Nitroxide Free Radicals: Spin Labels for Probing Biomolecular Structure

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Numerous organic molecules have been bound to biological macromolecules in efforts to determine molecular structure and biological function. As early as the 1930's antibodies and other proteins were selectively labeled with fluorescent or colored molecules to locate high concentrations in living cells.[1] Naturally, the approach has become more sophisticated over the years. Proteins are now commonly tagged at active sites or other well-defined positions, and some results of tagging experiments have been correlated with X-ray crystallographic data. The diversity of the probes has also increased, and there are now labels designed to be observed by a variety of spectroscopic techniques including fluorescence, optical absorption, and electron spin resonance (esr).

Each technique has its advantages. Fluorescence, for example, is particularly useful when very low concentrations of label must be used or when rotational mobility is of interest. A fluorescent label is also very sensitive to the local environment. Optically absorbing labels are frequently less sensitive to the environment, and this can be an advantage when sorting out the causes of an optical shift. Optically absorbing labels are convenient to use because instruments for both stationary-state and kinetic experiments are readily available in most laboratories. The labels in esr experiments are free radicals (spin labels). The main advantages here are sensitivity to the local environment, ability to measure very rapid molecular motion, and, usually, absence of interfering signals from the environment. Spin labels can be used equally well in optically transparent or optically opaque solutions. It is obvious that these various types of probes are complementary. The choice of technique will depend on the questions being asked and the system under investigation.

The chemistry involved in preparing and applying the various types of probes also has a common underlying theme. In each case an organic molecule with the desired spectroscopic properties is synthesized and then allowed to interact either covalently or noncovalently with the macromolecule of interest. Many examples of fluorescent and optically absorbing probes may be found elsewhere.[2]

Some examples of nitroxide free radicals are given in Figure 1. Nitroxides I through VII are representative of the basic structures which have become available through the pioneering efforts of Rozantzev, Neiman, Hoffmann, Rassat, and others.[3-7] The relatively unreactive N–O group contains the unpaired electron necessary to produce an esr signal, but it is the reactive functional group on the other end of each molecule that provides the chemical handle needed in preparing useful spin labels. The spin label VIII, for example, is prepared from ketone I using textbook procedures for synthesizing phenylhydrazones.[3] Similarly, nitroxides IX–XIV, XVIII, and XIX were prepared from II, III, V, and VI using well-known reactions of alcohols, amines, and acids.[8-13] In a different approach, general methods have recently been developed by Keana and others for converting carbonyl groups into useful nitroxides such as XV, XVI, and XVII.[14,15] General methods for converting other functional groups to spin labels will undoubtedly be developed. It is apparent, then, that a sufficiently wide variety of nitroxide free radicals are becoming available to make nitroxides competitive with the more familiar optical labels.

The first description of a labeling study using nitroxides was reported in 1965 by Stone, *et al.*[16] The chemistry of nitroxides and the earlier spin-labeling studies of proteins and nucleic acids has been reviewed by Hamilton and McConnell.[17] The purpose of the present Account is to acquaint the potential user with

(1) A. H. Coons, H. J. Creech, and R. N. Jones, *Proc. Soc. Exptl. Biol. Med.*, **47**, 200 (1941), and references contained therein.

(2) G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, **1**, 65 (1968); R. Steiner and H. Edelhoch, *Chem. Rev.*, **62**, 457 (1962); S. A. Bernhard and G. L. Rossi in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Ed., W. H. Freeman and Co., San Francisco, Calif., 1968.

(3) E. G. Rozantzev and M. B. Neiman, *Tetrahedron*, **20**, 131 (1964).

(4) R. Briere, H. Lemaire, and A. Rassat, *Bull. Soc. Chim. France*, 3273 (1965).

(5) E. G. Rozantzev and Yu. V. Kokhanov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1477 (1966).

(6) E. G. Rozantzev and L. A. Krinitzkaya, *Tetrahedron*, **21**, 491 (1965).

(7) A. K. Hoffmann and A. T. Henderson, *J. Am. Chem. Soc.*, **83**, 4671 (1961).

(8) O. H. Griffith, J. F. W. Keana, S. Rottschaefer, and T. A. Warlick, *ibid.*, **89**, 5072 (1967).

(9) O. H. Griffith, J. F. W. Keana, D. L. Noall, and J. L. Ivey, *Biochim. Biophys. Acta*, **148**, 583 (1967). There is an omission in the reported synthesis of this compound. In the actual synthesis of X, triethylamine (2.6 mmol) was added to 2.6 mmol of the nitroxide acid in anhydrous ether. The addition of triethylamine was accidentally deleted in the final version of the manuscript.

(10) A. S. Waggoner, A. D. Keith, and O. H. Griffith, *J. Phys. Chem.*, **72**, 4129 (1968).

(11) O. H. Griffith and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S.*, **55**, 8 (1966).

(12) S. Ohnishi, J. C. A. Boeyens, and H. M. McConnell, *ibid.*, **56**, 809 (1966).

(13) S. Ogawa and H. M. McConnell, *ibid.*, **58**, 19 (1967).

(14) J. F. W. Keana, S. B. Keana, and D. Beetham, *J. Am. Chem. Soc.*, **89**, 3055 (1967).

(15) J. H. Osiecki and E. F. Ullman, *ibid.*, **90**, 1078 (1968).

(16) T. J. Stone, T. Buckman, P. L. Nordio, and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S.*, **54**, 1010 (1965).

