot \\
\text{Free radical reaction} & \\
\cdot \cdot & \rightarrow \cdot \\
\text{C-N bond cleavage} & \\
\end{align*}
\]

\[
\begin{align*}
2 \cdot \cdot \cdot & \rightarrow 2 \cdot \cdot + 2 \cdot \cdot \\
\text{Oxidation} & \\
\cdot \cdot & \rightarrow \cdot \cdot \cdot \\
\text{Reduction} & \\
\cdot \cdot & + 2 \text{HCl} \rightarrow \cdot \cdot + \cdot \\
\text{Reaction with acid} & \\
\end{align*}
\]

Reaction (1) may be easily disposed of as a problem for spin labeling by using only nitroxides that bear tertiary carbon atoms adjacent to the nitroxide nitrogen. [The paramagnetic resonance spectra of the unstable nitroxides bearing α hydrogens have additional characteristic splittings, however, and this gives rise to the interesting subject of "spin trapping" whereby transient free radicals are identified (Janzen, 1971; Adevih and Lagercrantz, 1970).] Reactions (2)–(4) are primarily of concern in the synthesis of spin labels in that they may compete with the nonradical portion of the nitroxide molecule for consumption of reagents and may lead to unwanted products. Reaction (3) is a special case of photolysis of stable nitroxides (Keana et al., 1971; Keana and Baitis, 1968). In the nitroxide ring structures that have no unsaturation, reactions of type (2) account for the predominant products in photochemical reactions (Keana et al., 1971).

\[
\begin{align*}
\begin{array}{ccc}
\text{I} & \text{II} & \text{III} \\
\text{OH} & \text{OH} & \text{OH} \\
\text{N} & \text{N} & \text{N} \\
\text{O} & \text{O} & \text{O} \\
\end{array}
\end{align*}
\]

From the spin-labeling point of view, reactions (5) and (6) present the most serious complications, although the use of specific reductants in biological systems to produce a signal decay can be used to good advantage, as will be discussed at the end of this section. Nitroxides are readily reduced to hydroxylamines by hydrogen and platinum catalyst and further to secondary amines when palladium on carbon is used as a catalyst (Rozantsev, 1970). Lithium aluminum hydride reductions of other functional groups in a
5. THE CHEMISTRY OF SPIN LABELS

probably proceeds via disproportionation of the protonated nitroxide, a species that has been observed under anhydrous conditions (Hoffman and Eames, 1969).

\[
\begin{align*}
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\end{align*}
\]

Nitroxides are reduced rapidly by aqueous solutions of sodium ascorbate (in excess). This reduction has been used effectively to differentiate labels on the inside and outside surfaces of phospholipid membranes and to measure the rate of motion of lipids from one side of the membrane to the other (Kornberg and McConnell, 1971).

The signal decay of nitroxides in the presence of biological systems lends itself to kinetic analysis and has proven very useful in some cases (Corker et al., 1966; Stier and Sackmann, 1973; Stier and Reitz, 1971).

Di-i-butyl nitroxide has been used to trap free-radical intermediates in photosynthesis (Corker et al., 1966). Rapid and irreversible loss of the spin-label signal in the presence of chloroplasts and photosynthetic organisms was observed. The signal decay was faster in light than dark. This study also lists a number of organic materials capable of free-radical or oxidation-reduction reactions that react with the paramagnetic nitroxide.

The enzyme system consisting of cytochrome P₄₅₀, cytochrome P₄₅₀ reductase (12:1) may be used to oxidize 2,2,6,6-tetramethylpiperidine to 1-oxyl-2,2,6,6-tetramethylpiperidine (TEMPO)† or, in the presence of NADPH, to reduce TEMPO (Stier and Sackmann, 1973; Stier and Reitz, 1971). This enzyme system is a component of microsomal membranes. The NADPH-dependent reduction has been employed as a new method of measuring phase transitions in biological membranes (Stier and Sackmann, 1973). For this measurement, a lipophilic spin label is used. The rate of reduction of the spin label by the enzyme depends on the lateral mobility of the label and of the membrane components within the plane of the membrane. A plot of the rate of reduction of the lipophilic label versus temperature showed a sharp break at about 32°C. As a control, a similar plot for a nonlipophilic spin label showed no break.

II. THE SYNTHESIS OF SPIN LABELS

In this section, representative syntheses of different classes of spin labels are discussed. The numerous reviews on spin labeling (Berliner, 1974; Fehér, 1969; Gaffney, 1974; Griffith and Wagonner, 1969; Hamilton and McCon-

† Editor's note: The use of this acronym is found rather frequently in the literature and refers to the basic piperidine nitroxide moiety of a spin-label structure.
A. Isotopically Labeled Nitroxides

The synthesis of isotopically labeled nitroxides is discussed here as an illustrative example of the synthetic procedures used for preparation of the basic structures from which spin labels are derived. Isotopic substitution of nitroxides (Chiarelli and Rassat, 1973) is also of interest because it offers several relatively unexplored possibilities for future spin-label experiments. Labels that are both paramagnetic and radioactive would have numerous applications in the study of biological problems—for instance, for quantitative measurement of label concentration. The syntheses that have been developed for introduction of deuterium (Chiarelli and Rassat, 1973; McFarland and McConnell, 1971) and $^{13}$C (Briere et al., 1968, 1971) may equally well be used as a means of preparing tritium and $^{14}$C substituted spin labels. Another reason for preparing isotopically substituted spin labels is to achieve a varied paramagnetic resonance spectrum. The replacement of nitroxide $^{14}$N (nuclear spin quantum number $I = 1$) by the isotope $^{15}$N ($I = \frac{1}{2}$) is of particular interest because the paramagnetic resonance spectrum is simplified from three spectral lines to two (Briere et al., 1965; Antiferova et al., 1970; Stryukov and Rozantsev, 1973; Williams et al., 1971). In certain cases where detailed theoretical analysis of resonance spectra is required, the $^{15}$N nitroxide spectrum could simplify the analysis. In addition, the existence of spin labels with two fundamentally different resonance spectra allows one to consider double label experiments and measurement of relative spectral intensities.

The first stable nitroxide was prepared by oxidation of triacetoneamine (Lebedev and Kazarnovskii, 1959). This synthesis of triacetoneamine has been reinvestigated (Chiarelli and Rassat, 1973), in order to optimize yield and conditions for work with isotopic precursors and is illustrated here for the preparation of triacetoneamine $d_{18}$ (VIII $d_{18}$):

$$\text{ND}_2 + 3 \text{D}_3\text{C}=\text{O} \rightarrow \text{H}_2\text{O} \rightarrow \text{D}_3\text{C}=\text{D}_2\text{O} \rightarrow \text{D}_3\text{C}=\text{D}_2\text{O} \rightarrow \text{D}_3\text{C}=\text{D}_2\text{O}$$

Oxidized triacetoneamine, often referred to as TEMPO, has been labeled with $^{13}$N (Briere et al., 1965; Williams et al., 1971) and deuterium (Chiarelli and Rassat, 1973).

Deuterium-substituted di-$r$-butynitroxide (XIV $d_{18}$) (Chiarelli and Rassat, 1973) has been prepared by a multistep synthesis that begins with
labeled acetone and ends with a step identical to the original method used by Hoffman et al. (Kaplan et al., 1973) for preparation of di-s-butyl nitroxide from t-nitrobutane:

\[
\begin{align*}
\text{D}_2\text{C} = \text{C} = \text{O} \quad \text{CD}_3 & \quad \text{CD}_3 \\
& \quad \text{CD}_3 \\
\text{VII-d}_4 
\end{align*}
\]

A third general class of nitroxides, the \(N\)-oxylxazolidines, (Keana et al., 1967), has been prepared with deuterium substitution, (Chiarelli and Rassat, 1973). This type of label is prepared from a ketone precursor and 2-amino-2-methylpropanol. Deuterium-substituted \(N\)-oxylxazolidine derivatives of acetone-\(d_6\) (Chiarelli and Rassat, 1973) and 5-ketoalmitic acid-4,4,6,6-\(d_4\) (McFarland and McConnell, 1971) have been prepared. The syntheses require, in addition to deuterium exchange of the protons adjacent to the ketone carbonyl, the preparation of 2-amino-2-methylpropanol-\(d_8\) as outlined:

\[
\begin{align*}
\text{D}_2\text{C} = \text{C} = \text{O} \\
& \quad \text{Ni} \\
\text{VIII-d}_4 
\end{align*}
\]

B. Synthetic Precursors

The majority of spin labels that have been prepared are derivatives of a few cyclic nitroxide precursors that are themselves derivatives of triacetoneamine. The reaction schemes leading to spin-label precursors derived from

Fig. 1. Reaction schemes leading to spin-label precursors.
triacetoneamine are summarized in Fig. 1† The experimental procedures for preparation of most of these molecules are described in detail in the book by Rozanskev (1970). The syntheses of the remaining precursors are given in Section III of this chapter.

C. Spin-Labeled Protein Modification Reagents

Spin-labeled derivatives of many of the known protein modification reagents have been prepared (Hirs, 1972; Barker, 1971). These labels are enumerated in Chapter 8 of this book. The spin-label protein modification reagents that have received by far the most use are the maleimide XXXII (a, b) (Griffith and McConnell, 1966) and iodoacetamide labels XXXIII (a, b) (Ogawa and McConnell, 1967; McConnell and Hamilton, 1968).

\[
\begin{align*}
\text{XXXII} \quad & R = \text{XXXIII(a)*} \\
& \text{or} \\
& \text{XXXIII(a, b)} \\
& \text{XXXIII(a)*} \\
\end{align*}
\]

In the synthesis of the maleic acid derivative, although formation of the thermodynamically more stable maleimide XXXII(b) is favored when the cyclization of the intermediate maleamic acid is carried out in acetic anhydride and sodium acetate, cyclization with dicyclohexylearbodiimid in acetonitrile affords the isomaleimide (XXXIV) (Barratt et al., 1971a; Wilcox and Stratigos, 1967). This is of concern because both maleimide and isomaleimide derivatives react with proteins, and the labels are capable of reacting at different sites to give paramagnetic resonance spectra exhibiting varying degrees of immobilization (Barratt et al., 1971a). Experimental conditions have been worked out for preparation of both piperidinyl and pyrrolidinyl maleimides in good yield. The presence of isomaleimide impurity can be detected easily by characteristic infrared bands (1710 cm\(^{-1}\) for maleimide and 1795 and 1690 cm\(^{-1}\) for isomaleimide (Barratt et al., 1971a)]. The isomaleimide also may be converted to the maleimide at elevated temperatures (Barratt et al., 1971a).

† Editor's note: Several of these are now commercially available. See Appendix III for a listing of manufacturers.

5. THE CHEMISTRY OF SPIN LABELS

The iodoacetamide spin labels are prepared via the more stable bromo- or chloroacetamides (XXXV) (McConnell et al., 1969). In general, the iodoacetamides react with sulphydryls somewhat more slowly than the maleimide labels and in some cases, this may influence the specificity achieved in labeling sulphydryl versus amino groups. Thus, the number of S-carbamethyalted cysteines resulting from the reaction of the iodoacetamide XXXIII with hemoglobin (two reactive sulphydryls per tetramer) was found...
to be 2.0 ± 0.25 moles of labeled sulphydryls per mole of protein after reaction for 28 hr at pH 7.8 and 2°C (Ogawa, 1967). Other groups on the protein could only be labeled after prior blocking with —SH reagents and prolonged reaction times. On the other hand, in maleimide spin-labeled hemoglobin, both the β-93 sulphydryl and other sites are labeled in a ratio of 5 to 1 (Ohnishi et al., 1966).

In the case of reaction with ribonuclease A, which has no sulphydryl groups, bromoacetamide labels (XXXV; X = Br) may react with histidyl and lysyl residues when the protein is denatured in 8 M urea at pH 5.5 (Smith, 1968).

The following series of iodoacetamide, bromoacetamide, and maleimide spin labels of varying length is commercially available (Syva Associates, 1970):

\[
\begin{align*}
O & \text{N} \quad \{X_n\} \quad \text{R} = \\
O & \text{N} \quad \{X_n\} \quad \text{R} = \\
\text{NH} & \text{CO} \quad \text{CH}_3 \quad \text{I} \\
\text{NH} & \text{CO} \quad \text{CH}_3 \quad \text{Br}
\end{align*}
\]

\[n = 0 \text{ to } 7\]

In addition to protein-labeling studies, the iodoacetamide and maleimide labels have been very useful synthetic intermediates in reactions with small molecule mercaptans. They have led to syntheses of spin-labeled ATP (Cooke and Duke, 1971), and to a thiogalactoside (see Section III.X) and a thiocholesterol derivative (Huang et al., 1970).

A spin-labeled isothiocyanate (XXXVI) has been prepared (see Section III.L) in order to overcome the stability problems encountered with the similar isocyanate label (Stone et al., 1965). A simple procedure (see Section III.L) involving addition of freshly distilled thiophosgene to a basic aqueous solution of TEMPOamine (XX) avoided the experimental difficulties, including destruction of paramagnetism, which accompanied the standard isothiocyanate synthesis involving (1) carbon disulfide and (2) chloroethyl carbonate (Crossley, 1955). The isothiocyanate is insoluble in the aqueous reaction solvent and may be collected simply by filtration.

\[
\begin{align*}
\text{NH}_2 & \quad \text{Cl}\text{C} = \text{S} \\
\text{Cl}\text{C} = \text{S} & \quad \text{(aq, NaOH)} \\
\text{N} & \equiv \text{C} \equiv \text{S}
\end{align*}
\]

\[
\begin{align*}
\text{XXXVI} & \\
\text{XXXVII} \\
\text{XXXVIII}
\end{align*}
\]

5. THE CHEMISTRY OF SPIN LABELS

The reactions of the isothiocyanate spin label have not been investigated in detail, but in studies of the molecular control of muscle contraction, it was noted that the isothiocyanate label reacted with groups (presumably —NH$_2$) quite different from those labeled by iodoacetamide and maleimide spin labels (Tonomura et al., 1969; Cooke and Morales, 1969). In reaction with troponin as well as with myoglobin (Shimshick, 1973), lysozyme, (Shimshick, 1973), and hemoglobin (Shimshick, 1973), the isothiocyanate label produced weakly immobilized signals, implying reaction with peripheral amino groups. The isothiocyanate label probably resembles phenyl isothiocyanate in reaction with N-terminal amino groups (Edman, 1956).

A series of isothiocyanate spin labels with varying numbers of atoms between the spin label ring and isothiocyanate groups have been prepared and are commercially available (Syva Associates, 1970).

Several fluorophosphonate labels have been prepared by the reaction of methyl phosphonodifluoridate (Morrisett et al., 1969) or phosphorous oxydichlorofluoride (Hsia et al., 1969; Hoff et al., 1971) with TEMPO alcohol (2,2,6,6-tetramethylpipеридин-4-ол-1-оксил). The labels are selective for the active site of serine enzymes such as acetylcholinesterase.

\[
\begin{align*}
\text{Ser-OH} & \quad \text{F-P-O} \quad \text{N-O} \\
& \quad \text{CH}_3 \\
& \quad \text{XXXVII}
\end{align*}
\]

Work with these reagents should be carried out with extreme care because fluorophosphonates are potent cholinesterase inhibitors (Saunders and Stacey, 1948; Chapman and Saunders, 1948). Diisopropyl fluorophosphonate is a more potent cholinesterase inhibitor than eserine. One part per million of this fluorophosphonate produced noticeable effects lasting at least seven days on human volunteers (Saunders and Stacey, 1948).

Several spin-label acylating reagents have been prepared. For example, the spin-label hydroxysuccinimide ester XXXVIII reacts with valyl-t-RNA

\[
\begin{align*}
\text{XXXVIII} & \\
\text{XXXVIII} & \quad \text{H}_2\text{N}_2\text{R} \\
& \quad \text{(R = Val-t-RNA)}
\end{align*}
\]

to give a spin labeled aminoacyl-t-RNA (Hoffman et al., 1970). Mixed carboxylic–carbonic anhydride acylating reagents (XXXIX, XL) have similar reactivity (Griffith et al., 1967; Barratt et al., 1971b).
D. Lipid Spin Labels

Spin-labeled derivatives of steroids (Huang et al., 1970; Hubbell and McConnell, 1969; Keana et al., 1967), fatty acids (Hubbell and McConnell, 1971; Seelig, 1970; Waggoner et al., 1969), and phospholipids (Aneja and Davies, 1970; Devaux and McConnell, 1974; Hubbell and McConnell, 1971) have been prepared. In the case of the steroid labels, all but one are derived via the procedure developed by Keana for conversions of ketones to nitro-

![Chemical structure](image)

ides (Keana et al., 1967). Other steroid nitrooxides which have been prepared by Keana's procedure are XLIII, XLIV, and XLV (Hubbell and McConnell, 1969). An alternative procedure has been used to prepare a thiocholesterol spin label (Huang et al., 1970). The oxazolidine nitroxides are prepared in three steps, illustrated below.

![Chemical structure](image)

In general, the first two steps (water removal and heating) are accomplished by heating the appropriate components for several days in refluxing toluene with water removal via a Dean–Stark trap. However, this reaction can also be accomplished by heating the reaction components in a sealed tube in the absence of solvent (Chiarelli and Rassat, 1973). The latter approach was used in preparation of the deuteriated dimethyloxazolidine derivative of acetone-d$_5$. The oxazolidine was isolated in 61% yield in this case. The maximum yield of oxazolidine is limited by the equilibrium between ring-opened (XLIX) and closed (L) structures (Elderfield, 1957). Oxidation of the oxazolidine L to the nitroxide LII may be accomplished by hydrogen peroxide–sodium tungstate if the material is water soluble (Chiarelli and Rassat, 1973). The oxidation may also be achieved with m-chloroperbenzoic acid (Keana et al., 1967).

A recent variation on the oxazolidine spin-label preparation has been used to create a spin-label cholesterol biradical in good yield (Keana and Dinerstein, 1971).

![Chemical structure](image)

The fatty acid spin labels LV(m, n) have been synthesized in most combinations of $m + n + 3 = 16$ or 18 and are prepared by the Keana procedure for converting ketones to N-oxoxyoxazolidines (Hubbell and McConnell, 1971; Waggoner et al., 1969; Seelig, 1970).
The ketoester precursors are synthesized by the following general scheme:

\[
\begin{align*}
\text{CO}_2\text{CH}_3 & \quad \text{CO}_2\text{CH}_3 \\
\text{(CH}_2)_{n} & \quad \text{(CH}_2)_{n}
\end{align*}
\]

For \( n \) less than 7, the half acid ester LVI results from acid-catalyzed equilibration of the corresponding diacid and diester (Hubbell and McConnell, 1971). For \( n \) greater than 7, the diester is half hydrolyzed in methanolic barium hydroxide to give LVI (Durham et al., 1963). The ketoester LVIII(5, 10)† may be prepared somewhat more easily by Raney nickel oxidation of the natural product, 12-hydroxystearic acid (Freedman and Applewhite, 1966).

The synthesis of a new type of spin-labeled fatty acid has recently been reported (Williams et al., 1971).

5. THE CHEMISTRY OF SPIN LABELS

and phosphatidic acid, respectively. In both syntheses, the spin-labeled alcohol was coupled to phosphatidic acid in the presence of 2,4,6-trisopropylbenzenesulfonyl chloride (TPS). Lecithins bearing spin-labeled fatty acid chains have been prepared by the reaction of the fatty acid anhydrides with lysolecithin in the absence of solvent (Hubbell and McConnell, 1971; Robles and Van den Berg, 1969). One of these spin-labeled derivatives (LXV) also served as a starting point for synthesis of spin-labeled phosphatidylethanolamine (LVII) (Devaux and McConnell, 1974; Barzilay and Lapidot, 1971).

The biosynthetic incorporation of spin-labeled fatty acids has been studied as a method of preparing spin-labeled phospholipids. Preparation of spin-labeled mycoplasma lipids polar lipids (Tourtellotte, 1970), phosphatidic acid (Stanacev et al., 1972), lecithin (Colbeau et al., 1972), and phosphatidylethanolamine (Colbeau et al., 1972) have been reported.

† Editor's note: A common nomenclature using the acronym "doxyl" for the oxazolotriene-N-oxyl moiety is now used frequently in the literature and in later chapters. For example, the nitroxide from the 12-keto stearic acid is 12-doxyl stearic instead of LV(5, 10).
E. Spin-Labeled Nucleotides

A spin-label derivative of AMP has been prepared in high yield (74%) by the reaction of 6-chloropurine ribotide (LXVIII) with TEMPOamine (XX) (Atkinson et al., 1969). Conversion of the monophosphate to the triphosphate was achieved by DCC coupling of the AMP label and tributylammonium pyrophosphate under rigorously anhydrous conditions.

Another derivative of ATP has been prepared by the reaction of TEMPO iodoacetamide with 6-mercapto ATP (Cocce and Duke, 1971). In a similar reaction, the pyrrolidinyl iodoacetamide XXXIII(a) was coupled to 4-thiouridine 2',(3')-phosphate as well as to the thiouridine of several E. coli t-RNAs (Hara et al., 1970).

F. Spin-Labeled Cofactors and Prosthetic Groups

Spin-labeled derivatives of (1) NAD (Weiner, 1969), (2) corrinoids and vitamin B₁₂ (Buckman et al., 1969; Law et al., 1971), and of the hemes of (3) cytochrome c peroxidase, myoglobin, hemoglobin, and horseradish peroxidase (Asakura et al., 1969, 1971), and of (4) cytochrome c (Raykhman et al., 1972), have been prepared. These syntheses are outlined below.
G. Spin-Labeled Sugars

A tritiated galactoside spin label (Struve and McConnell, 1972), two \( N \)-acetyl glycosides (Wien et al., 1972), and two thioglycosides (see Section III) have been prepared by employing reactions well known in carbohydrate chemistry. The syntheses of these labeled glycosides are summarized below.

[Diagrams showing chemical structures and reactions are displayed here.]

LXXI

LXXXII* (derivative of galactose)

LXXXVII* derivative of chitobiose
good examples of syntheses of a series of reagents with a variable chain length between the “reporter” group and the group bound by the protein. Several of these series as well as a series of protein modification reagents (Syva Associates, 1970), are shown below.

Substrates for \( \alpha \)-chymotrypsin

\[
\text{XCIII} \quad \text{(Kosman et al., 1969)}.
\]

Substrates for carboxic anhydrase

\[
\text{XCIII} \quad \text{(Erlich et al., 1973)}.
\]

Protein modification reagents

\[
\text{XCIII} \quad \text{(Syva Associates, 1970)}.
\]

I. Spin Labels in Immunochemistry

Spin-labeled nitrophenyl haptenes have been the subject of many spin label studies involving antibodies (Hsia and Piette, 1969; Hsia and Little, 1971; Stryer and Griffith, 1965). Some of these structures were summarized in Section II.H.
A spin-labeled morphine derivative (XCIV) is the basis of a new general immunoassay technique (Leute et al., 1972). This label produces a broad, immobilized paramagnetic resonance spectrum when bound to antibodies to carbomethylmorphine-BSA conjugate, but an intense, sharp signal when it is freely rotating in solution. The assay procedure depends on the competition of unlabeled morphine with XCIV for the antibody-binding site.

