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Physics and chemistry of spin labels

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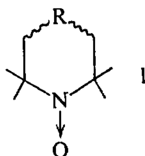
I. INTRODUCTION

Biological systems provide the physical chemist with an abundance of interesting, challenging and significant problems. One example is the problem of the molecular basis of co-operative or allosteric interactions between distant ligand or substrate binding sites in hemoglobin and in enzymes. This problem has been discussed recently in *This Journal* by Eigen (1968) and by Wyman (1968). Another particularly challenging problem is the molecular organization of biological membranes. Such problems tend to be particularly resistant to solution by the straightforward application of most spectroscopic techniques, in large part because of the enormous chemical and spectroscopic complexity of biological macromolecules. This spectroscopic complexity has stimulated the use of various 'probes' that can be introduced into selected sites in complex systems to provide spectroscopic signals that are comparatively free from interference. The use of heavy metal atoms ('isomorphous replacement') in X-ray studies of protein crystals (Green, Ingram & Perutz, 1954), and fluorescent dyes in the study of proteins in solutions (Weber, 1953; Steiner & Edelhoch, 1962) are early examples. Spin labels represent a new member of the family of spectroscopic structural probes. A spin label is a synthetic paramagnetic organic free radical, usually having a molecular structure and/or chemical reactivity that results in its attachment or incorporation at some particular target site in a biological macromolecule, or assemblage of macromolecules (Ohnishi & McConnell, 1965; Stone *et al.* 1965). This type of probe is being used in our laboratory to study allosteric interactions in proteins, and molecular dynamics and organization in membranes.

Most spin labels that have been used thus far in biophysical studies are based on nitroxide radicals having the general formula,



I

In this molecule the odd-electron is localized almost entirely on the nitrogen and oxygen atoms (Stone *et al.* 1965; Hamilton & McConnell, 1968); the four methyl (or alkyl) groups attached to the tertiary carbon atoms are necessary to reduce the chemical reactivity of the free radical

to the point where it is unreactive to many biochemical substances in aqueous solution of neutral pH. The group R serves to direct the free radical to the appropriate target in a macromolecular system, where it may be directly incorporated through, for example, covalent bond formation and/or polar and/or hydrophobic interactions or indirectly incorporated through one or more enzymatic steps in which the group R acts as a susbstrate. For an early example of the enzymatic attachment of a spin label, see Berliner & McConnell (1966). One very important feature of the nitroxide spin labels is the very great biochemical specificity that can be achieved through the use of suitable chemical groups R. In other words, even though the N $\rightarrow$ O group and the surrounding carbon atoms and methyl groups can hardly be considered to have a negligible size, it has now been clearly demonstrated that this group is none the less an entirely tolerable perturbation in a great variety of selected biochemical situations including, for example, the active sites of enzymes. A crucial property of the sterically protected nitroxide group is that it is neither strongly hydrophobic nor hydrophilic, and this important characteristic of the molecule is easily dominated by the group R. A second important feature of the nitroxide spin labels is the comparative simplicity and high accuracy of the spin quantum mechanics that relates the physical state of the label to its paramagnetic resonance spectrum. Here the 'physical state' of the label signifies its orientation in space if it has a fixed orientation, and the nature of its motion if it is moving. This simplicity also carries over to the use of spin labels as structure specific perturbations on nuclear resonance spectra, and on nuclear relaxation times.

A third important feature of spin labels in biological systems is that their paramagnetic resonance spectra, as well as their effects on nuclear resonance spectra, are usually completely free from interference. This is because the vast majority of the molecules in biochemical systems are diamagnetic and not paramagnetic.

Three reviews dealing with spin labels have already appeared in the literature (Hamilton & McConnell, 1968; Griffith & Waggoner, 1969; Ohnishi, 1968) and other articles dealing with specific biological applications of spin labels, and their use for the study of membranes, are in preparation (McConnell, 1970; McConnell & Hubbell, 1971). In the present paper we shall concentrate on certain relatively recent *physical* and *chemical* aspects of this technique, in particular, the structural and kinetic parameters that can be obtained from the magnetic resonance

spectra of biological systems containing spin labels, as well as some of the synthetic methods that have proven particularly useful in the preparation of labels. (The reader is referred to the two forthcoming papers cited above for a discussion of the more biological aspects of spin-label studies.) Finally, we give some examples to justify our above expressed contention that the nitroxide group is a tolerable perturbation in a variety of biochemical situations.

II. Analysis of the magnetic resonance spectra of biological systems containing spin labels

Structural and kinetic information can be obtained from electron and nuclear magnetic resonance spectra of biological systems containing spin labels. In the present section we summarize briefly some of the physical principles that relate magnetic resonance spectra to molecular structure and kinetics. Space does not permit us to review the basic principles of magnetic resonance spectroscopy; excellent books are available at both the elementary and advanced levels. See for example, Carrington & McLachlan (1967); Slichter (1963); Abragam (1961). However, in so far as possible our mathematical equations are supplemented with qualitative physical discussion.

First, we consider the paramagnetic resonance of nitroxide spin labels having the general formula I, assuming R does not contain a second paramagnetic center. As a first approximation we may say that paramagnetic resonance absorption by a free radical is observed when the following condition is met.

$$h\nu = g|\beta|\,|\mathbf{H}+\mathbf{H}_{\text{loc}}|. \tag{1}$$

Here h is Planck's constant, and ν is the frequency of the quantum of radiation that is absorbed. g is the so-called spectroscopic splitting factor, or simply g-factor, and $|\beta|$ is the absolute value of the electronic Bohr magneton, $0{\cdot}93 \times 10^{-20}$ erg/G. The total magnetic field acting on the odd electron of the free radical is $\mathbf{H}+\mathbf{H}_{\text{loc}}$, where $\mathbf{H}$ is the strength of the externally applied magnetic field, and $\mathbf{H}_{\text{loc}}$ is the field acting on the electron due to local sources, especially that due to the magnetic moment of the nitrogen nucleus (nuclear hyperfine interaction). A dominant feature of the paramagnetic resonance of nitroxide-free radicals is the splitting of the resonance spectra into three lines. This splitting arises because the nitrogen nucleus has a spin-angular momentum of one,

$I(N^{14}) = 1$, and the component of the nitrogen nuclear spin angular momentum in the external field direction can take on three values $I_H = 1, 0, -1$,. Thus, the local field acting on the odd electron, $\mathbf{H}_{loc}$, takes on three values, and the paramagnetic resonance spectrum is a triplet. Most paramagnetic resonance spectra are observed using a klystron as a source of microwave radiation, with a frequency that is fixed at some value in the vicinity of 9000 Mc/sec. Resonance absorption is observed by varying the applied field strength **H**, until various values of **H** and $\mathbf{H}_{loc}$ satisfy equation (1). The resonance spectra are typically in the vicinity of $|\mathbf{H}| \simeq 3200$ G when $\nu \simeq 9000$ Mc/sec. The local hyperfine fields are of the order 15–30 G for radicals I. The above features of the paramagnetic resonance spectra of nitroxide free radicals can be put into quantitative form through the use of the spin Hamiltonian, $\mathscr{H}$.

$$\mathscr{H} = |\beta|\mathbf{S}.\mathbf{g}.\mathbf{H}+h\mathbf{S}.\mathbf{T}.\mathbf{I}-g_N\beta_N\mathbf{I}.\mathbf{H}. \qquad (2)$$

The observed resonance spectra arise from allowed transitions between the eigenstates of this Hamiltonian. In this equation, **S** and **I** are mathematical operators that represent the electron- and nuclear-spin angular momenta, **g** is the *g*-factor tensor, **T** is the nuclear hyperfine tensor that includes both the anisotropic (electron-spin)—(nuclear-spin) dipolar interaction and the isotropic Fermi contact interaction, g_N is the *g*-factor for the N^{14} nucleus, and β_N is the nuclear Bohr magneton. The first term in equation (2) represents the interaction between the electron-spin magnetic moment and the external field, corrected for electron spin-orbit effects, the second term the electron-nuclear magnetic hyperfine interaction, and the last term gives the interaction between the nitrogen nucleus and the applied field.

A. *Orientation of spin labels in simple host crystals*

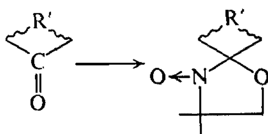
The quantities **g** and **T** are tensors. The principal axis systems for these tensors have been found to be parallel to each other in a number of experiments with a variety of spin labels (Griffith, Cornell & McConnell, 1965; McConnell & Hamilton, 1968; Hubbell & McConnell, 1969*a*). Let the principal axis system be *x*, *y*, *z*. The elements of the tensors g_x, g_y, g_z; T_x, T_y, T_z can be determined most accurately by incorporating the appropriate spin label, or a suitable nitroxide analogue, in an appropriate diamagnetic host single crystal, preferably one with a simple and known crystal structure. The first studies of this type were carried out by

Griffith *et al.* (1965). They found, for example, the following values for these tensor elements for di-*tert*-butylnitroxide.

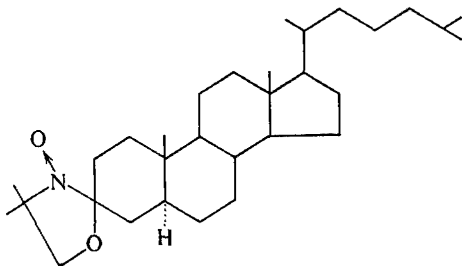
$$g_x = 2{\cdot}0089, \quad g_y = 2{\cdot}0061, \quad g_z = 2{\cdot}0027 \pm 0{\cdot}001$$

$$T_x \simeq T_y \simeq 14\text{–}17 \text{ Mc/sec}, \quad T_z = 87 \text{ Mc/sec}.$$

These determinations were carried out by measuring the resonance spectra of the nitroxide radicals in the single crystals as a function of crystal orientation, relative to the applied magnetic field direction, and making reasonable assumptions about the orientation of the radicals, which were substitutional impurities in host crystals with known structure. The elements of these tensors do depend to a small extent on the structure and composition of the group R. As a first rough approximation one can often neglect the dependence of these parameters on R. However, for precise quantitative work it is desirable to determine the parameters of the spin Hamiltonian from a single crystal study of a guest nitroxide in a host crystal that is chemically and structurally as close as possible to the spin label of interest. For example, Keana, Keana & Beetham (1967) have recently introduced a particularly useful synthetic method whereby *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of a variety of ketones can be prepared:



The parameters of the spin Hamiltonian for the oxazolidine nitroxide group were determined by Hubbell & McConnell (1969*a*) from an analysis of the paramagnetic resonance spectra of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-cholestan-3-one,



dissolved in single crystals of cholesteryl chloride. The parameters obtained for the spin Hamiltonian in this case are

$$g_x = 2.0089 \pm 0.001, \quad g_y = 2.0058 \pm 0.001, \quad g_z = 2.0021 \pm 0.001$$

$$T_x = 16.2 \pm 2 \text{ Mc/sec}, \quad T_y = 16.2 \pm 2 \text{ Mc/sec}, \quad T_z = 86 \pm 2 \text{ Mc/sec}.$$

In these single crystal studies, the principal axes are easily determined experimentally, since the corresponding resonance line positions are extrema for each of the principal axis directions (i.e. $\mathbf{H} \parallel \mathbf{x}$ or $\mathbf{H} \parallel \mathbf{y}$ or $\mathbf{H} \parallel \mathbf{z}$). For example, the largest hyperfine splittings for II (86 Mc/sec or

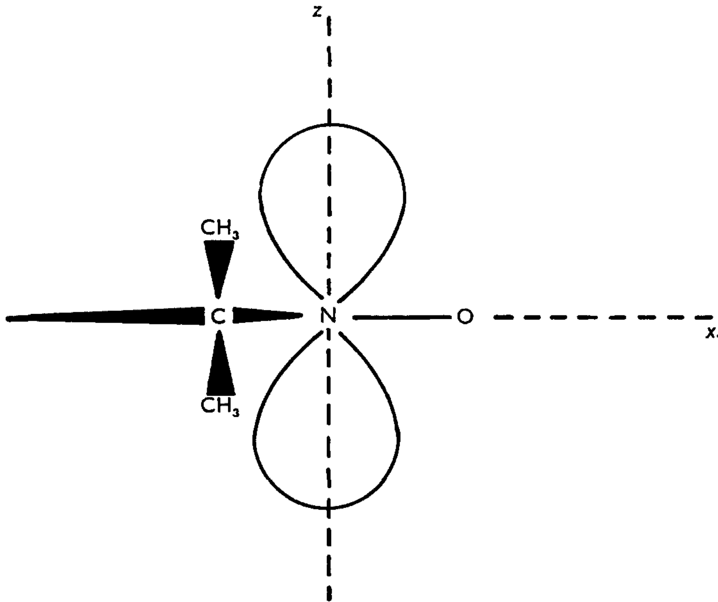


Fig. 1. Sketch of the principal axis system x, y, z of the spin Hamiltonian for a nitroxide radical. In this drawing it is assumed that the nitrogen, oxygen and two tertiary carbon atoms are all coplanar (see text).