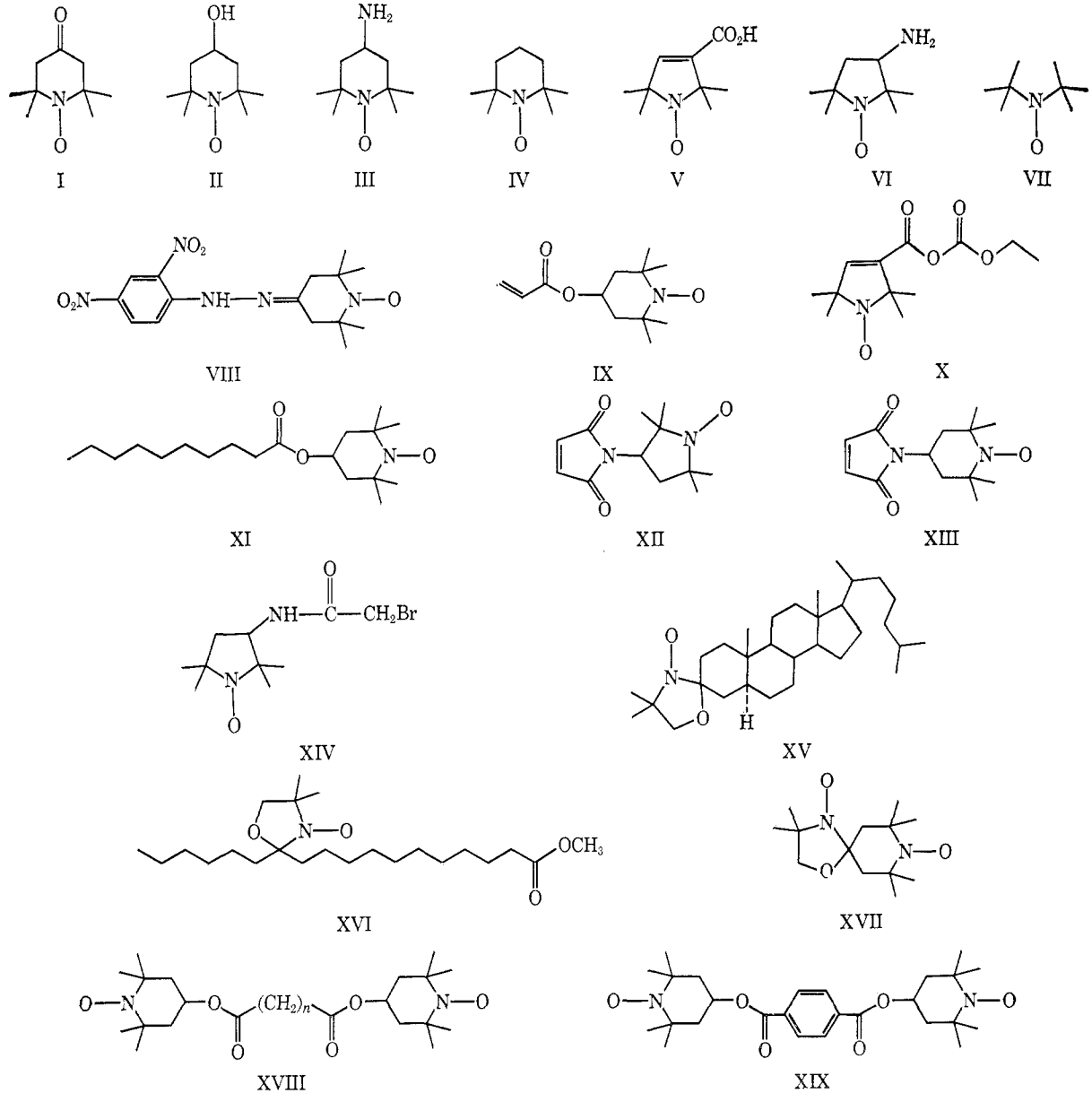

Figure 1. Some examples of nitroxide molecules which have been used as spin labels or as precursors for the synthesis of spin labels. Two methyl groups signified by straight lines are attached to the carbon atoms adjacent to the N–O group of each nitroxide. (Useful references: I,[3,4] II,[4] III,[5,45] IV,[3,4] V,[6] VI,[6] VII,[7] VIII,[3,36] IX,[8,46] X,[9] XI,[10] XII,[11] XIII,[12] XIV,[13,47] XV,[14] XVI,[52,53] XVII,[27] XVIII,[28,29] XIX.[28,29])

the kinds of information that have been obtained or could, in our opinion, be obtained with nitroxide labels. Esr is clearly the main method of detection, but a number of other spectroscopic techniques are also mentioned because the multiple technique approach has a growing place in labeling studies.

The examples that we have chosen to illustrate spin labeling emphasize membrane model and membrane studies partly because of the recent progress in this area and partly because it is the main interest in this laboratory. No exhaustive coverage of spin-labeling studies is attempted. In fact, a general definition of spin labeling could include all esr studies in which small paramagnetic probes are used to obtain information on the surrounding environment (in contrast to studies aimed at determining the structure of the paramagnetic species). This is indeed a large topic. It would encompass the frequently elegant esr studies of copper and manganese ions bound to biomolecules, the studies of the porphyrin–metal complexes of hemoglobin, chlorophyll, cytochromes, and other proteins, and some studies of organic free radicals produced by irradiating biomolecules. Irradiation studies are usually undertaken to identify the free radicals, but some information regarding the environment of the radical probes is frequently obtained from the esr spectra. Fortunately, most of these topics have received attention in recent review articles.[18,19]

(17) C. L. Hamilton and H. M. McConnell in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Ed., W. H. Freeman and Co., San Francisco, Calif., 1968, p 115.

(18) P. Hemmerich, *Proc. Roy. Soc.* (London), **A302**, 335 (1967).