![Image of XCIV](image)

A novel use of spin labels in immunochemistry has recently been reported (Humphries and McConnell, 1974). In this case, the spin label is contained in high concentration in the aqueous interior of red blood cell ghosts. These spin-label-loaded ghosts are subject to complement-mediated immune lysis in the presence of antibodies. When the highly concentrated spin label is contained within the ghosts, the paramagnetic resonance signal is very broad. Upon lysis of a ghost, spin label becomes diluted in the medium with a resulting increase in sharp signal intensity. This approach constitutes a new spin-label immunoassay technique by which as little as $10^{-11}$ antigen in 20 μl may be detected. A particular virtue of this immunoassay technique is that no separation of ghosts from supernate is required, and thus, the assay may be performed quite rapidly.

III. EXPERIMENTAL PROCEDURES FOR PREPARATION OF SPIN LABELS

A. Triacetoneamine-$d_{16}$ (VIII-$d_{16}$) (Chiarelli and Rassat, 1973, translated by B. Gaffney)

![Image of Triacetoneamine-$d_{16}$](image)

Liquid ammonia-$d_3$ (3.5 ml, 0.12 mole) was added to 25 ml acetone-$d_6$ (99.7% $d$, 0.31 mole) and 8 g CaCl$_2$ in an autoclave cooled in liquid nitrogen. The mixture was sealed and stirred and heated in the autoclave at $50^\circ$C for 24 hr. The reaction products were placed in a 50 ml flask and heated for 5 hr at $70^\circ$C to drive off ammonia. To the remaining red-brown liquid was added 0.5 ml of water, and the mixture was cooled to $-15^\circ$C and stirred continually, whereupon the hydrate of triacetoneamine-$d_{16}$ crystallized. The product was collected by filtration, recrystallized from ether, and then sublimed under vacuum. The yield was 5.1 g (30%) of triacetoneamine-$d_{16}$ (VIII-$d_{16}$, m.p. 58°C).

B. α-Aminoisobutyric acid-$d_6$ (XV-$d_6$) (Chiarelli and Rassat, 1973)

![Image of α-Aminoisobutyric acid-$d_6$](image)

A solution of 15 gm NaCN in 50 ml of deuterium oxide was added drop by drop to a solution of 20 ml acetone-$d_6$ (16 gm, 0.25 mole) and 60 gm NH$_4$CO$_3$ (0.60 mole) in 150 ml deuterium oxide. The reaction mixture was kept at $50^\circ$C for 48 hr. After continuous extraction with ether for 48 hr, 33 gm of 5,5-dimethylhydantoin-$d_6$ (XIV-$d_6$, quantitative yield) was obtained.

The $d_6$-hydantoin (25 gm) and 250 gm of Ba(OH)$_2$ were dissolved in 500 ml water. The solution was heated at $130^\circ$C for 3 days. After cooling, 1 N H$_2$SO$_4$ was added until the pH of the solution was 5–6. Barium sulfate precipitated and was removed by filtration. The filtrate was evaporated in a rotary evaporator and the resulting amino acid was recrystallized from ethanol–water (80–20). The yield of α-aminobutyric acid-$d_6$ (XV-$d_6$) was 18.4 gm (85% yield), m.p. 337°C (sublimes from 260°C).

C. 2-Methyl-2-aminopropanol-$d_8$ (XVI-$d_8$) (Chiarelli and Rassat, 1973)

![Image of 2-Methyl-2-aminopropanol-$d_8$](image)

α-Aminobutyric acid-$d_6$ (XV-$d_6$) (10 gm) was added in small amounts to a suspension of 8 gm of lithium aluminum deuteride in 600 ml tetrahydrofuran. The suspension boiled gently during addition. When boiling ceased, the solution was heated under reflux for 5 days. The reaction was terminated by addition of 8 ml deuterium oxide, 8 ml 15% sodium deuterioxide and 24 ml deuterium oxide. Tetrahydrofuran was evaporated from the organic phase and the residue was distilled. The fraction boiling between 162° and 166°C was collected to give 5.8 gm of 2-amino-2-methylpropanol-$d_8$ (XVI-$d_8$) (70% yield).
A mixture of 4 gm 2-amino-2-methylpropanol-\textit{d}$_4$ (XVI-\textit{d}$_4$), 200 mg p-toluenesulfonic acid, and 25 ml acetone-\textit{d}$_4$ was sealed in a tube, and the tube was heated in an autoclave at 60°C for 5 days. The reaction mixture was dried over Na$_2$SO$_4$ and taken up in 100 ml petroleum ether. A stream of dry HCl was bubbled into the solution and the resulting hydrochloride was removed by filtration. The hydrochloride was dissolved in water and neutralized with dilute NaOH and extracted with ether. The ether was distilled slowly through a Vigreux column. The residue was distilled yielding 3.1 gm (61% yield) of 2,2,4,4-tetramethylloxazolidine-\textit{d}$_{14}$ (bp 122°-126°C).

In order to oxidize the oxazolidine, 0.5 ml 5% sodium tungstate solution and 40 mg disodium EDTA were added to a 20 ml flask containing 0.5 gm 2,2,4,4-tetramethylloxazolidine-\textit{d}$_{14}$ and cooled in an ice bath. To the cooled mixture was added 0.5 ml of 10% H$_2$O$_2$. After 6 hr, the mixture was extracted with ether, the ether layers dried over Na$_2$SO$_4$, and the ether evaporated slowly. The residue was purified by chromatography on alumina (50 gm, Woelm, activity III). A mixture of 90% pentane and 10% ether was used to elute the product, XVII-\textit{d}$_{14}$ 143 mg (28% yield).

E. 1-Oxyl-2,2,5,5-tetramethyl-3-hydroxymethylpyrrolidine (XXVII) (B.J. Gaffney, unpublished)

Three grams (0.016 mole) of 1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidine carboxylic acid (XXVI) (Rozantszev, 1970) was dissolved in 40 ml dry tetrahydrofuran, and the solution was cooled in an ice-water bath. Lithium aluminum hydride (0.70 gm, 0.024 equivalents; freshly opened can) was added to the cooled solution in small portions. The addition of lithium aluminum hydride required 1 hr. The reaction mixture was stirred at room temperature for 4 hr. Excess lithium aluminum hydride was decomposed by careful addition of a saturated aqueous solution of Na$_2$SO$_4$. The Na$_2$SO$_4$ solution was added until the white salts coagulated. The tetrahydrofuran layer was decanted and dried over Na$_2$SO$_4$. The dried solution was filtered and evaporated to give 2.48 gm (89% yield) of 1-oxyl-2,2,5,5-tetramethyl-3-hydroxypropyrlidine (XXVII) (mp 112°C).

F. 1-Oxyl-2,2,5,5-tetramethyl-3-bromomethylpyrrolidine (XXVIII) (B.J. Gaffney, unpublished)

The alcohol above (XXVII, 1.72 gm, 0.01 mole) was dissolved in 3 ml (0.037 mole) pyridine and the solution was cooled in an ice bath. To the cooled solution was added 1 ml methanesulfonyl chloride. The reaction mixture was allowed to stand at room temperature for 18 hr. Water (10 ml) was added to the reaction mixture, and the resulting solution was extracted three times with 10 ml portions of ether. The combined ether layers were extracted once with cold dilute HCl and once with saturated NaCl solution. After it was dried over Na$_2$SO$_4$, the ether solution was filtered and evaporated to give the methane sulfonate as an oil. The methane sulfonate was used for the next step without further purification.

The crude methane sulfonate (2.4 gm) was dissolved in 20 ml dimethylformamide. Lithium bromide (2.6 gm, dried prior to use for 12 hr at 120°C) was added to the solution of the methane sulfonate, and the reaction mixture was protected from moisture by Drierite. The reaction was carried out at 70°C for 2 hr. Water (10 ml) was added to the cooled mixture. The aqueous layer was extracted four times with ether, and the ether layers were extracted once with saturated NaCl solution. The ether solution was dried over Na$_2$SO$_4$ and evaporated to give a yellow oil. Thin-layer chromatography (Eastman prepared silica gel plate, benzene : chloroform, 1 : 4) showed two spots. The components of the mixture were separated by column chromatography on 30 gm silica gel, eluting with 50% chloroform in benzene. The second of two fast-moving bands was collected and evaporated (without heating) to give 0.85 gm of the bromide (XXVII) as an oil that crystallized after storage for 2 days in a desiccator over P$_2$O$_5$ (mp 43-45°C).

Anal. calcd. for C$_9$H$_{17}$NOBr:

C, 46.00; H, 7.23; N, 5.95; Br, 34.01

Found: C, 45.95; H, 7.20; N, 5.69; Br, 34.08.
G. TEMPOmonophosphate (XXIII) (4-phospho-2,2,6,6-tetramethylpiperidine-1-oxyl) (Weiner, 1969).

The phosphorylation of TEMPO alcohol (XXII) was based on the procedure outlined by Tener (1961). To 1 mmole XXII (172 mg) was added 4 ml 1 M cyanoethyl phosphate in aqueous pyridine. The solution was evaporated to dryness and reconstituted with 5 ml pyridine. This was repeated four times to remove the water. To the final mixture, in 5 ml pyridine, was added 1.5 g of N,N'-dicyclohexylcarbodiimide. After 3 days at room temperature, the reaction was terminated by adding 2 ml water. The urea formed was removed after 1 hr by filtration and washed with 25 ml water. The wash was combined with the yellow solution, and the resulting solution was made 0.4 M in LiOH. The basic solution was refluxed for 1 hr to hydrolyze the cyanoethyl group, then filtered. To the yellow supernate was added 6 ml water and additional precipitate was removed. The yellow solution was passed through a 2 × 28 cm Dowex 50X-8 ion-exchange column to remove the lithium. The phosphorylated product was made basic with pyridine and used without further purification.

H. TEMPO triphosphate (Ogata, 1971)

TEMPO monophosphate (~ 10 mmoles) was concentrated at 40°C to 25 ml using a rotary evaporator. Tributylamine (2.4 ml) and 100 ml anhydrous pyridine were then added, and the solution was concentrated as before to ~ 10 ml. Another 100 ml anhydrous pyridine were added, and the solution was again concentrated to 10 ml. Forty milliliters anhydrous N,N-dimethylformamide (DMF) were then added, and the solution was again concentrated to 10 ml. After repeating the last step once, 50 ml anhydrous DMF and 8 g (50 mmoles) of fresh 1,1'-carbonyldimidazole (dissolved in 100 ml dry DMF) were added to the residue. The reaction flask was then tightly stoppered, shaken vigorously for 30 min, then placed in a desiccator and allowed to stand at room temperature for 1-2 days. Methanol (3.3 ml) was then added. After standing for 30 min, 40 mmoles tributylammonium pyrophosphate dissolved in 200 ml dry DMF were added and the mixture shaken vigorously for 5 min.

The tightly stoppered flask was again placed in a desiccator and the mixture was filtered and the residue washed with two 10 ml portions of dry DMF. To the combined filtrates was added an equal volume of methanol, and this was concentrated on a rotary evaporator to a heavy dark yellow oil. The oil was dissolved in a small volume of 0.01 M triethylammonium bicarbonate (TEABC) applied to a 3.4 × 40 cm column of DEAE-Sephadex (HCO₃⁻) and eluted with a linear gradient of TEABC 0.01 to 0.4 M in 4 liters. The progress of the column could be followed visually (the nitroxide is yellow) and four very well separated bands were collected. Material from the first band was chromatographically indistinguishable from the starting nitroxide monophosphate [Whatmann no. 3MM paper developed in ethanol : 1 M ammonium acetate (5 : 2)]. The second band exhibited a five-line paramagnetic resonance spectrum characteristic of a nitroxide biradical and is thus likely to be a dimer of the monophosphate. Band three was assumed to be the diphasate, and band four (the principal band) the triphosphate. Band four was concentrated to 5 ml, 250 ml ethanol was added, and the solution was concentrated again to remove TEABC. This was repeated twice. Methanol (~ 50 ml) and a solution of 20.5 g sodium perchlorate in 250 ml acetone were then added to the residue. The precipitated sodium salt of the triphosphate was collected by centrifugation, washed with four 20 ml portions of acetone, and then dried in a desiccator over P₂O₅. The solid obtained was then dissolved in 50 ml water and the solution adjusted to pH 8.5 with 0.1 M Ba(OH)₂. The precipitated barium pyrophosphate was carefully and completely removed by centrifugation, and the light orange supernate was then passed through a column (~ 100 ml bed volume) of AG 50X2 ion exchange resin. The eluent was made basic with triethylamine, concentrated to 5 ml, freed of triethylamine by repeated addition and evaporation of absolute ethanol, and precipitated with sodium perchlorate as before. The precipitate was then dried exhaustively over P₂O₅ at 0.1 Torr.

In the first synthesis, the resulting bright red product had a total phosphate to labile phosphate ratio of 3 : 2.04 and a spin (measured by comparing the paramagnetic resonance spectral amplitude of a solution of the triphosphate with that of a standard solution of 2,2,6,6-tetramethyl-4-piperidinol) to phosphate ratio of 0.15 (expected for a nitroxide triphosphate is 0.33). The total phosphorus found was 18.3% calculated for C₉H₁₉O₁₁NP₂Na₂ is 18.6%.
5. THE CHEMISTRY OF SPIN LABELS

in about 500 ml of absolute ethanol and filtered. The filtrate was evaporated under reduced pressure and redissolved in 20 ml water. Fifteen milliliters of this solution was applied to a column containing 60 ml AG 50W-X8 (Bio-Rad Laboratories, ammonium ion form), and eluted with 250 ml 0.25 M ammonium bicarbonate and 400 ml 1 M ammonium bicarbonate. The first 300 ml of 1 M ammonium bicarbonate eluate was evaporated under reduced pressure and the residual solids were dispersed in 100 ml of absolute ethanol and filtered. The filtrate was evaporated under reduced pressure and the residual solids were twice dispersed in 100 ml absolute ethanol and evaporated under reduced pressure. The pH of a solution of the residual solids in 50 ml water was adjusted to 2.9 by the addition of 6 ml 1.2 M HCl. The water was removed under reduced pressure. The orange solid was dried to 2.1 gm under vacuum over P_2O_5. It appeared as a single spot on a cellulose thin-layer chromatogram that was developed in propanol ammonium hydroxide (14.8 M)–water (6 : 3 : 1, v/v/v) and stained with iodine vapor.

Anal. calcd. for C_{13}H_{24}N_4O_4Cl:
C, 55.80; H, 10.09; N, 10.01; Cl, 12.67.
Found: C, 54.76; H, 9.97; N, 9.95; Cl, 12.48.

J. N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide [XXXII(a)] (C. L. Hamilton and H. M. McConnell, unpublished)

Maleic anhydride (0.33 gm, 3.4 mmole) was dissolved in 20 ml anhydrous ether in a 50 ml round bottomed flask equipped with a magnetic stirrer. 2,2,6,6-Tetramethyl-4-aminopiperidine-1-oxyl (0.58 gm, 3.4 mmole) was dissolved in a few ml anhydrous ether; this solution was added dropwise to the stirred maleic anhydride solution from a dropping funnel. The mixture was then stirred at room temperature for an hour, and the product (0.75 gm, 92%) was collected by filtration and washed with several portions of ether. Further purification was unnecessary.

A mixture of the above N-nitroxylnaleamic acid (0.96 gm, 3.6 mmole), 0.15 gm (1.8 mmole) of anhydrous sodium acetate, and 15 ml of acetic anhydride were heated for 2–3 hr in a boiling water bath. The acetic anhydride was removed by distillation in vacuo. The residue was dissolved in benzene as much as possible; the solids remaining were removed by filtration. The residue obtained after removing the solvent was distilled at 5 × 10^{-3} mm

I. TEMPOcholine chloride (XXI) (Kornberg, 1972; Kornberg and McConnell, 1971)

(a) 4-(N,N-Dimethylamino)-2,2,6,6-tetramethylpiperidine was prepared by the method of Ike and Wisegarver (1955). Twenty-five grams 4-amino-2,2,6,6-tetramethylpiperidine (XX) (Aldrich Chemical Co.) was added with magnetic stirring to 38 gm 98% formic acid in an ice bath. The mixture was removed from the ice bath, combined with 28.5 gm 37% formaldehyde, refluxed for about 10 hr, cooled in an ice bath, and combined with 18.2 gm concentrated HCl. Formic acid, formaldehyde, and water were removed under reduced pressure. The residue was combined with about 100 ml of water and about 50 gm NaOH. The lower phase was extracted twice with diethyl ether. The upper phase and ether extracts were dried over BaO. The ether was removed by distillation at atmospheric pressure. Vacuum distillation (at 29–36°C and less than or about 1 Torr) yielded 18 gm (67%) of clear, colorless liquid.

Anal. calcd. for C_{11}H_{24}N_2: C, 71.67; H, 13.13; N, 15.20.
Found: C, 71.55; H, 12.99; N, 14.95.

(b) 2-Bromoethyl acetate was prepared by the reaction of 2-bromoethanol with acetic anhydride. Excess acetic anhydride (about 100 gm) was added with magnetic stirring to 100 gm of 2-bromoethanol (Aldrich Chemical Co.) in an ice bath. The mixture was heated on a steam bath for 1 hr, cooled in an ice bath, and repeatedly extracted with cold 1% NaHCO_3 until the pH of the upper phase was about 7. The lower phase was then dried over anhydrous Na_2SO_4. Vacuum distillation yielded a clear, colorless liquid: IR (film), band at 1740 cm^{-1} (carboxylic ester).

(c) A mixture of 8.52 gm 4-(N,N-dimethylamino)-2,2,6,6-tetramethylpiperidine and 15.5 gm 2-bromoethyl acetate was kept in the dark at room temperature for 3 days. The white solid product was dispersed in anhydrous diethyl ether, filtered and washed with anhydrous diethyl ether by suction, and dried to 11.6 gm (72%) under vacuum over P_2O_5. A mixture of 6.15 gm of the dry solid, 1.70 gm EDTA, 0.93 gm NaOH, 2 gm sodium tungstate dihydrate, 25 ml 30% H_2O_2 were added. After 2 days, the remaining H_2O_2 was destroyed by vigorous magnetic stirring. The water was removed under reduced pressure. Benzene was added and then removed under reduced pressure. The residual solids were dispersed
(140°-160°C) to yield solid material (72%), mp 99°C. Thin-layer chromatography (Eastman chromagram, chloroform) indicated normal maleimide (IR; 1690 cm⁻¹) plus small amounts of isomaleimide (IR; 1790, 1690 cm⁻¹).

K. N-(1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (XXXIII)

Procedure (1) (McConnell et al., 1969)

\[
\begin{align*}
\text{N-} & \quad \text{NH} - \text{CO} - \text{CH}_3 \\
\end{align*}
\]

15.6 gm 2,2,6,6-tetramethyl-4-aminopiperidine (Aldrich Chemical Co.) was acetylated with an equivalent amount of chloroacetyl chloride. The reaction mixture was extracted with dilute HCl, and the extract was neutralized with aqueous NaOH. The white solid that precipitated was collected on a filter and suspended in about 600 ml water; 3 gm sodium tungstate, 3 gm disodium EDTA, and 60 ml 30% H₂O₂ were added to the suspension, and the resulting mixture was stirred until all solid material dissolved. The solution was allowed to stand for 2 hr, and then the resulting orange solution was saturated with NaCl and extracted with ether. The ether phase was washed with water to remove excess peroxide. The dark orange crystals obtained after drying over MgSO₄ and removal of the ether in vacuo were dissolved in acetone, and a slight excess of NaI dissolved in acetone was added. After standing overnight, the mixture was filtered and the acetone was removed in vacuo. The dark oil remaining was dissolved in 200 ml hot toluene, and after 3 days, the product (XXXIII) crystallized as dark orange needles that melted at 114°-117°C.

Analyzed for C₁₁H₂₀IN₂O₂: C, 39.0; H, 5.9; I, 37.4.

Found: C, 39.3; H, 6.2; I, 37.5.

Procedure (2) (Procedure of B. J. Gaffney)

To a solution of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (XX) (5 gm, 0.029 mole) and NaOH (1.17 gm) in 50 ml water was added chloroacetyl chloride (3.33 gm, 0.029 mole) over a period of 1 hr. The reaction mixture was stirred for 1 hr, and the orange precipitate was removed from the mixture by filtration and dried overnight over P₂O₅ to give 2,2,6,6-tetramethyl-4-chloroacetamidopiperidine-1-oxyl (2.00 gm, 28% yield). One recrystallization from benzene yielded red prisms (mp 120°-121°C).


Found: C, 53.68; H, 8.32; N, 10.96

5. THE CHEMISTRY OF SPIN LABELS

A solution of 2,2,6,6-tetramethyl-4-chloroacetamidopiperidine-1-oxyl (1.95 gm, 0.0079 mole) and NaI (1.18 gm, 0.0079 mole) in 20 ml acetone was allowed to stand for 48 hr at 15°-20°C. The white precipitate of NaCl was removed by filtration and washed with acetone and dried (0.35 gm). To the remaining acetone solution was added an additional 3 gm NaI. After 48 hr, the acetone was removed under vacuum at 20°C. To the remaining red oil was added 5 ml water, and the mixture was rapidly extracted three times with ether. The ether solution was dried (Na₂SO₄) and evaporated to give a red oil that solidified on trituration with benzene. The red crystals were collected by filtration and dried (2.10 gm, 78% yield; mp 121°-123°C). This sample was recrystallized twice from benzene to give an analytical sample.

Analyzed for C₁₁H₂₀IN₂O₂: C, 38.95; H, 5.94; N, 8.26.

Found: C, 40.69; H, 5.95; N, 8.55.

L. 1-Oxy-2,2,6,6-tetramethyl-4-isothiocyanatopiperidine (XXXVI) (B. J. Gaffney, unpublished)

\[
\begin{align*}
\text{O-N} & \\
\end{align*}
\]

To 150 ml of a 5% NaOH solution of 1-oxyl-2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (6.42 gm, 0.0375 mole) at 0°C was added freshly distilled thiophosgene (4.32 gm, 0.0375 mole, 3 ml; bp 73°-75°C/758 Torr) over a period of 15 min. The resulting mixture was stirred for 10 min, and the orange precipitate was removed by filtration, washed three times with water, and dried under vacuum over P₂O₅ to give a red powder (mp 107°-112°C, 5.7 gm, 71% yield). This powder was recrystallized from ethanol and water to give red prisms (1.6 gm, mp 124°-125°C). Two further recrystallizations from benzene afforded an analytical sample (mp 127°-128°C):

Analyzed for C₁₀H₁₅N₂OS: C, 56.30; H, 8.03; N, 13.13.

Found: C, 56.24; H, 8.06; N, 12.97.

