*SPIN-LABELED BIOMOLECULES**

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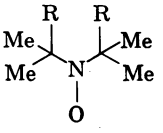
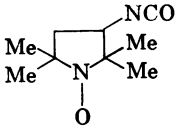
In the present work we show that structural and kinetic information can be obtained from the paramagnetic resonance spectrum of a rather simple stable radical chemically bonded to biomolecules. Paramagnetic nitroxide radicals containing the group



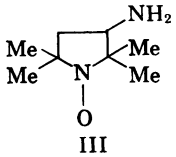
I

are remarkably stable and inert,[1] and show sharp, well-resolved, and simple paramagnetic resonance spectra[2] that are sensitive to molecular motion,[3] and, to a lesser extent, sensitive to polarity of the molecular environment.[4] As shown below, such nitroxide radicals containing an isocyanate group can be bound to proteins and synthetic polypeptides in a manner similar to that used previously to attach fluorescent dyes.[5] Here we report a preliminary study of the paramagnetic resonance of a nitroxide radical bonded to bovine serum albumin and to poly-L-lysine. It is evident from the present study that there are many other potential applications of this method to biological systems.

Materials and Methods.—Preparation of 2, 2, 5, 5-tetramethyl-3-isocyanatopyrrolidine-1-oxyl (II): 2, 2, 5, 5-Tetramethyl-3-aminopyrrolidone-1-oxyl (III) was synthesized from triacetonamine by the method of Rosantzev and Krivitzkaya.[6]



II



III

A saturated solution of III in dry benzene was added dropwise at 0°C to a stirred solution of 12.5% phosgene in benzene (1 mole III to 2 moles phosgene). The isocyanate II proved to be too unstable to purify extensively; solvent was removed *in vacuo* at room temperature and the crude product immediately added to a buffered solution containing the protein or polypeptide.

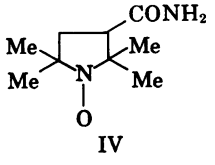
Reaction of II with bovine serum albumin (BSA) and poly-L-lysine: (i) *Poly-L-lysine:* The reaction product from 0.1 gm of III with phosgene was dissolved in 20 ml of 1% sodium bicarbonate solution, and this solution added to 1 gm of poly-L-lysine HBr in 30 ml of 1% sodium bicarbonate solution. The resulting solution was stirred for 3 days at room temperature and then dialyzed against distilled water to remove the bicarbonate and unreacted nitroxide. From the observed resonance intensities it is estimated that there are 1.2 molecules of nitroxide per polymer molecule (mol wt 50,000).

(ii) *BSA:* The reaction product from 1 gm of III with phosgene was dissolved in 25 ml of 1% sodium bicarbonate solution, and this solution added to 2.5 gm of crystalline BSA in 75 ml of 1% sodium bicarbonate solution. The resulting solution was stirred for 3 days at 0°C and dialyzed against distilled water. There were one or two attached nitroxide groups per BSA molecule.

Pepsin digestion of nitroxide-labeled BSA: A solution of 6 ml of a 2.5% solution of nitroxide-labeled BSA was digested at pH 2.1 with 0.2 mg of pepsin for 15 min. The action of pepsin was stopped by dilution 20:1 with phosphate buffer of pH 7.5.

Spectra: All the paramagnetic resonance spectra reported in the present paper were obtained at 9,500 Mc using a Varian spectrometer. Spectra were also obtained at 35,000 Mc, and, in general, these spectra differ in appearance from those obtained at 9,500 Mc. These differences are entirely consistent with our following interpretations of the 9,500-Mc spectra.

Results.—The paramagnetic resonance spectra of radicals in solution depend on the rate of molecular tumbling.[7-10] This is illustrated in Figure 1 for the nitroxide radical IV.