30·7 G) are observed when the applied field **H** is parallel to the principal axis **z**. This principal axis direction also corresponds to a minimum g factor (2·0021), so for this orientation the $I_z(N^{14}) = O$ hyperfine component occurs at the highest field strength.

The single crystal studies, as well as theoretical electronic structure calculations (Hamilton & McConnell, 1968) show clearly that in general

the x-axis must be nearly parallel to the N → O bond direction, and the z-axis is nearly parallel to the axis of the $2p\pi$-orbital that holds the odd electron. This orbital has a large amplitude on the nitrogen atom

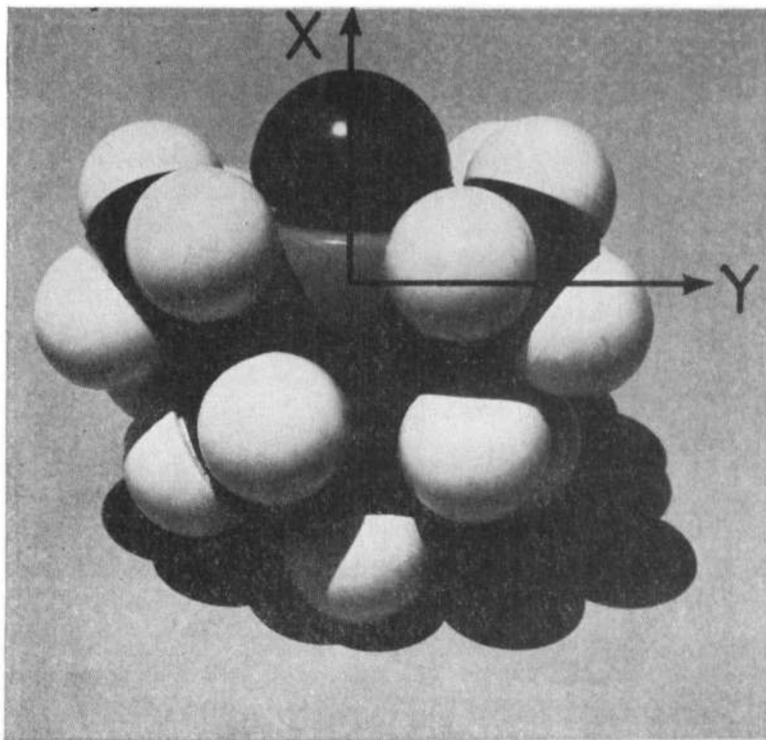
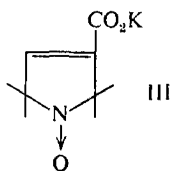


Fig. 2. Approximate orientation of the principal axes x and y of the spin Hamiltonian in 2,2,6,6-tetramethylpiperidine-1-oxyl.

(Hamilton & McConnell, 1968). A sketch of the axis system is shown in Fig. 1. In Fig. 1 it is assumed that the nitrogen, oxygen and two tertiary carbon atoms are exactly coplanar. This is known to be true in 2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl (III) (Kruger & Boeyens, 1968).



and only approximately so in 2,2,6,6-tetramethylpiperidine-4-ol-1-oxyl (IV) (Lajzerowicz-Bonneteau, 1968).

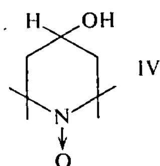
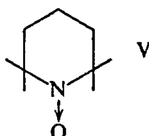


Fig. 3. Principal hyperfine axes *y* and *z* in Pauling–Corey–Koltum model of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 12-keto-stearic acid (right). A Pauling–Corey–Koltum model of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative at 5-ketostearic acid is shown on the left.

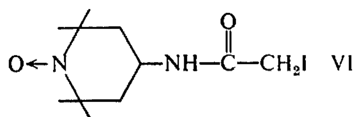
A rough sketch of these principal axes in relation to a PCK model of 2,2,6,6-tetramethylpiperidine-1-oxyl (V) is shown in Fig. 2.



The orientation of the *y* and *z* principal axes in another spin label is shown in Fig. 3. This label is the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 12-ketostearic acid which will be discussed later.

B. *Orientation of spin labels in protein crystals*

When nitroxide labels take up fixed orientations in anisotropic assemblages of biological macromolecules, then information on these orientations can be obtained from the anisotropy of the resonance spectra. In the case of labels attached at highly specific sites on pure proteins in single crystals, the resonance spectra are typically quite simple and highly anisotropic and can be analyzed in terms of the label orientation(s) relative to the crystal axis. One example of such a study is that carried out by McConnell & Hamilton (1968) and by McConnell, Deal & Ogata, (1969). These investigators used the spin label *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide (VI) to alkylate the reactive sulfhydryl groups at positions cysteine *β*93 in horse hemoglobin.



The hemoglobin was then crystallized and the resonance spectra recorded as a function of crystal orientation in the applied field. Examples of resonance spectra from spin-labeled single crystals are shown in Fig. 4 for two particular hemoglobin derivatives, carbonmonoxyhemoglobin and methemoglobin (ferrihemoglobin). Fig. 5 shows a plot of the resonance positions in these spectra as a function of the orientation of the applied magnetic field **H** relative to the crystallographic axes. The reader is referred to the original publications for the quantitative analysis and discussion of these spectra. Suffice it to say here that from such measurements one can easily and accurately determine the orientation of the principal axis (or axes) *z* relative to the crystallographic axes.

These experiments on spin-labeled hemoglobin crystals were designed to settle one simple question: are the protein structures in the vicinity of cysteines β93 identical in two hemoglobin derivatives, carbonmonoxyhemoglobin and methemoglobin? The answer to this question proved to be an interesting and unexpected one. At each cysteine β93 site, the label was found to take up two distinct and well defined orientations, A and B (indicated 1 and 2 in Figs. 4, 5). The A orientations were found to be

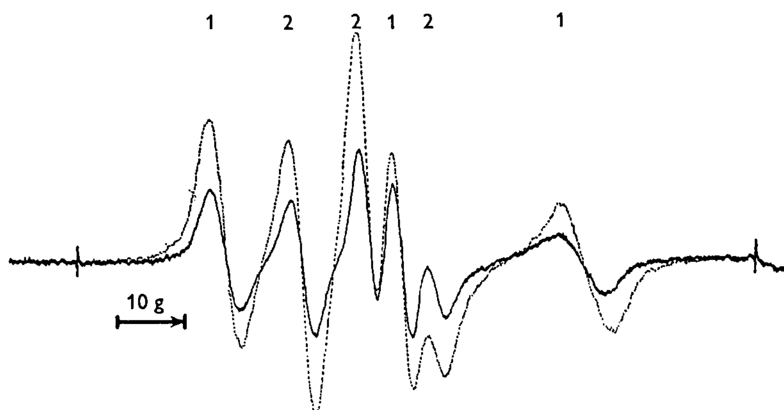


Fig. 4. The paramagnetic resonance spectra of a crystal of spin-labeled horse carbonmonoxyhemoglobin (——) and a crystal of horse methemoglobin (- - -) at pH 7·0. In both cases the applied field is perpendicular to the crystallographic twofold **b** axis, and makes an angle of 45 ± 3° with the monoclinic **a** axis and an angle of 24 ± 3° with the **c** axis. Signals from two (chemically distinct) radical orientations are designated 1 and 2. The relative intensities of signals 1 and 2 depend on the ionic composition of the crystallizing solutions, and are *not* identical for the two crystalline derivatives in equilibrium with identical ionic solutions.

identical in both hemoglobin derivatives as were the B orientations. Thus it could be concluded that the protein conformations in the vicinity of β93 were very nearly the same in the two derivatives. This conclusion was in fact not surprising in view of the X-ray studies by Perutz & Mathews (1966) who have concluded that the quaternary protein structure is identical for the two derivatives. The spin-label studies did show, however, that the local protein structure in the vicinity of β93 is *not exactly identical* in the two cases, since the relative populations of labels in the A and B states are not identical in the two derivatives (McConnell *et al.* 1969).

Spin-label studies of protein crystals can provide other types of inter-

esting information. Since one can observe the resonance spectra of labels attached to proteins in single crystals, and in solutions, there is the possibility of making a direct physical comparison of local protein

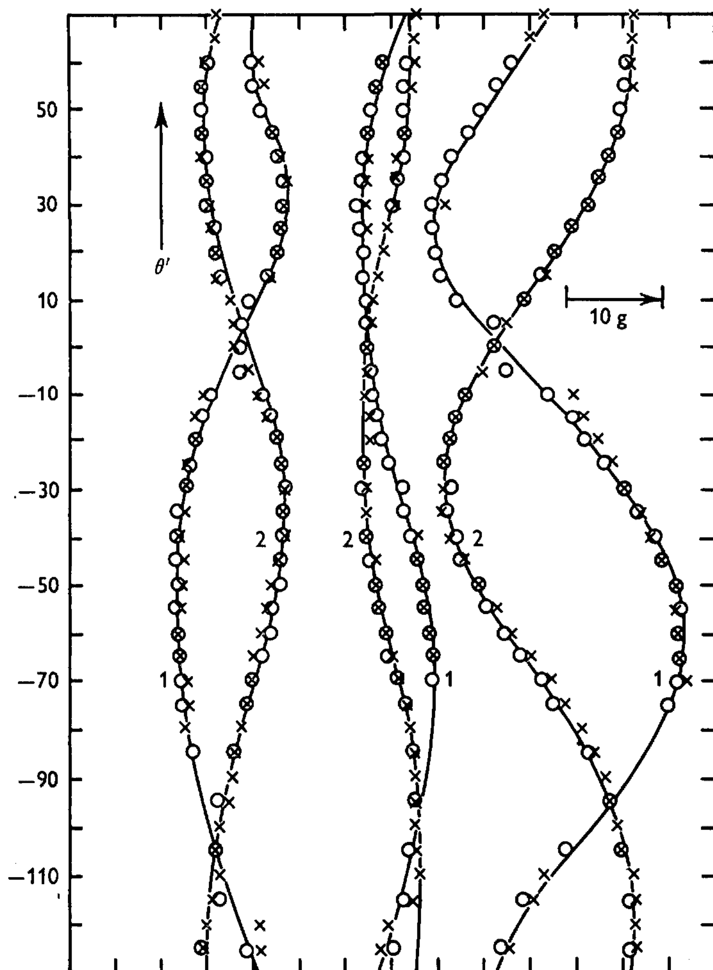


Fig. 5. Resonance line positions of a single crystal of spin-labeled horse carbonmonoxyhemoglobin (*o*) and methemoglobin (*x*) when the applied field is perpendicular to the twofold **b** axis, and makes an angle of $-\theta'$ with the **a** axis and an angle of $69° + \theta'$ with the **c** axis.

structure in the two cases. This has, of course, been an age-old point of discussion between different groups interested in protein structure. A comparison of the resonance spectra of the labeled hemoglobin in

solutions and in single crystals shows clearly that there can be no major difference in protein structure in the vicinity of β93 in the two cases, and indeed that the resonance changes that were observed are of the same order-of-magnitude as produced by changes in ionic composition of the solvent (McConnell *et al.* 1969).