(19) M. T. Jones and W. D. Phillips, *Ann. Rev. Phys. Chem.*, **17**, 323 (1966).

Esr of Nitroxide Free Radicals

Spin Hamiltonian and Anisotropic Effects. The approximate spin Hamiltonian, $\hat{\mathcal{H}}$, for a collection of nitroxide free radicals is[20]

$$\hat{\mathcal{H}} = |\beta|\hat{\mathbf{S}}\cdot\boldsymbol{g}\cdot\mathbf{H} + \hat{\mathbf{S}}\cdot\boldsymbol{T}\cdot\hat{\mathbf{I}} + \left[\begin{matrix}\text{electron–electron}\\ \text{dipole terms}\end{matrix}\right] + \left[\begin{matrix}\text{electron–electron}\\ \text{exchange terms}\end{matrix}\right]$$

where β, $\mathbf{H}$, $\boldsymbol{g}$, $\boldsymbol{T}$, $\hat{\mathbf{S}}$, and $\hat{\mathbf{I}}$ are the electron Bohr magneton, the laboratory magnetic field, the g matrix, the hyperfine matrix, the electron spin operator, and the nuclear spin operator, respectively. The nuclear Zeeman term has been omitted and, for clarity, small second-order effects are not discussed here. The first term in $\hat{\mathcal{H}}$ is the electron Zeeman term and represents the interaction of the electron spin with the external magnetic field. This large interaction gives rise to the useful relation $h\nu = g\beta H$, or ν (in kMHz) = $2.8H$ (in kG), where ν is the microwave frequency of the esr spectrometer. Currently, most esr spectrometers operate at ν = 9.5 kMHz (X-band), but an increasing number can also perform at ν = 35 kMHz. The majority of published nitroxide spectra, therefore, are recorded at 3.4 kG, and a few are taken at 12.5 kG. There is very little difference between the spectra recorded at the two frequencies providing the nitroxide free radicals are rapidly tumbling. For reasons discussed below, the spectra are frequency dependent if the nitroxide is slowed down by a viscous solvent (or biomolecule) or if it is held in a rigid lattice.

The second term in the Hamiltonian represents the interaction between the unpaired electron and the nitrogen nucleus of the nitroxide N–O group. This term, although much weaker than the Zeeman interaction, yields the important rule that a nuclear spin, I, will split the hypothetical single Zeeman line into $2I + 1$ lines. For nitrogen, $I = 1$, and the result is three lines of equal intensity (Figure 2). The sample responsible for spectra a, b, and c of Figure 2 was an organic single crystal doped with a small amount of di-*t*-butyl nitroxide (VII). The crystal lattice served to orient the nitroxide free radicals and prevent nitroxide–nitroxide interactions. The remaining spectrum, d, was recorded using a dilute solution of the same nitroxide. The four spectra of Figure 2 differ, of course, but the appearance of each one can be characterized by the line width and two important parameters, the g value and the coupling constant, a. The g value determines the center of the spectrum, and a is simply the distance between two adjacent lines. It is important to recall that increasing or decreasing g will shift the entire three-line spectrum to lower or higher magnetic fields, respectively, without altering the distance between lines. Similarly, variations in a expand or contract the pattern without altering the position of the center line. These effects are illustrated in Figure 2. Spectra a, b, and c were recorded with the laboratory magnetic field along the three principle axes, x, y, and z, respectively, of the nitroxide free radical. The spectra show that the a and g parameters are anisotropic in the sense that they vary with the orientation of the crystal in the magnetic field. The coupling constants, a_{xx} and a_{yy}, of the first two spectra are obviously nearly equal to each other but are much smaller than the coupling constant, a_{zz}, of the third spectrum. The g values of all these spectra are different, as can be seen by comparing the amount each center line is shifted from the dashed reference line. The actual measured values are $a_{xx} = a_{yy} = 6$ G, $a_{zz} = 32$ G, $g_{xx} = 2.0089$, $g_{yy} = 2.0061$, and $g_{zz} = 2.0027$.[21] The corresponding quantities of spectrum d, the solution spectrum, are $a^0 = 15$ G and $g^0 = 2.0060$.[21] The parameters of the solution spectrum clearly lie between the extremes for the single crystal. The quantitative relations between the solution and single-crystal data are simply $a^0 = (1/3)(a_{xx} + a_{yy} + a_{zz})$ and $g^0 = (1/3)\cdot(g_{xx} + g_{yy} + g_{zz})$. In spin labeling, this anisotropy is important in two respects. The anisotropy can be used directly to sense preferential orientation of a collection of spin labels in crystals and membranes or other biological structures. This application is reminiscent of optical birefringence. A good general rule is that

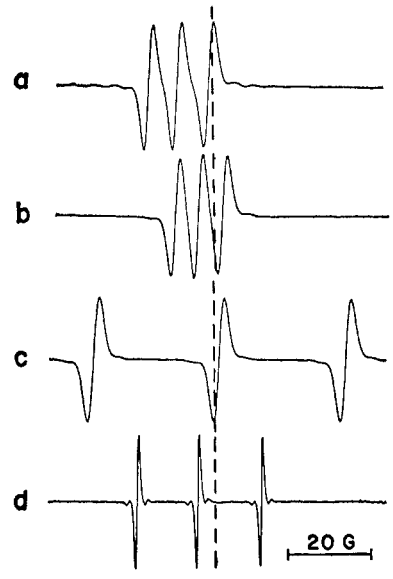

Figure 2. X-Band esr spectra illustrating the a and g anisotropy of di-*t*-butyl nitroxide (nitroxide VII of Figure 1.) Spectra a, b, and c are obtained from a tetramethyl-1,3-cyclobutanedione host crystal doped with a small amount of nitroxide VII. The host crystal orients the nitroxide molecules so that their respective molecular axes (x axis parallel to the N–O bond, z axis parallel to the nitrogen p orbital, and y axis perpendicular to both the x and the z axes) are aligned in certain directions relative to the crystal axis. The crystal has been rotated to align the laboratory magnetic field with the x, y, and z axes of the oriented nitroxides producing spectra a, b, and c, respectively. Spectrum d is obtained from nitroxide VII (10^{-5} M) dissolved in di-*t*-butyl ketone. The vertical dotted line represents g = 2.00036, the g value of a 2,2-diphenyl-1-picrylhydrazyl reference sample.