IV

Figure 1*c* shows the spectrum of IV in glycerine at 77°K. The observed line widths are a measure of anisotropies of the hyperfine interaction and g factor. These anisotropies are more and more effectively averaged out in solutions of lower viscosity, as illustrated in Figure 1*a* and *b*. The anisotropies of the N^{14} nuclear hyperfine interaction and g factor for the nitroxide group have been studied by Maki[3] and, in more detail, by Griffith, Cornell, and McConnell.[11] The anisotropic N^{14} hyperfine Hamiltonian is

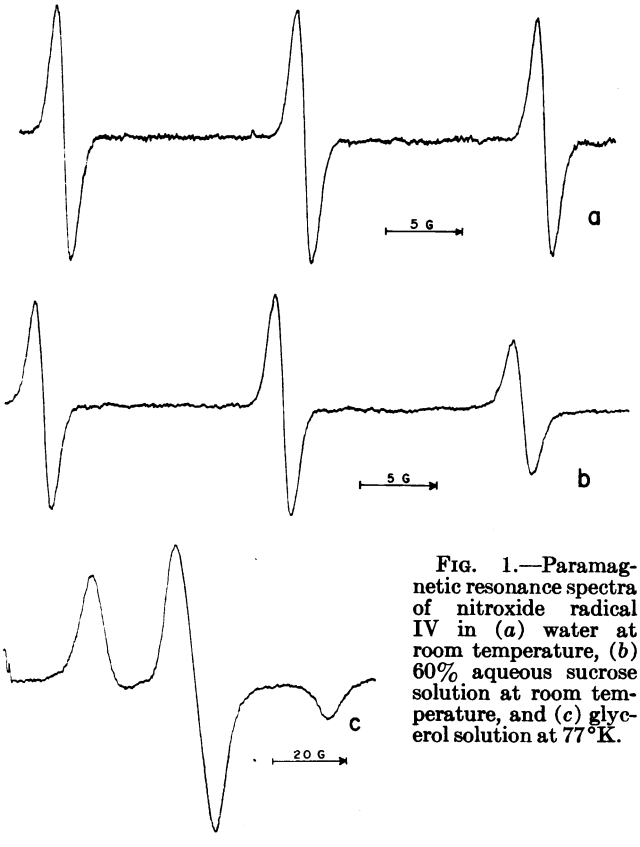


FIG. 1.—Paramagnetic resonance spectra of nitroxide radical IV in (*a*) water at room temperature, (*b*) 60% aqueous sucrose solution at room temperature, and (*c*) glycerol solution at 77°K.

$$\mathcal{H} = hAS_zI_z + hBS_xI_x + hCS_yI_y, \tag{1}$$

where approximate values for A, B, and C are[11]

$$|A| \simeq 87 \text{ Mc}, \qquad |B| = |C| \simeq 14 \text{ Mc},$$

and A, B, and C have the same sign. These values for A, B, and C can change slightly ($\sim$15%) in going from one compound containing the nitroxide group to another by changing R in I, or by changing the polarity of the solvent. In equation (1), the principal Cartesian axes x and z lie along the NO bond direction and along the π-orbital of the nitrogen atom that holds the unpaired electron. In the particular case of di-tert-butyl nitroxide, where R = CH_3 in I, the observed[11] elements of the anisotropic g tensor are $g_x = 2.0089 \pm 0.0002$, $g_y = 2.0061 \pm 0.0002$, and $g_z = 2.0027 \pm 0.0002$. The apparent splitting between the outermost lines in the rigid glass spectrum in Figure 1*c* is roughly 180 Mc, and is equal to $2A$ for IV in glycerine glass. In the solutions of low viscosity in Figure 1*a* and *b*, where the rate of molecular tumbling, τ^{-1}, is large compared to the anisotropies in the hyperfine interaction, the observed splitting, $\sim$43 Mc, is the isotropic component of the hyperfine interaction, $|a|$,

$$|a| = {}^1/_3|A + B + C|. \tag{2}$$

The observed magnitudes of $|a|$, and $|A|$, $|B|$, and $|C|$, as well as the near equality of $|B|$ and $|C|$, make it clear the unpaired spin in the nitroxide group is largely localized in a π-orbital on the nitrogen atom (estimated spin density, $\rho \sim 0.9$),[12, 13] and thus the most appropriate valence structure for the nitroxide group is