In principle, the resonance spectra of labels attached to proteins in single crystals can detect the presence of non-crystallographic molecular symmetry axes in protein molecules built up from equivalent subunits (McConnell & Boeyens, 1967). The following systematic procedure is suggested: it is often easy to find the principal axis direction z for an oriented nitroxide radical in a single crystal even in the presence of other radicals with other orientations. If a protein molecule containing two equivalent subunits is labeled at equivalent sites, one can then locate the two principal axes z' and z'', one for each subunit. Any two axes $\pm z'$ and $\pm z''$ are always related to each other by three mutually perpendicular twofold axes. One of these must be the authentic twofold axis. The authentic and pseudo axes can be distinguished through the use of two or more labels with different molecular structures. A preliminary study of the non-crystallographic twofold axis in α-chymotrypsin has been made using this approach (Berliner, 1967).

C. *Orientation of spin labels in biological membranes*

A study of the orientation of spin labels in intact biological membranes has been carried out by Hubbell & McConnell (1969*c*). The orientation of selected labels relative to the surface of membranes is obviously a parameter of great structural interest. Two interesting complications have been encountered in the analysis of the resonance spectra of labeled membranes in terms of label orientations. First, labeled membranes have thus far shown a rather broad distribution of label orientations, in contrast to the small number of discrete orientations encountered in the case of specifically labeled proteins in single crystals, as described above. A broad distribution of label orientations in the case of membranes is not surprising in view of the great chemical (and presumably structural) complexity of intact biological membranes, and the difficulty of obtaining highly oriented arrays of membranes. A second interesting complication in the study of labeled membranes is the rapid anisotropic motion of some labels in certain membranes. We briefly consider the first of these interesting problems here. Anisotropic motion is considered in the next section.

In discussing anisotropic distributions of label orientations encountered in the study of membranes, let us first describe the completely *isotropic* case. Consider a single nitroxide radical where the applied field direction **H** makes polar and azimuthal angles of θ and ϕ in the x, y, z principal axis system of the radical. Let the absorption spectrum of this radical be a (**H**, θ, ϕ).

$$a(\mathbf{H},\theta,\phi) = a_1(\mathbf{H},\theta,\phi)+a_0(\mathbf{H},\theta,\phi)+a_{-1}(\mathbf{H},\theta,\phi). \tag{3}$$

In $a_m(\mathbf{H},\theta,\phi)$, m refers to the projection of the N^{14} spin angular momentum in the direction of the local field acting at the N^{14} nucleus. Judging from observed resonance spectra of oriented radicals in single crystals, the individual resonance absorption lines can be approximated by gaussian line-shape functions, with a half-width of the order of 5 G. For example,

$$a_1(\mathbf{H}, \theta\, \phi) = \exp-\{(H-H_r^{(1)}(\theta,\phi))^2/\Delta^2\}. \tag{4}$$

Here $H_r^{(1)}(\theta, \phi)$ is the center of the resonance signal, which, of course, depends on θ and ϕ. The absorption curve $A(\mathbf{H})$ for a *distribution* of molecular orientations with probability function $p(\theta, \phi)$ is then

$$A(\mathbf{H}) = \sum_m A_m(\mathbf{H}), \tag{5}$$

$$A_m(\mathbf{H}) = \int_{\phi=0}^{2\pi}\int_{\theta=0}^{\frac{1}{2}\pi} p(\theta,\phi)\, a_m(\mathbf{H},\theta,\phi)\,\mathrm{d}\theta\,\mathrm{d}\phi. \tag{6}$$

For an isotropic distribution, $p(\theta, \phi) = (\frac{1}{2}\pi)\sin\theta$. Calculated line shapes $A(\mathbf{H})$ for the isotropic case have been made by Itzkowitz (1967) using a computing machine. The calculated line shapes are in good agreement with the observed spectra of isotropic samples containing spin labels. Such spectra are often termed 'polycrystalline' since they can be observed using an isotropic powder of labeled single crystals, and the spectra are also termed 'strongly immobilized' to emphasize that any molecular motion of the spin label is so slow as not to affect the resonance spectra.

Fig. 6 shows the isotropic resonance spectrum of a strongly immobilized radical. The central signal receives contributions from all the radicals for which $I_z(N^{14}) = 0$. The low and high field signals arise from radicals for which (*a*) $I_z(N^{14}) = +1$ and $= -1$ and (*b*) the principal axis **z** is parallel or nearly parallel to the applied field **H**. The separation of the outer signals is $2T_z$. The outer low- and high-field signals thus arise from the $\mathbf{H} \parallel \mathbf{z}$ extrema in the resonance spectra. This accumulation of intensity in the $\mathbf{H} \parallel \mathbf{z}$ extrema is, of course, due to the fact that the

resonance line positions do not change rapidly with θ when θ is small. In typical strongly immobilized isotropic spectra the $\mathbf{H} \parallel \mathbf{x}$ and $\mathbf{H} \parallel \mathbf{y}$ extrema are not normally resolved, partly because the corresponding resonance lines overlap one another and also overlap the $I_z(N^{14}) = 0$ hyperfine component. This is evident from the resonance line positions shown in Fig. 7.

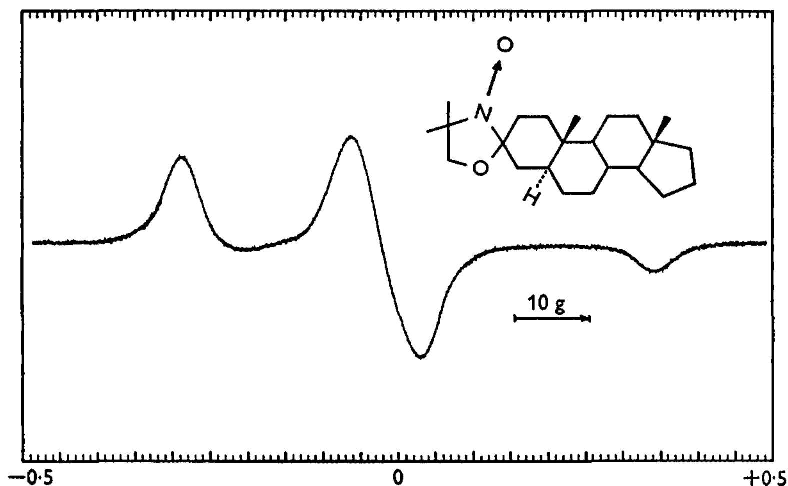


Fig. 6. Isotropic paramagnetic resonance spectrum of a 'strongly immobilized' spin label. The label is the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-androstan-3-one in a 1:1 isopentane-hexane mixture at −180 °C.

In the analysis of strongly immobilized resonance spectra, the low- and high-field lines are very important. Although such spectra can always be reproduced using a computing machine and an appropriate spin Hamiltonian, it is useful to have a simple analytic 'feeling' for the shape and intensities of these outer lines. This can be obtained as follows.

Fig. 7 shows the resonance line positions taken from the work of Ohnishi, Boeyens & McConnell (1966). In this case crystallized horse hemoglobin is labeled at the cysteine β93 positions with a maleimide label, VII.

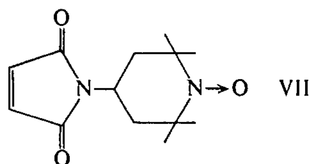


Fig. 7 shows resonance line positions as the applied field **H** is rotated in two principal axis planes of one radical, the **zx** plane, and the **zy** plane. It is clear from these data that the $I_z = \pm 1$ signals do not depend very strongly on ϕ except when θ is relatively close to 90°. Indeed, for a line width of 5 G the $\phi = 0°$ and $\phi = 90°$ signals are not well resolved from one another for values of θ less than 70°. Therefore let us calculate

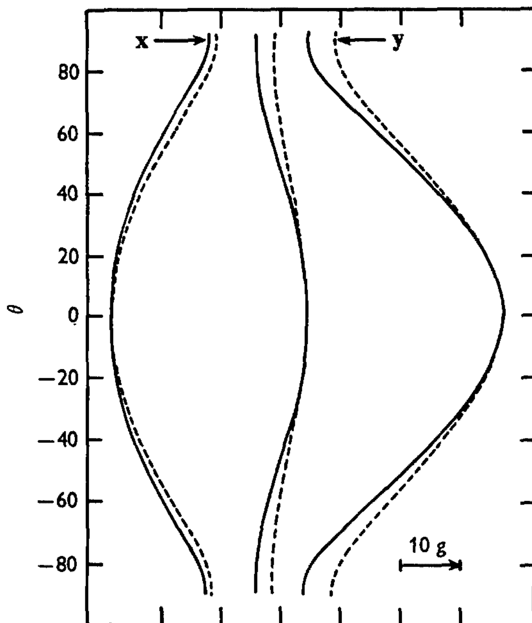


Fig. 7. Angular variation of the resonance positions in crystals of horse hemoglobin labeled with VII (see text). Planes of rotation are (approximately) the **zx** and **zy** principal axis planes of the nitroxide spin Hamiltonian. The solid curve (——) represents a rotation of the applied field **H** in the **zx** plane ($\phi = 0°$, variable θ) and the dotted curve (· · · ·) represents a rotation of the applied field in the **zy** plane ($\phi = 90°$, variable θ).

the absorption curve $A_1(\mathbf{H})$ using the following analytical expression for the resonance position.

$$H_r^{(1)}(\theta, \phi) = H_0^{(1)}(1-\lambda_1 \cos\theta). \tag{7}$$

This expression accurately represents the resonance positions for $0 \leqslant \theta \lesssim 70°$, and is quite inaccurate for values of θ much outside this range. $H_0^{(1)}$ and $H_0^{(-1)}$ represent the low- and high-field extrema of the $I_z = +1$ and $I_z = -1$ hyperfine states. If we now consider the isotropic case, $p(\theta, \phi) = (\frac{1}{2}\pi) \sin\theta$, combine equations (4), (6) and (7),

calculate $dA^{(1)}/dH$, and carry out the integration over θ and ϕ, we readily obtain the result

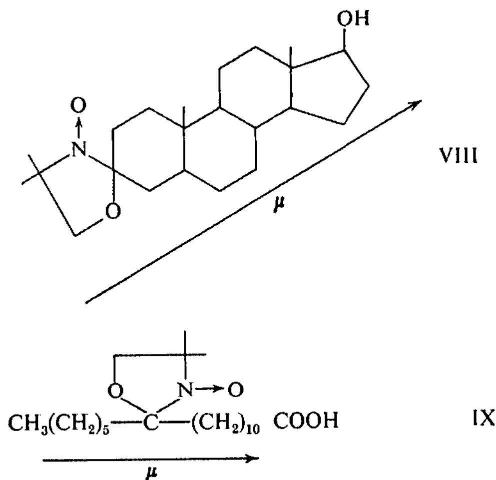
$$\frac{dA_1(\mathbf{H})}{dH} = \frac{1}{H_0\lambda_1}\exp -\{(H - H_0^{(1)}(1-\lambda_1))^2/\Delta^2\} + \ldots. \tag{8}$$

Here $+\ldots$ represents a signal centered around $H_0(1+\lambda_1)$ that arises from contributions to the integral in regions of θ where equation (7) is obviously not valid, and these terms are to be dropped. Thus, the expression in equation (8) gives the derivative of the resonance absorption for the $\mathbf{H} \parallel \mathbf{z}$ extrema. It is interesting to note that these derivative curves have the *shapes* of the absorption curves themselves, and the *amplitudes* of the derivative extrema depend inversely on the curvatures of the line positions of the extrema. Observed derivative curve line shapes, widths, and amplitudes are in agreement with equation (8) for strongly immobilized, isotropic nitroxide spectra. The class of nitroxide radicals giving rise to these high- and low-field extrema must therefore have their principal axes lying within a cone of angle Ω about the applied field direction, where

$$H_0\lambda_m(1-\cos\Omega) \sim \Delta. \tag{9}$$

The angle Ω is estimated to be of the order of 46° (26°) for the low-field (high-field) extrema of typical nitroxide radicals whose single crystal line widths are of the order of 5 G.