(20) A. Carrington and A. D. MacLachlan, "Introduction to Magnetic Resonance," Harper and Row, Publishers, New York, N. Y., 1967.

(21) O. H. Griffith, D. W. Cornell, and H. M. McConnell, *J. Chem. Phys.*, **43**, 2909 (1965).

any biological sample exhibiting changes in spectra (*e.g.*, Figure 2) as the sample is rotated in the magnetic field is worthy of further study. Secondly, anisotropy is responsible for the important effects of rotational motion on the spectra. The anisotropic values of a and g mentioned above are required in formulas calculating molecular tumbling rates from esr line shape data.

The Effects of Rotational Motion on the Esr Spectra. The anisotropic effects discussed above are very nearly averaged to zero if the free radicals are tumbling rapidly in water or other nonviscous solvents (Figures 2d and 3a). If, however, the viscosity of the solution is increased, the rotational motion of the nitroxides decreases and the spectra become increasingly asymmetric, as shown in Figure 3. The bottom spectrum of Figure 3 is the limiting case approached as the viscosity becomes very large. This spectrum could be obtained, for example, after grinding up the crystal of Figure 2a–c or after rapidly freezing the solution of Figure 2d. It could also be observed from an aqueous solution of nitroxides rigidly bound to large protein molecules. Although not obvious at first glance, this spectrum (Figure 3d) is simply the sum of the three extreme single-crystal spectra of Figure 2 and the spectra of all intermediate orientations in the magnetic field. It follows directly that the splitting between the outermost lines of Figure 3d is equal to the total splitting ($2a_{zz}$) of Figure 2c. Figure 3d is frequently referred to as the powder spectrum, the rigid glass spectrum, or the polycrystalline spectrum.

Between the two limits of very rapid motion and a rigid glass, the spectra are quite complex. This complexity provides much of the information that can be obtained about molecular motion using spin-labeling

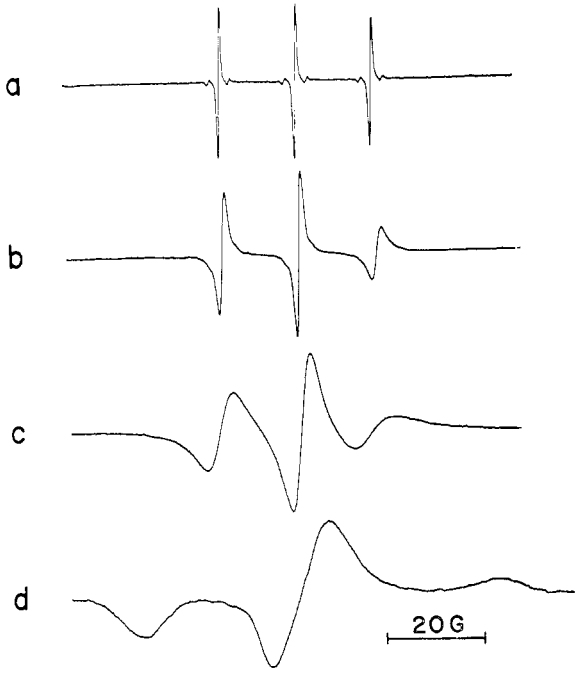

Figure 3. X-Band esr spectra of nitroxide VII (see Figure 1) dissolved in ethylene glycol at (a) 25°, (b) −25°, (c) −80°, and (d) −150°.

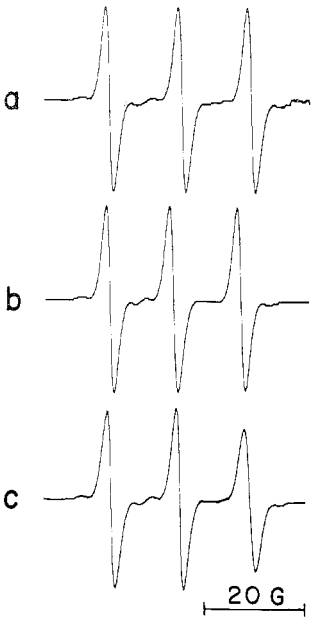

Figure 4. An illustration of anisotropic line broadening of an X-band esr spectrum obtained by summing the spectra of a nitroxide-doped host crystal (see Figure 2) oriented in two slightly different directions in the laboratory magnetic field. To obtain spectrum a, the host crystal containing nitroxide VII was oriented with the magnetic field about 60° from the z axis of the included nitroxides (see Figure 2). The angle was increased by about 3° to obtain spectrum b. Spectrum c is simply spectra a and b summed with a time-averaging computer. Note that the high-field line (far right) of spectrum c is appreciably shorter than the other two lines.

techniques. The mathematical manipulations are troublesome because the Hamiltonian is now time dependent and approximations are unavoidable. The reason behind the asymmetry is, however, quite straightforward. Consider Figure 4, for example. Spectra a and b are recorded at two slightly different orientations of a nitroxide-doped single crystal in the magnetic field. The a parameter of spectrum b is slightly smaller than that of a, while the reverse is true for the g values. In other words, the splitting of Figure 4b is smaller, but all three lines of b have been shifted slightly downfield. A simple addition of Figure 4a and 4b does not produce a symmetrical pattern (Figure 4c). In solution most of the anisotropic effects are removed by Brownian motion, but the residual effects remain as time-dependent perturbations.