The polar character of this group may account for the dependence of the hyperfine

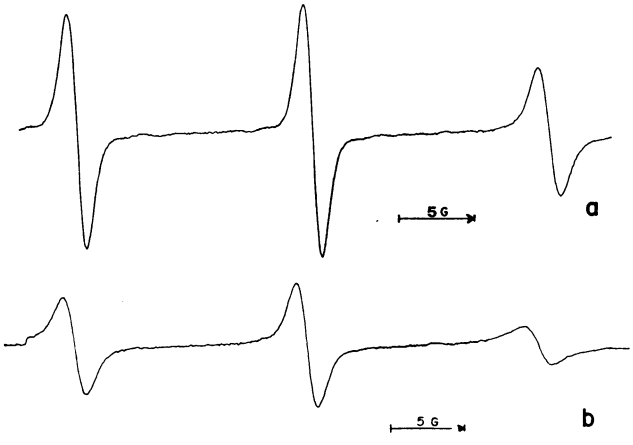


FIG. 2.—Paramagnetic resonance spectra of nitroxide radical labeled poly-L-lysine at (*a*) pH 8.0 and (*b*) pH 11.0.

interaction on solvent polarity, the more polar solvents stabilizing the negative charge on the oxygen and increasing the spin density on the nitrogen atom.

The variation in line width of the hyperfine multiplets seen in Figure 1*a* and *b* can be understood in terms of the interplay of hyperfine anisotropy and *g* factor anisotropy in producing paramagnetic relaxation characterized by a transverse line width parameter T_2.[3, 7-10] In the following discussion we shall use the notation of Kivelson.[8]

For the case of rapidly tumbling nitroxide radicals, the Lorentzian line width parameter T_2 can be related to the anisotropy of the hyperfine interaction, the anisotropy of the *g* factor, the nitrogen nuclear spin quantum number M, and the correlation time τ for isotropic molecular tumbling, by the equation

$$(T_2(M))^{-1} = \tau\left\{[3I(I+1) + 5M^2]\frac{b^2}{40} + \frac{4}{45}(\Delta\gamma B)^2 - \frac{4}{15}b\Delta\gamma BM\right\} + X. \quad (3)$$

In equation (3), B is the applied field strength in gauss, $I = 1$ is the nuclear spin quantum number for N^{14}, $b = 4\pi/3(A - B)$, and

$$\Delta\gamma = \frac{-|\beta|}{\hbar}[g_z - {}^1/_2(g_x + g_y)]. \quad (4)$$

The quantity X represents contributions from other broadening mechanisms that do not depend on M.

The above very simple equation for T_2 is expected to be adequate for hyperfine line shapes when (*a*) there is axial symmetry in the anisotropic hyperfine interaction; i.e., $B = C$; (*b*) the tumbling motion is isotropic; (*c*) the tumbling motion is sufficiently slow that $\omega^2\tau^2 \gg 1$, where $\omega = g|\beta|B\hbar^{-1}$; (*d*) $(\pi a)^2\tau^2 \ll 1$; (*e*) $b^2\tau^2 \ll 1$.

Equation (3) may be rearranged in the more convenient form,

$$T_2(0)/T_2(M) = 1 - \frac{4\tau}{15}b\Delta\gamma BT_2(0)M + \frac{\tau}{8}b^2T_2(0)M^2. \quad (5)$$

Experimental values for the ratio $T_2(0)/T_2(\pm 1)$ can be obtained conveniently from the square roots of the ratios of experimental derivative curve peak heights.