An example of an anisotropic distribution of label orientations can be seen in the resonance spectra in Fig. 8, which are taken from Hubbell & McConnell (1969*c*). In these experiments, the membranes of intact erythrocytes were labeled with the *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5α-androstan-3-one-17β-ol, VIII and 12-ketostearic acid, IX.



The spectra in Fig. 8 were observed while the erythrocytes were flowing through a small gap (~ 0·2 mm) between two flat quartz plates, and were thus oriented by hydrodynamic shear. The paramagnetic resonance spectra clearly have strongly immobilized, or nearly strongly immobilized components. The amplitudes of the outer hyperfine extrema depend on the orientation of the applied field relative to the hydrodynamic shear

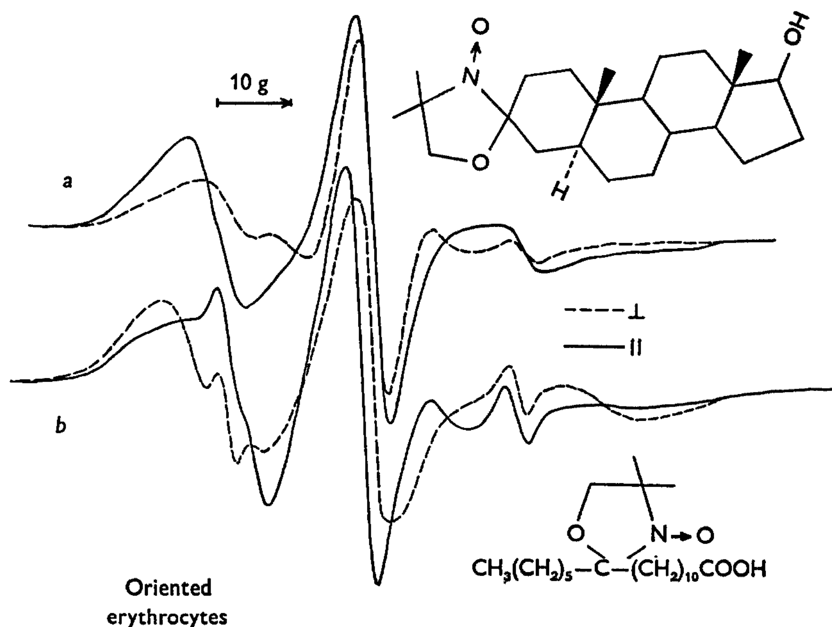


Fig. 8. Paramagnetic resonance of the *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of (*a*) 5α-androstan-3-one-17β-ol, and (*b*) sodium 12-ketostearate in erythorcytes oriented by hydrodynamic shear with the applied field perpendicular and parallel to the shear plane. In case (*a*) the center point *g*-factor is close to the isotropic value for both orientations, 2·0057. In case (*b*) the outer (inner) hyperfine extrema centre at $g = 2{\cdot}0027 \pm 0{\cdot}001$ ($2{\cdot}0057 \pm 0{\cdot}001$). The sharp signals at the isotropic splitting in case (*b*) are due to label IX in aqueous solution in equilibrium with the bound label.

plane. Let **P** be a unit vector perpendicular to the shear plane. The spin labels VIII and IX therefore have some anisotropic distribution relative to this plane. A rough indication of the anisotropy of the label orientation relative to **P** can be obtained by a comparison of the relative intensities of the outer extrema, corrected for relative instrumental sensitivity in the two orientations. It has been estimated roughly that for label VIII the $\mathbf{z} \perp \mathbf{P}$ orientation is more probable than the $\mathbf{z} \parallel \mathbf{P}$ orientation by a factor

of two, while for label IX the **z** ∥ **P** orientation is more favoured than the **z** ⊥ **P** orientation by 50% or more (Hubbell & McConnell, 1969*c*).

The preferred orientation of these labels relative to the plane of hydrodynamic shear has a straightforward qualitative explanation. The model of label VIII shown in Fig. 9 shows that the principal axis **z** is nearly perpendicular to the long amphiphilic axis $\boldsymbol{\mu}$. The label VIII

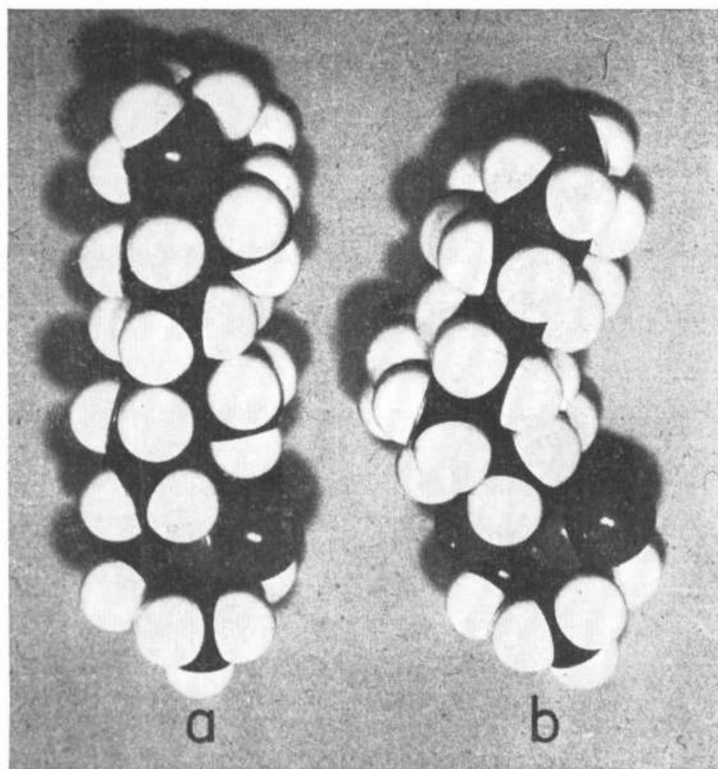


Fig. 9. Pauling–Corey–Koltum models of the *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of (*a*) 5α-androstan-3-one-17β-ol and (*b*) 5β-androstan-3-one-17β-ol.

takes up a preferred orientation in the erythrocyte with the long axis perpendicular to the membrane surface. Under hydrodynamic shear the erythrocyte membranes are preferentially oriented with the plane of the membranes parallel to the shear plane. This then leads to the preferred **z** ⊥ **P** orientation for label VIII. Just the opposite preferred orientation, **z** ∥ **P**, is found for label IX. This is expected since **z** is parallel to the

long amphiphilic axis μ in label IX. A model of label IX is shown in Fig. 3.

D. *Anisotropic motion of spin labels*

Certain spin labels have been found to undergo rapid anisotropic motions in some membranes and phospholipid dispersions (Hubbell & McConnell, 1969*b*, *c*). Let us consider briefly the general question of the effect of motion on the resonance spectra of nitroxide spin labels.

In the spin Hamiltonian in equation (2), the principal axes of **g** and **T** are molecule-fixed. In applied fields **H** of the usual strength, the secular or stationary components (as well as the harmonic components) of the electron-spin angular momentum are fixed relative to the laboratory axis system. In other words, the electron-spin precesses about the direction of the applied laboratory field, but is subjected to local fields that depend on the orientation of the molecule relative to the external field. Thus, in the presence of molecular motion, the Hamiltonian in equation (2) becomes time-dependent. This Hamiltonian may be written in the form,

$$\mathscr{H}(t) = \langle\mathscr{H}(t)\rangle + \{\mathscr{H}(t) - \langle\mathscr{H}(t)\rangle\}. \tag{10}$$

Here $\langle\mathscr{H}(t)\rangle$ is a suitable time or ensemble average of $\mathscr{H}(t)$, and $\{t\}$ gives the time-dependent fluctuation of $\mathscr{H}(t)$ about this average. Under favorable circumstances, $\langle\,\rangle$ provides the dominant features of the spectra, and $\{t\}$ can be treated as a (hopefully) small-time-dependent perturbation. The very fact that $\langle\,\rangle$ can account for the dominant features of the spectra may be extremely useful information in itself. For example, a nitroxide radical showing a sharp three line spectrum with hyperfine splittings equal to

$$a = \tfrac{1}{3}Tr(\mathbf{T}) = \tfrac{1}{3}(T_z + T_x + T_y), \tag{11}$$

immediately indicates a rapid isotropic tumbling motion with an inverse correlation time τ^{-1} that is large compared with the largest anisotropic term in the spin Hamiltonian, $|T_z - T_x|$. The effects of the explicitly time-dependent terms $\{t\}$ on paramagnetic resonance line shapes have been discussed extensively in the literature (McConnell, 1956; Kivelson, 1960; Freed & Fraenkel, 1963; Hudson & Luckhurst, 1969).

In recent work by Hubbell & McConnell (1968, 1969*b*, *c*) it has been found that very interesting information about molecular motion in membranes and phospholipid dispersions can be found by analyzing the resonance spectra in terms of an average of effective Hamiltonian $\mathscr{H}' = \langle\mathscr{H}(t)\rangle$. For example, Fig. 10 shows the paramagnetic resonance spectrum of label V in the presence of a rabbit vagus nerve fiber. Signal B

arises from label V dissolved in the aqueous solvent (Ringer's solution) surrounding the nerve fiber, and signal A arises from V dissolved in a hydrophobic environment (Hubbell & McConnell, 1968). In both cases the correlation time for tumbling is clearly very short (e.g. $\sim 10^{-9}$–10^{-11} sec.), showing this system of excitable membranes must contain *highly fluid* hydrophobic regions.

It was pointed out some time ago (McConnell, 1967) that if a spin label were to undergo a rapid anisotropic motion about some axis $\boldsymbol{\nu}$,

Fig. 10. The paramagnetic resonance of spin label V (see text) in the liquid-like hydrophobic region of the rabbit vagus nerve (A) and in the surrounding aqueous solution (B). The spin-label signals from these environments coincide for the other two hyperfine components of the spectrum.

then the resonance spectra should be accounted for by an effective or time-average Hamiltonian $\mathscr{H}'$.

$$\mathscr{H}' = |\beta|\, \mathbf{S}.\mathbf{g}'.\mathbf{H} + h\mathbf{S}.\mathbf{T}'.\mathbf{I} - g_N\beta_N\mathbf{I}.\mathbf{H}. \tag{12}$$

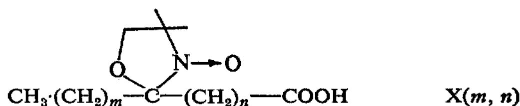
Here the elements of $\mathbf{g}'$ and $\mathbf{T}'$ are suitable averages of the elements of $\mathbf{g}$ and $\mathbf{T}$. For sufficiently rapid axial motion, $\mathscr{H}'$ must have axial symmetry about $\boldsymbol{\nu}$. In this case the elements of $\mathbf{g}'$ and $\mathbf{T}'$ are $g'_{\parallel}, g'_{\perp}, g'_{\perp}$ and $T'_{\parallel}, T'_{\perp}, T'_{\perp}$ where $\parallel$ and $\perp$ signify parallel and perpendicular to the symmetry axis $\boldsymbol{\nu}$. More generally, a label may undergo a more complicated motion still leading to axial symmetry of $\mathscr{H}'$ in some direction $\boldsymbol{\nu}$. One can easily show that in the case that rapid anisotropic motion of a nitroxide label leads to an effective axial symmetry axis $\boldsymbol{\nu}$, the elements of $\mathbf{T}'$ and $\mathbf{T}$ are related as follows.

$$T'_{\parallel} = \overline{\alpha^2}T_x + \overline{\beta^2}T_y + \overline{\gamma^2}T_z, \tag{13}$$

$$T'_{\perp} = \tfrac{1}{2}(1-\overline{\alpha^2})T_x + \tfrac{1}{2}(1-\overline{\beta^2})T_y + \tfrac{1}{2}(1-\overline{\gamma^2})T_z. \tag{14}$$

Here $\overline{\alpha^2}$, $\overline{\beta^2}$, $\overline{\gamma^2}$ are the time averages of the squares of the direction cosines of **ν** in the x, y, z principal axis system. For example, $\gamma^2 = \cos^2(\mathbf{\nu}, \mathbf{z})$ where $(\mathbf{\nu}, \mathbf{z})$ denotes the angle between **ν** and **z**. Similar equations hold for the elements of **g** and **g′**. We shall refer to $\cos^{-1}\sqrt{\overline{\gamma^2}}$ as the 'mean angular deviation' between **ν** and **z**. The spectra calculated from $\mathcal{H}'$ can be expected to be good approximations when the motion is described by inverse correlation times that are large compared with the appropriate anisotropic terms in $\mathcal{H}$.