The complexity of the problem is somewhat greater as one approaches either of the two extremes of very fast or very slow molecular tumbling. If the rate of tumbling is extremely rapid, then spin rotation interactions must also be considered. On the other hand, if the rotation is very slow, the time-dependent perturbations become uncomfortably large for normal perturbation calculations. Nevertheless, considerable progress has been made in quantitatively estimating molecular motion from esr line-shape data. Freed and Fraenkel[22] and Kivelson[23] have developed useful relations for the more rapid tumbling range (Figure

(22) J. H. Freed and G. K. Fraenkel, *J. Chem. Phys.*, **39**, 326 (1963).
(23) D. Kivelson, *ibid.*, **27**, 1087 (1957).

3a,b). Itzkowitz[24] later extended the range by treating the rotational motion as an angular random-walk problem. The relationships derived in the latter work are not precise, but this weakness is far outweighed by the fact that Itzkowitz was able to computer-simulate esr spectra over the full range of Figure 3. Other research groups[25] are currently engaged in obtaining more quantitative descriptions of the rotational motion, particularly in the slow rotation limit.

The aim of most calculations is to relate the esr line shapes to a parameter, τ, the rotational correlation time. A naive definition of τ is simply the average time required for a nitroxide undergoing Brownian motion to rotate through a significant arc (say, 40°). Actual line-shape calculations, of course, require τ to be defined more rigorously in terms of statistical fluctuations of angular variables. In any case, τ is inversely related to the tumbling rate of the molecule. The esr spectroscopist usually considers τ values of 5×10^{-11} sec and 5×10^{-8} sec as very fast and very slow tumbling rates, respectively. These two values represent approximate outside limits obtainable from solution esr studies.

Solvent Effects on the Esr Spectra. Both parameters a and g are solvent dependent.[4,26] The value of a^0 for a nitroxide is typically 1 or 2 G smaller in hydrocarbon solvents than in water. The corresponding change in g^0 in going from water to a hydrocarbon solvent is on the order of +0.0005. These solvent effects have important consequences in spin-labeling studies. They can be used, for example, much as optical solvent shifts are employed to determine the polarity of the immediate environment of the labels. If two different environments are accessible to a rapidly tumbling nitroxide, the amount of nitroxide in each environment can sometimes be measured (see membrane studies below). Solvent effects can, however, cause difficulty in measurements of rotational motion. The a and g values of many nitroxides are very nearly the same. This has led to the practice of using the anisotropic data of one nitroxide-doped single crystal to calculate τ values for various nitroxides in quite different environments. A better understanding of solvent effects on anisotropic a and g data is needed before absolute values of rotational correlation times can be considered accurate.

The Effects of Nitroxide–Nitroxide Interactions on the Esr Spectra. The electron–electron dipole and exchange interactions are important only when high concentrations (*i.e.*, $>10^{-3}$ *M*) of nitroxides are present. This condition is usually avoided in spin-labeling studies because of the complexity of dealing simultaneously with slow tumbling and electron–electron interactions. However, it is important to be aware of the effects because of the possibility of having small local regions of high nitroxide density in an over-all dilute solution. The line broadening can be quite complicated, but if the nitroxides are tumbling rapidly the sharp three-line spectrum simply broadens and finally coalesces as the nitroxide concentration is increased. The final exchange-narrowed single-line spectrum is characteristic of pure solid or liquid nitroxides. Another simple example of nitroxide–nitroxide interactions is observed in dilute solutions of dinitroxides such as XVII–XIX[27–30] and trinitroxides.[31] Nitroxide polymers have also been reported.[8,32,33]

Electron–electron exchange and dipole terms are potentially important in spin-labeling studies. Various membrane models could, for example, be tested by incorporating spin-labeled lipids or proteins into the membrane and determining the relative positions and orientations of the probes from the esr spectra. Even qualitative observations of aggregation effects are likely to provide useful information in complex lipid and lipoprotein spin-labeling experiments.

Combining Esr with Other Spectroscopic Techniques. Esr provides an excellent way of determining the rotational mobility of a nitroxide probe, the relative polarity of its environment, and its state of aggregation. However, the location of the probe in the system being investigated cannot usually be determined by esr. Fortunately, in many cases the nuclear magnetic resonance (nmr) spectrum of the system (rather than of the probe itself) contains information that may help to specify the position of the probe. The ability of paramagnetic metal ions[34] and stable free radicals[35] to shorten the relaxation times and, therefore, broaden the nmr absorption lines of nearby magnetic nuclei is well known. The broadening effects are a function of the separation and relative motion of the nuclei and the probe. It should be possible to identify the location of probes in some cases by observing the broadening of previously assigned nmr lines as the nitroxide is introduced into the system. Nmr has not been used to determine the location of nitroxide spin labels in membranes or other biomolecular structures. Nmr line broadening has been observed in a study of nitroxide–micelle interactions,[36] and a pulsed nmr (spin–echo) study of the effects of various concentrations of nitroxides on proton relaxation times has recently