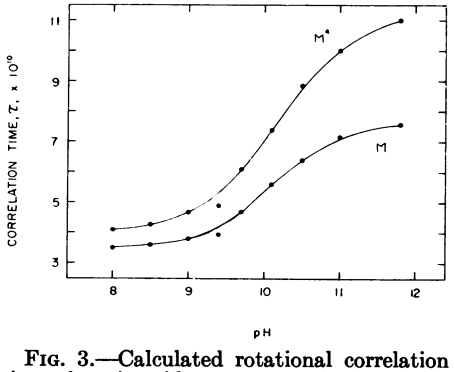


FIG. 3.—Calculated rotational correlation times for nitroxide group attached to poly-L-lysine as a function of pH. Upper curve gives values calculated from term quadratic in M, and lower curve gives values calculated from term linear in M.

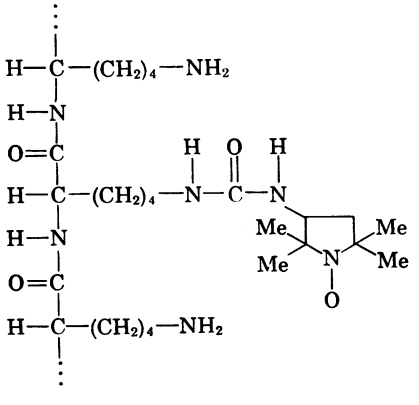
The calculation of the correlation time τ may be illustrated by reference to the spectrum in Figure 1*b*. Here the ratios $T_2(0)/T_2(1)$ and $T_2(0)/T_2(-1)$ are 1.052 and 1.94, respectively. $T_2^{-1}(0) = 18.67$ Mc; this paramagnetic relaxation rate is related to the peak-to-peak separation $\Delta\nu$ (sec^{-1}) for the central hyperfine component of the derivative curve in Figure 1*b* by the equation $T_2^{-1} = \pi\sqrt{3}\Delta\nu$. The identification of the $M = 1$ N^{14} nuclear spin quantum state with the low field hyperfine component can be based on (*a*) purely theoretical considerations that lead to a positive value of the isotropic hyperfine interaction,[13] or (*b*) the present line

width analysis together with the previously determined g factor anisotropy[11] for nitroxide radicals. Note that A must also be positive, as well as $b = 4\pi/3(A - B)$. The calculated correlation time τ obtained from the linear term is 3.0×10^{-10} sec, and the correlation τ obtained from the quadratic term is 3.4×10^{-10} sec. The correlation time for nitroxide radical IV in pure water obtained by a similar calculation from paramagnetic resonance spectra at 35,000 Mc is 2.5×10^{-11} sec.

Poly-L-lysine: Figure 2 shows the paramagnetic resonance spectra of nitroxide radical labeled poly-L-lysine at (*a*) pH 8.0 and (*b*) pH 11.0. At the lower pH the hyperfine line widths are considerably narrower than the line widths observed at the higher pH where the poly-L-lysine has a helical structure.[14] As expected, the spectra reveal more motion for nitroxide attached to the random coil configuration. Plots of the correlation time τ versus pH for poly-L-lysine are given in Figure 3. The two correlation times given for each value of pH correspond to times calculated using the linear and quadratic terms in M. The source of disagreement between the two calculated correlation times is not known; it may be due to anisotropic motion or to a solvent dependence of the hyperfine and g factor tensors which has not been taken into account in the analysis of the spectra. The general shape of the graphs in Figure 3 is remarkably similar to the graphs of specific rotation versus pH for poly-L-lysine obtained by Applequist and Doty.[14]

In the pH range 9–11 the helical content of poly-L-lysine has been found to vary between nearly 0 and 100 per cent.[14] Our paramagnetic resonance spectra show no evidence of distinguishable nitroxide groups in this region, corresponding to groups attached to lysines in helical regions, and lysines in random coil regions. Our failure to detect distinguishable nitroxide radicals of this sort *could* be due to a rapid interconversion of helical and random coil regions, but this is by no means necessary since the line widths for the nitroxide radicals in the two cases are not very different.