M. TEMPOphosphatidylcholine (LXIII) (Kornberg and McConnell, 1971; Kornberg, 1972)

\[
\begin{align*}
\text{R} & \quad \text{CO} - \text{O} - \text{CH} \quad \text{N} - \text{O}
\end{align*}
\]
(a) Two grams dipalmitoylphosphatidylcholine (derived from egg phosphatidylcholine by the methods of Brockeroff and Yurkowski (1965) and Baer and Buchnea (1959)) were dispersed in 50 ml water with a Branswill 20 kHz sonicator and intermittent cooling in an ice bath. The sonication was continued until further sonication did not produce further clearing of the homogenous opalescent dispersion. A mixture of 3.3 ml 1 M pH 5.6 acetic acid–calcium acetate buffer, 100 ml Savoy cabbage supernate [freshly prepared by the method of Davidson and Long (1958)], 100 ml alcohol-free diethyl ether (freshly distilled from P2O5), and 20 ml water was shaken with the dipalmitoylphosphatidylcholine dispersion for 13 hr at room temperature, then shaken with 35 gm citric acid (monohydrate) and 170 ml water and centrifuged. The aqueous phase was extracted three times with 500 ml portions of chloroform. The combined ethereal phase and chloroform extracts were evaporated under reduced pressure. The residue was dissolved in absolute ethanol, and then the ethanol and last traces of water were removed under pressure. The residue was dissolved in 15 ml chloroform and added dropwise to a magnetically stirred solution of 20 gm of barium acetate (monohydrate) and 400 ml methanol–water (1:1, v/v). The precipitate was collected by centrifugation, shaken for about 10 min at room temperature with 250 ml 0.5 N H2SO4 and 250 ml chloroform, and centrifuged. The lower phase was extracted once more with 250 ml 0.5 N H2SO4 once with 500 ml methanol–water (1:1, v/v), combined with an extract of the upper phase from the first 0.5 N H2SO4 extraction (the upper phase was extracted once with 250 ml 0.5 N H2SO4 and once with 250 ml water), and evaporated under reduced pressure. Traces of water were removed from the residue by repeated evaporation of absolute ethanol, and traces of ethanol were removed by repeated evaporation of toluene, yielding 1.83 gm (103%) white solid which was dissolved in 10 ml anhydrous chloroform. An oxalic acid-impregnated silica thin-layer chromatogram of this solution was developed in chloroform–methanol–HCl (12 M) (67:13:0.5, v/v/v). It showed one spot with the same RF value as phosphatidic acid derived from egg phosphatidylcholine by the method of Davidson and Long (1958).

(b) One-half of the anhydrous chloroform solution that resulted from the procedure in (a) was transferred to a two-necked flask, equipped for mechanical overhead stirring, which contained about 45 gm of glass beads (roughly 2 mm in diameter). 2.5 ml anhydrous pyridine, 0.55 gm TEMPOchlorine chloride (XXI) and 1.21 gm 24,6-trisopropylbenzenesulfonfyl chloride. The mixture was vigorously stirred for 5 hr at room temperature. Ten milliliters of water was added with cooling and then the mixture was stirred for 3 hr at room temperature, shaken with 250 ml chloroform and 150 ml of 0.5 N H2SO4, and centrifuged. The lower phase was washed successively with 150 ml 0.5 N H2SO4, 150 ml water, 225 ml 2% NaHCO3 in methanol–water (1:2, v/v), and 225 ml methanol–water (1:2, v/v), evaporated under reduced pressure, dissolved in 50 ml chloroform–methanol (1:1, v/v), applied to a column containing 600 ml alumina [E. Merck, activity I, washed extensively with chloroform–methanol (1:1, v/v) for the removal of fines, activated for 3 hr at 110°C, and packed in the column as a slurry in chloroform], and eluted with chloroform–methanol (1:1, v/v). The first 25 ml of orange eluate were discarded. The next 250 ml of orange eluate were concentrated to a small volume under reduced pressure, diluted with absolute ethanol, and centrifuged for removal of alumina particles. The ethanol was removed under reduced pressure. The orange solids were dissolved in 20 ml chloroform. This spin-labeled phosphatidylcholine solution contained 914 μmoles of phosphorus (78% of the total phosphorus in the solution that was applied to the alumina column). Silica thin-layer chromatograms of the solution were developed in chloroform–methanol–NH4OH (14.8 M)–water (70:30:4:1, v/v), chloroform–methanol–water (65:25:4, v/v), and chloroform–methanol–acetic acid–water (50:25:7:3, v/v). In each case there was only one spot. There were, however, two spots (RF values 0.42 and 0.47) on a silica-impregnated paper chromatogram developed in diisobutyl ketone–acetic acid–water (8:5:1, v/v) and sprayed with the phosphate reagent of Dittmer and Lester (1964). The spot corresponding to an RF value of 0.42 was about 10% as intense as the other spot. A 12% contamination of the spin-labeled phosphatidylcholine solution by some nonparamagnetic phospholipid would explain why the ratio of nitroxide/phosphorus was lower than expected. Analysis: Nitroxide/phosphorus, 0.88; palmitic acid/phosphorus, 2.00. The nonparamagnetic impurity was removed by silica column chromatography. Four milliliters of the chloroform solution of spin-labeled phosphatidylethanolamine were applied to a bed of silica (Mallinkrodt 100 mesh silicic acid, sieved to 60–140 mesh, activated for 2 days at 110°C) in chloroform, roughly 50 cm high, and eluted successively with 300 ml chloroform, 800 ml 5% methanol in chloroform, 400 ml 10%, 800 ml 12.5%, 800 ml 15%, and 2.5 liters of 17.5%. The second liter of 17.5% methanol in chloroform contained about 125 μmoles of phosphorus. The spot on the silica-impregnated paper chromatogram corresponding to an RF value of 0.42 was about 1% as intense as the other spot. Analysis: Nitroxide/phosphorus, 1.04. Palmitic acid/phosphorus, 2.00.

The quantitative hydrolysis of spin-labeled phosphatidylcholine—0.5 ml alcohol-free diethyl ether, 0.02 ml 1 M pH 5.6 acetic acid–calcium acetate buffer, and 0.5 ml of cabbage supernate were shaken for 15 hr at room temperature—yielded only phosphatidic acid [identified on an oxalic acid-impregnated silica thin-layer chromatogram developed in chloroform–
methanol–HCl (12 M) [87: 13: 0.5, v/v/v] by comparison with phosphatidic acid derived from egg phosphatidyleholine by the method of Davidson and Long (1958) and TEMPOcholine [identified on a cellulose thin-layer chromatogram developed in propanol–NH₄OH (14.8 M)-water (6: 3: 1, v/v) by comparison with TEMPOcholine chloride]. The Rₜ values of spin-labeled phosphatidylcholine on silica chromatograms in basic and acidic solvents were greater than the corresponding Rₜ values of phosphatidyleholine [Rₜ values of 0.67 and 0.40 for spin-labeled phosphatidyleholine and phosphatidyleholine, respectively, on a silica thin-layer chromatogram developed in chloroform–methanol–NH₄OH (14.8 M)-water (70: 30: 4: 1, v/v/v), and Rₜ values of 0.47 and 0.42 for spin-labeled phosphatidyleholine and phosphatidyleholine, respectively, on a silica-impregnated paper chromatogram developed in diisobutyl ketone–acetic acid–water (8: 5: 1, v/v/v)], that is, spin-labeled phosphatidyleholine was less polar than phosphatidyleholine by the criterion of silica chromatography.

N. The 1-oxyl-2,2-dimethyloxazolidine derivative of methyl-12-keto stearate (LVIII, 5, 10) (Procedure of Waggoner et al., 1969)

\[
CH₃-\left(\text{CH}_2\right)_m-\left(\text{CH}_2\right)_n-\text{COOCH}_3
\]

To form the oxazolidine compound, 3.0 gm (10 mmole) of methyl-12-keto stearate, 20 ml (200 mmole) of 2-amino-2-methyl-3-propanol (Alrich), and 35 mg (0.2 mmole) of p-toluenesulphonic acid monohydrate were dissolved in 200 ml xylene. The mixture was refluxed for 10 days using a Dean–Stark trap containing anhydrous CaSO₄ for continuous water removal. After refluxing, the solution was cooled and washed four times with 50 ml portions of saturated NaHCO₃ solution, once with 50 ml saturated NaCl solution, then dried over Na₂SO₄. The xylene was removed with a rotary evaporator leaving 2.7 gm of thick colorless liquid that contained about 25% ketone starting material, traces of other impurities, and about 75% oxazolidine as estimated by thin-layer chromatography using silica gel G as an adsorbent, hexane–diethyl ether (7: 3) as the developer, and H₃SO₄ spray followed with charring for detection of the spots. The ketone has Rₜ of about 0.7 and the oxazolidine an Rₜ of about 0.3. The \(-\text{CH}_2-\) protons on the oxazolidine ring are found at 3.43 ppm (TMS) in the nmr spectrum. The oxazolidine was not isolated but was oxidized directly to nitroxide.

The mixture containing the oxazolidine was dissolved in 300 ml anhydrous diethyl ether containing 1.5 gm m-chloroperoxybenzoic acid (Alrich Chemical Co.) (87% pure) was added dropwise to the oxazolidine solution over a period of 1 hr. The number of moles of m-chloroperoxybenzoic acid added was 1.50 times the number of moles of oxazolidine present (estimated by thin-layer chromatography as described above) in the 2.7 gm mixture of ketone and oxazolidine. As the peroxide was added, the solution slowly turned pale yellow. The ether solution was allowed to come to room temperature, and 48 hr later it was washed four times with 50 ml saturated NaHCO₃, once with water, dried over Na₂SO₄, evaporated to half its former volume, and placed in dry ice to precipitate excess ketone starting material. After evaporating the ether, the viscous, yellow, oily nitroxide was purified by preparative thin-layer chromatography again using hexane–ether (7: 3). The yellow nitroxide band lies between the starting material and the oxazolidine. The total nitroxide yield based on the starting material was about 35%.

Anal. calc. for CₓHᵧNₒₒ₄: C, 69.3; H, 11.1; N, 3.5.

Found in two separate determinations:
C, 69.3 (69.4); H, 11.0 (10.9); N, 3.5 (3.5).

The ir spectrum of the nitroxide in chloroform shows a typical ester carbonyl band at 5.78 µm. The number of unpaired electrons per mole of methyl stearate nitroxide was found to be 5.0 × 10⁻²³ ± 0.5 × 10⁻²³ based on two determinations using nitroxides of known purity.

O. The 1-oxyl-2,2-dimethyloxazolidine derivative of 5-keto palmitic acid (LVIII, 10, 3) (Hubbell and McConnell, 1971)

\[
CH₃-\left(\text{CH}_2\right)_m-\left(\text{CH}_2\right)_n-\text{COOCH}_3
\]

A solution of 68.2 gm undecyl bromide (0.29 mole) in 350 ml of dry diethyl ether was added in the usual way to 6.94 gm (0.29 gm-atom) of magnesium turnings in 100 ml of dry diethyl ether. After the addition, the reaction was refluxed until almost complete disappearance of the magnesium.

The Grignard solution was cooled in an ice bath and 27.5 gm (0.15 mole) finely powdered anhydrous CdCl₂ was added in one portion. The ice bath was removed, and after a period of 30 min the ether was almost completely removed by distillation. Dry benzene (350 ml) was added and 28 gm (0.23 mole) methyl-4-(chloroformyl)butyrate (Alrich Chemical Co.) in 100 ml dry benzene was added over a period of 10 min with rapid stirring. The mixture was then heated to reflux for 1 hr, after which time the mixture was cooled in an ice bath and approximately 100 ml water was added slowly with stirring. Then a large excess of 0.1 N H₂SO₄ was added until two distinct phases formed. The benzene was removed under reduced pressure
and the solid twice recrystallized from pentane, yielding 40 gm methyl 5-ketopalmitate; mp (uncorr) 49°–50°C; ir (KBr) bands at 1735 (carboxylic ester) and 1715 cm⁻¹ (ketone).

To 500 ml toluene were added 30 gm (0.1 mole) of the methyl 5-ketopalmitate, 100 ml (1.0 mole) of 2-amino-2-methyl-1-propanol, and 100 mg p-toluenesulfonyl acid monohydrate. The mixture was refluxed for 6 days using a Dean–Stark trap for water removal. The toluene phase was then washed with six 200 ml portions of saturated NaHCO₃ solution and four 200 ml portions of water and dried with anhydrous Na₂SO₄. The toluene was removed under reduced pressure, yielding a colorless, viscous liquid. The 4',4'-dimethylxazolidinone derivative of the methyl 5-ketopalmitate was not purified at this stage, but oxidized directly to the N-oxyl-4',4'-dimethylxazolidinone derivative.

The 4',4'-dimethylxazolidinone was dissolved in 500 ml diethyl ether and cooled to 0°C in an ice bath, and 100 ml diethyl ether containing 22.4 gm m-chloroperbenzoic acid (85%, Aldrich Chemical Co.) was added over a period of 2 hr. The mixture was allowed to stand for 12 hr, at which time the ether phase was washed four times with 200 ml portions of saturated Na₂CO₃ and four times with 200 ml portions of water and dried with anhydrous Na₂SO₄. The ether was removed under reduced pressure, and the yellow, viscous oil chromatographed on silica gel, eluting with benzene–ether (7:3 v/v). The center portion of the fast-moving yellow band was collected, yielding 10 gm of the N-oxyl-4',4'-dimethylxazolidinone derivative of methyl 5-ketopalmitate; ir (film) band at 1735 cm⁻¹ (carboxylic ester).

Anal. calcd for C₂₁H₄₀NO₄:  C, 68.07; H, 10.88; N, 3.78.
Found:  C, 68.19; H, 10.78; N, 3.73.

The nitroso ester was dissolved in 100 ml of dioxane, and 4% NaOH solution was added until an oil began to form. When the oil had dissolved, more of the NaOH solution was added until another oily phase formed. This was repeated until no precipitate formed on addition of NaOH solution. The solution was brought to pH 1 with concentrated HCl, 100 ml water was added, and the solution was extracted with ethyl acetate. The ethyl acetate phase was dried over anhydrous Na₂SO₄ and the ethyl acetate removed under reduced pressure to yield the corresponding N-oxyl-4',4'-dimethylxazolidinone derivative of 5-ketopalmitic acid: mp (uncorr) 45–46°C; ir (KBr) band at 1710 cm⁻¹ (carboxylic acid).

P. Acylation of lysolecithin (Hubbell and McConnell, 1971)

(a) LV(m, n) anhydrides. The anhydrides were prepared by the following general method of Selinger and Lapidot (1966). To 75 ml dry CCl₄ containing 10 mmole LV(m, n) was added 1.03 gm (5 mmole) dicyclohexycarbodiimide in 25 ml of dry CCl₄. After 12 hr, the precipitated dicyclohexylurea was removed by filtration through sintered glass and the CCl₄ removed under reduced pressure. The anhydrides were used without further purification: ir (CCl₄) bands at 1810 and 1750 cm⁻¹ (carboxylic anhydride).

(b) Egg lysolecithin. To 1 gm egg yolk lecithin in 300 ml ether was added 50 mg lyophilized Crotalus adamanteus venom (Pierce Chemical Co.) in 4 ml 5 mM CaCl₂ solution. At the end of 1.5 hr, 100 ml of ethanol was added and the volume reduced under vacuum to about 25 ml. The remaining solution was centrifuged at low speed to remove the precipitated enzyme. The precipitate was washed once with 2:1 v/v chloroform–methanol and the extract combined with the original supernate. The volume was reduced to approximately 2 ml and added to 40 ml diethyl ether. The precipitated lysolecithin was collected by centrifugation and washed twice with 40 ml of diethyl ether.

(c) Acylation of egg lysolecithin with LV(m, n) anhydrides. The method used here is that of Cubero Robles and Van den Berg (1969) and is illustrated by the acylation of egg lysolecithin with LV(10, 3) anhydride. To 50 mg (approximately 0.1 mmole) of egg lysolecithin in a 10 ml pear-shaped flask was added 0.27 gm (0.39 mmole) of LV(10, 3) anhydride and 3 mg (0.05 mmole) of finely powdered Na₂O. The flask was sealed and slowly rotated in an oil bath at 60°C. Periodically, the reaction mixture was examined by thin-layer chromatography. A gradual increase in the amount of lecithin was observed with a concomitant decrease in the lysolecithin, which had completely disappeared after 24 hr. A small amount (0.5 ml) of chloroform was then added to the reaction mixture and resulting solution applied to 7 gm Unisil (Clarkson Chemical Co., Williamsport, Pa.) that had been activated for 12 hr at 120°C. The fatty acids and excess anhydride were eluted with 2% methanol in chloroform and the yellow band collected. Evaporation of the solvent yielded 40 mg of a waxy yellow solid. The thin-layer chromatographic behavior of the lecithin prepared by this method [LVX(10, 3), LVX(7, 6), and LVX(5, 10) lecithins] is nearly identical with that of natural egg lecithin.

The spin-labeled lecithins hydrolyzed by phospholipase A to yield the corresponding fatty acid and a compound that cochromatographed with
natural lysolecithin. Phospholipase D degradation gave a new compound that behaved on thin-layer chromatography like authentic phosphatidic acid. It should be noted that while the LXXV(5,10) lecithin hydrolyzed by both enzymes at approximately the same rate as natural lecithin, the LXXV(7,6) and LXXV(10,3) lecithins were hydrolyzed at a much slower rate, the effect being largest with phospholipase A. The ir spectra of the lecithins are consistent with published spectra of synthetic lecithins, with bands at 1735, 1075, and 1250 cm⁻¹ (solid film).


(a) Preparation of 6-chloropurine riboside. A mixture of 2′,3′,5′-tri-O-acetylinosine (50 gm, 0.127 mole), dimethylamine (13.25 ml), and phosphoryl chloride (62.5 ml, 0.68 mole) in 62.5 ml chloroform was heated under reflux for 15 min. The reaction mixture turned light brown. The reaction mixture was poured into 500 ml ice water and the excess phosphoryl chloride was hydrolyzed. The organic layer was separated and washed twice with 500 ml 1 N HCl and with water until the pH of the aqueous layer was over 6. This extract was evaporated to a thick syrup and dissolved in 600 ml methanolic NH₄ and let stand overnight at 5°C. The mixture was evaporated under reduced pressure and methanol added. The crude 6-chloropurine riboside was crystallized and yielded 20.03 gm, 55%, mp 176.5°–177°C. The paper chromatogram of the crude product showed traces of adenosine, which were removed by repeating the crystallization from aqueous methanol.

(b) Phosphorylation of 6-chloropurine riboside. To a mixture of phosphorus oxychloride (1.38 ml, 15 mmole) in trimethyl phosphate, 6-chloropurine riboside (125 gm, 5 mmole) was added at 0°C and the mixture was kept for 2 hr with stirring at 0°C. The mixture was poured into 50 ml of ice water and was neutralized to pH 8 with 6 N NaOH. Trimethyl phosphate was removed by extraction twice with 50 ml of chloroform. The aqueous layer was concentrated to 10 ml at a temperature below 50°C, and methanol (10 ml) was added. (Disodium 6-chloropurine ribotide is soluble in 60% aqueous methanol.) Inorganic salts were separated on a filter and the filtrate was evaporated to dryness.

The residue was dissolved in 10 ml water and an excess of aqueous barium acetate was added. The precipitates of inorganic salts and a small amount of Ba₂IMP (side product) were separated on a filter. The barium salt of 6-chloropurine ribotide was crystallized by adding 20 ml ethanol. Barium acetate is soluble in 80% aqueous ethanol. Recrystallization was repeated in the same way. 1.39 g of pure barium salt was obtained. Yield was 52% based on the nucleoside. Absorbance 250 nm/260 nm = 0.80, 280 nm/260 nm = 0.164.

(c) Preparation of TEMPO-AMP from 6-chloropurine ribotide. The barium salt of 6-chloropurine ribotide was converted to the free acid by passing through a Dowex 50 column (H⁺ form). Then 200 μmoles of 6-chloropurine nucleotide in 2.6 ml was adjusted to pH 8.5 with TEMPO-amine and evaporated to dryness. 0.1 ml TEMPOamine and 3 ml t-butanol were added and heated to the boiling point to give a clear solution. The reaction was checked by electrophoresis in 0.05 M citrate pH 3.5. The slower component (TEMPO-AMP, Rf = 0.48 relative to 6-chloropurine ribotide) had λmax = 267 nm and the reaction went to 95% conversion.

2.4 ml of reaction mixture containing 162 μmoles was diluted to 100 ml with 5 mM TEABC (triethyl ammonium bicarbonate) pH 8.2 and was applied to a DEAE-Sephadex column (10 cm x 1 cm² DEAE-TEABC). The column was washed with 5 mM TEABC and then the product eluted with a 200 ml gradient (5–200 mM TEABC). TEMPO-AMP eluted at 50 mM. The pooled fractions were reduced to dryness four times from water, and dissolved in 2 ml water. Yield was 119 μmoles or 74% from 6-chloropurine ribotide. Pentose:organic phosphate:orthophosphate = 1.00 ± 0.04:0.99 ± 0.03:0.02; λmax = 267 nm, E₂₅₀ = 20700.

(d) Pyrophosphorylation of TEMPO-AMP. 119 μmoles TEMPO-AMP in 2 ml water was passed through Dowex-50 in the pyridine form. Five column volumes were collected and evaporated to near dryness. Then 119 μmoles of tributylamine (redistilled and anhydrous) in 3 ml pyridine was added to the residue. The residue was evaporated three times from pyridine and three times from acetonitrile (spectroquality, anhydrous). The residue was dissolved in 2 ml HMP (hexamethylphosphoric triamide, redistilled and anhydrous). 600 μmoles of carbonyldimidazole was added in
2 ml HMP. The reaction was allowed to proceed overnight at room temperature. The reaction was checked by electrophoresis on 0.05 M NH₄HCO₃ pH 8.0. The imidazole derivative had about 0.75 the mobility of the ribotide at pH 3.0. 1070 μmoles methanol was added in 0.5 ml HMP, and the mixture was allowed to stand for 4 hr. Then 1.19 ml HMP containing 600 μmoles of pyrophosphate (tributylamine salt) was added and the reaction proceeded at room temperature. After 4 hr, 1 ml of 1.2 M TEABC pH 8.2 and several grams of ice were added. The mixture was then diluted with 500 ml cold water and absorbed onto a DEAE-Sephadex column (as above). The product was eluted with a 200 ml, 0-450 mM TEABC gradient, pH 8.2. TEMPO-ATP eluted at 250 mM. The pooled fractions were evaporated to dryness two times from water and two times from methanol. Chromatography in n-propanol-NH₄₂-water (55:20:25) showed that the material was 97% TEMPO-ATP, with both a faster and a slower component (2.0% and 0.6% respectively).

R. 6-Thioacetamido-TEMPO-ATP (LXXI) \(6\text{-mercapto}[N-(1\text{-oxyl-2,2,6,6-}\text{tetramethyl-4-piperidinyl}]-\text{acetamido-9-β-D-ribofuranosylpurine-5'-triphosphate}\) (Cooke and Duke, 1971).

A typical preparation involved reaction of 10 mg (3.14 × 10⁻³ moles) of spin label dissolved in 10 ml of tris buffer (10⁻³ M) at pH 8.5 with 22 mg (3.42 × 10⁻³ moles) of 6-mercaptoATP. The mixture was stored in an oxygen-free atmosphere in the dark at 25°C. The progress of the reaction was followed by monitoring the disappearance of the absorption peak at 322 nm and the appearance of the peak at 282 nm. Upon completion of the reaction (72 hr), the reaction mixture was placed on a DEAE-cellulose column (9 × 2 cm) in the bicarbonate form at pH 7.6 and eluted with a 2 liter linear gradient (0.0-0.2 M) of tetraethylammonium bicarbonate. The eluent was monitored at 282 nm for the product that appeared in a well-defined peak separated from both unreacted SH-ATP and unreacted spin label. The fraction containing the product was evaporated, and the residue, dissolved in methanol, was precipitated with acetone containing a twentyfold molar excess of sodium perchlorate. The yellow precipitate was washed with acetone, then ether, and finally dried over P₂O₅. The yield was customarily 50% of the theoretical. The product had an extinction coefficient at 282 nm of 2.04 × 10⁴ in a solvent consisting of 0.05 M potassium acetate (pH 4.8) and exhibited an \(R_f\) value of 0.78 on thin-layer cellulose plates in an isopropanol-ammonium bicarbonate system (3:2, v/v).