(24) M. S. Itzkowitz, *J. Chem. Phys.*, **46**, 3048 (1967).
(25) For example, the research groups of R. G. Gordon, D. Kivelson, and J. H. Freed.
(26) T. Kawamura, S. Matsunami, and T. Yonezawa, *Bull. Chem. Soc. Japan*, **40**, 1111 (1967).
(27) A. S. Waggoner and O. H. Griffith, unpublished results. This diradical, like XVIII, exhibits a five-line spectrum in dilute solution.
(28) R. Briere, R. Dupeyre, H. Lemaire, C. Morat, A. Rassat, and P. Rey, *Bull. Chim. Soc. France*, 3290 (1965).
(29) H. R. Falle, G. R. Luckhurst, H. Lemaire, Y. Marechal, A. Rassat, and P. Rey, *Mol. Phys.*, **11**, 49 (1966).
(30) S. H. Glarum and J. H. Marshall, *J. Chem. Phys.*, **47**, 1374 (1967).
(31) M. B. Neiman, E. G. Rozantzev, and V. A. Golubev, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 548 (1965).
(32) E. G. Rozantzev and G. F. Pavelko, *Vysokomol. Soedin., Ser. B*, **9**, 866 (1967).
(33) G. Drefahl, H. H. Hoerhold, and K. D. Hofmann, *J. Prakt. Chem.*, **37**, 137 (1968).
(34) N. Bloembergen, E. M. Purcell, and R. V. Pound, *Phys. Rev.*, **73**, 679 (1948).
(35) H. S. Gutowsky and J. C. Tai, *J. Chem. Phys.*, **39**, 208 (1963).
(36) A. S. Waggoner, O. H. Griffith, and C. R. Christensen, *Proc. Natl. Acad. Sci. U. S.*, **57**, 1198 (1967).

been completed.[37] Nitroxides are less effective in shortening water–proton relaxation times than are certain paramagnetic metal ions (*e.g.*, Fe^{3+}, Mn^{2+}). In a related approach, Sternlicht[38] has suggested the possibility of using spin labels to assign lines of complex protein nmr spectra. One can expect more frequent use of nmr in future spin-labeling studies. Some experiments will be influenced by the impressive nmr studies of manganese ion binding to biomolecules.[39]

Nitroxide molecules exhibit two characteristic optical absorptions, one in the visible and one in the uv region of the spectrum. A strong, relatively solvent-insensitive π–π^* transition is found at about 240 mμ (ϵ ~2000), and at 400 to 500 mμ a much weaker (ϵ ~5–15), solvent-sensitive n–π^* transition is observed.[4,26] In principle, the optical absorption at longer wavelengths could be utilized much as the solvent a^0 and g^0 value shifts to study the environment of the nitroxide probe. However, few experiments of this type have been performed because the low extinction coefficient of this transition requires high nitroxide concentrations and because the absorption peak may be obscured by the much larger π–π^* peak. Perhaps a more useful method of combining optical shifts with esr in order to probe biological systems is to attach a chromophore moiety to the nitroxide label. An example is the molecule VIII which has been used in an esr investigation of micelle solubilization.[36]

Another property of the nitroxide free radical is its profound effect on fluorescent and triplet-state molecules.[40,41] Apparently, the interaction responsible for paramagnetic quenching of fluorescence is effective only over very short distances. This offers a possible means of determining the position of spin labels in biological systems containing fluorescent groups such as tryptophan. A tryptophan residue of a protein, for example, may not fluoresce after a nearby site has been tagged with a nitroxide free radical.

Examples of Spin Labeling of Membrane Models and Biological Membranes

Membrane Models. *The Incorporation of Nitroxides into Micelles.* Many surface-active molecules aggregate into micelles when the concentration exceeds a certain value, the critical micelle concentration. In aqueous solution the hydrocarbon portions of the surface-active molecules tend to form a hydrophobic phase and the polar head groups extend into the aqueous environment. A somewhat idealized micelle of sodium dodecyl sulfate (NaDS) molecules is shown in Figure 5. Historically, micelles are of biochemical interest because they demonstrate that surface-active molecules

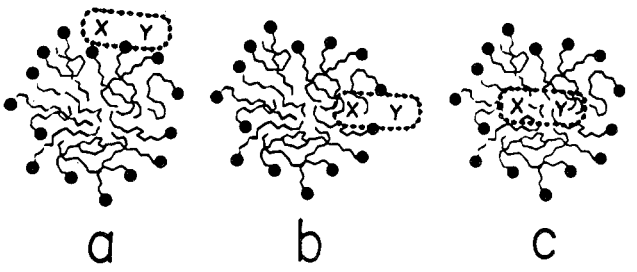

Figure 5. Three hypothetical models of micelle solubilization. The solubilized molecule, X–Y, is nitroxide VIII of Figure 1, and the aggregated hydrocarbon chains with polar ends represent sodium dodecyl sulfate molecules.

can spontaneously form membrane-like units in aqueous solution. Of more importance at present is that these aggregates can bring into solution, or solubilize, molecules that are otherwise nearly insoluble in water. Thus, micelles or similar structures can play a role in the transport of hydrophobic molecules. Spin labeling has recently been used by Waggoner, *et al.*,[10,36] to test the three common models of solubilization (Figure 5a–c). In the first model the probe is visualized as being adsorbed rigidly onto a rigid micelle, in the second model the probes are oriented radially in a micelle, and in the third model the probes are considered to be dissolved in the hydrocarbon interior of the micelle. In principle these models can be distinguished by solubilizing a nitroxide bonded to an optical chromophore. To test the first model (Figure 5a), for example, the esr rotational correlation times can be compared to values calculated from the simple Stokes law relation[42]