Our results on poly-L-lysine show that the nitroxide group has remarkably free motion in both the helical and random coil configurations. The mode of attachment of the nitroxide radical is almost certainly to an ϵ-amino group,[5] to give a substituted urea as sketched below.



The very rapid molecular motion of the nitroxide group that is required to produce the observed spectra need not be spherically symmetric; a number of rapid but more restricted types of motion can also produce the observed isotropic averaging.

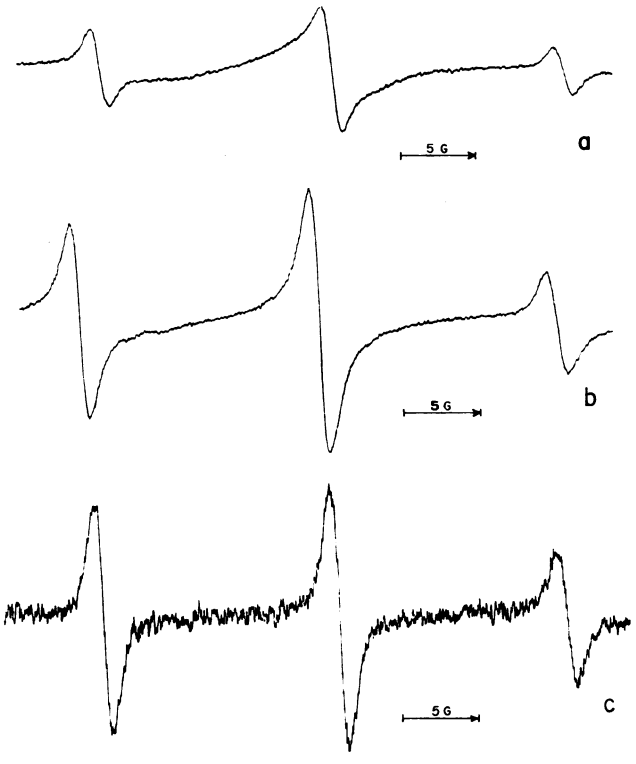


FIG. 4.—Paramagnetic resonance spectra of nitroxide radical labeled bovine serum albumin at (*a*) pH 7.5, (*b*) pH 2.2, and (*c*) in pH 7.5 phosphate buffer after digestion with pepsin.

Bovine serum albumin: There is apparently a large increase in the effective volume of BSA between pH 4 and 2 which has been studied previously by changes in fluorescent polarization,[15, 16] optical birefringence,[17] optical rotation,[18] viscosity,[18] and sedimentation rate.[16] When a 2.5 per cent solution of nitroxide-labeled BSA is titrated with HCl from pH 8.5 to 1.7, there are significant changes in the resonance spectra of the radical which correlate well with those observed in the other physical properties mentioned above. The spectrum at pH 7.5 is reproduced in Figure 4*a*. At first glance this spectrum appears to be approximately the same as that observed for the nitroxide group attached to the poly-L-lysine (Fig. 2*a*) and for the free nitroxide in viscous solution (Fig. 2*b*). However, a closer examination reveals a broad tailing of the central line, and the same spectrum taken at much higher gain (Fig. 5) shows two additional broad resonance lines flanking the high and low field lines of the sharper hyperfine multiplets. A superposition of the spectrum of the nitroxide attached to poly-L-lysine with that of free nitroxide IV in a rigid glass (Fig. 1*c*) would reproduce the observed BSA spectrum very well, except for a small discrepancy (~14 Mc) between the splitting of the high and low field broad lines in the BSA sample and that of the corresponding lines in the glass. Thus there exist at least two distinct types of binding of the nitroxide isocyanate derivative to the protein; one to sites where the nitroxide group is free to tumble quite rapidly, as evidenced by its low correlation time, and a second to sites where the nitroxide

