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*A NITROXIDE-MALEIMIDE SPIN LABEL**

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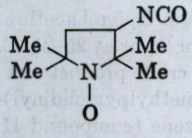
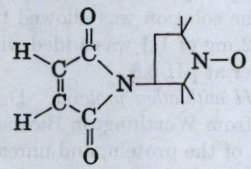
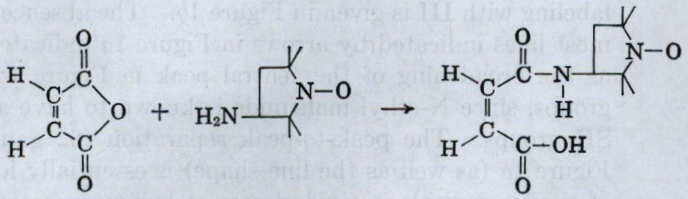
In previous work it has been shown that certain chemical, structural, and kinetic information can be obtained from the paramagnetic resonance of synthetic organic free radicals ("spin labels") that are attached to biomolecules.[1–3] For example, the nitroxide-isocyanate (2,2,5,5-tetramethyl-3-isocyanatopyrrolidine-1-oxyl)



was chemically bonded to poly-L-lysine and to bovine serum albumin (BSA) and showed a paramagnetic resonance spectrum that was sensitive to known structural changes in these molecules, such as those produced by changes in pH.[2] This nitroxide-isocyanate is unfortunately not an ideal spin-labeling reagent since it is unstable and thus difficult to purify, and moreover the isocyanate group is rapidly hydrolyzed by water in competition with the labeling reaction. In the present paper we describe the preparation and some labeling properties of a new, much more stable and generally useful nitroxide spin-labeling compound, N-(1-oxyl-2,2,5,5-tetramethyl pyrrolidinyl)-maleimide,



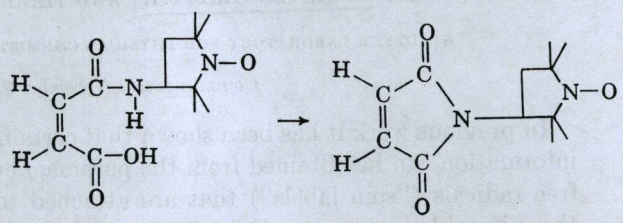
Materials and Methods.—Preparation of N-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-maleamic acid (II):



I II

2,2,5,5-tetramethyl-3-amino-pyrrolidine-1-oxyl (I) was prepared by the procedure of Rozantzev and Krivitzkaya.[4] An equimolar amount (0.40 gm) of I in 1 ml of anhydrous diethyl ether was slowly added with stirring to a room-temperature solution of 0.25 gm of maleic anhydride in 5 ml anhydrous diethyl ether. The N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleamic acid (II) immediately precipitated and after 3 hr of stirring at room temperature the precipitate was filtered, washed ten times with 0.4 ml of anhydrous diethyl ether, and dried; yield 97%. *Analysis:* Calc. for $C_{12}H_{19}N_2O_4$: C, 56.5; H, 7.5; N, 11.0%. Found: C, 56.0; H, 7.5; N, 11.3%.

Preparation of N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide (III):



II III

A mixture of 0.63 gm II, 5 ml acetic anhydride, and 0.2 gm sodium acetate were stirred in a tightly closed container for 24 hr at 25–35°C. The acetic anhydride was removed *in vacuo* at room temperature, and the crude product was obtained as a viscous oil which solidified on standing. (N-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide (III) was purified by sublimation or by recrystallization from benzene (compound II is insoluble in benzene). *Analysis:* Calc. for $C_{12}H_{17}N_2O_3$: C, 60.7; H, 7.2; N, 11.8%. Found: C, 60.6; H, 7.3; N, 11.8%. Preparations of other substituted (nonparamagnetic) maleamic acids and maleimides are given in the literature.[5–7]

Reaction of III with bovine serum albumin: Solid III (1.2 mg) was added to BSA (0.34 gm) dissolved in 5 ml of 0.1 *M* phosphate buffer, pH 6.8. After stirring for several hours at 0°C the solution was dialyzed against 0.1 *M* phosphate buffer at 5°C. This procedure was repeated using BSA dissolved in 0.1 *M* phosphate buffers at pH 4 and at pH 10, followed by dialysis against 0.1 *M* phosphate buffer, pH 6.8. The resulting spectra were essentially the same for all three solutions (Fig. 1*a*). Compound III is evidently unstable in water since solutions of BSA added to aqueous solutions of III were not spin-labeled. Compound II does not react with BSA under the above conditions (pH 6.8).

Reaction of BSA with N-ethyl maleimide and III: N-ethyl maleimide (1.8 mg) was added to 10 ml of 0.1 *M* phosphate buffer (0°C, pH 6.8) containing 0.69 gm BSA. After stirring several hours at 0°C the solution was allowed to stand overnight at 5°C. The solution was dialyzed at pH 6.8 (5°C), 2 mg of III was added with stirring, and the solution was again dialyzed against a phosphate buffer at pH 6.8.

Reaction of III with other proteins: Details of these reactions will be given elsewhere. Enzymes were obtained from Worthington Biochemical Corporation. In general, solid III was added to a stirred solution of the protein, and unreacted radicals were removed by dialysis against an appropriate buffer.