S. Preparation of coordinate nitroxide corrinoids (LXXV) (Buckman et al., 1969)

\[
\begin{align*}
O-N & \begin{array}{c}
\text{OH} \\
\text{CO} \text{CH}_2
\end{array} \\
\text{HO} & \text{OH}
\end{align*}
\]

Aquo-Co(III)-nitroxylcobinamide was prepared by allowing a tenfold excess of either 2,2,6,6-tetramethylpiperidine-N-oxyl or 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl with 10.0 mg diaquocobinamide to stand at room temperature for 10 days in 20 ml ethanol. Diaquocobinamide was synthesized by exhaustive aerobic photolysis of methylcobinamide. Methylcobinamide was synthesized and purified by the method described by Wood and Wolfe (1966). Aquo-Co(III)-nitroxycobinamide was crystallized by addition of excess acetone, followed by a 2 day growth period at 4°C. The crystals (7.0 mg) were centrifuged and Soxhlet-extracted for 48 hr with diethyl ether at a rate of 5 ml/min. When the colorless ether layer, dried over CaSO₄, was evaporated to dryness, no residue remained. The coordinate complex was stored at -15°C over CaSO₄. The ultraviolet-visible spectrum of this derivative indicates that water was not displaced from the upper axial ligand.
5. THE CHEMISTRY OF SPIN LABELS

Spin-labeled protohemin, labeled at positions 6 and 7 of the porphyrin ring, was prepared from protohemin and 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl as follows. Protohemin (13.2 mg) dissolved in 20 ml of tetrahydrofuran was mixed with the nitroxide in a molar ratio 4 to 6 radicals per hemin in the presence of N,N-dicyclohexylcarbodiimide (50 mg, Eastman Kodak Company). The reaction mixture was kept in a dark room for 24 hr at 23°C. The progress of the reaction was traced by thin-layer chromatography (2.6-lutidine-water, 10:1, v/v). Protohemin that did not move in the lutidine-water system gradually changed to mononitroxide compounds and finally to spin-labeled protohemin that has nitrooxyl free radicals at positions 6 and 7 of the porphyrin ring. After the reaction, the solution was mixed with 200 ml of ethyl acetate and washed twice with water, once with 2 N Na₂CO₃ to remove unreacted protohemin, and again several times with water. By these washings, more than 95% of the unreacted spin labels were removed. The solution was then evaporated to dryness. The dried material was further purified by chromatography on Sephadex LII-20. Spin-labeled protohemin was prepared by the same technique as mentioned above. Spin-labeled protoporphyrin gave a clear single spot in thin-layer chromatography with an Rf value of 1.0, corresponding to protohemin dimethyl ester. The spin-labeled protoporphyrin was soluble in organic solvent, but completely insoluble in aqueous alkaline solution, indicating that no free carboxyl groups remained. By optical and epr measurements, the ratio of hemin to the nitrooxyl free radicals was calculated as approximately 1:2. The pyridine hemochromogen spectrum of the spin-labeled protoporphyrin had the same absorption maximum at 555 nm as either protohemin or proto-

hemin dimethyl ester, suggesting that positions 2 and 4 of the porphyrin ring were intact. All these results suggest that the nitrooxyl free radicals were bound at positions 6 and 7 of the porphyrin ring.

U. Spin-labeled NAD (adenosine 5'-diphosphate 4(2,2,6,6-tetramethylpiperidine-1-oxyl) (LXXIII) (Weiner, 1969)

The solution of 1-oxyl-2,2,6,6-tetramethyl-4-phosphopiperidine (XXIII) in pyridine was evaporated to dryness many times. To the flask was added 5 ml dry pyridine and 600 mg AMP morpholidate (Calbiochemical Co.); the resulting solution was evaporated to dryness and 5 ml dry pyridine was added and the evaporation was repeated five more times. The reaction proceeded at room temperature in 5 ml pyridine for 5 days and was terminated by the addition of 15 ml water. The solvents were removed under vacuum on a rotary vacuum evaporator, keeping the temperature below 40°C. More water was added and the evaporation was continued until the odor of pyridine was absent. Finally the precipitate was dissolved in 20 ml water and the insoluble material was removed by filtration. To the aqueous solution was added 350 mg barium acetate and 100 ml methanol and the solution was left at -15°C for 12 hr. The white precipitate was collected and dissolved in pH 6, μ = 0.006, phosphate, and the barium salts were removed by centrifugation. The product was separated from AMP by chromatography on a DEAE-cellulose column using a linear gradient between μ = 0.006 and 0.2 phosphate.†

The product was homogeneous on paper chromatography using ethanol-1.0 M ammonium acetate (pH 7.5, 7:3) or n-butanol-acetonetic acid-5% NH₄OH (4.5:1.5:1:1:2). The migration of the spin-labeled ADP was identical with that of commercial ADP ribose (Sigma Chemical Co.). The molar extinction coefficient for spin-labeled ADP is ε₂₅₀ 17,000.

† Recent work indicates that the work-up time can be accelerated by separating the products on a 2 m Sephadex G-10 column rather than DEAE. Thus, the reaction is terminated with water as before; the water-pyridine is evaporated and the residue is dissolved in 1 ml pH 6 pyridine-acetate buffer. Insoluble material is removed by centrifugation and the products are separated on a G-10 column. (H. Weiner, personal communication.)
V. \( (1\text{-Oxyl-2,2',6,6'-tetramethyl-d}^{[3]}\text{H]-piperidinyl})\beta\text{-D-galactoside (LXXXII)} \)
(Struve and McConnell, 1972)

The tritiated alcohol (prepared by NaBT₄ reduction of 1-oxyl-
triacetoneamine) \((1.89 \text{ gm}, \sim 60 \text{ mCi}) , 3.04 \text{ gm silver carbonate}, 10 \text{ gm Drier-
te}, \text{ and 10 ml dry, alcohol-free chloroform were placed in a three-necked}
round-bottom flask equipped with a sealed mechanical stirrer, a drying tube,
and a dropping funnel. The flask was wrapped in black paper and stirred for
1 hr. Then 0.41 gm anhydrous silver perchlorate was added as a catalyst.
Acetobromo-\( \alpha\text{-D-galactose (4.13 gm), synthesized from D-galactose (Lemiex, 1963), was dissolv}
ed in 15 ml dry, alcohol-free chloroform, and the solution added to the stirred mixture over a period of 1 hr. Stirring was
continued for 4 hr and the mixture filtered. The residue was washed with 10 ml chloroform and the combined filtrates were concentrated under
reduced pressure. The compound was purified by chromatography on a silica
gel column (Grace, grade 62, 60–200 mesh) \(3.5 \text{ cm} \times 31.6 \text{ cm} \), eluted with dichloromethane-ethyl ether \((8:2)\). Thin-layer chromatography in this
solvent system separates the bromide, alcohol, and product from each other.
The first orange band \((270–330 \text{ ml})\) from the column was collected and evaporated
to dryness. Thin layer chromatography in the same solvent showed only one spot \((R_f = 0.5)\) by uv absorption, radioactivity, or by
ashing with \(50\% \text{ H}_2\text{SO}_4\). The optical rotation was \([\alpha]_D^{24} = -9.9 \pm 0.2^\circ \text{C} (c = 6.7, \text{ methanol})\).

The residue was dissolved in 10 ml of anhydrous methanol and 40 \(\mu\text{l of}
1 \text{ M sodium methoxide in methanol was added. After 3 hr at room temperature,}
the sodium methoxide was neutralized by addition of 20 mg anhydrous Dowex 50 \((\text{H}^+ \text{ form})\). The mixture was filtered after stirring for \(\frac{1}{2} \text{ hr. The}
filtrate was evaporated to a small volume and applied to Whatman 3MM paper.
The chromatogram was developed by descending chromatography w
with ethyl acetate-\( \alpha\text{-propanol-water (5:3:2 v/v)}\). The uv absorbing band
nearest the origin was eluted with water. The eluate was evaporated
to dryness and the residue was dissolved in a small amount of absolute ethanol.
A slight precipitate that formed was removed by filtration and the filtrate
evaporated to dryness. Yield: 0.74 mole \((7.4\%)\).

5. THE CHEMISTRY OF SPIN LABELS

W. \( (1\text{-Oxyl-2,2',5,5'-tetramethyl-3-pyrrolidinyl)methyl-\beta-chitobiose (LXXXIV)} \)
(Wien et al., 1972)

The procedure for the preparation of this compound is a modification of
the method described by Kuhn and Kirschenlohr (1953). Acetochloroi-
chitobiose \((314 \text{ mg})\) prepared by the method of Dahlquist and Raftery (1969)
was intimately mixed with 344 mg of \( (1\text{-oxyl-2,2',5,5'-tetramethyl-3-pyr}
rolidinyl)\text{carbinol}\) and 504 mg of \( \text{Hg(CN)}_2\) using a mortar and pestle. This
mixture was transferred to a 25 ml flask and dried \(\text{in vacuo}\) for 5 hr. The
reaction was initiated by adding 3 ml of chloroform (freshly distilled from
\(\text{P}_2\text{O}_5\)). The reaction mixture was stirred at room temperature for 72 hr,
then evaporated to dryness under reduced pressure at less than 40°C. The
residue was redissolved in chloroform and excess mercuric cyanide removed
by filtration. The yellow filtrate was washed exhaustively with water to
remove excess \(\text{IV}\), dried over \(\text{MgSO}_4\), and evaporated to dryness yielding a
yellow glass. This was applied to a column \((0.6 \times 10 \text{ cm})\) of SilicAR,
\(\text{CC-4, 100–200 mesh (Mallinkrodt Chemical Works)}\) equilibrated with
methylene chloride. The column was developed by eluting successively with
methylene chloride, methylene chloride–chloroform \((1:1)\), chloroform,
and chloroform–methanol \((10:1)\). The desired blocked glycoside was eluted last.
Evaporation to dryness afforded a yellow glass that could not be crystallized.
The infrared spectrum exhibited carbonyl bands at \(1745 \text{ cm}^{-1}\) (acetate)
and \(1660 \text{ cm}^{-1}\) (acetamide and no hydroxyl absorption). This material was
decaylated by dissolving in 20 ml methanol saturated with \(\text{NH}_3\) at 5°C.
After 24 hr, the reaction mixture was evaporated to dryness at less than 30°C.
The yellow syrup was completely soluble in water; lyophilization afforded
91 mg of a yellow hygroscopic powder. The infrared spectrum exhibited
carbonyl absorption at \(1655 \text{ cm}^{-1}\) (acetamide) but not at \(1730-1750 \text{ cm}^{-1}\)
(acetate).

Anal. calcd. for \(\text{C}_{25}\text{H}_{44}\text{N}_3\text{O}_{12}\text{NH}_2\): \(\text{C}, 50.41; \text{H}, 7.95; \text{N}, 9.40\).

Found: \(\text{C}, 50.56; \text{H}, 8.07; \text{N}, 10.04\).
X. (1-Oxyl-2,2,6,6-tetramethyl-4-amidopiperidinyl)methyl-β-D-thiogalactoside (LXXXIX) (S. J. Opella and B. J. Gaffney, unpublished)

Tetra-O-acetyl-α-D-galactosyl bromide (Lemieux, 1963) was converted to tetra-O-acetyl-β-D-thiogalactose and then to the thiogalactoside by the method of Černý and Pacák (1959). To a solution of 0.76 gm (0.002 mole) acetyl-β-D-thiogalactoside and 0.7 gm (0.002 mole) 1-oxyl-2,2,6,6-tetramethyl-4-iodoacetamidopiperidine in 14 ml acetone was added 2 ml water and 0.27 gm K₂CO₃. The mixture was stirred at room temperature for 4 hr, at which time thin-layer chromatography [chloroform–ether, 1:1, Kieselgel plates (Merck)] showed no remaining tetraacetylthiogalactose. The reaction mixture was filtered and evaporated to complete dryness. The anhydrous, acetylated reaction product was dissolved in 5 ml anhydrous ethanol, and 20 mg sodium methoxide was added to this mixture to achieve deacetylation. After 5 min, 5 ml water was added. The cation exchange resin IR-120 (H⁺ form) was added to the deacetylation mixture to remove cations, and after 5 min, the suspension was filtered. The eluate was evaporated to dryness, taken up in 2 ml water, extracted three times with ether, and again evaporated to dryness. Thin-layer chromatography on Kieselgel plates [5 x 20 cm, solvent: isopropanol–ethyl acetate–water (7:1:2); charred with 50% H₂SO₄] gave single spots for each of the following: the deacetylated thiogalactoside spin label, the product of the reaction of iodoacetamide spin label with tetraacetylthiogalactose, and the fully acetylated spin-labeled thiogalactoside (acetylated with acetic anhydride–sodium acetate) (R₇ values: 0.61, 0.66, and 0.71).

Anal. calc. for C₁₅H₂₁N₂O₇: C, 50.10; H, 7.67; N, 6.87.
Found: C, 49.46; H, 8.33; N, 5.53.

Y. (1-Oxyl-2,2,5,5-tetramethylpyrroldinyl)-β-D-thiogalactoside (LXXXVIII) (B. J. Gaffney, unpublished)

This procedure follows that of Černý and Pacák (1959) for preparation of β-D-thioglucosides. To a solution of 0.32 gm (0.89 x 10⁻³ moles) tetra-O-acetyldithio-β-D-galactose in 1.5 ml acetone was added 0.23 gm (0.98 x 10⁻³ moles) of 2,2,5,5-tetramethyl-3-bromomethylpyrroldinyl bromomethane and 0.12 gm K₂CO₃ dissolved in 1 ml water. A crystal of NaI was added to the reaction mixture, and the mixture was heated at 35°C for 3 hr in a stoppered flask. After addition of 10 ml water and 2 ml chloroform, the organic and aqueous layers were separated and the aqueous layer was extracted three times with 1 ml chloroform. The organic layers were dried over Na₂SO₄ and evaporated to give 0.45 gm of a yellow oil. A portion (0.23 gm) of this oil was purified by preparative thin-layer chromatography (three plates, 20 x 20 cm, 0.75 mm thick, Merck Kieselgel HF₂₅₄, dried 30 min at 120°C and allowed to stand 20 hr in air) using chloroform–methanol (180:1) as solvent R₇ values for the product, tetraacetylthio-β-D-galactoside, and nitroxide bromide were 0.17, 0.33, 0.47). The yield was 0.14 gm (59%).

Deacetylation was achieved by dissolving 0.14 gm of the spin-labeled acetyl sugar in 1 ml anhydrous methanol containing a trace of sodium methoxide. The solution was filtered through charcoal and evaporated to give 0.084 gm of (1-oxyl-2,2,5,5-tetramethylpyrroldinyl)thio-β-D-galactoside.

Z. 1-Oxyl-2,2,5,5-tetramethyl-3-[3-[[p-sulfamoylphenyl]carbamoyl]propionamide]-1-pyrroldine [XCH(c)] (Procedure of Erlich et al., 1973)

Sulfanilamide (2.0 gm; 12 mmoles) and succinic anhydride (1.16 gm; 12 mmoles) was dissolved in acetone (10 ml), and the reaction mixture was allowed to stand overnight. The precipitate (2.5 gm) was removed by filtration recrystallized from methanol to give colorless needle crystals, mp 208°C–210°C.

Calculated: C, 44.11; H, 44.44; N, 10.29.
Found: C, 43.77; H, 4.47; N, 10.03.
5. THE CHEMISTRY OF SPIN LABELS


For reviews of spin labeling, see:

General References


ACKNOWLEDGMENTS

I would like to thank Professor H. M. McConnell and the colleagues with whom I have worked during a number of years in his laboratory for the numerous inspiring conversations which have brought to my attention many of the papers cited in this chapter.

I am very grateful to Professor A. Kornberg for permission to include the unpublished synthesis of 6-amino-TEMPO-ATP which he, D. Brutlak, and the late M. Atkinson developed.

Some of the synthetic procedures included were developed in the laboratory of Professor K. Nakashima, Tohoku University, Sendai, Japan, while I was a Varian Associates Postdoctoral Fellow (1967-1968).

The assistance of Robert S. Schepp and Sandi Hanson in preparation of the manuscript is gratefully acknowledged.

Preparation of this manuscript has been supported by the NSF under grant GB3301X-1, and by the NIH under grant NS-08038-06 (to H. M. McConnell) and by the NIH under grant 1R01 CA 15997-01 (to myself).

REFERENCES

For reviews of nitroxide chemistry, see:

5. THE CHEMISTRY OF SPIN LABELS

5. The Chemistry of Spin Labels


Molecular Structures of Nitroxides

J. LAJZEROWICZ-BONNETEAU

LABORATORY OF PHYSICAL SPECTROMETRY
UNIVERSITY OF SCIENCE AND MEDICINE OF GRIGNOLLE
GRIGNOLLE, FRANCE

I. X-Ray Analysis of Single Nitroxide Crystals 239
II. Experimental Results 244
III. Conclusions on the Conformations of Nitroxides 244
IV. ESR Spectra of Single Nitroxide Crystals 246
   A. Role of the Exchange Interaction 246
   B. Experimental g Tensor Values 246
   C. Relative Orientation of the g Tensor and the $^{13}C_6N=O \cdot$ Group 246
References 248

I. X-RAY ANALYSIS OF SINGLE NITROXIDE CRYSTALS

 Appropriately substituted nitroxyl derivatives of piperidine, pyrrolidine, pyrroolidine, and oxazolidine are solids at room temperature and crystallize quite readily. The crystal and molecular structures of a number of these derivatives have been determined by X-ray diffraction methods (Strout and Jensen, 1968; Woolfson, 1970). The dozen structures that have been determined by this method are listed in Fig. 1.

There are generally two, four, or eight molecules per elementary crystal unit cell derived from each other by symmetry operations. This leads to the determination of the coordinates of approximately thirty atoms. These data allow determination of molecular stereochemistry and conformation (interatomic angles and distances) as well as the relative orientation of different molecules within the crystal lattice. These structures have been determined
Fig. 1. Structures of nitrosoxide molecules determined by crystallographic analysis.

I 2,2,6,6-Tetramethylpiperidine-1-oxyl (Bordeaux et al., 1973; Capionmont et al., 1972b)
II 2,2,6,6-Tetramethylpiperidine-1-oxyl-4-ol (Lajzerowicz, 1968; Berliner, 1970)
III 2,2,6,6-Tetramethyl-4-piperidinone-1-oxyl (Shibaeva et al., 1972; Bordeaux and Lajzerowicz, 1974a)
IV Dl(2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl) sulfate (Capionmont, 1972)
V Iminoxyl organosilicon tetra radical (Shibaeva et al., 1973)
VI Caryophyllene iodonitrosite (Hawley et al., 1968)
VII 9-Azabicyclo[3.3.1]nonane-3-one-9-oxyl (Capionmont et al., 1971)
VIII 1,5-Dimethyl-8-azabicyclo[3.2.1]octane-3-one-8-oxyl (Capionmont, 1973)
IX 2,2,5,5-Tetramethyl-3-carbamidopyrrole-1-oxyl (Turley and Boer, 1972)
X (+)-3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl (Ament et al., 1973)
XI Potassium 2,2,5,5-tetramethyl-3-carboxy pyrrole-1-oxyl (Boeyens and Kuiper, 1970)
XII 2,2,5,5-Tetramethyl-1-aza-3-cyclo pentanene-1-oxyl-3-azic (Chion et al., 1972)

to various degrees of accuracy; the standard deviation for bond distances between carbon, nitrogen, and oxygen atoms varies from $3 \times 10^{-3}$ to $2 \times 10^{-2}$ Å. Bond angles vary from 0.2° to 1°. The positions of hydrogen atoms have not been determined for all of the twelve molecules in Fig. 1. For those molecules whose hydrogen atom positions are known, the standard deviations are often as much as ten times greater than those for the larger atoms. These standard deviations will be quoted in parentheses immediately after the number to which they refer: e.g. C(1)—C(2) = 1.536(3).

Fig. 2. (a) 2,2,6,6-Tetramethylpiperidine-1-oxyl bond angles and distances (Bordeaux et al., 1973), with permission of Heyden & Son, Ltd.) (b) Projection of the molecule on its symmetry plane (hydrogen atoms are represented by small circles).

Fig. 3. (a) 2,2,6,6-Tetramethylpiperidine-4-piperidinone-1-oxyl bond angles and distances. (b) Projection of the molecule parallel to its mean plane; the binary axis $O_2 N_1 C_6 O_2$ is in the projection plane. (Bordeaux and Lajzerowicz, 1974a.)
6. MOLECULAR STRUCTURES OF NITROXIDES

![Chemical Structures](image)

**Fig. 4.** (a and b) Bond distances and angles of 2,2,5,5-tetramethyl-3-carbamidopyrrolino-1-oxyl. The pyrrolino ring and the nitroxide group are coplanar. (Tulley and Boer, 1972.)

**Fig. 5.** (a and b) Bond distances and angles of the molecules (+)-carboxy-2,2,5,5-tetramethyl-1-pyryrolidin-oxyl.

![Chemical Structures](image)

**Fig. 5c.** Projection of the molecule parallel to its mean plane. The pseudobinary axis of the cycle (O,N) is in the projection plane. (Ament et al., 1973.)

**TABLE I**

<table>
<thead>
<tr>
<th>Nitroxide</th>
<th>Symmetry of the ring</th>
<th>N-O bond length (Å)</th>
<th>Angle $\angle_(deg)$</th>
<th>Angle $\phi$ between the N-O bond and the CNC plane (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chair form, mirror plane</td>
<td>1.296 (5)</td>
<td>123.5 (3)</td>
<td>19.4</td>
</tr>
<tr>
<td>II</td>
<td>Chair form, mirror plane</td>
<td>1.291 (7)</td>
<td>125.4 (5)</td>
<td>15.8</td>
</tr>
<tr>
<td>III</td>
<td>Twist form, two-fold axis</td>
<td>1.276 (3)</td>
<td>123.3 (2)</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>Chair form, mirror plane</td>
<td>1.276 (5)</td>
<td>123.9 (5)</td>
<td>18.2</td>
</tr>
<tr>
<td>V</td>
<td>Chair form</td>
<td>1.31</td>
<td>126</td>
<td>20</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>1.31 (2)</td>
<td>121 (1)</td>
<td>24</td>
</tr>
<tr>
<td>VII</td>
<td>Mirror plane</td>
<td>1.289 (4)</td>
<td>114.2 (3)</td>
<td>30.5</td>
</tr>
<tr>
<td>VIII</td>
<td>Mirror plane</td>
<td>1.274 (4)</td>
<td>107.4 (3)</td>
<td>24.9</td>
</tr>
<tr>
<td>IX</td>
<td>Planar</td>
<td>1.267 (5)</td>
<td>114.8 (5)</td>
<td>0</td>
</tr>
<tr>
<td>X</td>
<td>Twist form</td>
<td>1.272 (3)</td>
<td>116.2 (2)</td>
<td>3.3</td>
</tr>
<tr>
<td>XI</td>
<td>Planar</td>
<td>1.277 (8)</td>
<td>114.8 (9)</td>
<td>0</td>
</tr>
<tr>
<td>XII</td>
<td>Planar</td>
<td>1.263 (13)</td>
<td>114.7 (7)</td>
<td>0</td>
</tr>
</tbody>
</table>

* References to the structures are found in the text.
The conformation obtained is that of the molecule in the crystal. The interactions between molecules are generally of the van der Waals type, and it is reasonable to consider that the conformation of the molecule in the crystal differs little from that of the molecule in solution. Apart from the actual conformation of the molecule, there are several other noteworthy features:

1. In molecules containing groups that may participate in hydrogen bonding (e.g., \( \text{OH}, \text{NH}_2 \)), the oxygen atom in the \( \text{N} - \text{O} \) group participates in a weak intermolecular hydrogen bond (II, IX, X), a phenomenon that might occur in solution as well.