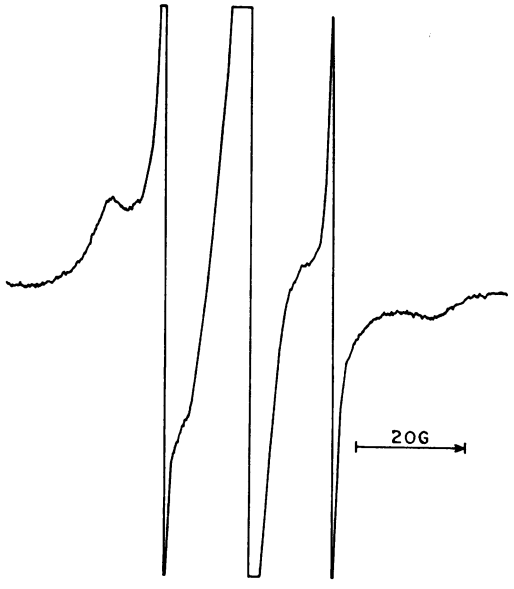


FIG. 5.—High gain paramagnetic resonance spectrum of nitroxide radical labeled bovine serum albumin at pH 7.5 showing broad lines due to strongly hindered and slowly rotating nitroxide groups.

group has much less freedom of motion. These much less mobile nitroxide radicals can still tumble at the same rate as the entire BSA molecule, but since the BSA rotational frequency ($\sim 10^6$ sec^{-1})[17] is small compared to the hyperfine anisotropy, the rotational motion of the whole BSA molecule has little effect on the paramagnetic resonance line widths.

The spectrum of the radical labeled BSA at pH 2.2 is reproduced in Figure 4*b*. Here the intensity of the short correlation time nitroxide spectrum has increased considerably. The same spectrum taken at high gain shows that the high and low field broad lines have disappeared almost completely. Evidently more of the rapidly tumbling nitroxide has been formed at the expense of that with restricted motion. A plot of the change in intensity of the high field line which should be due only to the rapidly moving nitroxide groups is given in Figure 6. The intensity changes very little from pH 7.5 to 4.0 and then rises rapidly to pH 2.1 where it begins to fall again. The changes in resonance spectra with pH were shown to be reversible by back titration. The curve in Figure 6 is quite similar to those obtained for the variation of viscosity and optical rotation of BSA with pH in this range.[18]

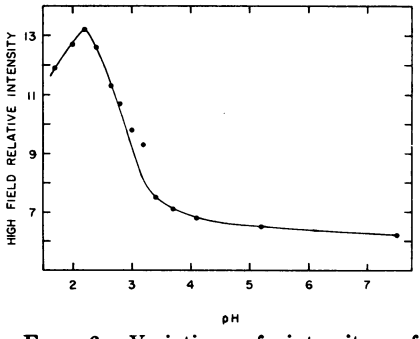


FIG. 6.—Variation of intensity of high field hyperfine components of nitroxide labeled bovine serum albumin as a function of pH.

If the portion of nitroxide which has restricted motion is deep inside the BSA molecule, the expansion of the protein associated with the pH 4–2 transition evidently leads

to a considerable decrease in the steric inhibition to rotation at these binding sites. Thus, the intensity of the broad portion of the spectrum decreases and that of the narrower hyperfine lines increases as more and more of the bound nitroxide is allowed to tumble freely with the opening up of the protein.

A similar change in the spectrum of nitroxide-labeled BSA was observed on digestion with pepsin for a short time. This enzymic reaction has been studied extensively by Weber and Young.[19] The first stage is a degradation of BSA into three equal fragments. The spectrum in pH 7.5 phosphate buffer before and after digestion shows changes similar to those observed for the acid pH transition (Fig. 4*c*). In the spectrum of the digested protein the intensity of the three principal lines has increased while the tailing of the central line has decreased. It appears that breaking the protein into the three fragments has also exposed nitroxide groups formerly tightly held in place within the protein.

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† NATO postdoctoral fellow, 1964–1965.

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