Spin label VIII as well as the N-oxyl-4′,4′-dimethyloxazolidine derivatives of the straight chain $n+2$ keto acids having $m+n+3$ carbon atoms (X (m, n)) have been found to undergo rapid anisotropic motions in certain membranes and phospholipid dispersions.



For example, the paramagnetic resonance of X(17, 3) in a sonicated dispersion of phospholipids is shown in Fig. 11. Although we have not

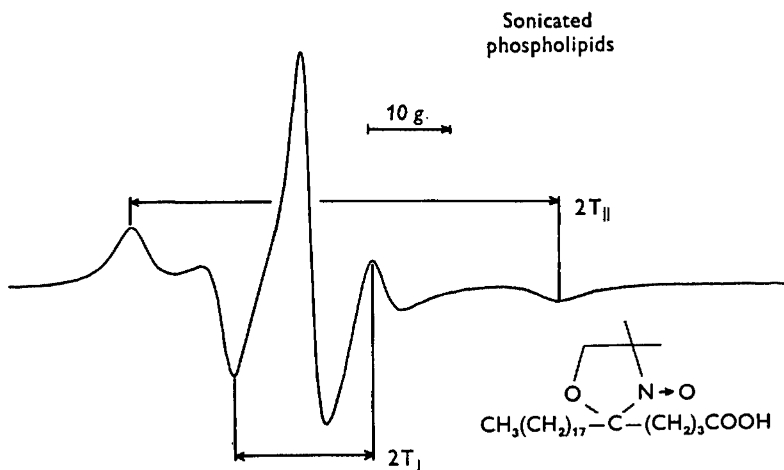


Fig. 11. Paramagnetic resonance of the N-oxyl-4′,4′-dimethyloxazolidine derivative of sodium 5-ketotricosanoate (label X (17, 3)) in a sonicated dispersion of purified soybean phosphatides, pH 8·0.

yet carried out a machine computation that reproduces the line shape in Fig. 11 in terms of a spin Hamiltonian having axial symmetry, it is quite clear from previous calculations of line shapes for ions having aniso-

tropic but axially symmetric g-factors (Schoffa, 1964) that the observed shape in Fig. 11 is indeed associated with axial symmetry. Note that both the outer and inner hyperfine extrema are well resolved, leading to direct determinations of $T'_{\parallel}$ and $T'_{\perp}$: $T'_{\parallel} = 73$ Mc/sec. $T'_{\perp} = 23{\cdot}8$ Mc/sec. (Independent checks on such spectral assignments are afforded by the requirement that Trace ($\mathbf{T}$) = Trace ($\mathbf{T}'$).) Since the outer hyperfine extrema have a separation $2\mathbf{T}'_{\parallel} = 2 \times 72$ Mc/sec that is close to $2\mathbf{T}_z = 2 \times 86$ Mc/sec for the oxazolidine nitroxide, it is clear that whatever the details of the rapid anisotropic motion may be, the molecule-fixed principal axis $\mathbf{z}$ must be relatively close to the axial symmetry axis $\boldsymbol{\nu}$. The mean angular deviation between $\mathbf{z}$ and $\boldsymbol{\nu}$ is in fact found to be 26°. Since the hyperfine tensor for the oxazolidine ring is already (accidentally) axially symmetric the one interaction that must be averaged to zero by the rapid motion is the x–y g-factor anisotropy. In other words, the axial motion must at least be rapid compared with

$$h^{-1} \mid \beta(g_x - g_y) \mid H \sim 10^7 \text{ sec}^{-1}.$$

This rapid anisotropic motion of X(17, 3) is doubtless predominantly about the long amphiphilic axis $\boldsymbol{\mu}$; in other words, $\boldsymbol{\mu} \simeq \boldsymbol{\nu}$. A similar rapid anisotropic motion has been deduced from the resonance spectrum of VIII in the walking leg nerve fiber membrane of *Homarus americanus*. The label X(17, 3) also shows evidence for rapid anisotropic motion in this nerve fiber. Fig. 12 shows the resonance of X(17, 3) in this nerve fiber for $\mathbf{H} \perp \mathbf{A}$ and $\mathbf{H} \parallel \mathbf{A}$ where $\mathbf{A}$ is the common axonal cylinder axis of the nerve fiber. An analysis of these resonance spectra shows that the $\boldsymbol{\nu} \perp \mathbf{A}$ orientation is preferred over the $\boldsymbol{\nu} \parallel \mathbf{A}$ orientation by a factor of five. This evidently signifies that the long amphiphilic axis of X(17, 3) is preferentially perpendicular to the local membrane surface; alternatively we may say the plane of the oxazolidine ring is preferentially parallel to the local membrane surface.

It is extremely interesting to note that the motion of the oxazolidine ring is effectively rapid and isotropic in X(5, 10), suggesting that the central region of lipid bilayers in nerve membranes is very fluid. Recall from the previous section that this is *not* the case for X(5, 10) in erythrocyte membranes, where the oxazolidine ring is nearly 'strongly immobilized'.

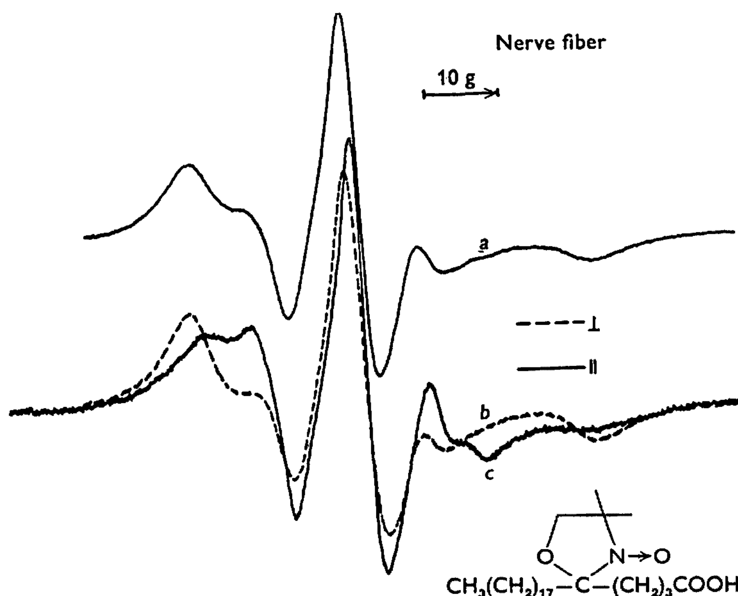
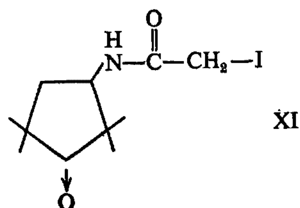


Fig. 12. Paramagnetic resonance of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of sodium 5-ketotricosanoate (label X (17,3)) in the walking leg nerve fiber of *Homarus americanus*. (*a*) Minced nerve; (*b*) applied field perpendicular to cylinder axis of nerve fiber; (*c*) applied field parallel to cylinder axis of nerve fiber. The *g*-factor for the center of the outer (inner) hyperfine extrema is $2{\cdot}0031 \pm 0{\cdot}001$ ($2{\cdot}0064 \pm 0{\cdot}001$).

E. *Conformational changes in labeled macromolecules*

The general dependence of resonance spectra on the molecular motion of labels enables one to use these paramagnetic molecules as very delicate indicators of conformation changes in macromolecules.

The most extensively investigated example of this is the case of spin-labeled hemoglobin (Ogawa & McConnell, 1967; Ogawa, McConnell & Horwitz, 1968; McConnell, Ogawa & Horwitz, 1968). Fig. 13 shows the oxygen-dependence of the paramagnetic resonance spectrum of horse hemoglobin alkylated at positions β93 by the iodoacetamide spin label



XI

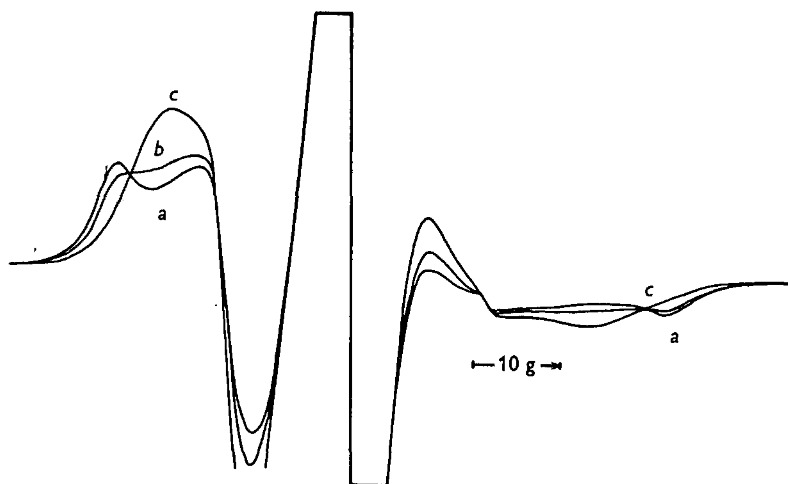
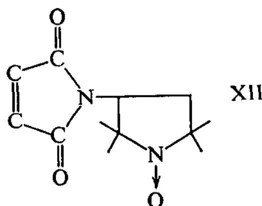


Fig. 13. The paramagnetic resonance spectra of spin-labeled horse hemoglobin (*a*) 100% oxygenated; (*b*) 71% oxygenated; (*c*) deoxygenated. The label is the iodoacetamide XI.

These spectra arise from the labeled hemoglobin in *aqueous solution* at room temperature. The spectrum of this label attached to the hemoglobin molecule depends on the motion of the label relative to the local protein structure and not on the rotational diffusion of the whole hemoglobin molecule. (This can be demonstrated quite convincingly from the spectrum of hemoglobin labeled at $\beta 93$ with a maleimide label (Boeyens & McConnell, 1966).



This shows that the over-all rotational diffusion of the hemoglobin tetramer is too slow to average out the anisotropies in the spin Hamiltonian since the observed spectrum in this case is clearly of the 'strongly immobilized' type.)

Spectra such as those illustrated in Fig. 13 show sharp *isosbestic* points. This result signifies that these labels sense two, and only two local protein conformations during the course of the oxygenation of the

molecule. The change in resonance spectrum was found to be linear in the degree of heme group oxygenation, indicating that each label XI reflects the structure of the β subunit to which the label is bound.