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

where r is the particle radius. If this model is correct there should be at least order-of-magnitude agreement between the experimental τ and τ calculated for a rigid sphere the size of a micelle. In evaluating the second and third models (Figure 5b,c), solvent-shift data are more useful than rotational correlation times. Figure 5c, for example, requires that the probe be located in a hydrocarbon environment. The requirement of Figure 5b is simply that the environment of the chromophore (Y) be different from the environment of the nitroxide (X). The double label, X–Y, is only needed in testing this model. The data of this study indicate that none of the three models is an adequate description of solubilization. The measured tumbling rates were too rapid for the rigid micelle model, and the solvent shift data suggest both X and Y experience the same relatively polar time-average environment. Therefore the main conclusion of this study was that solubilization is a dynamic process (*e.g.*, $\tau = 5 \times 10^{-10}$ sec for nitroxide VIII in 5% NaDS). The conclusions are, of course, valid only for the specific nitroxides solubilized, and there were a number of assumptions made in obtaining the rotational cor-

(37) S. B. Roeder, W. K. Wun, and O. H. Griffith, submitted for publication.
(38) H. Sternlicht and D. Wilson, *Biochemistry*, **6**, 2881 (1967).
(39) R. A. Dwek and R. E. Richards, *Ann. Rev. Phys. Chem.*, **18**, 99 (1967).
(40) A. L. Buchachenko, M. S. Khloplyankina, and S. N. Dobryakov, *Opt. Spektrosk.*, **22**, 554 (1967).
(41) L. A. Singer and G. A. Davis, *J. Am. Chem. Soc.*, **89**, 158 (1967).
(42) G. E. Pake, "Paramagnetic Resonance," W. A. Benjamin, Inc., New York, N. Y., 1962, Chapter 5.

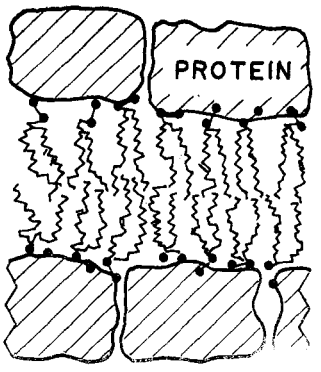

Figure 6. A cross-section view of the often-used bilayer model for biological membranes. The large outer layers of protein form a sandwich containing a bimolecular layer of lipid molecules. In this model the polar heads (dark) of the lipids are presumably binding in an ionic fashion to the outer protein layer. The hydrocarbon part of the lipids extends into the central portion of the membrane.

relation time data. Nevertheless, this study does illustrate the type of information that can be obtained using double-labeling techniques.

Membranes. *Noncovalent Labeling Experiments.* Figure 6 illustrates one possible configuration of a biological membrane. This hypothetical structure consists of a lipid bimolecular leaflet sandwiched between two layers of protein. Membranes from various sources undoubtedly differ in structure, and the above hypothetical model may or may not resemble any real membrane. Nevertheless, there is some evidence in support of this type of structure,[43] and Figure 6 provides a convenient starting point for a discussion of membrane spin-labeling experiments. There are several ways one could use spin labeling to provide information about the membrane of Figure 6. For example, small unreactive nitroxides could be diffused into membrane model systems (see above) or into biological membranes. Alternatively, spin labels could be covalently bonded to the protein components. In yet a third approach one could attach nitroxides to the lipid components.

Hubbell and McConnell[44] recently took the first approach and diffused nitroxide IV into several membranous systems. They observed that when IV is present in both aqueous and hydrophobic environments of low viscosity the high-field line of the normal three-line spectrum is split into two lines. (This results from a simple sum of two spectra as explained in the discussion of Figure 4.) The intensities of the two high-field lines provide a measure of the amount of nitroxide tumbling rapidly in each environment. Hubbell and McConnell found that phospholipid suspensions and certain excitable membrane systems including the vagus nerve of rabbit, the walking leg nerve of *Homarus Americanus* (Maine lobster), and the excitable membrane of muscle exhibit two high-field lines, whereas a number of other membrane systems exhibit only the normal three-line nitroxide spectrum. The conclusion drawn from these observations and supporting experiments is either that excitable membranes contain phospholipid bilayers (see Figure 6) similar to those present in phospholipid vesicles or that the excitable membranes contain other components with very similar hydrophobic regions. The authors also observe changes in the relative heights of the two lines as other molecules are dissolved in the membranes. Another small nitroxide, di-*t*-butyl nitroxide (VII), also can exhibit a high-field doublet, and the general requirements appear to be rapid tumbling and reasonable solubility in both aqueous and nonaqueous environments.

In a seemingly unrelated study, Falle, *et al.*,[29] have analyzed the spectrum of dinitroxides XVIII and XIX in a liquid crystal of *p*-azoxyanisole. The spectrum clearly shows the dinitroxide is oriented in the nematic solution. Similar experiments on oriented membranes (*e.g.*, myelin) could provide valuable information regarding the dynamic structure of the lipid layers.

Spin Labeling of Protein Components. It is now a straightforward approach to spin label proteins using one of the many established procedures. Protein spin-labeling experiments prior to 1967 are adequately discussed elsewhere.[17] More recently, experiments with spin-labeled proteins and nucleic acids have been completed using III,[45] IX,[46] XIII,[47-49] and XIV.[47,48] In spite of advances in labeling techniques, it is not easy to obtain useful information about the structure and function of membranes from the esr spectra of spin-labeled membrane proteins. Some progress toward this goal has been made in a recent study by Sandberg and Piette.[50] The purpose of this work was to investigate drug–membrane interactions. Bovine erythrocyte ghosts were first labeled with maleimide nitroxide XIII, and the ghosts were treated with chlorpromazine. The maleimide was shown by blocking experiments to be bonded predominantly to SH groups. As is frequently the case, the spectrum indicated the presence of both weakly and strongly immobilized labels. The effect of the drugs was to decrease reversibly the concentration of weakly immobilized labels (presumably by hindering their rotation and thus converting them to strongly immobilized spin labels). The authors suggest that the changes occur because the labels are near the binding site of

(43) See, for example, discussions in the Ciba Foundation Symposium, "Principles of Biomolecular Organization," G. E. W. Wolstenholme, Ed., J. and A. Churchill, Ltd., London, 1966.