2. When studying the crystal of a compound containing one or more centers of asymmetry, it is possible to obtain the absolute configuration of the molecule; such is the case for the nitroxide X (Ament et al., 1973).

3. Some nitroxides have been found to be polymorphic; i.e., they have various crystalline forms. This is the case with compound I (Capionmont et al., 1972). In the stable phase between 20° and 40°C, the molecules are in chair-chair inversion movement; this inversion, which also exists in solution, must be capable of occurring when the nitroxide is bound to a macromolecule.

II. EXPERIMENTAL RESULTS

The detailed results for four nitroxides of different structural types are:

- Nitroxide I: Piperidine chair-form ring
- Nitroxide II: Piperidine twisted-form
- Nitroxide IX: Pyrrolidine
- Nitroxide X: Pyrrolidine

For each compound we give a projection of the molecule on a particular plane (Figs. 2b, 3b, 5c) and diagrams with interatomic angles and distances (Figs. 2a, 3a, 4a, 4b, 5a, 5b). In addition, Table 1 gives all the data on the \( \text{C} - \text{N} - \text{O} \) nitroxide group: N-O bond distance, CNC angle, and angle \( \alpha \) between the N-O bond and the CNC plane.

III. CONCLUSIONS ON THE CONFORMATIONS OF NITROXIDES

All the five- and six-membered rings have the expected form and symmetry, but are nevertheless very deformed. The dimensions of the group \( \text{C} - \text{N} - \text{O} \) are variable. This group is planar when the ring is either planar or contains a binary axis of symmetry; otherwise the group is pyramid shaped.

6. MOLECULAR STRUCTURES OF NITROXIDES

Angle CNC is particularly sensitive to the constraints of the ring; its value changes from 125° for piperidine rings to 107° for the bicyclic compound VIII. Diagrammatically, two models for the nitroxides can be proposed (Fig. 6).

Fig. 6. Diagrams of the nitroxide group and its overall van der Waals thickness. (a) Five-membered ring, the nitroxide group is planar; (b) six-membered ring, the nitroxide group is bent.

Nonempirical methods have been used for calculating wave functions of the elementary radical \( \text{H}_2\text{NO} \) (Salotto and Burnette, 1970; Ellinger et al., unpublished results). These calculations lead to an aminoxyl group without any well-defined intrinsic geometry. In particular, the total electronic energy varies only slightly with the torsion of the group (two nominal minima are found for \( \phi = \pm 17° \)). The theoretical model predicts the N-O bond length to be 1.27 Å and the CNC angle to be 123°. Both values are in excellent agreement with the experimental values found for nitroxides in which the N-O group is not highly constrained (e.g., six-membered rings).

Semiempirical calculations have been done on compound I (PCILQ method—perturbative configuration interaction using localized orbitals). Here, too, the calculated geometry agrees well with the experimentally
determined structure. The energy difference between the planar form and the pyramidal configuration is 1.05 kcal/mole (Ellinger et al., unpublished results).

From studies on polarization parameters and spin density distribution in the N—O+ group, Hayat and Silver (1973) find that bending is necessary to account for the observed values. They obtain an angle \( \alpha = 17^\circ \) for the nitroxide III, which is found planar in the crystalline state.

IV. ESR SPECTRA OF SINGLE NITROXIDE CRYSTALS

A. Role of the Exchange Interaction

The ESR studies of single nitroxide crystals are useful for obtaining information about molecular orientations in the unit cell and therefore facilitate crystal structure determinations. In addition, these studies allow precise measurements of the principal values of the \( g \) tensors. ESR spectra of single nitroxide crystals contain only a single line, which is Lorentzian in shape and about 10 G in width. These spectral properties are exhibited by all nitroxide single crystals regardless of the number of radicals per unit cell or the orientation of the crystal in the magnetic field (Bordeaux et al., 1973). In these crystals the distances between the \( \text{C}^\text{N—O+} \) groups are about 6 Å.

Consequently, the exchange interaction between neighboring spins is very large compared to the difference of absorption energies of these spins themselves. Thus, for each orientation, the measured experimental \( g \) factor \( (g_{\text{exp}}) \) is the mean of the different individual nitroxide \( g \) factors (Capron et al., 1974).

B. Experimental \( g \) Tensor Values

From these ESR studies, principal values of the \( g \) tensors \( g_{xx}, g_{yy}, g_{zz} \) have been found and are shown in Table II. The estimated accuracy is \( \pm 0.0002 \). These components cover a very wide range of values, and there does not seem to be a relation between them and the molecular conformation. The lower mean values for \( (g_{xx} + g_{yy} + g_{zz}) \) are for the nitroxides II and IX, which exhibit hydrogen bonding in the crystal.

C. Relative Orientation of the \( g \) Tensor and the \( \text{C}^\text{N—O+} \) Group

When the nitroxide group is planar, the principal axes \( X, Y, \) and \( Z \) coincide respectively with the \( N—O+ \) bond, the \( \text{C—C} \) direction, and the perpendicular to axes \( X \) and \( Y \), which passes through the long axis of the

<table>
<thead>
<tr>
<th>Compound</th>
<th>( g_{xx} )</th>
<th>( g_{yy} )</th>
<th>( g_{zz} )</th>
<th>( (g_{xx} + g_{yy} + g_{zz}) )</th>
<th>Relative orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.0103</td>
<td>2.0069</td>
<td>2.0030</td>
<td>2.0067</td>
<td>Coincidence</td>
</tr>
<tr>
<td>II</td>
<td>2.0096</td>
<td>2.0064</td>
<td>2.0027</td>
<td>2.0062</td>
<td>Deviation</td>
</tr>
<tr>
<td>III</td>
<td>2.0104</td>
<td>2.0074</td>
<td>2.0026</td>
<td>2.0068</td>
<td>Coincidence</td>
</tr>
<tr>
<td>IV</td>
<td>2.0112</td>
<td>2.0083</td>
<td>2.0039</td>
<td>2.0071</td>
<td>Coincidence</td>
</tr>
<tr>
<td>VIII</td>
<td>2.0104</td>
<td>2.0066</td>
<td>2.0038</td>
<td>2.0069</td>
<td>Deviation</td>
</tr>
<tr>
<td>IX</td>
<td>2.0086</td>
<td>2.0066</td>
<td>2.0028</td>
<td>2.0061</td>
<td>Coincidence</td>
</tr>
<tr>
<td>XII</td>
<td>2.0101</td>
<td>2.0088</td>
<td>2.0028</td>
<td>2.0065</td>
<td>Coincidence</td>
</tr>
</tbody>
</table>

\(^a\) A. Capron et al., 1974.
\(^b\) Editor's note: A complete table of all nitroxide \( g \) and hyperfine tensors reported to date is compiled in Appendix II, p. 564, and in Chapter 3, Table I, p. 73.

2p \( \pi \) orbital. In other cases it is sometimes necessary to assume a noncoincidence of the \( X \) axis and the \( N—O+ \) bond in order to interpret the ESR results.

The examples of compound I and II are instructive (Bordeaux et al., 1973). The crystallographic structures are isomorphic with only one molecule in the unit cell. The symmetry plane of the crystals is the molecular mirror plane, so the orientation of the principal \( g \) axes is easy to find. In compound I the \( X \) axis and \( N—O+ \) bond coincide. In compound II a displacement of 8.5° is found between these directions (Fig. 7). The intermolecular hydrogen bond present in compound II is probably responsible for this effect.

![Fig. 7. 2,2,6,6-tetramethyl-4-piperidinol-1-oxyl deviation of the principal axes of the \( g \) tensor (X and Z) from the N—O+ bond. (Bordeaux et al. (1973), with permission of Heyden & Son, Ltd.)](image)

Crystal and molecular structures of two \( N+oxyl \) oxazolidine nitroxides have been determined: XIII, 1,4-bis(4,4'-dimethyl-oxazolidine-\( N+oxyl \))cyclohexane (Gleason, 1973); XVII, spiro(1,2'-(4,4'-dimethyl-oxazolidine)cyclohexane) (Bordeaux and Lajzerowicz, 1974b). In both compounds the
N—O group is planar (strictly for XIV). The NO bond lengths are 1.25 (1) XII and 1.259 (4) XIV. The principal values of the g tensor for the compound XIV are 2.0089, 2.0076, and 2.0036.

ACKNOWLEDGMENTS

Thanks are due to Dr. S. Ament, J. Wetherington, J. Moncrief, K. Flohr, M. Mochizuki, E. Kaiser, Y. Ellinger, J. Dauady, A. Rassat, and M. Sobra for communicating their results prior to publication, and to Drs. D. Bordeaux, A. Capiomont, and B. Chion and Professor A. Rassat and associates for helpful discussions.

REFERENCES


6. MOLECULAR STRUCTURES OF NITROXIDES


Instrumental Aspects of Spin Labeling

PATRICIA JOST and O. HAYES GRIFFITH
INSTITUTE OF MOLECULAR BIOLOGY
AND
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF OREGON
EUGENE, OREGON

I. ESR Instrumentation and Instrumental Artifacts
   A. ESR Spectrometers
   B. The Microwave Cavity and the Sample
   C. Receiver Gain, Field Modulation Amplitude, and Microwave Power
   D. Scan Time, Filter Time Constant, and Field Inhomogeneity
   E. Degassing and Concentration Effects

II. The Use of Computers to Process Spin Labeling Data
   A. Time Averaging
   B. Scaling Experimental Spectra
   C. Integration of the Experimental Spectrum
   D. Spectral Titration
   References

I. ESR INSTRUMENTATION AND INSTRUMENTAL ARTIFACTS

A. ESR Spectrometers

A number of books are available which describe the design, construction, and sensitivity of ESR spectrometers (Ayscough, 1967; Poole, 1967; Alger, 1968; Wilmhurst, 1968). Wertz and Bolton (1972) give a good description of

7. INSTRUMENTAL ASPECTS OF SPIN LABELING

the basic instrumentation of electron spin resonance. These are useful to the expert in the field but most potential users have a commercial ESR spectrometer available and must use it as it is. Our aim here is to provide the nonexpert with background to help in the use of the ESR spectrometer as a black box. Particular emphasis is placed on instrument settings, since most operator errors are made here. Any comparison among the many commercial spectrometers is deliberately avoided because the various spectrometers are constantly being upgraded and because the authors have not made a careful comparison of the various instruments available. All spectra displayed in this chapter were recorded on Varian ESR spectrometers, most of them on a Varian E-3.†

A schematic diagram of a simple ESR spectrometer is shown in Fig. 1. The basic parts are the klystron, magic tee, cavity, detector-amplifier combination, and a recording device. (Modern commercial instruments use a microwave circulator instead of the magic tee to accomplish the same purpose.) The klystron, in conjunction with the fixed isolator and adjustable attenuator, provides the operator with a variable source of microwave energy. The magic tee is the microwave equivalent of the familiar Wheatstone bridge. The spectrometer is constructed so that the impedances of arms 2 and 4 are nearly matched and very little power reaches the detector on arm 3. The sample is situated in the microwave cavity, a region of high microwave field $H_1$. The klystron frequency is fixed during the experiment and the magnetic field is scanned. As the resonance condition is approached, the sample absorbs microwave energy, creating an imbalance in the bridge. This imbalance is detected as increased power in arm 3 and is amplified and displayed on a recorder. The modulation coils also play an important role. They are needed in the conversion of the dc signal to an ac voltage, which can then be easily amplified using the powerful methods of phase sensitive detection. The modulation coils are frequently imbedded in plastic and fastened to the sides of the microwave cavity, out of sight of the operator. The use of modulation causes the spectra to appear as a first derivative of the microwave absorption, and provides the operator with one more control that must be adjusted properly, the modulation amplitude control.

B. The Microwave Cavity and the Sample

The cavity and sample are of special importance. Many cavity designs are available, including rectangular cavities, cylindrical cavities, and special slow-wave structures. Rectangular cavities are the most common type at present and all spectra presented here were recorded using a standard Varian rectangular cavity. However, any high-quality (high-$Q$) cavity can be used in spin labeling studies. The user should be aware of which type of cavity is available since this determines the geometry of the sample holder. A typical cavity will have an opening 1 cm in diameter, but the actual liquid or solid sample is much smaller than this opening. As one might expect, the optimum sample geometry for a rectangular cavity is a flat, rectangular cell and the best geometry for a cylindrical cavity is a cylindrical sample tube. More care must be exercised when dealing with aqueous samples than with organic materials because the dielectric loss of water tends to lower the spectrometer sensitivity by lowering the cavity $Q$. Commercial quartz flat aqueous sample cells have approximate dimensions $6 \text{ cm} \times 1 \text{ cm} \times 0.3 \text{ mm}$ i.d. These useful cells are equipped with stoppered ports at the top and the bottom. When dealing with moderate concentrations of spin label, $10^{-3} \text{ M}$ to $10^{-6} \text{ M}$, less expensive cells may be used. For example, the aqueous samples may be drawn into 0.4-mm-i.d. thin-walled capillary tubes made of

† Editor's note: See Appendix III, p. 566, for a compendium of commercial equipment and supplies.
ordinary flint (soft) glass or Pyrex. The capillary tubes are available from standard laboratory supply companies. After filling, these capillary tubes may be sealed at the dry end with a flame or with a soft wax such as Universal Red Wax available from Central Scientific Company (note that some nitroxides are soluble in wax). The filled capillaries are dropped into standard 2-4-mm-i.d. quartz ESR sample tubes. The combination is then placed in the cavity. After the experiment the capillary tube is discarded and the quartz tube is ready for reuse. The quartz tubes are easily made from stock quartz tubing, but special care must be taken since standard quartz tubing frequently has an intrinsic ESR signal. It is more convenient to avoid this problem by purchasing the quartz ESR sample tubes.

Dry tissues, powders, hydrocarbon solvents, and other samples that do not contain water are less troublesome. Large-diameter flint glass or Pyrex capillaries make useful sample tubes. Disposable pipettes sealed at the small end make inexpensive tubes that are easy to degas. Of course, the quartz flat cells and ESR sample tubes may be used with organic samples as well as aqueous samples.

Many special sample holders have been constructed. These range from a simple glass cover slip used to support phospholipid multilayers, to the complex arrangement shown in Fig. 2. The sample holder of Fig. 2 was designed to permit simultaneous nerve excitation and ESR signal recording. Note that the sample itself (the lobster nerve) is quite small and most of the supporting structure is made of thin plastic and other dielectric materials.

![Diagram of ESR sample cell](image)

**Fig. 2.** One example of a specialized ESR sample cell. A nerve chamber for stimulating and recording the action potential in the ESR cavity. This sample configuration permits the observation of spin-labeled nerves during nerve stimulation. (a) Top view, (b) side view. [From Calvin et al. (1969)].

With reasonable care, electrodes and other small objects may be placed in the cavity. The presence of moderate quantities of metal can drastically alter the characteristics of the microwave cavity. Another kind of sample holder, designed for reproducible sample insertion into the cavity, is described by Gaffney and McNamee (1974).

Some experimental applications require controlling the humidity of the sample. This is readily accomplished by placing the sample over a reservoir containing an appropriate saturated salt solution for which the equilibrium relative humidity has been reported (see “Handbook of Chemistry and Physics,” CRC Press, any edition). A simple chamber can be improvised from a 1-cm Pyrex tube approximately 20 cm long, and a small salve jar, as shown in Fig. 3a. The middle 8–10 cm of the Pyrex tube is etched with hydrofluoric acid to reduce the amount of glass in the cavity. The sample is supported in the tube either on glass wool (Fig. 3b) or on a thin glass cover slip held in a split tubing, the other end of which is slipped over a plastic rod with a rubber stopper at the top to close the chamber (Fig. 3a). The Pyrex tube, with sample inserted, is joined to the salve jar containing the salt solution with a one-hole rubber stopper, and the sample is allowed to equilibrate. The whole assembly is then inserted into the cavity from the bottom, supported so that the sample, surrounded by the thinned glass wall, is in the center of the cavity. Standard taper fittings can also be modified for this use (see Fig. 3b). This amount of glass reduces the cavity Q somewhat, but for many spin labeling purposes this much loss of sensitivity can be tolerated. A similar chamber made of polyethylene or quartz may be preferable when higher sensitivity is important.

![Diagram of chamber setup](image)

**Fig. 3.** Example of chambers that can be inserted in the ESR cavity when it is desirable to maintain a spin-labeled sample in equilibrium with a vapor phase.
C. Receiver Gain, Field Modulation Amplitude, and Microwave Power

The gain, modulation amplitude, and microwave power controls are grouped together because they strongly influence the signal amplitude. If any or all three of these controls are set at zero, no signal will be observed. As each variable is increased, the signal amplitude increases, passes through an optimal range, and then either decreases or becomes distorted. The optimal range will depend on the sample and, to a lesser extent, on the ESR spectrometer. The receiver gain needs no elaboration since this control is encountered in all chemical instruments. The receiver gain (also called amplifier gain or signal level control) may be increased until the amplifier becomes unstable and the signal-to-noise ratio decreases. The effect of modulation amplitude is more dramatic, as illustrated in Fig. 4. As the modulation amplitude increases, the ESR lines first increase in height, then broaden, and finally become greatly distorted. A useful rule of thumb is to set the modulation amplitude equal to or less than the ESR linewidth in gauss. In Fig. 4 the peak-to-peak linewidth is 0.5–1.0 G. As seen in Fig. 4, the best choice for the modulation amplitude is also 0.5 G. (For convenience, spectra for this chapter were usually recorded at 1.0 G modulation amplitude. For very accurate line shape studies somewhat lower settings are usually optimal.) A word of caution is needed here. This rule of thumb and other generalizations mentioned in this chapter can be misused. If careful line shape measurements are being made, the investigator should always decrease the modulation amplitude (or other instrument setting) and determine if this has an effect on the line shape. Only if the change in line shape or relative peak heights is insignificant can the setting be considered appropriate.

The effects of microwave power on nitrooxide ESR spectra have not been carefully investigated. In any system, relaxation processes are present that allow the spins to return to the ground state after absorption of microwave energy. If the power becomes too large, however, the relaxation processes are unable to return the spin system to equilibrium, and saturation takes place. The power required to saturate depends on the spin–lattice relaxation time. Saturation and relaxation effects will play an important role in future spin labeling experiments involving electron–nuclear and electron–electron double resonance. In most conventional ESR studies, however, saturation should be avoided. It is difficult to determine precisely the point at which saturation becomes significant. One test involves measuring the signal height as a function of the microwave power. Below saturation, the amplitude of the signal varies linearly with the power. As saturation sets in, the amplitude increases at a lower rate and eventually either flattens out or decreases as the power is increased. Some effects of saturation are illustrated.

Fig. 4. The ESR spectrum of a typical nitrooxide as a function of field modulation amplitude. The sample is an aqueous solution containing $5 \times 10^{-4}$ M piperidone nitrooxide ($2,2,6,6$-tetramethyl-piperidine-1-oxyl-4-one) at room temperature. The microwave power, scan time, scan range, and filter time constant are 5 mW, 4 min, 100 G, and 0.3 sec, respectively.
in Fig. 5. Figures 5a and 5b represent nitroxides at room temperature in water and in an aqueous sodium dodecyl sulfate solution, respectively. The relative peak heights change with increasing microwave power. Rotational correlation times are calculated from peak heights (or line shapes) and will be in error if saturation occurs. Figure 5c illustrates another possible pitfall. These spectra were recorded for a doxylstearic acid in an aqueous phospholipid dispersion. The concentration and pH of the solution were adjusted so that the nitroxide was present in both the aqueous phase (sharp lines) and in the phospholipid vesicles (broad lines). As the microwave power is increased, the sharp lines saturate more rapidly than the broad lines. Equilibrium constants based on relative line heights at higher powers would underestimate the nitroxide concentration in the aqueous phase. Microwave power of 1-5 mW appears to be acceptable for room-temperature spin labeling studies. Some saturation may be occurring at these power levels, but the effects evidently are not serious. For spectra of nitroxide free radicals at liquid nitrogen temperatures the power level should be reduced to 1 mW or lower to avoid saturation effects. Once again, the investigator should check the results by decreasing the power and looking for changes in the ESR spectrum.

D. Scan Time, Filter Time Constant, and Field Inhomogeneity

Improper adjustments in these three experimental parameters produce similar line shape distortions. The scan time or sweep time is the time required to vary the dc magnetic field slowly over a specified interval (the scan range). The effect of varying the scan time on a typical nitroxide ESR spectrum with a 100-G scan range is illustrated in Fig. 6. The 4.0- and 8.0-min scans are almost superimposable, whereas the 0.5-min scan is badly distorted. There is a tradeoff here between the quality of the spectrum and the amount of time required to record it. In this example a 4.0-min scan appears to be a good choice, but if the spin-labeled sample is stable, the investigator may choose the 8.0-min scan time. It is interesting to note that the same effect is evident in the much broader spectrum of a membrane model system (Fig. 6b). Of course, in both samples the distortion caused by shortening the scan time can also be obtained by increasing the scan range while holding the scan time constant.

Nearly all chemical instruments have filter networks to increase the signal-to-noise ratio, and an ESR spectrometer is no exception. The effect of varying the filter time constant, given a fixed scan time and scan range, is shown in Fig. 7. In this example distortion occurs in all but the top spectrum. The rule to follow is that the time constant must be much shorter than the time required to sweep through the ESR line. In this example the scan
Fig. 6. The dependence of nitroxide ESR on scan time. (a) Spectra for 5 × 10⁻⁴ M piperidone nitroxide in water at room temperature. (b) Spectra for a room-temperature aqueous dispersion containing 10⁻⁴ M 12-doxylstearate, methyl ester, and 1 wt % egg lecithin (pH 7). For all spectra the microwave power, modulation amplitude, scan range, and filter time constant are 5 mW, 1 G, 100 G, and 0.3 sec, respectively. The gain setting is the same for all spectra.

Time was 4 min and the scan range was 100 G. One ESR line covers about 2 G. Therefore it takes about (4/100) × 60 = 2.4 sec to scan through each ESR line. It is easy to see why the 0.1-sec time constant was the only reasonable choice. To avoid distorted spectra, it is good practice to check the time constant, scan time, and scan range experimentally. The bottom spectrum of Fig. 7 represents a special case of signal distortion. In this example the time constant of the filter network is so large that the line shape approximates the integral of the first derivative curve. This behavior is expected of a simple filter circuit (consider, for example, a resistor in series with a capacitor). However, as discussed below, the digital computer provides a better method of integration.

The distortion caused by field inhomogeneity is illustrated in Fig. 8. The inhomogeneity of the dc magnetic field decreases from the top spectrum to the bottom spectrum. The inhomogeneity was created by moving the cavity various distances from the center of the magnet. Field inhomogeneity of this magnitude is not ordinarily observed in commercial 9.5-GHz instruments.

Fig. 7. An example of distortions introduced through improper choice of the time constant. The sample is the same as for Fig. 3. The microwave power, modulation amplitude, scan time, and scan range for all spectra are 5 mW, 1 G, 4 min, and 100 G, respectively.

Fig. 8. Nitroxide spectra distorted by an inhomogeneous magnetic field. The sample is the same as for Fig. 3. The microwave power, modulation amplitude, scan time, scan range, and filter time constant are 5 mW, 1 G, 4 min, 100 G, and 0.3 sec, respectively. The gain setting is the same for all spectra, and the field inhomogeneity increases from top to bottom.