Theoretical arguments based in part in the crystal structures of horse hemoglobin due to Perutz and co-workers suggested that some other label attached to β93 might show a significant spectral deviation from isosbesty during the course of oxygenation. This was found to be the case for the iodoacetamide label VI attached to positions β93 (Ogawa

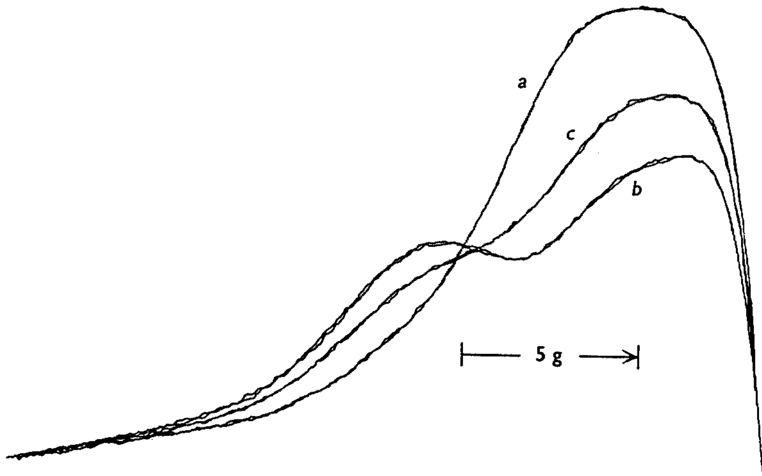


Fig. 14. The paramagnetic resonance of a 1 % human hemoglobin solution labeled with the iodoacetamide VI; 0·1 M-phosphate, pH 7·5, 18 °C. (*a*) deoxy; (*b*) oxy; (*c*) intermediate oxygenation. Each spectrum is recorded twice to indicate reproducibility.

et al. 1968). The spectra are shown in Fig. 14. These results then indicate the existence of a local region of the hemoglobin molecule that has a structure that depends on the state of oxygenation of more than one heme group, and this is qualitatively just the type of interaction one expects to be involved in allosteric interactions. The significance of these results in relation to models of allosteric interactions has been discussed elsewhere (McConnell *et al.* 1968). It is clear that many studies of allosteric effects and conformational changes in macromolecules are possible using spectral changes of this type. It is to be understood, of course, that more than one label may have to be tried before one can find a label whose spectrum is sufficiently sensitive.

F. *Nuclear relaxation in biological systems containing spin labels*

It is well known that nuclear relaxation rates in liquids and solids are enhanced in the presence of paramagnetic ions and molecules (Abragam, 1961). In the case of liquids, the relaxation rate of nuclei in a diamagnetic molecule D generally depends on how frequently and how closely D encounters the paramagnetic ions or molecules P in the solution containing both D and P. The enhanced nuclear relaxation has its origin in the very large and fluctuating magnetic field that acts on a nucleus when it is close to one (or more) unpaired electron(s); this field is of the order of magnitude of $|\beta|/r^3$, when r is the electron-nucleus distance. When $r = 10^{-8}$ cm this field is approx. 10^4 G. Theories of the relaxation of nuclei in the immediate environment of paramagnetic ions (or molecules) have been given by Solomon (1955) and by Bloembergen (1957). The electron-nuclear dipolar contribution to the nuclear relaxation rate of a nucleus in the immediate vicinity of a paramagnetic ion was shown by these authors to be

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{g^2\beta^2 g_N^2\beta_N^2}{r^6}\left[3\tau_c + \frac{7\tau_c}{1+\omega_e^2\tau_c^2}\right]. \tag{15}$$

Here τ_c is the correlation time for the dipolar interaction and ω_e is 2π times the electron-spin resonance frequency.

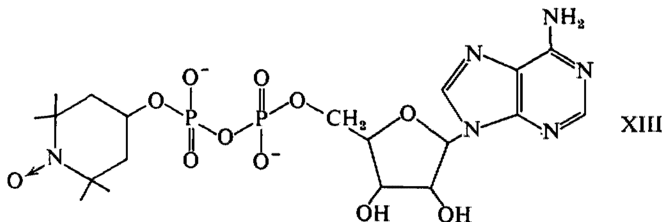
The Bloch equations for nuclear magnetic resonance spectra can be modified to include the effects of chemical exchange between diamagnetic and paramagnetic environments (McConnell, 1958). These modified equations yield the following simple expression for the paramagnetic contributions to the relaxation rate of a nucleus which can exchange back and forth between diamagnetic and paramagnetic environments (Swift & Connick, 1962; Luz & Meiboom, 1964).

$$\frac{1}{T_{1p}} = \frac{pq}{T_{1M}+\tau_M}. \tag{16}$$

Here T_{1p}^{-1} is the contribution to the nuclear relaxation rate arising from the paramagnetic species. T_{1M}^{-1} is the nuclear relaxation rate when the molecule containing the nucleus is at a distance of closest approach to the unpaired electron(s) (PD complex), p is the ratio of the number of paramagnetic ions or molecules to the number of nuclei undergoing resonance, q is the number of molecules that can co-ordinate the paramagnetic species, and τ_M is the lifetime of the strong magnetic encounter —that is, the lifetime of the PD complex.

Water proton relaxation in aqueous solutions of manganous and other ions is enhanced in the presence of DNA (Eisinger, Shulman & Szymanski, 1961) and is also enhanced in the presence of proteins and substrates (Cohn & Leigh, 1962). By determining the temperature dependence of such enhanced relaxation rates it is sometimes possible to obtain quantitative information on both τ_M and T_{1M} in equation (16). The lifetime τ_M provides a direct measure of a chemical kinetic rate under steady state conditions. The relaxation time T_{1M} can provide information on molecular geometry through its strong dependence on the electron-nuclear distance in equation (15), providing the correlation time τ_c can be estimated. A review of this use of nuclear relaxation enhancement by paramagnetic species to study enzyme kinetics and geometry has been written recently by Mildvan & Cohn (1970).

Weiner (1969); Mildvan & Weiner (1969*a*, *b*) have carried out a most interesting study of the electron and nuclear magnetic resonance of alcohol dehydrogenase using the following spin-label analog of nicotinamide-adenine dinucleotide (XIII).



These authors showed that (*a*) this spin label competes with NADH for the same binding site in the enzyme, (*b*) the paramagentic resonance of the bound label corresponds to 'strong immobilization', (*c*) the *bound* label produces an enhancement in water proton relaxation rate by a factor of eighty, (*d*) this water proton relaxation enhancement decreases in the presence of alcohol and (*e*) the nuclear resonance of the alcohol methyl protons is specifically broadened by interaction with the nitroxide electron spin in the active site of the enzyme. These studies lead to the immediate qualitative conclusion that the alcohol binds close to the 2,2,6,6-tetramethylpiperidine-*N*-oxyl ring (as expected from the biochemical role of the corresponding nicotinamide ring in NAD), and that alcohol binding limits the access of water to this region in the active site. These investigators were moreover able to determine the kinetics of alcohol binding (from τ_M) and made estimates of the (methyl-proton)–

(electron-spin) distance (3·6 A) and (methylene-proton)–(electron-spin) distance (4·1 A) at the active site (from T_{1M} and equation (15)).

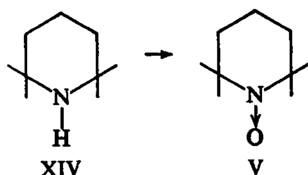
It is quite clear that measurements of just this type can be carried out on many other enzyme-substrate systems containing suitable spin labels. It is also clear that there are many other types of systems where (electron-spin)–(nuclear-spin) interactions can provide significant structural and/or kinetic information. For example, Roberts, Hannah & Jardetsky (1969) have synthesized a spin-label monophosphate, 2,2,6,6-tetramethyl-9-hydroxypiperidine-1-oxyl monophosphate (XXXV—see below) which appears to occupy a site similar to that which inorganic phosphate occupies close to the active site of ribonuclease. The local magnetic field due to the spin label produces a strong broadening of the proton nuclear resonance of various amino acids in the enzyme, and the broadening can therefore be used to obtain structural information on the environment of the label. Other possibly significant electron-nuclear interactions will be mentioned later in Part V.

III. Synthesis of nitroxide spin labels

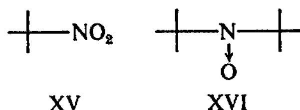
The first synthetic free radical employed as a probe of biomolecular structure was the chlorpromazine radical cation (Ohnishi & McConnell, 1965). However, the utility of the chlorpromazine radical was limited by its low stability under conditions appropriate to biological samples and by its relatively complex magnetic resonance spectrum. Two recent reviews of stable radicals (Buchachenko, 1963; Forrester, Hay & Thomson, 1968) list very few radical species which can be used for spin-label studies. Of the known stable free radicals, the di-*t*-alkylnitroxides represent a seemingly unique solution to the problems of chemical stability and simplicity of the resonance spectra. (In the literature, organic free radicals are commonly (and loosely) referred to as 'stable' if they are long-lived in the system of interest. This signifies that the radical is not rapidly destroyed by spontaneous decomposition, by dimerization or polymerization, or by chemical reaction with other chemical components in the system of interest.)

A. *Aliphatic nitroxide radicals*

Lebedev & Kazarnovskii (1960) identified the first di-*t*-alkylnitroxide V, from the hydrogen peroxide oxidation of the secondary amine, 2,2,6,6-tetramethylpiperidine XIV.

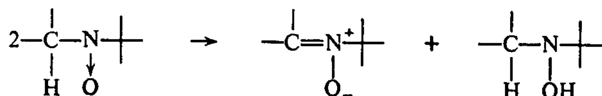


Shortly thereafter, the simplest of this class of compounds, di-*t*-butylnitroxide XVI, was prepared by alkali metal reduction of *t*-nitrobutane XV and its remarkable stability was noted (Hoffman & Henderson, 1961).



The preparation of stable nitroxides by the above methods, as well as in free radical reactions of *t*-nitrosoalkanes and alkyl nitrites and by mild oxidation (e.g. silver oxide) of di-*t*-alkylhydroxylamines, have been reviewed (Forrester *et al.* 1968). For purposes of spin-label synthesis, the hydrogen peroxide oxidation of a di-*t*-alkylamine has been most often employed. The oxidation is carried out in water or water–methanol at neutral or basic pH with either phosphotungstic acid or sodium tungstate as catalyst (Hamilton & McConnell, 1968). Alternatively, the same amines can be oxidized in inert solvents by organic peracids such as *m*-chloroperbenzoic acid (Keana *et al.* 1967).

The di-*t*-alkylnitroxides are stable by almost all criteria for defining free radical stability. Obviously, electron delocalization is not a major factor in determining their stability; in fact, the di-*t*-alkylnitroxides are more stable than some aromatic nitroxides. The feature which distinguishes the stability of di-*t*-alkylnitroxides from the other dialkylnitroxides is the inability of the former to undergo disporportionation.



Although the di-*t*-alkylnitroxides show no tendency to dimerize,

apparently because of steric bulk of the four α-methyl groups, dimers of several of their analogues have been obtained. Fremy's salt is a dimer in the solid state. Sulfur analogues of stable nitroxides XVII are dimers at room temperature and are in reversible equilibrium with the monomeric radicals only at elevated temperatures (Bennett, Sieper & Tavs, 1967).

O= N–(S)$_n$–N =O ⇄ O= N→S XVII

Ditrifluoromethylnitroxide is a monomer at room temperature but dimerizes at −160° (Makarov *et al.* 1965).

Soon after their discovery, it was noted that the di-*t*-alkylnitroxides are sufficiently stable to allow a variety of chemical conversions to be carried out selectively on a second functional group in the molecule (Rozantsev & Neiman, 1964; Briere *et al.* 1965 *a*, *b*). The many syntheses of spin labels which have been developed serve well to illustrate the diverse conditions to which the nitroxide group is chemically inert.