(44) W. L. Hubbell and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S.*, **61**, 12 (1968).

(45) An attempt has been made to study the rotational mobility of protein carboxyl groups by coupling the amine III to the carboxyl groups using water-soluble carbodiimides. However, under the conditions chosen, only about one molecule of III was bound to each protein molecule (R. A. MacQuarrie, O. H. Griffith, and J. F. W. Keana, unpublished results).

(46) Molecule IX has been found to react in low yield with a mixture of tRNAs. This observation was not pursued further (S. Rottschaefer and O. H. Griffith, unpublished results).

(47) I. C. P. Smith, *Biochemistry*, **7**, 745 (1968).

(48) I. C. P. Smith and T. Yamane, *Proc. Natl. Acad. Sci. U. S.*, **58**, 884 (1967).

(49) G. L. Grigorian, A. E. Kalmanson, E. G. Rozantzev, and V. S. Suskina, *Nature*, **216**, 927 (1967).

(50) H. E. Sandberg and L. H. Piette, *Agressologie*, **9**, 1 (1968).

the drug molecules or that the drugs intercalate into the protein–lipid complex, thus causing a change in surface structure. A third mechanism suggested by the authors is that the phenothiazine derivatives cause a conformational change in the membrane structure. Since the effect is very small, there is some question as to whether the labels involved are those bonded to SH groups or those located at less specific sites. Some results using chlorpromazine free radicals are also presented, and this study does indicate one approach in labeling membranes. In a related study, Barratt, *et al.*,[51] labeled proteins with the maleimide XII and observed the esr spectra of complexes of the proteins with phospholipids.

Spin-Labeled Lipids. A third approach in the investigations of membranes is to attach the nitroxide directly to a lipid. The lipid would then be incorporated by diffusion or, preferably, by enzymatic uptake into the membrane structure. Ideally, the nitroxide would not interfere with the normal reactions of the important lipid carboxyl group. This goal may or may not be obtainable, but a good starting point is to attach the nitroxide at some distance from the carboxyl end of the lipid. Recently the first synthesis of a spin-labeled lipid (XVI) has been accomplished by Waggoner, *et al.*[52] This methyl ester stearate and the corresponding acid are stable in the presence of a wide variety of substances including, for example, sodium dodecyl sulfate micelles, lecithin, myelin, and bovine serum albumin. Furthermore, the nitroxide moiety is bonded rigidly to the hydrocarbon chain of the lipid. This provides insurance that the esr spectra accurately reflect motion of the lipid. As can be seen in Figure 1, the spirane structure of nitroxide XVI aligns the z principal axis (direction of maximum splitting) along the hydrocarbon chain, a property that could be of some value in orientation experiments.

Keith, *et al.*,[53] have demonstrated the uptake of spin-labeled lipid XVI by *Neurospora crassa*, the common bread mold. The spin label was introduced into the medium, and after some hours of growth the *Neurospora* was harvested, the mitochondria were extracted, and the mitochondrial lipids were separated into phospholipid, neutral lipid, and free fatty acid fractions. Esr signals obtained from each of these three lipid classes indicate that some of the spin-labeled lipid had been biosynthetically incorporated into phospholipids and other lipid components of the mitochondria. The esr experiments were accompanied by control experiments using ^{14}C-labeled stearic acid. The incorporation of spin label was undoubtedly far from quantitative.

These incorporation experiments suggest new approaches in spin-labeling experiments. For example, it may well be possible to introduce spin labels into membranes in good yield simply by introducing the spin-labeled lipid into the growth medium of a fatty acid mutant (*i.e.*, an organism which cannot synthesize a given fatty acid class and so is more likely to incorporate high levels of the nitroxide analog). In any case, it should prove possible to synthesize chemically well-defined spin-labeled phospholipids and triglycerides, starting with saturated or unsaturated lipid spin labels, or it might prove possible to synthesize such complex lipids biosynthetically by the choice of an appropriate organism. Studies of the association and rotational mobility of these lipid components may contribute to the eventual understanding of such problems as what the rotational freedom of a lipid is in various membranes, why certain structures require unsaturated fatty acids, and what structural alterations accompany membrane excitation.

Nitroxides and Living Organisms. *The Problem of Coexistence.* If spin labeling is to become useful in such areas as membrane structure and neurobiology, some understanding is needed of the effects of organisms on nitroxides and *vice versa*. Several *in vivo* experiments involving nitroxides have been published,[54–58] but the conditions differ, and it is difficult at this point to gain a clear picture of the coexistence problem. In a study[59] designed to examine the compatibility of bacterial cells (*Escherichia coli*) with a dilute solution of nitroxide I, both the colony-forming ability of the cells and the survival of the esr signal were followed with increasing times of exposure. It appears that the water-soluble nitroxide I and this biological system are relatively incompatible. The cells survive best under conditions which result in destruction of the signal, and the signal persists in circumstances that impair the viability of the organism. The result may not be general (see ref 58, for example), but it does suggest the necessity for caution in utilizing these probes in intact biological systems. Some relatively water-insoluble nitroxides may be compartmentalized in the cell so that a similar marked loss of the signal will not occur, as suggested by the *Neurospora* lipid spin-label study mentioned earlier.

Thus, under appropriate conditions, the use of spin labels in the study of biomolecular structures, in either *in vitro* systems or *in vivo* incorporation, has the potential of providing new approaches to problems of biomolecular organization.

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