In 35-GHz spectrometers, special dual-cavity applications, or when using older magnet systems, the problem is common. It can usually be corrected by adjusting the position of the microwave cavity. The reason for including this effect is to point out that the bottom spectrum of Fig. 8 is almost identical to the top left spectrum of Fig. 6 and the third spectrum of Fig. 7. Fortunately, this is one of the few examples of a line distortion that has several possible causes.

E. Degassing and Concentration Effects

Anyone performing spin labeling experiments soon encounters oxygen-nitroxide interactions and nitroxide-nitroxide interactions. The ground state of molecular oxygen is a triplet and oxygen is therefore paramagnetic. It is rarely observed directly by ESR, but oxygen does interact with the spin label through exchange and dipolar mechanisms. The result is the well-known oxygen broadening, illustrated in Fig. 9. The top spectra are of the
Fig. 9. Effects of oxygen broadening on the ESR spectrum. The spectra were taken on $10^{-4}$ M solutions of piperidone nitroxide in (a) water, (b) methanol, and (c) chloroform, respectively. The top spectra were recorded on samples equilibrated with the atmosphere. The bottom spectra were recorded after nitrogen was bubbled through the solutions at room temperature for 30 sec. The microwave power, modulation amplitude, scan time, scan range, and filter time constant are 5 mW, 1 G, 4 min, 100 G, and 0.3 sec, respectively. All spectra were recorded at room temperature and at the same gain setting. Reversibility was tested by bubbling air through the sample to verify that concentrations were not altered by the degassing procedure.

Piperidone nitroxide dissolved in water, methanol, or chloroform at room temperature. Although it is not obvious from the spectra, the three solutions contain approximately equal concentrations of the nitroxide. Considerably more oxygen broadening is observed in methanol and chloroform (and other hydrocarbon solvents) than in aqueous solutions. The oxygen may be removed either by the freeze–thaw method or by bubbling a good grade of nitrogen or argon gas through the samples. The bottom spectra of Fig. 9 were recorded after nitrogen gas was passed through the solutions.

Fig. 10. Concentration effects on nitroxide ESR spectra. (a) Spectra recorded on varying concentrations of a long-chain nitroxide, piperidinol nitroxide ester of dodecanoic acid, in dodecane (degassed) at room temperature. (b) Spectra taken using varying concentrations of the same nitroxide in an aqueous solution containing 0.5 wt % sodium dodecylsulfate at room temperature. The nitroxide concentration is the same for the two spectra in each row and the actual value is written between the two spectra. The microwave power, modulation amplitude, scan time, scan range, and filter time constant for all spectra are 5 mW, 1 G, 4 min, 100 G, and 0.3 sec, respectively, with variable gain settings.
for approximately 30 sec. The effects are completely reversible. After passing air through these same samples, the spectra are indistinguishable from the top row of Fig. 9.

Nitroxide–nitroxide interactions also occur via the familiar dipolar and exchange mechanisms. The effect of increasing the nitroxide concentration of piperidino nitroxide ester of dodecanoic acid in degassed decane at room temperature is shown in Fig. 10a. The sharp three-line spectrum remains essentially unchanged until the concentration exceeds $10^{-3}$ M. As the concentration is increased beyond this point, the three lines gradually broaden and move together. At $6 \times 10^{-3}$ M, the three lines are still evident. The exchange-narrowed, single-line spectrum of the pure nitroxide is shown at the bottom of the figure for comparison.

The values given in the center of Fig. 10 represent average concentrations. Local concentrations can exceed these average values, producing unusual results. For example, if the nitroxide were not soluble to the extent of $1 \times 10^{-3}$ M, solid flakes of nitroxide would be present and a superposition of three sharp lines and a single broad line would result. A more subtle example is given in Fig. 10b. In this case the same water-insoluble nitroxide is introduced into an aqueous solution containing small clusters (micelles) of sodium dodecyl sulfate. The equilibrium concentration of nitroxide molecules is very much higher in the micelles than in the aqueous phase. The net result is that the local concentration of nitroxide is much greater than the average bulk concentration, and nitroxide–nitroxide broadening is observed at lower bulk concentrations. At $1 \times 10^{-4}$ M the spectrum consists of three sharp lines. The heights are unequal due to slower rates of tumbling but this effect is not of primary concern here. At $1 \times 10^{-3}$ M all three lines are already greatly broadened. At $5 \times 10^{-3}$ M the three lines are disappearing, and the $3 \times 10^{-2}$ M solution gives a spectrum very similar to that of the pure nitroxide. The point is that it is important to watch for nitroxide–nitroxide interactions even when the average concentration of nitroxide is low. With reasonable care, errors in interpretation can be avoided and nitroxide–nitroxide interactions will provide valuable information about the system under investigation.

II. THE USE OF COMPUTERS TO PROCESS SPIN LABELING DATA

Small computers appropriate for interfacing with spectrometers are commercially available, and the use of these computers in spin labeling can be very profitable. Many choices are available, but our experience has been with the Varian 620/i computer, equipped with 8K memory, teletype, and

7. INSTRUMENTAL ASPECTS OF SPIN LABELING

general interface, connected directly to the ESR spectrometer, and more recently with the Varian 620/L-100 16K system used in a time-sharing mode among three spectrometers. Since this section was originally written, many other systems have been developed and marketed for digitizing and manipulating spectroscopic data. Given the memory to store several sizable arrays in addition to the necessary program and the software capability for redefining the start and end of arrays (operationally this amounts to horizontally shifting one spectrum into register with another spectrum), in principle the data manipulations described here are quite straightforward. They can be performed either with a dedicated minicomputer or with a central computer with interactive graphics. In the University of Oregon system, developed by Prof. Charles Klopferstein, the computer controls the ESR spectrometer through commands from the teletype and digitizes the data as the spectrum is collected. Various data treatments described below can be performed, either as the points are collected or immediately after collection. The data can also be transferred to paper or magnetic tape and reentered into the computer for later processing. With ESR spectra reduced to digital form, it is possible to perform a number of operations that are tedious or impossible to perform by hand. Among these are time averaging, correction for a slanting baseline, scaling spectra, integration, and spectral titration.

A. Time Averaging

The earliest application was time averaging and was first used in ESR by Klein and Barton (1963). In this application, several approaches are possible, and all improve the signal-to-noise ratio in a very noisy spectrum by repeated scanning. The change in signal-to-noise ratio is proportional to $n^{1/2}$, where $n$ is the number of scans. We have found most useful the procedure of adding successive scans, dividing each point by the number of scans, and instructing the computer to list the number of scans on the teletype. The resulting spectrum can be plotted out at any time during the repeated scans, either on an external recorder or on the spectrometer recorder itself. It is preferable to increase the signal-to-noise ratio by introducing more label or concentrating the sample, but, if this is not feasible, time averaging can be very useful in improving the quality of the spectrum and revealing otherwise inaccessible details.

B. Scaling Experimental Spectra

One simple application that we have routinely found useful is the scaling of experimental spectra. This is illustrated in Fig. 11, where three spectra were collected using the same sample. The sample is the spin label 5-doxylosteic acid in an aqueous dispersion of lecithin: cholesterol (molar
The instrumental variables of scan range, gain, and phase reversal contribute to the apparent differences in the experimental spectra (left column, Fig. 11). In the right column these spectra have been replotted to the same horizontal scale and normalized to an arbitrary vertical scale. It is now clear that all three experimental spectra are identical. In practice, such large variations may not be encountered, but when comparing closely related spectra, this procedure makes clear small differences (conversely, small apparent differences frequently disappear). Scaling can also be proportional to the relative spin label concentrations. This involves integration of the spectra and examples will be given in Section C.

Frequently experimental spectra are compared to computer-calculated theoretical line shapes, in order to estimate molecular motion or order in the samples. In principle, the calculated line shapes can be scaled individually to each experimental spectrum. In practice, it is simpler to scale the experimental spectra to the calculated spectra. A typical example is given in Fig. 12.

C. Integration of the Experimental Spectrum

Figure 13 illustrates integration of first derivative ESR spectra. We include this example to show how difficult it is to estimate by eye the relative concentrations from peak heights. The nitroxide concentration present varies as the product of the linewidth squared times the height of the first derivative ESR lines. Thus, if the ESR linewidth increases by a factor of 10 upon binding of the spin label, the height of the bound spectrum will be reduced 100-fold and small concentrations of rapidly tumbling spin label can almost obscure a much larger concentration of the bound spin label present. The bottom row of Fig. 13 consists of the familiar first derivative ESR spectra, representing examples of three different mobilities of nitroxide spin labels. Integrating once yields the absorption spectra, analogous to the familiar absorption spectra of optical absorption or nuclear magnetic resonance spectroscopy (middle row of Fig. 13). A second integration (i.e., integrating the absorption spectrum) has been performed to determine relative concentrations, and the results are shown in the top spectra of Fig. 13. The heights $I_0$, $I_b$, and $I_c$ are proportional to the concentrations of the probe, and $I_b : I_c : I_0$ is approximately 1 : 19 : 40. For purposes of illustration, the middle line of each of the first derivative spectra in Fig. 13 has been arbitrarily adjusted to the same height before integration. When this height is held constant, the relationship between ESR linewidth and concentration is dramatically evident. A small concentration of freely tumbling nitroxide has a relatively large first derivative peak height. It can be readily seen from Fig. 13 that quite a low concentration of unbound spin label in the presence of bound label may dominate the composite spectrum obtained.
7. INSTRUMENTAL ASPECTS OF SPIN LABELING

If the data are digitized, combinations of spectral subtraction and subsequent double integration can be used to determine relative concentrations of label in the two environments. It is necessary to obtain or simulate the spectrum of at least one component. For example, the spectrum of the spin label in the aqueous phase can be recorded separately, or samples with and without the protein or lipid-depleted samples can be used. The next step is to adjust the spectra for slanting baseline and offset so that the baseline value is zero. The spectra are then shifted horizontally relative to each other so that they are in register. Finally, incremental subtractions are carried out until an obvious endpoint is reached (e.g., a negative baseline or phase reversal of peaks). An example is given in Fig. 14. To simulate a typical experimental

![Diagram](image.png)

**Fig. 13. Integration by computer.** (a) Freely tumbling nitroxide: the same sample as for Fig. 3; (b) moderately immobilized nitroxide: 16-doxylstearic acid in an aqueous dispersion of lecithin; (c) strongly immobilized nitroxide: 5-doxylstearic acid in an aqueous dispersion of lecithin: cholesterol, molar ratio 2:1. All spectra were recorded at room temperature. The middle lines of all three first derivative ESR spectra are of the same height.

**D. Spectral Titration**

Another problem frequently encountered in spin labeling experiments is the occurrence of composite spectra. This happens whenever the nitroxide partitions between two or more environments. For example, when a lipid spin label is diffused into an aqueous suspension of membranes a complex spectrum is often obtained. It may be due to the sum of the two spectra arising from equilibrium concentrations of aqueous and membrane-associated probes. Composite spectra are also encountered when the lipid spin label partitions between the fluid bilayer phase and sites on the hydrophobic protein surface within the membrane. Other examples occur in antibody-antigen studies and enzyme spin labeling experiments. A set of composite spectra is often obtained as a result of some experimental variable such as concentration, temperature, or pH. The related spectra may exhibit common points of intersection when the spectra are superimposed. These isoclinic points are usually the result of isokinetic points in the absorption spectra and may well indicate the presence of a simple equilibrium, although some caution must be used in this kind of interpretation (Marriott and Griffith, 1974).

![Diagram](image.png)

**Fig. 14. Spectral subtraction by computer.** (a) A room-temperature, composite spectrum. Two capillary tubes were placed in the cavity, one containing an aqueous dispersion of $1.5 \times 10^{-3} M$ 5-doxylstearic acid in lecithin, the second containing $5 \times 10^{-3} M$ piperidine nitroxide in water. (b)-(e) Are traces produced by subtracting increasing amounts of the sharp three-line spectrum from the composite spectrum, with total subtraction occurring in spectrum (d). In spectrum (e), too much of the sharp three-line spectrum has been subtracted (note the three sharp lines of this spectrum are phase-reversed). [From Jost et al. (1971)]
7. INSTRUMENTAL ASPECTS OF SPIN LABELING

concentrations of the spin label present. In Fig. 15, $I_c = 236$, $I_b = 7$, and $I_a = 222$ in arbitrary units. Note that $I_c$ very nearly equals $I_b + I_a$. Since this figure was made we have added enough memory to use double precision arrays to store the integrals (i.e., each point in the array can be used without prior division by a constant) and the accuracy has been somewhat improved. A common source of much larger error is, of course, the presence of a nonzero baseline in the first derivative spectrum, which is easily detected by displaying or plotting the first integral. If the last 5–10 G of the absorption curve gives a nonzero average value, this way of determining concentration would be inappropriate.

The actual concentrations of nitroxides in the two capillary samples used in this example were $C_a = 5 \times 10^{-5}$ M and $C_b = 1.5 \times 10^{-3}$ M. As another check the ratio $I_a/I_b = 32$ is in good agreement with the known ratio of concentrations $C_a/C_b = 30$.

We have discussed our general system and the software we use elsewhere (Klopfenstein et al., 1972; Jost et al., 1971), and the interested reader can consult these for more detail and other applications.

ACKNOWLEDGMENTS

We are grateful to Miss Des Brightman for technical assistance and to Professor Charles Klopfenstein for helpful discussions. This investigation was supported by Public Health Service Research Grant No. CA-10337 from the National Cancer Institute.

REFERENCES


---

The Use of Spin Labels for Studying the Structure and Function of Enzymes

JOEL D. MORRISETT

DEPARTMENT OF MEDICINE
BAYLOR COLLEGE OF MEDICINE
HOUSTON, TEXAS

I. Introduction
II. Reagents for Spin Labeling Enzymes
   A. Covalently Binding Spin Labels
   B. Noncovalently Binding Spin Labels
III. Laboratory Techniques for Spin Labeling Enzymes
   A. Labeling the Enzyme
   B. Properties of Spin-Labeled Enzymes
   C. Sample Measurement
IV. Information Obtainable from Spin-Labeled Enzymes
   A. Rates of Catalysis
   B. Mechanisms of Denaturation
   C. Distances between Different Groups
   D. Polarity of Binding Sites
   E. Protein Symmetry
   F. Active-Site Geometry
   G. Changes in Active-Site Conformation Caused by Ligand Binding or Proteolysis
   H. Rotational Correlation Times of Enzymes
V. Detailed Studies of Spin-Labeled Enzymes
   A. Lysozyme
   B. Ribonuclease
   C. DNA Polymerase
   D. Citrate Synthase
   E. Phosphofructokinase
   F. Creatine Kinase
   G. Alcohol Dehydrogenase
   H. d-Glyceraldehyde-3-phosphate Dehydrogenase (GPDH)
   I. Chymotrypsin
   J. Trypsin
   K. Subtilisin

274
the achievement of an impressive series of accomplishments in this area. One of the techniques that has made highly significant contributions to our understanding of enzyme structure is spin labeling.

Many reagents for the chemical modification of specific side chains in proteins had been developed by the time the first spin-label experiment was reported (Ohnishi and McConnell, 1965). Several of these reagents were quickly and easily altered to include the nitroxyl moiety, thereby generating a series of covalent spin-label reagents. Some of these reagents were found to react with several side chains, giving rise to mixed EPR spectra, which could not always be interpreted unambiguously. This problem was solved in part by the refinement of site-specific methodology (Singer, 1967), which has culminated in the elegant technique of affinity labeling.

Although the successful application of spin labeling to protein structure determination has depended heavily on the state of the art of protein chemistry, the single most important factor responsible for the widespread use of this method in any area has been the intrinsic magnetic properties of the nitroxyl radical itself. These magnetic properties of nitrooxides have, in turn, allowed the measurement of a variety of important structural properties of enzymes, a description of which is the subject of this chapter.

II. REAGENTS FOR SPIN LABELING ENZYMES

A. Covalently Binding Spin Labels

A wide selection of covalently binding spin labels has been synthesized, and many of these have been used to study various enzyme systems. The more common ones are commercially available from such companies as Syva, Aldrich, or Frinton.† Procedures or references for the synthesis of many of these labels are given in Chapter 5. Covalently binding spin labels may be divided into four general categories according to the type of reaction they undergo: alkylation (Fig. 1), acylation (Fig. 2), sulfonation (Fig. 3), and phospho(acy)ylation (Fig. 4).

I. ALKYLATING SPIN-LABEL REAGENTS

The reaction of proteins with spin-labeled alkyl halides is conveniently monitored by measuring the release of halide ion with a halogen electrode. The analogues of bromoacetamide (I, VIII in Fig. 1) are especially useful because acid hydrolysis of a protein labeled with either reagent yields the carboxymethyl derivative of the alkylated amino acid(s) (see Section III.A.1). The most common sites of reaction for these reagents are the

† Editor's note: Addresses for these and other manufacturers may be found in Appendix III, p. 566.
8. SPIN-LABELLED ENZYMES

sulphydryl group of cysteine, the imidazole group of histidine, the ε-amino group of lysine, and the thioether of methionine. The carboxymethyl derivatives of each of these amino acids have been isolated and characterized (Gundlach et al., 1959). These derivatives exhibit characteristic elution times on the amino acid analyzer. Their quantitation allows estimation of the site and extent of spin-label incorporation.

The dibromoketone (II, Fig. 1) may be useful for cross-linking two reactive groups which are near each other (e.g., the two active site histidine residues (His 12 and His 119) of bovine pancreatic ribonuclease (Wyckoff et al., 1970) or the adjacent thiol (Cys 25) and imidazole (His 159) in papain (Drenten et al., 1970). Another attractive possibility is the use of this reagent to join two sulphydryl groups resulting from the reductive cleavage of a specific disulfide bond in a protein (Sperling et al., 1969).

The carbodiimide spin label (III, Fig. 1) has been used by Azzi et al. (1973) to inhibit membrane-bound ATPase. Presumably, an unusually nucleophilic group at the active site of this enzyme adds across one of the double bonds of the carbodiimide. This recently developed reagent may also prove useful in studies of enzymes involved in nucleic acid metabolism, since the reaction of a similar carbodiimide with uridine and guanosine bases in nucleic acids has been reported (Metz and Brown, 1969).

The piperidinyl and pyrrolidinyl derivatives of N-ethylmaleimide (IV and V, Fig. 1) have been employed extensively. However, serious problems can attend the use of these labels. The nucleophilic groups (sulphydryl and amino) that normally react with these reagents can either add across the carbon–carbon double bond or attack one of the carbonyl groups. In the latter case, the imide ring opens, thereby increasing the number of “spacer” atoms between the nitroxyl ring and the bond which connects the reagent to the protein. If both reaction mechanisms occur during labeling of a single group on the protein, the resulting EPR spectrum will most likely be a mixed one containing two components. In some cases, workers have interpreted such spectra as binding of the label at two or more different sites on the protein. In aqueous solutions, the pyrrolidinyl derivative (V, Fig. 1) has been found to hydrolyze rapidly to an unreactive product (Smith, 1968). Furthermore, Barratt et al. (1971) have shown that unless the reaction conditions are carefully controlled during the synthesis of maleimide V (Fig. 1), the isomaleimide VI will also be produced. Albumin spin-labeled with maleimide V (Fig. 1) gave an EPR spectrum quite different from that obtained when the isomaleimide (VI, Fig. 1) was used.

Reagent VII (Fig. 1) is an active-site-directed analogue of p-toluene sulfonilphenylalanine chloromethyl ketone (TPCK), which selectively alkylates His 57 of chymotrypsin (Kosman, 1972). While several noncovalently
binding affinity spin labels have been synthesized (e.g., Fig. 5), few such covalent derivatives have been made. These specific reagents are of great value in obtaining high levels of labeling at a single site, an important consideration in distance-measuring experiments.

Caryophyllene iodonitrosite (IX, Fig. 1) contains a very bulky alkyl substituent that might be useful for studying spacious active or binding sites (Hawley et al., 1967). However, due to extensive proton coupling, the paramagnetic resonance lines of this reagent are rather broad (3.5 G), thereby limiting its usefulness (Morrisett, unpublished data). At the other extreme is X (Fig. 1), which represents one of the smallest covalent spin labels yet synthesized (Motherwell and Roberts, 1972). This molecule, like XV (Fig. 1), enjoys the advantage of having only an interposed methylene group between the nitroxyl ring and the bond joinin it to the protein. Such spin labels are often more sensitive to conformational changes of proteins than compounds that have several intervening atoms between the ring and the protein. Spin labels that are closely attached to a protein will often have their motion strongly impeded. In such cases, these labels can be used to determine the rotational correlation time of the protein molecule (Shimshick and McConnell, 1972).

Cyanuric chloride has been derivatized with 1-oxy-2,2,6,6-tetramethyl-4-aminopiperidine to give XII (Fig. 1). This reagent has been used by Likhentshtein and Bobodzhanov (1969) to tag histidine and lysine side chains of albumin. It, like the dibromoketone (II, Fig. 1), possesses two potentially reactive halogens and may be useful as a cross-linking spin label.

The isothiocyanate nitroxide (XIII, Fig. 1) may be used to carbamylate amino groups (McConnell and McFarland, 1970). This reagent is considerably more stable than the corresponding isocyanate originally prepared by Stone et al. (1965).

1-Oxy-2,2,6,6-tetramethylpiperidine-4 (XIV, Fig. 1) reacts with a free amino group to form a Schiff base, which upon reduction by sodium borohydride, yields a stable N-alkyl derivative (Wagner and Hsu, 1970). There is no apparent reduction of the nitroxide under the conditions used by these workers (excess sodium borohydride, several hours at pH 9 and 0°C).

Compound XVIII (Fig. 1) has been used to label the active site SH group of D-glyceradldehyde-3-phosphate dehydrogenase (Balthasar, 1971). This reagent, like I and VIII (Fig. 1), is a derivative of haloacetate acid, and therefore will give a stable carbonamino acid that is quantifiable by amino acid analysis. The aldehyde XVII (Fig. 1) has been used to alkylate the same enzyme. Both of these spin labels might be useful for studying the active sites of lipid hydrolyzing enzymes such as phospholipase A, lecithin : cholesterol acyltransferase, or lipoprotein lipase.

2. ACYLATING SPIN-LABEL REAGENTS

The acylating spin-label reagents have been used extensively to tag reactive hydroxyl, thiol, or amino functionalities of proteins. These compounds generally exhibit greater specificity than the alkylation reagents but less than the sultonolating or phosphorylating reagents. They do not allow an advantage afforded by many of the alkylation spin labels, namely, the formation of amino acid derivatives that are stable to conditions used for total protein hydrolysis (i.e., 6 N HCl, 110°C, 24 hr).

The succinic amide ester, succinic diester, and maleic diester (I, II, III in Fig. 2) have been used by Kosman et al. (1969) to study the active site geometry of α-chymotrypsin. These reagents, along with IV, V, IX, and X (Fig. 2) contain the nitrophenyl leaving group. The release of this chromophore may be monitored spectrophotometrically (Flohr et al., 1972), providing a convenient method for studying the kinetics of the labeling reaction.