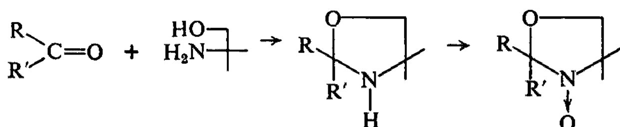
B. *Synthesis of labels*

Three general approaches have been employed in spin-label syntheses. First, a functionally substituted nitroxide I may be subjected to chemical reactions which modify the group R so that it will exhibit a desired specific chemical or biological activity. For this approach, five nitroxide precursors, IV and XVIII–XXI, which were first described by Rozantsev and co-workers (1964–65), are readily prepared from the corresponding commercially available amines.

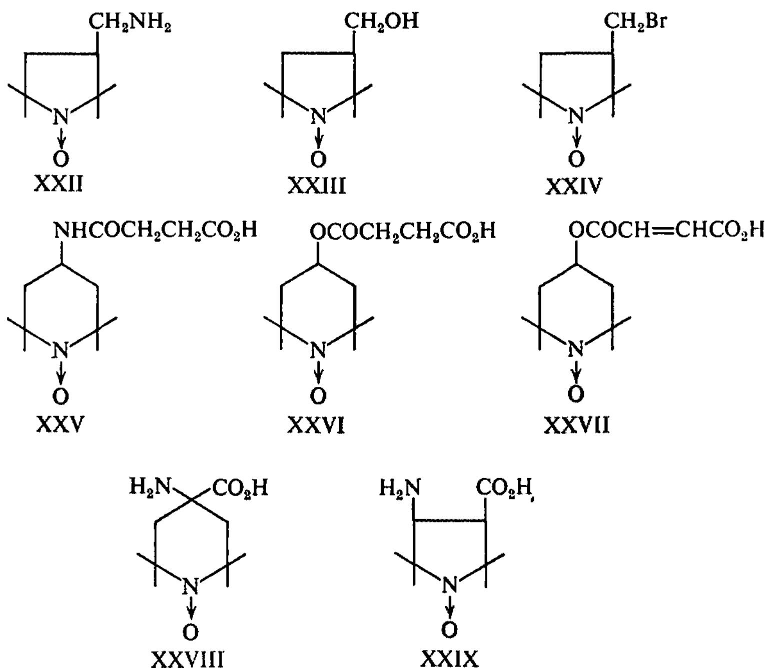
XVIII (O, N→O) IV (OH, N→O) XIX (NH_2, N→O)

XX ($CONH_2$, N→O) XXI ($CONH_2$, N→O)

Second, since nitroxides are unstable, for example, to strong acids and some reducing agents, spin-label preparations may also begin with modification of the R group followed by oxidation to the nitroxide as a last step. A third approach to spin label synthesis is provided by the discovery of a convenient means of converting ketones to their *N*-oxyl-4′,4′-dimethyloxazolidine derivatives and subsequent oxidation to stable nitroxides (Keana *et al.* 1967).



By far the majority of spin labels have been prepared by the first method mentioned above; by chemical conversions of five nitroxides, IV and XVIII–XXI. Several derivatives of these molecules have been prepared recently and should also be generally useful as synthetic intermediates. These include the primary amine XXII (Hsia & Piette, 1969), the primary alcohol and bromide, XXIII and XXIV, (B. G. McFarland, unpublished, 1969), several carboxylic acids XXV–XXVII (Kosman, Hsia & Piette, 1969; W. Balthasar, unpublished, 1969) and the amino acids, XXVIII and XXIX (Rassat & Rey, 1967).



Functional modification of R in the nitroxide precursors, IV and XVIII–XXI, has led to spin labels designed to react specifically with sulfhydryl, terminal- and ε-amino, and histidine tyrosyl and serine hydroxyl residues of proteins, to spin labels which act as enzyme substrates and inhibitors, and to spin labels which bind to biomolecules by non-covalent interactions. The early developments of this approach were reviewed (Hamilton & McConnell, 1968; Griffith & Waggoner, 1969; Ohnishi, 1968), at which time it had been amply demonstrated that nitroxides were stable to conditions which involve derivatization of ketones, formation of esters employing diazomethane or dicyclohexylcarbodiimide, formation of amides employing normal acylating reagents under basic or neutral conditions, and condensations and hydrolyses in strong bases.

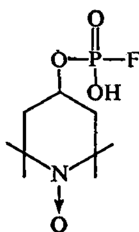
Three of the sulfhydryl specific reagents described earlier (Hamilton & McConnell, 1968) have gained wide use in recent spin-label studies. The maleimide VII has been used in studies of lipid-protein complexes (Barratt, Green & Chapman, 1968), serum albumin (Grigorian *et al.* 1967) and muscle contraction (Cooke & Morales, 1969; Tonomura, Watanabe & Morales, 1969).

The iodoacetamides, VI and XI, which were not fully developed as spin labels at the time of the earlier review, have proved highly effective, particularly in studies of hemoglobin. Considerable insight into the allosteric oxygen binding of hemoglobin has been derived from hemoglobin spin-labeled with the iodoacetamides at the sulfhydryl group of cysteine β93 (see Section II E). Lichtenstein *et al.* (1968) have employed a cysteine directed mercurial spin label to investigate conformational changes in bovine hemoglobin.

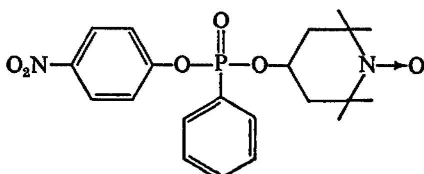
A number of new spin-label reagents for acylating the serine hydroxyl group have been prepared. Among these are the fluorophosphate, XXX*a* (L. H. Piette, private communications, 1969), the *p*-nitrophenylphosphonate, XXX*b* (W. G. Struve, unpublished, 1969) and a series of *p*-nitrophenyl esters, XXXI (Kosman *et al.* 1969); for an earlier investigation, see Berliner & McConnell (1966).

A series of bromo acid and bromo amide nitroxide labels have been employed in studying the conformational properties of bovine pancreatic ribonuclease A (Smith, 1968).

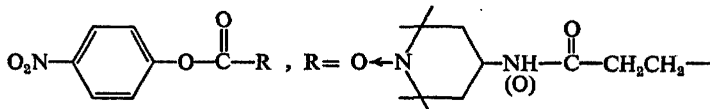
Several spin-label reagents have been designed to react with free amino groups. The isothiocyanate, XXXII (B. G. McFarland, unpublished, 1968), is very stable at temperatures below 70°, in contrast



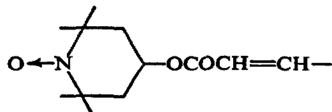
XXXa



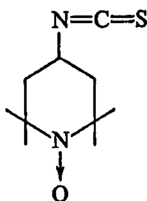
XXXb



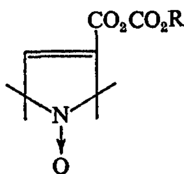
XXXI



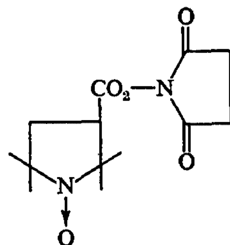
with the previously prepared isocyanate (Stone *et al.* 1965). The isothiocyanate has been employed in muscle contraction studies (Cooke & Morales, 1969; Tonomura *et al.* 1969). The nitroxide mixed carboxylic-carbonic acid anhydrides, XXXII (Griffith *et al.* 1967), are another example of amino group-directed spin labels. A third reagent, a spin-label analogue XXXIV of the *N*-hydroxysuccinimide ester of acetic acid has been used to label the α-amino group of valyl-*t*-RNA (Hoffman, Schofield & Rich, 1969).



XXXII



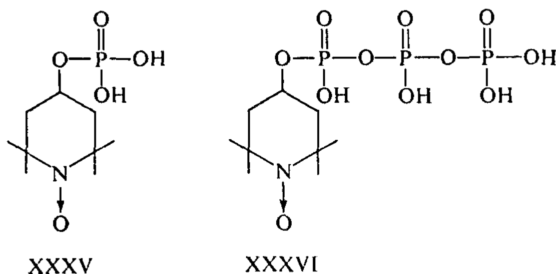
XXXIII



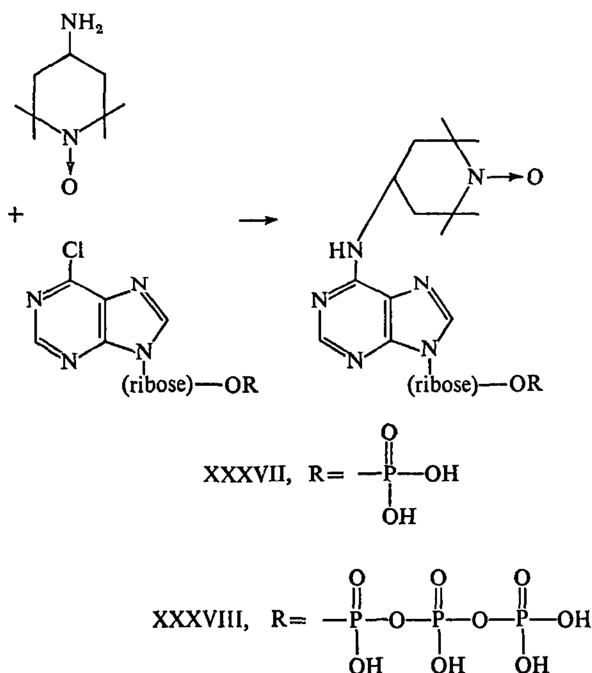
XXXIV

The compatibility of nitroxides with many of the reaction conditions employed in synthetic phosphate chemistry has been utilized in the recent syntheses of three spin-label analogues of enzyme cofactors. The nitroxide alcohol, IV, has been phosphorylated by reaction with

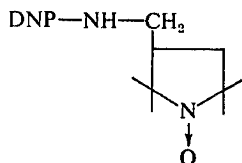
β-cyanoethylphosphate (Weiner, 1969; R. T. Ogata & A. Horwitz, unpublished results, 1969) or with dimorpholinophosphobromidate (Roberts *et al.* 1969). The monophosphate, XXXV, has been found to occupy a site similar to the inorganic phosphate binding site of ribonuclease (Roberts *et al.* 1969). In addition, it has been converted to the triphosphate. The spin-label triphosphate, XXXVI, acts as a cofactor for oxygen unloading of hemoglobin analogous to 2,3-diphosphoglyceric acid (R. T. Ogata & H. M. McConnell, unpublished, 1969).



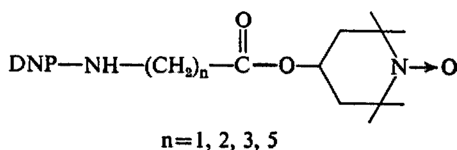
The monophosphate has also been converted to an analogue of NAD, XIII, see section II F. This spin-label has allowed detailed studies



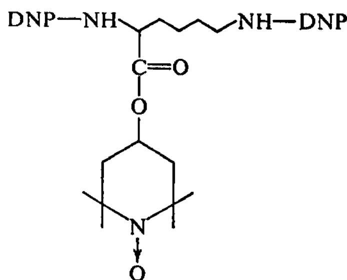
to be made of the binary and ternary complexes of alcohol dehydrogenase (Mildvan & Weiner, 1969*a*, *b*). Spin-label analogues of AMP, XXXVII, and ATP, XXXVIII, which bind to DNA polymerase, have recently been synthesized (M. R. Atkinson, D. L. Brutlag & A. Kornberg, unpublished, 1969).