![Chemical structures for acylating spin label reagents that have been used to probe the structure and function of enzymes. Some pertinent references to either the synthesis and/or use of these molecules are: I. Kosman et al. (1969); II. Kosman et al. (1969); III. Kosman et al. (1969); IV. Berlinsky and McConnell (1966); Flohr et al. (1971); Flohr and Kaiser (1972); Ament et al. (1973); V. Berlinsky (1972); VI. Griffith et al. (1967); VII. Barchatt et al. (1969); VIII. Hoffman et al. (1969); IX. Balthasar (1971); X. Sparrow (1973).](image-url)
The mixed carboxylic-carbonic acid anhydride VI (Fig. 2) is a highly reactive reagent that has been used to acylate the ε-amino groups of poly-L-lysine (Griffith et al., 1967). Apparently, the acylation reaction successfully competes with the hydrolysis reaction in aqueous solution. A possible disadvantage of this reagent is that the nonparamagnetic R group, instead of the paramagnetic nitroxy moiety, may become attached to the protein or peptide, in which case undetectable urethane formation would occur. A spin-labeled analog of N-acetylimidazole (VII, Fig. 2) has been synthesized by Barratt et al. (1969) and used to tag the phenolic hydroxyl groups of poly-L-tyrosine. This reagent appears to exhibit some degree of specificity since treatment of an equimolar mixture of poly-L-tyrosine and poly-L-lysine resulted in preferential labeling of tyrosine residues. This reagent might be useful for studying the active site of carboxypeptidase A, which bears a tyrosine side chain at this locus (Lipscomb et al., 1970).

The N-hydroxysuccinimide ester of a spin-labeled carboxylic acid (VIII, Fig. 2) has been used to acylate the ε-amino group of valyl-RNA. The attached label was used to monitor thermal unfolding of the amino acid–nucleic acid complex (Hoffman et al., 1969).

The spin-labeled carbonate X (Fig. 2) is a very convenient acylating agent, which upon reaction with amino groups yields a urethane. This reagent has been used to label the deblocked amino terminus of peptide polymers obtained during the solid phase synthesis of apolipoprotein fragments (Sparrrow, 1975). The accessibility of the amino terminus of a growing peptide (on the polymer) has been estimated from the EPR spectrum and has been studied as a function of the swelling solvent and the type of polymer support. An obvious advantage of this reagent is that the group attached to the amino terminus can be removed by standard deblocking procedures and the synthesis continued.

3. SULFONYLATING SPIN-LABEL REAGENTS

An array of fifteen different ortho-, meta-, and para-substituted spin-labeled sulfonating agents has been synthesized by Berliner and colleagues to study and compare the active sites of trypsin and α-chymotrypsin (Berliner and Wong, 1974; Wong et al., 1974). Several of these compounds were insoluble in water alone and also underwent hydrolytic decomposition in aqueous solutions, making it necessary to add them as dioxane solutions and portionwise over a period of time. These technical problems prevented carrying out accurate kinetic measurements of the inhibition reaction. The release of the paramagnetic center from the reagent before sulfonation of the enzyme resulted in a mixture of sulfonlated enzyme molecules that did and did not carry a spin label. Once the intact reagent became attached to

the protein, there appeared to be no release of the nitroxide from the aromatic moiety. There was, however, slow desulfonation, a process that had been documented earlier by Fahrney and Gold (1963). In spite of these difficulties, Berliner and Wong (1974) were able to obtain very meaningful data by use of these labels (Fig. 3).

![Chemical structures of sulfonating spin-label reagents used by Berliner and Wong (1974) and Wong et al. (1974) in studies comparing the active site geometries of α-chymotrypsin and trypsin.](image-url)

Fig. 3. Chemical structures of sulfonating spin-label reagents used by Berliner and Wong (1974) and Wong et al. (1974) in studies comparing the active site geometries of α-chymotrypsin and trypsin.
4. PHOSPHONYLATING SPIN-LABEL REAGENTS

The organophosphorus spin labels were first introduced by Hsia et al. (1969) and Morrisett et al. (1969). These reagents are highly specific for the nucleophilic serine residue at the active site of many enzymes. However, there are at least two known exceptions to this specificity. Reagent I (Fig. 4) has been used to spin label the unusually reactive tyrosine residues of papain and lysozyme (Morrisett, unpublished experiments). The nonparamagnetic reagent diisopropylphosphorofluoridate (DFP) has been shown to react with Tyr 123 of papain (Chaiken and Smith, 1969) and with Tyr 20 and Tyr 23 of lysozyme (Kato and Murachi, 1971). Retention of activity by the

\[ \text{Fig. 4. Chemical structures of phosphorylating (phosphonylating) spin-label reagents which have been used to study the active site region of serine esterases and/or proteases. Pertinent references are: I. Morrisett et al. (1969); Morrisett and Broomfield (1971, 1972); Berliner and Wong (1973); Hoff et al. (1971). II. Morrisett and Broomfield (1971); Shmismick and McConnell (1972). III. Berliner and Wong (1973). IV. Hsia et al. (1972). V. Hsia et al. (1969). VI. Hsia et al. (1969). VII. Struve and Goldstein (1971). VIII. Hsia et al. (1972). IX. Grigoryan et al. (1973). X. Grigoryan et al. (1973).} \]

...phosphorylated enzymes and X-ray crystallographic data of the native molecules (Drenth et al., 1970; Phillips, 1967) indicate that none of these tyrosine residues is involved in catalysis.

Most of the phosphorylating agents shown in Fig. 4 are extremely toxic and should be handled with rubber gloves in a well-ventilated hood. It is wise to keep 1.0 M NaOH nearby for rinsing glassware exposed to the agent and hydrolyzing any spilled material. Generally, these reagents react...
very rapidly. For example, 125 mg of chymotrypsin (5 μmoles) in 5 ml buffer was inhibited by 50 μmoles of I (Fig. 4) to the extent of about 95% after 5 min (Morrisett and Broomfield, 1972).

The phosphor(n)ylating and sulfonfylating reagents give spin-labeled protein derivatives that are usually more resistant to hydrolysis (i.e., hydrolysis that results in regeneration of active enzyme) than the acylating reagents. This is graphically illustrated in Fig. 5, which compares the time dependence of nitroxide released at pH 6.6–6.8 (as determined by increase in amplitude of the narrow line component) from a-chymotrypsin spin labeled with piperidinyl phosphate I (Fig. 4) and pyrrolidinyl carboxylate IV (Fig. 2). According to the model of Steliz et al. (1969), this stability of the phosphor(n)yl and sulfonfyl analogues of acyl enzymes is due to the steric exclusion of an activated water molecule in the vicinity of the atom (phosphorus or sulfur) which is normally attacked.

Phosphor(n)ylated enzymes can undergo a process known as “aging,” whereby an alkyl or alkoxy group is released from the enzyme while the phosphorous moiety remains attached. This process may be acid or base catalyzed. In the case of acid catalysis, a carbonyl ion is released (Fig. 6), whereas when the process is base catalyzed, an alkoxide ion or an olefin is released. The aging phenomenon can give rise to mixed EPR spectra that contain narrow line components which are not the result of hydrolytic dephosphor(n)ylation, since the aging is not accompanied by return of enzymatic activity. This problem was essentially ignored by Hoff et al. (1971) in a spin-label study of atropinesterase, subtilisin, and chymotrypsin. Aging has been shown to be an important consideration in studies on acetycholinesterase (Morrisett et al., 1970a,b) inhibited with phosphorylating reagents I or II (Fig. 4). The effect of pH, ionic strength, and temperature on the aging of phosphorylated cholinesterases is well known (Kejier et al., 1974).

Reagents I and II (Fig. 4) have been used by Morrisett and Broomfield in studies on the mechanism of guanidine-induced denaturation of α-chymotrypsin (Morrisett and Broomfield, 1971) and in studies comparing the active site geometries of trypsin, chymotrypsin, subtilisin, elastase, and acetycholinesterase (Morrisett and Broomfield, 1972). Phosphorylating reagent I and phosphorylating reagent III (Fig. 4) have been employed by Berliner and Wong (1973) to elucidate a subtle autolysis effect observed with spin-labeled trypsin. The biradical phosphate IV and mono-radical phosphate VIII (Fig. 4) have been used by Hsia et al. (1972) and Kosman and Piette (1972) in active-site studies of cholinesterase, chymotrypsin, trypsin, elastase, and thrombin. Phosphor(n)ylating reagents for which the leaving group is p-nitrophenoxide (rather than fluoride) have been employed by Grigoryan et al. (1973) to investigate the urea denaturation of α-chymotrypsin (IX, X, Fig. 4) and by Struve and Goldstein (1971) to spin label serine esterases in synaptic membranes (VII, Fig. 4).

B. Noncovalently Binding Spin Labels

Spin-labeled ligands that are bound to proteins by forces other than covalent chemical bonds (e.g., hydrophobic, ionic, and hydrogen bonding) undergo chemical exchange with unbound species. The strength of these interactions will govern the residence time of the ligand and may affect the motional freedom of the nitroxy group attached to that ligand. If the affinity of the protein for the ligand is great enough, an EPR spectrum of only the bound species may be obtained (e.g., Chignell et al., 1972). For lesser affinities, a mixed spectrum of bound and unbound species may result (e.g., Ogata and McConnell, 1972). When the association constant is quite low, the spectrum of the bound species will be masked by that of the unbound species. In favorable cases where the spectral amplitude of the bound label does not significantly increase the amplitude of the unbound species,

† Editor's note: The name "spin probe" is also used to distinguish noncovalent from (covalently bound) spin labels.
the latter quantity may be used to calculate an association constant for the ligand (Wien et al., 1972). The affinity and specificity requirements of noncovalently binding spin labels are major considerations in designing experiments that employ these molecules. Both of these parameters must be accurately known for distance-measuring experiments to be meaningfully interpreted (Wien et al., 1972).

A number of spin-labeled coenzymes have been prepared and gainfully utilized. A nitroxyr derivative of ATP (Fig. 7) has been used by Krugh (1971) to measure distances between groups in the ATP and AMP binding sites on DNA polymerase. A phosphate group of ADP has been esterified with 1-oxyl-2,2,6,6-tetramethylpiperidin-4-ol giving an analogue of NAD (II, Fig. 7) which was used in a study of alcohol dehydrogenase (Weiner, 1969; Mildvan and Weiner, 1969a,b). These workers were able to measure the distance from the coenzyme binding site to the sites occupied by substrates or inhibitors such as ethanol, acetaldehyde, and isobutyramide. By coordination of the nitroxy oxygen to the cobalt atom of cobinamides, spin-labeled derivatives of methylcobinamide and S'-deoxyadenosylcobinamide (III, Fig. 7) have been prepared (Law et al., 1971). An analogue of vitamin B₁₂ has been prepared by alkylation of Co⁺⁺ 5,6-dimethylbenzimidazolylcobamid with the piperidinylbromonamide VIII (Fig. 1). Homolytic cleavage of the cobalt–carbon bond could be brought about by photolysis, and EPR could be used to follow the kinetics of this process (Buckman et al., 1969). Two analogues of coenzyme A (VII and VIII, Fig. 7) have been synthesized and used to study the active site of citrate synthase (Weidman et al., 1973).

Glycosides of N-acetylglucosamine (V) and di-N-acetylglicosamine (IV) with 1-oxyl-2,2,5,5-tetramethyl-4-hydroxymethylpyrrolidine as the aglycone have been prepared by Wien et al. (1972). These spin-labeled sugars were used to measure the distance from subite D to His 15 on lysozyme. The pseudosugar piperidinylacetamide VI (Fig. 7) was originally expected to bind at one of the six subsites in the catalytic region, but X-ray crystallographic data indicated that its highest occupancy was at an anomalous binding site near Trp 123 (Berliner, 1971).
8. SPIN-LABELED ENZYMES

III. LABORATORY TECHNIQUES FOR SPIN LABELING ENZYMES

The success of any spin-label study is highly dependent on careful experimental design and execution. This is especially true of studies designed to yield information about the structure of an enzyme and to correlate that information with what is known about the enzyme's biological function. With all probe techniques that involve the use of an extrinsic reporter group such as a spin label, it is essential not only to anticipate but to evaluate the effect of that group on the structure and function of the system under study. Only when such an evaluation has been made can the results of probe experiments be placed in full perspective and their meaning correctly interpreted. In this section are outlined several techniques that have proven useful in minimizing ambiguity and maximizing information content of results from spin-labeled enzyme experiments.

A. Labeling the Enzyme

1. COVALENT LABELING

The enzyme of interest may be labeled by either covalent attachment or noncovalent binding. The usefulness of covalent labeling depends on the presence and reactivity of amino acid side chains at or near the active site. Covalent labeling experiments usually fall into one of two different categories: those in which the label is attached near the active site and does not severely impair biological function (e.g., Cohn et al., 1971; Jones et al., 1973) and those in which the label is attached to a group in the active site and destroys most or all catalytic activity (e.g., Berliner and Wong, 1974; Morrisett and Broomfield, 1972).

In the ideal case, a single functional group at a desirable location is labeled 100%, and no other groups in the protein are labeled at all. The attainment of such specificity may require special conditions of pH, ionic strength, temperature, reaction time, or reagent concentration. Often this high level of specificity can be achieved by using a spin-label reagent whose structure closely resembles that of a substrate or pseudosubstrate (e.g., Kosman, 1972). A working knowledge of affinity labeling (Singer, 1967) is very useful in the design (Means and Feeney, 1971; Baker, 1967) and synthesis (Rozantsov, 1970; Forrester et al., 1968) of such molecules. Reaction conditions for covalent binding of spin labels will generally be the same as those used for any protein-modifying reagent. However, there are a few buffer constituents that should be carefully considered before use. Thiols such as mercaptoethanol or glutathione (Morrisett and Drott, 1969) can be...
oxidized by spin labels with concomitant reduction of the nitroxy moiety and loss of paramagnetism. Nitroxide reduction is virtually instantaneous with ascorbic acid (Korzub and Mcconnell, 1971). High hydrogen ion concentration (pH < 2) for extended periods leads to destruction of the paramagnetic center. Paramagnetic ions can give rise to EPR spectra that overlap the nitroxide spectrum. Such ions can also enhance electronic relaxation of the spin label, resulting in diminution of spectral amplitude (Taylor et al., 1969; Leigh, 1970). Cobaltous ion has been shown to complex to the nitroxy group (Beck et al., 1967; Buckman et al., 1969; Law et al., 1971). In cases where it is necessary to remove divalent cations from spin-labeled proteins, this can be done by adding excess ethylenediaminetetraacetate (EDTA) and removing the EDTA-Co(II) complex. In experiments where the spin concentration is of critical importance, it is not sufficient merely to add excess EDTA, since EDTA alone can cause anomalous changes in spectral amplitude.

Upon completion of the labeling reaction, excess unbound label can be removed by any one of several convenient techniques. Dialysis (Craig and Chen, 1969) involves minimal equipment but has several disadvantages, including possible loss of protein due to adsorption to the dialysis tubing or escape through the pores, and slow or incomplete removal of unbound label. These difficulties can be minimized by use of Spectrapor™ tubing (Spectrum Medical Industries), which can be obtained with molecular weight cutoffs of 3500, 6000–8000, and 12,000–14,000. Gel filtration (Reiland, 1971) over Sephadex G-10 (Pharmacia) or Bio-gel P-2 (Bio-Rad) removes unbound label much more rapidly and completely than dialysis. In cases where a small amount of unbound label is present due to either incomplete removal or hydrolytic release from the protein (Berliner and McConnell, 1966; Morrisett and Broomfield, 1971), a mixed EPR spectrum containing narrow and broad components may be obtained. In such cases, the narrow spectral component can be removed by computer subtraction techniques (Jost et al., 1971).

At this point in the experiment it is necessary to compare the physical, chemical, and biological properties of the labeled and native enzyme. Changes in secondary, tertiary, or quaternary structure may be assessed by such techniques as ultracentrifugation (Schachman and Edelstein, 1973), circular dichroism (Adler et al., 1973), and intrinsic fluorescence (Brand and Witkop, 1967). The sequence position of modified residues may be determined from trypptic (Canfield and Anfinsen, 1963) or cyanogen bromide (Gross and Witkop, 1962) fragmentation. Which amino acid side chain(s) has actually been tagged can sometimes be determined from a change in the amino acid composition (Spackman et al., 1958). In cases where the bond joining the amino acid and the spin label is labile in 6 N HCl a change in the analysis will not be observed. In some favorable cases, however, a derivative amino acid will result which appears on the analysis chromatogram at a position not masked by other species. For example when ribonuclease was tagged with pyroxlidinylbromocetamide I (Fig. 1), its amino acid composition decreased by one histidine residue and increased by one carboxymethylhistidine residue (Morrisett, 1969; Daniel et al., 1973), which eluted immediately after proline. While the extent of labeling may be determined from the amino acid analysis, values so obtained are not as reliable as those obtained by using a radioactive spin label or by counting the number of incorporated spins. In the latter case, a known amount of accurately determined spin-labeled protein is hydrolyzed in 1.0 M NaOH for 24 hr at 60°C; a portion of the hydrolysate is transferred to a Corning 100 μl capillary sealed at one end, its EPR spectrum recorded under a standard condition, and the spectral amplitude compared to that of a standard solution of the corresponding spin label of known concentration (Wien et al., 1972). The number of incorporated spin labels may also be obtained by double integration of the first-derivative spectrum. Measurements of the biological activity of the labeled protein by at least one and preferably several different assay methods should be carried out and the results of these measurements compared to those obtained from the unlabel protein.

Some reactions may give rise to several differently labeled proteins, in which case the interpretation of spectral changes can be difficult if not impossible. Such a mixture can often be resolved into its components by use of ion-exchange chromatography (Himmelman, 1971), isoelectric focusing (Vesterberg, 1971), or polyacrylamide gel electrophoresis (Shuster, 1971). By using covalently binding spin-label reagents, one exposes himself to the same pitfalls encountered by anyone involved in the general area of protein modification. These include: (a) failing to perform complete amino acid analyses on the modified protein; (b) assuming that retention or loss of enzyme activity toward one class of substrate implies similar behavior toward all classes, and (c) concluding that demonstrated modification or survival of particular residues in model peptides or other proteins may be extrapolated to the case in question. Such pitfalls deserve careful consideration before proceeding to detailed studies on the labeled enzyme.

2. Noncovalent Labeling

Many of the techniques described above for covalently spin-labeled enzymes are equally useful for enzymes to which labels are noncovalently bound. While the spin labels in this latter case need not carry reactive functionalities, they must bear moieties that will confer on the total molecules sufficient affinity for the binding site of interest on the protein. Such spin labels are exemplified by the analogues of ATP (I, Fig. 7; Krugh,
B. Properties of Spin-Labeled Enzymes

The contribution of a nitroxy group to the ultraviolet absorption of the protein bearing it is dependent on the structure of the group and the wavelength of measurement. As shown in Fig. 8, the $\lambda_{max}$ of the oxazolidinyl-, piperidinyl-, and pyrrolidinyl-1-oxyl rings are in the region of 230-340 nm, well removed from the $\lambda_{max}$ of most proteins, which occurs at about 280 nm. The molar extinction of a nitrooxide at this latter wavelength is in the range of 200-800 as compared to 1100 for tyrosine and 5200 for tryptophan. Hence, for a protein such as chymotrypsin, which contains four tyrosines and eight tryptophans (Matthews et al., 1967) and bears one spin label at the active site (Morrisett and Broomfield, 1971), the contribution of the label's absorbance to that of the total molecule is less than 1%.

The ability of nitroxides to induce fluctuating magnetic fields that enhance nuclear relaxation is a well-known phenomenon. The resonance lines of nuclei on a protein become greatly broadened when a spin label is covalently attached. Hence, this effect makes it difficult if not impossible to measure distances between a covalently bound spin label and a nucleus on the same protein molecule. However, the effect is extremely useful for measuring distances to binding sites of chemically exchanging nuclei (Wien et al., 1972; Morrisett et al., 1973).

In some cases it is desirable to concentrate the spin-labeled protein in order to obtain a required optical density or resonance signal amplitude. This is achieved by such techniques as lyophilization, pressure ultrafiltration (Amicon or Millipore), suction ultrafiltration using collodion bags (Schleicher and Schuell), or simply adding an appropriate amount of dry Sephadex to the enzyme solution. While these methods for concentrating samples may be useful, they may also lead to aggregation of the protein. Aggregation, in turn, may give rise to significant changes in the correlation time $\tau_c$ of the spin label and/or particular nuclei on the protein.

C. Sample Measurement

There are a variety of cells, both commercial and home made, that can be used to contain spin-labeled protein solutions. The uniform size of counting calibrated capillaries makes them ideal for spin-count determinations where the sample must be reproducibly oriented in the spectrometer cavity. For routine measurements at room temperature, disposable Pasteur pipets sealed at the smaller end are adequate. For experiments that require use of the temperature controller and maximum signal level, the flat quartz microcell (Scanlon, Wilmad, or Varian) is appropriate. When the controller is not necessary but maximum signal is, the larger quartz flat cell (Varian) is best. Normally a standard 0.5 x 3 x 250 mm EPR tube is not satisfactory for aqueous samples because of high dielectric loss. Selection of proper spectrometer conditions is governed to a large extent by the nature of the sample and the true width of the resonance lines. The effects of different spectrometer settings on the nitroxide spectrum are illustrated in Chapter 7 and in a recent review by Jost and Griffith (1972). The use of computer methods to separate narrow- and broad-line spectral components (corresponding to mobile and immobile spin labels) and to subtract the spectral
contribution from unbound labels (Jost et al., 1971) has been mentioned earlier. Motion parameters (e.g., 2T_{ij}, 2T_{ij}, τ_{ij}, and τ_{ij}) can be rapidly obtained by computer methods (McFarland, 1974).

A spin-labeled enzyme may be obtained in the crystalline form by either labeling the enzyme in solution then crystallizing the labeled protein, or by labeling the enzyme in the crystalline form. For experiments involving crystals of spin-labeled protein (McConnell and Boeyens, 1967; Berliner and McConnell, 1971), reproducible orientation of the sample can be achieved by use of a crystal goniometer (see specifications in Berliner, 1967; such goniometers are commercially available from Varian). In order that the Q of the cavity be retained, the sample is not rotated in the cavity; rather, the laboratory magnetic field is rotated about the cavity. Thus, the larger spectrometers with rotating magnets are most suitable for this type of work.

IV. INFORMATION OBTAINABLE FROM SPIN-LABELLED ENZYMES

Nitroxy radicals exhibit a number of chemical and physical properties that make them extremely useful molecules for studying biochemical systems. These properties render the EPR spectrum of a nitroxide sensitive to various environmental conditions. For example, the spectrum can be affected by the motional constraint and orientation of the spin label in the system, by the polarity of the system, and by the presence of other species in the system such as reducing agents and paramagnetic ions. Conversely, a spin label can affect the properties of the system. It can bring about changes in the relaxation times of nuclei and the lifetimes of fluorophores in excited states.

The diverse properties of these molecules is unquestionably one of the major reasons for their tremendous popularity and wide use in solving biochemical and biophysical problems. The various types of information about the structure and function of enzymes that spin labels can reveal is described in this section.

A. Rates of Catalysis

The first study illustrating that spin labels can be used to study enzyme kinetics was carried out by Berliner and McConnell (1966). In that study, α-chymotrypsin was acylated at pH 4.5 with a p-nitrophenyl ester of a spin-labeled carboxylic acid (IV, Fig. 2). The EPR spectrum of the strongly immobilized, covalently bound label contained only broad-line components (upward arrows in Fig. 9). However, when the pH of the acyl–enzyme intermediate was raised to 6.8, the spin-labeled acyl group was released, giving a narrow-line spectrum for the freely rotating nitroxide (Fig. 9b). The time-dependent increase in narrow-line amplitude or decrease in broad-line amplitude can be used to measure the rate of decaylation (Fig. 5b). However, the spectral amplitude of a freely tumbling nitroxide is considerably greater than that of a strongly immobilized one. Hence, growth of the narrow-line component is a more sensitive indicator of decaylation. For the decaylation step, a first-order rate constant of (1.55 ± 0.1) × 10^{-3} \text{ sec}^{-1} was calculated.

The same technique has been used to compare the relative magnitudes of the individual rate parameters for both enantiomers of asymmetric substrates (Flohr and Kaiser, 1972):

\[
E + S \xrightarrow{k_1} E \cdot S \xrightarrow{k_3} ES + P_1 \xrightarrow{k_4} E + P_2
\]  

From visible absorption measurements of the rate of release of p-nitrophenol and from paramagnetic resonance measurements of the release of spin-labeled carboxylic acid (see Table I), values of K_2, k_2, and k_3 were calculated for both R and S isomers of IV (Fig. 2). Values of k_3 determined by
the two different methods agree to within 8–13% and indicate that the rate of hydrolysis of the dextrorotatory isomer is about twenty times greater than that of the levorotatory one (Table I). The absolute configuration of the dextrorotatory molecule resulting from the hydrolysis of IV (Fig. 2) has recently been determined by X-ray methods (Ament et al., 1973). Similar kinetic experiments have been carried out on the model enzyme systems cyclohexa-
amylase (Flohr et al., 1971) and cycloheptanamylose (Paton and Kaiser, 1970). A comparison of rate data indicate similar enantiomeric specificities of chymotrypsin and cyclohexaamylase in the catalysis of nonspecific ester substrates such as IV (Fig. 2). Cycloheptanamylose, however, shows no appreciable enantiomeric specificity in the hydrolysis of such substrates.

1-Oxyl-2,2,6,6-tetramethylpiperidinyl-4-phosphate (X, Fig. 7) is a substrate for alkaline phosphatase from *Escherichia coli* that gives the corresponding alcohol as the only paramagnetic cleavage product. The pronounced difference in resolution of the proton superhyperfine splitting of the EPR spectra of the substrate and cleavage product serve as the basis for a semiquantitative assay of alkaline phosphatase activity (Mushak et al., 1972). For the assay, the mixture of enzyme and substrate is quickly degassed, and the catalyzed reaction is monitored by loss of the superhyperfine structure of the spectrum and/or the upfield shift of the signal (Fig. 10).

The ability of certain biological molecules such as ascorbate (Kornberg and McConnell, 1971) and glutathione (Morrisett and Drotz, 1969) to reduce nitroxides and destroy their paramagnetism is well known. Certain enzyme systems also produce this effect. For example, the reduction kinetics of the spin-labeled phosphate X (Fig. 7) and 3-doxystearic acid (XXIII, Fig. 7) by microsomal membranes containing the cytochrome *P*₄₅₀-cytochrome *P*₄₅₀ reductase hydroxylating enzyme system have been studied by Stier and Sackmann (1973). An Arrhenius plot of the reduction rate constants revealed a significant difference in the behavior of the watersoluble and lipid-soluble spin labels. The activation energy of the fatty acid reduction decreased abruptly from 30.8 to 8.7 kcal/mole at about 32°C. No such break occurred in the Arrhenius plot of spin-labeled phosphate reduction (activation energy = 13.8 kcal/mole). These results were interpreted to indicate that the reducing enzyme was bound by a rather rigid phospholipid halo that is in a quasicrystalline structure below 32°C.

The conversion of a nonparamagnetic amine to a nitroxide (just the reverse of the process described in the preceding paragraph) is a novel technique that has been gainfully utilized by Stier and Reitz (1971) 2,2,6,6-
Tetramethylpiperidine (XXI, Fig. 7) was found to be oxidized by rat and rabbit liver microsomes to the corresponding nitroxide (XXII, Fig. 7) in a reaction requiring O₂ and NADPH. An oxidation sequence (Fig. 11) leads from XXI (Fig. 7) over the corresponding hydroxylamine (step 1) to the nitroxide (step 2), which is further oxidized to an unknown product (step 3). In addition to these oxidation processes, the nitroxide XXII (Fig. 7) is reduced over the N-hydroxy derivative to the starting secondary amine (Fig. 11, step 5). The authors deduce this sequence of events by measuring stationary concentrations of radicals formed and by examining the effect of inhibitors, stimulators, and temperature on the overall reaction and single reaction steps that they feel involve three different enzymes.
B. Mechanisms of Denaturation

Spin labels may be used to study unfolding of the active sites of enzymes. This application is illustrated by the chymotrypsin system, which has been examined by two groups (Morrisett and Broomfield, 1971; Berliner, 1972). In the former study, chymotrypsin was spin-labeled at the active site serine by piperidinyl phosphonate I and androstyl phosphonate II (Fig. 4). The denaturation of each enzyme derivative as a function of guanidine hydrochloride concentration was then studied by paramagnetic resonance. The EPR spectral changes that were observed are illustrated in Fig. 12. These changes may be expressed as changes in the ratio of resonance line amplitudes. For the experiment illustrated by Fig. 12, the ratio of high field to center field line heights was used. When this ratio was plotted against denaturant concentration, a sigmoidal curve was obtained (Fig. 13). Results obtained in this manner indicated that the concentration of denaturant required to unfold the active site region of \( \alpha \)-chymotrypsin labeled with I (Fig. 4) is significantly lower than that required when the enzyme is labeled with II (Fig. 4). For each of the two derivatives, the denaturant concentration required for unfolding is essentially pH independent. In a similar study employing chymotrypsin labeled with piperidinyl phosphorane III (Fig. 4) or pyrolyl carboxylate V (Fig. 2), Berliner (1972) found that when urea was used as the denaturant, the unfolding was \( \text{pH} \) dependent. These results implied that effects involving charged residues that were involved in active site conformation were swamped out by the ionic denaturant, whereas the neutral denaturant did not obliterate all of these effects.

Mushak and Coleman (1972) have performed a urea denaturation study of human carbonic anhydrase to which the piperidinylsulfonamide XIII (Fig. 7) was bound. While 6 M urea caused a relatively rapid denaturation of the enzyme as determined by optical rotatory dispersion, circular dichroic, or aromatic difference spectra, only 30% of the sulfonamide was released. At lower urea concentrations, lower levels of spin label were released. To release all of the spin-labeled sulfonamide, 8 M urea was required. Significantly, the stable fraction of the enzyme that was present at urea concentrations below 5 M could be observed even when the spin label was added after exposing the native enzyme to urea. These data are consistent with either a stepwise denaturation mechanism or a rapid equilibrium between folded and unfolded conformers at the lower urea levels.
C. Distances between Different Groups

The intrinsic paramagnetism of spin-labeled molecules creates perturbations in the magnetic and electronic environment of certain ions and other molecules. Since the magnitude of this effect depends on the distance between the paramagnetic label and the perturbed group, estimates of that distance can be made. Basically, there are four types of experiments that can be used to estimate distances between different groups at the catalytic site (and other sites) on an enzyme. These four types are currently at various stages of development.

1. Distance between a Paramagnetic Spin Label and a Diamagnetic Nucleus

Spin labels, like other paramagnetic species, create fluctuating magnetic fields that enhance the relaxation rate of nuclei within their sphere of influence. (See Chapter 9 for a quantitative treatment of this phenomenon.)

The possibility of gainfully utilizing spin labels to perturb nuclear resonances and thereby measure the intervening distance has been recognized for some time, but only recently has it been exploited. Experiments of this type were first attempted by Sternlicht and Wheeler (1967) and by McConnell et al. (1967). In these early studies, a general broadening was observed for all proton resonances of the protein to which a spin label was covalently attached. Because this broadening was so nonspecific, no detailed conclusions could be drawn concerning proximity of particular nuclei to the nitroxyl free electron. In a more fruitful experiment, Roberts et al. (1969) found that the noncovalent binding of piperidinyl phosphate X (Fig. 7) to ribonuclease was accompanied by a significant broadening of the C-2 proton resonances assigned to His 12 and His 119. The broadening for the His 12 resonance was greater, indicating that the average location of the unpaired electron on the spin label was closer to His 12 than His 119. A numerical value for the intervening distances was not calculated. In a more quantitative study, Milden and Weiner (1969a), using a spin-labeled analogue of nicotinamide adenine dinucleotide (II, Fig. 7) with the enzyme alcohol dehydrogenase, measured the relaxation rates of bound substrates or pseudosubstrates. From these measurements they were able to calculate distances from the protons of bound ethanol, acetaldehyde, or isobutyramide to the paramagnetic label. In a similar study, Krugl (1971) observed changes in the relaxation rates of purine protons of adenosine monophosphate (AMP) when bound to DNA polymerase that simultaneously bound spin labeled adenosine triphosphate (ATP) (I, Fig. 7). From these changes, he deduced that the AMP and ATP binding sites were contiguous and that the distance between the nitroxyl moiety on spin-labeled ATP and the C-2 proton of AMP was 7.1 ± 0.6 Å. In a detailed study of internuclear distances on lysozyme, site-directed spin labels were used to perturb the NMR spectra of specific protons on the enzyme and on bound substrates (Wien et al., 1972). Lysozyme covalently tagged with pyrrolyl bromoacetamide I (Fig. 1) at His 15 broadened the nuclear resonance spectra of N-acetyl-α-D-glucosamine and di-N-acetyl-α-D-glucosamine bound at the active site. Using these broadenings, the distance from the nitroxide at His 15 to the acetamido methyl group of these saccharides was calculated. The measurement was then made in the opposite direction by using spin-labeled saccharides (IV and V, Fig. 7) bound at the active site to broaden the resonance of the C-2 proton at His 15. In a third type of experiment, the pseudosugar piperidinylacetamide VI (Fig. 7), which binds anomalously near Trp 123 (Berliner, 1971), was used to estimate the distance from that residue to the acetamido methyl groups of bound saccharides and to the C-2 proton of His 15. The distances calculated from the resonance data were in good agreement with those distances determined from a molecular model of lysozyme built...
according to X-ray crystallographic coordinates. Recently, Jones et al. (1973) have performed distance-measuring experiments on phosphofructokinase which was covalently tagged with piperidinylhomoacetamide VIII (Fig. 1) at a single reactive sulphydryl group. The labeled enzyme retained a high level of catalytic activity and ATP binding capacity. The longitudinal and transverse relaxation times of specific protons in Mg-ATP bound to spin-labeled phosphofructokinase were shorter than those times measured with the native enzyme. This shortening of relaxation times due to dipolar spin-spin interaction was used to calculate distances of 7.2, 7.5, and 7.1 Å from the attached nitroxide to the H-2, H-8, and H-1' nucleotide protons, respectively. Spin labels enhance the relaxation rate not only of protons on enzymes and bound substrates or inhibitors, but also of protons on water molecules. Studies illustrating this principal have been carried out on liver alcohol dehydrogenase (Mildvan and Weiner, 1969a,b), creatine kinase (Cohn et al., 1971), and citrate synthase (Weidman et al., 1973).

2. Distance Between Two Different Spin Labels Attached to the Same Enzyme

When two nitroxy radicals are attached to the same molecule, their unpaired electrons may undergo magnetic interaction. The magnitude of this interaction depends on the distance between the nitroxy nitrogens and the motion of the radicals relative to each other. This interaction is large for separations less than 10 Å and small for separations greater than 14 Å (Calvin et al., 1969). When the spin-spin exchange frequency is large compared to the hyperfine frequency, a five-line EPR spectrum with relative line intensities of 1:2:3:2:1 is obtained. As the exchange frequency decreases, the intensity ratio becomes 1:1:1:1:1, then 2:1:2:1:2. When there is no exchange interaction, a simple three-line spectrum (1:2:1) like that of a single nitroxide is obtained. (A rigorous treatment of spin-spin exchange interactions is presented in Chapter 4.) When a reagent carrying two spin labels (e.g., IV, Fig. 4) is attached to the active site of an enzyme, the distance that separates the nitroxy nitrogens may be governed by the size of that site. Since only small changes in this distance can cause rather large changes in the EPR spectrum, this method has great potential for detecting subtle changes in active site conformation and geometry. This sensitivity is illustrated by changes in the spectrum of biradical IV (Fig. 4) attached to Ser 195 of α-chymotrypsin when Met 192 or Met 192 and Met 180 are oxidized (Kosman and Piette, 1972). Even more dramatic differences are observed when the spectra of this label attached to chymotrypsin, trypsin, elastase, and thrombin (Fig. 14) are compared (Hsia et al., 1972). Although double spin labels can exceed single spin labels in sensitivity to changes in their environment, they usually give complicated spectra that are much more difficult to interpret than monoradical spectra. For this reason, biradical spin labels have not seen the wide use that their monoradical counterparts have. Another application of spin-spin interaction to the study of protein structure involves the covalent attachment or noncovalent binding of two monoradicals at different loci on a protein or peptide. The success of such an experiment requires (a) that two and only two sites are
labeled, and (b) that the extent of their occupancy is known. Ovchinnikov (1973) has used this method to estimate the distances between the two ornithine side chains of gramicidin S and six of its structural analogs. In similar fashion, Filatova et al. (1973) have employed this technique for measuring the distance between spin labels attached to Arg 1 and Ser 6 of bradykinin.

3. DISTANCE BETWEEN A SPIN LABEL AND A PARAMAGNETIC ION

When a spin label and a paramagnetic ion are present in the same system, the effect of that ion on the resonance spectrum of the nitroxide is not always a mere broadening. When these groups are rigidly bound to a macromolecule so that their reorientation time is long compared to the electronic relaxation time, the observed line width of the EPR signal is given by

\[ \delta H = \frac{g \beta \mu^2 r}{r^3} \left( 1 - 3 \cos^2 \theta_k \right)^2 + \delta H_0 \]  

where \( g \), \( \beta \), and \( \hbar \) have their usual meanings, \( r \) is the correlation time for the dipolar interaction, \( \mu \) is the magnetic moment of the dipole, \( r \) is the distance between the ion and the nitroxyl nitrogen, \( \theta_k \) is the angle between the applied magnetic field and the line joining the two electronic spins, and \( \delta H_0 \) is the residual line width of the nitroxide in the absence of the paramagnetic ion (Leigh, 1970). Actually, the dipolar effect is observed as a diminution of the signal amplitude rather than a general broadening of the whole spectrum for the reason that Eq. (2) is sensitive to orientation changes. For a certain fraction of orientations, the first term on the right side of the equation will be small compared to the second term on that side, and the signal from this fraction will be governed largely by \( \delta H_0 \) and will yield an unperturbed spectrum. For most other orientations, the first terms are large compared to the second, and the resulting spectra are too broad to observe. Hence, the composite of these two effects is a fraction of the original signal. By determining the extent of signal diminution, one can calculate the distance between the two paramagnetic species using Eq. (2). Taylor et al. (1969) have applied this technique to the creatine kinase system. When this enzyme was spin labeled with I (Fig. 1) at the essential SH2 group, the estimated distance from the nitroxide to bound Mn2+ in the MnADP-spin-labeled kinase was 7–10 Å. Jones et al. (1973) have used the same method to map the active site of phosphofructokinase. When this enzyme was labeled with piperidinylbromoacetamide VIII (Fig. 1) at a single SH group, the estimated distance from the nitroxyl free electron to manganese in an ATP-containing ternary complex was 12.0 Å.

4. DISTANCE FROM A SPIN LABEL TO A FLUOROPHORE

The ability of species with electron spin multiplicities greater than zero to quench excited electronic states has been known for some time (Birks, 1970). Several groups have used di-t-butyl nitroxide as a quencher of excited triplet states (see Green et al., 1973 and references therein). Possible mechanisms for this quenching process include (a) resonance-excitation transfer; (b) collisional or energy-exchange transfer; (c) charge-transfer excited state; (d) electron-exchange-induced intersystem crossing, and (e) vibrational quenching (Green et al., 1973). However, these possibilities can be narrowed down to either (d) or (e). Viscosity studies suggest that this quenching phenomenon is operative over a distance of 4–6 Å. One application of spin-label-induced fluorophore quenching in a biological system involves the tryptophan quenching of albumin by a spin-labeled fatty acid. It has been shown that unlabeled long-chain fatty acids (e.g., oleic acid), upon binding

Fig. 15. Stern-Volmer plots of intrinsic tryptophan fluorescence of defatted bovine serum albumin in the presence of fatty acids. The order of quenching efficiency for the bound fatty acid is 5-doxylstearate (12,3-SLFA) (Δ) > 16-doxylstearate (14,1-SLFA) (○) > oleate (□) > 12-doxylstearate (5,10-SLFA) (●). (From Morrisset et al., 1973.)
to bovine albumin, diminish the intrinsic tryptophan fluorescence intensity (Spector and John, 1968). The magnitude of this effect varies only slightly when 16-doxystearate, 12-doxystearate, or oleate is used (Morrissett et al., 1975). However, when the nitroxy group is moved up to the C-5 position, which is near one of the tryptophan residues, a striking decrease in intensity (increase in quenching) is observed (Fig. 15). This experiment demonstrates the dependence of nitroxide-induced quenching on the spin label-fluorophore distance and illustrates how the effect may be used to map enzyme active sites.

This phenomenon was also demonstrated by Koblin et al. (1973) in a study of erythrocyte membranes. Normally, diamagnetic local anesthetics analogous to XXIV (Fig. 7) enhance the fluorescence of 1-anilinonaphthalene-8-sulfonate (ANS) in these membranes. However, when the paramagnetic anesthetic XXIV (Fig. 7) was used, it diminished the ANS fluorescence intensity by about 30%. When ascorbate was added to reduce the nitroxide, the intensity increased about 3.5-fold. These results suggested that local anesthetics occupy sites in the membrane which are very near those sites where ANS is bound.

D. Polarity of Binding Sites

The principal values of the hyperfine tensor of a nitroxide are dependent on the polarity of the environment. A hydrophobic environment will shift the equilibrium of Eq. (3) toward the right, yielding a species with much of the unpaired electron density associated with the oxygen atom:

\[
\begin{array}{c}
\vdots \\
N-O^-
\end{array} \quad \longrightarrow \quad \begin{array}{c}
\vdots \\
\vdots \\
N=O
\end{array}
\]

(3)

In this case, interaction of the free electron with the nitrogen nucleus is minimal resulting in a relatively low isotropic coupling constant, \( A_0 \). When the environment becomes more polar, the equilibrium shifts toward the left, electron-nitrogen nuclear interaction increases, and the coupling constant increases. This effect is clearly seen when \( A_0 \) of di-i-butylnitroxide is measured in solvents of differing polarity (Griffith et al., 1974). In Table II it is seen that for the nonpolar solvent n-hexane, the hyperfine splitting is 15.10 G, whereas in water this value increases to 17.16 G. The isotropic coupling constant for a spin label attached to a protein can be determined only when the label retains a high level of motional freedom. If the attached label is strongly immobilized, \( A_0 \) is no longer measurable. However, it has been shown that from spectra of these strongly immobilized nitroxides, the splitting between the low field maxima and high field minima (2\( A_{zz} \)) can

### Table II

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent</th>
<th>( A_0 )</th>
<th>( \theta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>15.10</td>
<td>2.0061</td>
</tr>
<tr>
<td>2</td>
<td>Heptane-pentane (1 : 1)†</td>
<td>15.13</td>
<td>2.0091</td>
</tr>
<tr>
<td>3</td>
<td>2-Hexene</td>
<td>15.17</td>
<td>2.0061</td>
</tr>
<tr>
<td>4</td>
<td>1,5-Hexadiene</td>
<td>15.30</td>
<td>2.0051</td>
</tr>
<tr>
<td>5</td>
<td>Di-n-propylamine</td>
<td>15.32</td>
<td>2.0061</td>
</tr>
<tr>
<td>6</td>
<td>Piperidine</td>
<td>15.40</td>
<td>2.0061</td>
</tr>
<tr>
<td>7</td>
<td>n-Butylamine</td>
<td>15.41</td>
<td>2.0050</td>
</tr>
<tr>
<td>8</td>
<td>Methyl propionate</td>
<td>15.45</td>
<td>2.0061</td>
</tr>
<tr>
<td>9</td>
<td>Ethyl acetate</td>
<td>15.45</td>
<td>2.0051</td>
</tr>
<tr>
<td>10</td>
<td>Isopropylamine</td>
<td>15.45</td>
<td>2.0050</td>
</tr>
<tr>
<td>11</td>
<td>2-Butanone</td>
<td>15.49</td>
<td>2.0050</td>
</tr>
<tr>
<td>12</td>
<td>Acetone</td>
<td>15.52</td>
<td>2.0061</td>
</tr>
<tr>
<td>13</td>
<td>Ethyl acetate saturated with water</td>
<td>15.59</td>
<td>2.0050</td>
</tr>
<tr>
<td>14</td>
<td>N,N-Dimethylformamide</td>
<td>15.63</td>
<td>2.0050</td>
</tr>
<tr>
<td>15</td>
<td>EPA (5 : 5 : 2)†</td>
<td>15.63</td>
<td>2.0050</td>
</tr>
<tr>
<td>16</td>
<td>Acetonitrile</td>
<td>15.68</td>
<td>2.0060†</td>
</tr>
<tr>
<td>17</td>
<td>Dimethylsulfoxide</td>
<td>15.74</td>
<td>2.0059</td>
</tr>
<tr>
<td>18</td>
<td>N-Methyloproprionamide</td>
<td>15.76</td>
<td>2.0059</td>
</tr>
<tr>
<td>19</td>
<td>2-Methyl-2-butanol</td>
<td>15.78</td>
<td>2.0059</td>
</tr>
<tr>
<td>20</td>
<td>EPA (5 : 5 : 10)†</td>
<td>15.87</td>
<td>2.0059</td>
</tr>
<tr>
<td>21</td>
<td>1-Decanol</td>
<td>15.87</td>
<td>2.0059</td>
</tr>
<tr>
<td>22</td>
<td>1-Octanol</td>
<td>15.89</td>
<td>2.0059</td>
</tr>
<tr>
<td>23</td>
<td>N-methylformamide</td>
<td>15.91</td>
<td>2.0059</td>
</tr>
<tr>
<td>24</td>
<td>2-Propanol</td>
<td>15.94</td>
<td>2.0059</td>
</tr>
<tr>
<td>25</td>
<td>1-Hexanol</td>
<td>15.97</td>
<td>2.0059</td>
</tr>
<tr>
<td>26</td>
<td>1-Propanol</td>
<td>16.05</td>
<td>2.0059</td>
</tr>
<tr>
<td>27</td>
<td>Ethanol</td>
<td>16.06</td>
<td>2.0059</td>
</tr>
<tr>
<td>28</td>
<td>Methanol</td>
<td>16.21</td>
<td>2.0058</td>
</tr>
<tr>
<td>29</td>
<td>Formamide</td>
<td>16.33</td>
<td>2.0058</td>
</tr>
<tr>
<td>30</td>
<td>1,2-Ethandiol</td>
<td>16.60</td>
<td>2.0058</td>
</tr>
<tr>
<td>31</td>
<td>Ethanol-water (1 : 1)†</td>
<td>16.69</td>
<td>2.0057</td>
</tr>
<tr>
<td>32</td>
<td>Water</td>
<td>17.16</td>
<td>2.0056</td>
</tr>
<tr>
<td>33</td>
<td>10 M LiCl aqueous solution</td>
<td>17.52</td>
<td>2.0056</td>
</tr>
</tbody>
</table>

* Griffith et al., 1974.
† All data measured at room temperature (23°-24°C). Estimated uncertainties are ±0.02 G and ±0.0001 for \( A_0 \) and \( \theta \), respectively, relative to the standard dilute aqueous solution of di-i-butylnitroxide for which \( A_0 = 17.16 \) and \( \theta = 2.0056. 
‡ By volume.
† EPA designates a mixture of ethyl ether (diethyl ether), isopentane (2-methylbutane), and alcohol (ethanol).