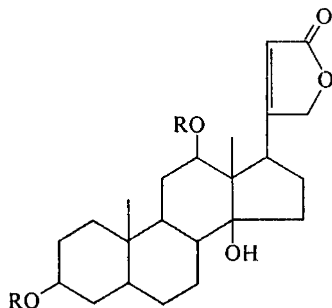
XXXIX



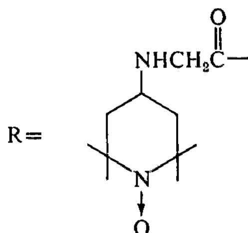
XL



XLI



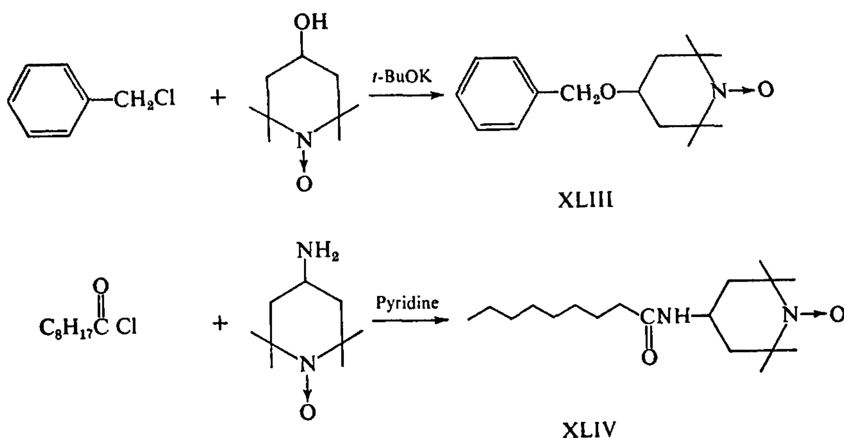
XLII



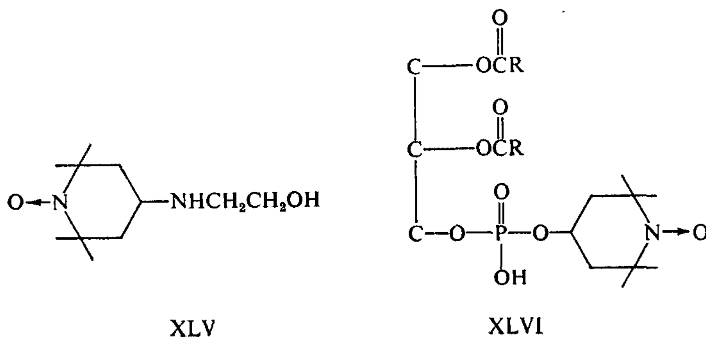
Progress has been made in the synthesis of several other types of spin labels which exhibit specific biological activity. A series of dinitrophenyl (DNP) spin labels, XXXIX–XLI, has been prepared for mapping antibody active sites (Hsia & Piette, 1969). (For previously prepared DNP spin labels, see Hamilton & McConnell, 1968.)

The bifunctional reagent, chloroacetyl chloride, has been employed to link the nitroxide amine, XIX, to digoxigenin to give a cardiac–glycoside spin label, XLII, which binds to the sodium–potassium activated ATPase of cerebral cortex cell membranes (R. E. Barnett, unpublished, 1969).

The chemical stability of the nitroxide precursors has also been employed recently in the synthesis of two new classes of spin labels. The spin labels, XLIII and XLIV, prepared as below, were found to act as local anesthetics in nerves (W. L. Hubbell & H. M. McConnell, unpublished, 1969).

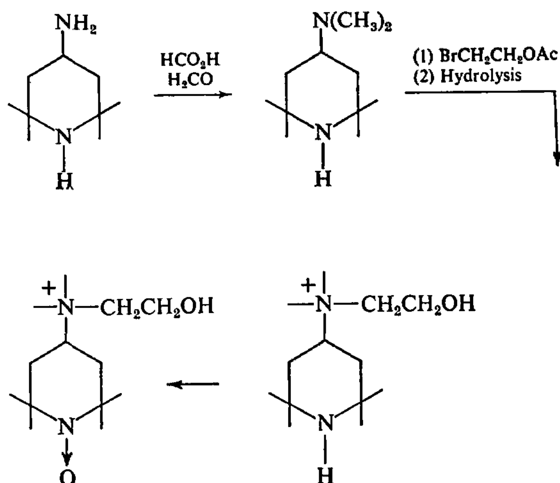


In addition, for studies of membrane structure, several spin-label analogues of membrane components have been synthesized and are currently being investigated. Besides a choline label, the synthesis of which is described later, spin-label derivatives of ethanolamine XLV



and phosphatidic acid XLVI have been prepared (R. Kornberg, unpublished, 1969). The ethanolamine spin label was prepared by reductive alkylation of the amine, XIX, by glycolaldehyde, and the phosphatidic acid derivative was prepared by dicyclohexylcarbodiimide coupling.

In spite of their considerable stability, nitroxides *are* sensitive to strong acids and some reducing conditions (the free radical is not affected by two electron reductants such as borohydride (Rozantsev, 1966) or by Wolff–Kishner reduction (Rozantsev & Neiman, 1964)). These difficulties can be circumvented by performing a synthetic sequence on the unoxidized spin-label precursor, followed by oxidation as a last step. This approach was employed in the synthesis of a spin-label analogue of choline, XLVII (W. L. Hubbell & R. Kornberg, unpublished, 1969).

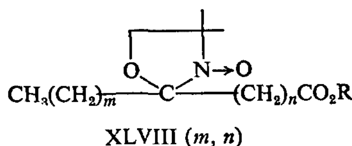


The preparation of the choline label, XLVII, is also one of numerous examples that the four α-methyl groups provide sufficient steric hindrance to render the 1-amino group virtually unreactive to reagents which normally react with secondary amines.

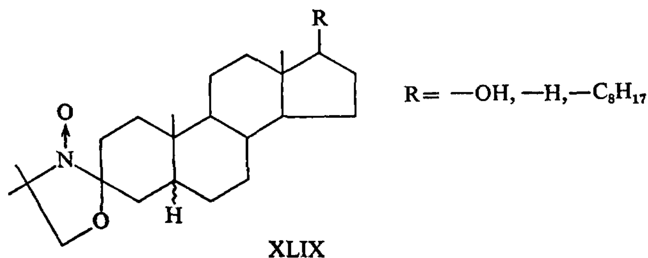
The above technology has been applied as well to the synthesis of nitroxide biradicals (Briere *et al.* 1965*a*) which also may be employed as spin labels to yield information on molecular conformation (Calvin *et al.* 1969; S. Ohnishi, private communication, 1969; W. L. Hubbell & H. M. McConnell, 1968, unpublished).

The conversion of ketones to the dimethyloxazolidine nitroxides

(Keana *et al.* 1967) is an approach which has been found particularly effective for converting readily available starting materials to a series of spin labels which bind to macromolecular systems by hydrophobic interactions. These include long-chain fatty acids, X(*m*, *n*), and esters, XLVIII(*m*, *n*) and steroids, XLIX (Hubbell & McConnell, 1969*a*, *b*; Keith, Waggoner & Griffith, 1968; J. Seelig, unpublished results, 1969).



XLVIII (*m*, *n*)



XLIX

C. *Enzymatic synthesis and biosynthesis of labels*

The enzymatic and biosynthetic preparation of spin labels is of considerable interest. An investigation of phospholipase D catalyzed exchange of the choline spin label, XLVII, for choline in lecithin is currently in progress as part of an effort to prepare membrane components (R. Kornberg, unpublished, 1969). The spin-labeled methyl stearate analogue, XLVIII (5, 10), has been biosynthetically incorporated into the membranes of *Neurospora crassa* (Keith *et al.* 1968).

D. *Reactivity of nitroxide radicals in biological systems*

As has been discussed in section III B, nitroxides are stable and unreactive under a wide variety of experimental conditions. However, in some instances, particularly in the presence of living cells, the free radical is destroyed, sometimes slowly and sometimes rapidly, probably by reduction or possibly by reaction with free radical biochemical intermediates. The reductions of nitroxides are currently being investigated in this laboratory, with their possible application to spin-

label studies in mind. For instance, nitroxides are rapidly reduced by ascorbic acid (A. Horwitz, unpublished, 1968). Ascorbate reduction can be reversed by air oxidation, and thus does not represent a serious stumbling block to studies of intact systems when ascorbate is present. In addition, nitroxides are also catalytically reduced by cysteine in the presence of traces of ferric ion, presumably through the ferrous-cysteine complex which results from ferric oxidation of cysteine (B. G. McFarland and H. M. McConnell, unpublished, 1968). This reduction can be prevented by agents which complex more strongly with iron than does cysteine (EDTA for example). It is of interest in the above reductions that the five-membered cyclic nitroxides are less reactive than the six-membered spin labels (A. Horwitz, unpublished, 1968). Growth of spin-labeled deoxyhemoglobin crystals led to another method of combating nitroxide reduction. When the crystals are grown in a crystallizing solution containing ferrous citrate, the spin label is reduced. However, the bound nitroxide can be regenerated in the crystal by exchange with a solution of unbound label (A. Horwitz & W. J. Deal, unpublished, 1968).

The chemical reactions of nitroxides, with loss of free radical, can also be used to advantage. For instance, it was possible to preload *E. coli* with spin-label phosphate, XXXV, and then study the rate of leak by observing the disappearance of magnetic resonance signal with ascorbic acid added to the surrounding medium (A. Horwitz, unpublished, 1969). The reactions of nitroxides with photo-induced radicals in chloroplasts and with model chemical reagents have been reported (Corker, Klein & Calvin, 1966). Similar studies were made with *chlamydomonas* (Weaver & Chon, 1966).

IV. A CRUCIAL QUESTION

A crucial question that can always be asked concerning the use of spin labels to study a biological problem is whether or not the spin-label molecule or group is a tolerable perturbation. That is, does the nitroxide group so perturb the system under study that the resonance results are no longer relevant to the properties of the unperturbed system? There is obviously no general answer to this quesion—each problem must be critically studied on an individual basis. There are several strategies that can be employed to demonstrate the relevance of results from spin-label studies to the unperturbed systems, and this has been done in a

number of cases. For example, hemoglobin alkylated at cysteines $\beta 93$ with VI and XI still binds oxygen co-operatively, so the labeled protein clearly has essential structural features in common with the unlabeled protein. The response of the oxygenation curve of the labeled protein to certain organic phosphates is also very similar to that of the unlabeled protein (B. G. McFarland & H. M. McConnell, unpublished, 1969). Likewise, the binding of the spin-label triphosphate, XXXVI, to hemoglobin as a function of oxygenation (R. T. Ogata & H. M. McConnell, unpublished, 1969) mimics closely the natural 'cofactor' for oxygen unloading, diphosphoglyceric acid (Benesch & Benesch, 1969). The spin-label analogue of NAD discussed in Part II above obviously must have a geometry in the active site of the alcohol dehydrogenase that is very similar to that of NAD. The biosynthetic incorporation of spin-labeled fatty acids into the phospholipids of *neurospora* mitochondria (Keith *et al.* 1968) provide another example of the compatability of spin labels with biological structures. As a further example, a spin-label analogue of ATP binds to DNA polymerase with an affinity similar to that of ATP itself (M. R. Atkinson, D. L. Brutlag & A. Kornberg, unpublished, 1969). These and other examples make it perfectly clear that spin labels are tolerable structural perturbations in many selected biochemical situations.

V. Preview

This section has been reserved to mention very briefly three areas of 'spin-label physics' which are almost totally unexplored at the present time, and which appear to us to have considerable promise for future biological applications of spin labels.

First, one can readily power saturate the paramagnetic resonance of a spin-labeled protein such as hemoglobin, and different regions of the spectrum saturate differently. This opens the possibility of using paramagnetic relaxation as a structural and kinetic probe (H. M. McConnell, unpublished, 1969). Second, electron–electron double resonance (Hyde, Chien & Freed, 1968) may be useful in getting distances between labels in macromolecular systems. Finally, the ease with which nitroxide labels attached to macromolecules can be saturated with microwaves suggests that such systems should show strong Overhauser effects, which in turn should provide further information on the geometry and chemical kinetics of electron–nucleus interactions.

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