Results.—The paramagnetic resonance of BSA spin-labeled with III is shown in Figure 1*a*. The spectrum of BSA reacted with N-ethyl maleimide before spin labeling with III is given in Figure 1*b*. The absence in Figure 1*b* of the two outermost lines indicated by arrows in Figure 1*a* indicates that these two lines, as well as the broadening of the central peak in Figure 1*a*, are due to spin-labeled SH groups, since N-ethyl maleimide is known to have a high degree of specificity for SH groups. The peak-to-peak separation (62 gauss) of the outermost lines of Figure 1*a* (as well as the line shape) is essentially identical to the powder spectra of similar radicals in rigid glasses at low temperatures. From these observations we conclude that the maleimide spin label attached to the SH group is immobilized in space for times of the order of, or longer than, the inverse of the anisotropy of the nuclear hyperfine interaction, $\sim 10^{-8}$ sec.[2, 8] This implies, of course, that not only is the rotatory diffusion time of the BSA molecule much longer than 10^{-8} sec (which is well known[9]) but also that the spin-label molecule must also be highly immobilized in its motion relative to the protein.[10, 11] At a pH of 2.1, where BSA is (reversibly) denatured, the intensity of the signal due to the immobilized spin label is almost zero, whereas the intensity of the mobile spin labels has increased

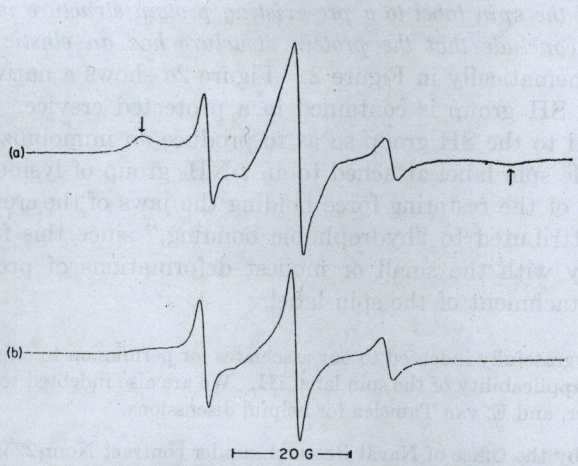


FIG. 1.—Paramagnetic resonance of bovine serum albumin spin-labeled with the nitroxide-maleimide III. Labeling procedures for samples yielding spectra (*a*) and (*b*) were identical, except that in (*b*) the SH group of the serum albumin was blocked by reaction with N-ethyl maleimide before spin-labeling.

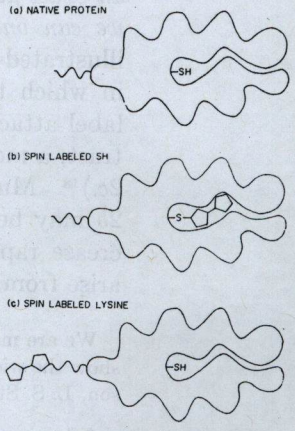


FIG. 2.—Schematic drawing of (*a*) native protein, (*b*) protein with labeled SH group yielding an immobile spin, and (*c*) protein with labeled lysine group yielding mobile spin.

by a factor of about two. Thus, under the conditions of our labeling procedure, only about half of the spin label reacts specifically with the SH group in BSA.

In preliminary experiments in these laboratories the nitroxide-maleimide III has been used successfully to spin-label a number of other proteins in solution, including (*a*) alcohol dehydrogenase,[12] (*b*) aldolase,[12] (*c*) catalase,[13] (*d*) carboxypeptidase,[14] (*e*) ceruloplasmin,[15] (*f*) α-chymotrypsin,[14] (*g*) creatin kinase,[12] (*h*) cytochrome *c*,[13] (*i*) hemoglobin,[16] (*j*) lactate dehydrogenase,[17] (*k*) lipase,[13] (*l*) lysozyme,[12] (*m*) papain, (*n*) poly-L-lysine, and (*o*) trypsin.[14] In all cases, the observed spectra had three sharp components, corresponding to the labeling of sites where the spin label has a great deal of motional freedom, such as ϵ-NH_2 groups of lysine residues on the surface of the protein. In addition to BSA, two proteins containing reactive SH groups (*g*, *i*) showed quite strong signals corresponding to immobilized spin labels. Other proteins (*b*, *c*, *j*, *k*, *m*) containing SH groups showed rather weak resonance signals due to immobilized spins. In the case of hemoglobin and papain, the immobilized spin-label signals were of reduced intensity when the SH groups were previously blocked with N-ethyl maleimide, and Hg^{++}, respectively. In the case of alcohol dehydrogenase, attachment of the spin label produces an immobilized spin only at acid pH.[12] Proteins without SH groups (*f*, *h*, *l*, *n*, *o*) showed no immobilized spin signals.

Perhaps the most interesting result from the spin-labeling experiments thus far has been the observation of immobilized spin labels in such a large variety of proteins. As indicated above, the usual site of immobile attachment is to an SH group, presumably involving addition of the SH across the carbon-carbon double bond of III. The remarkable feature of the attachment is that the protein structure must be sufficiently open as to admit the spin label for reaction with the reactive group (e.g., SH) and, on the other hand, the structure in the vicinity of the spin label must be sufficiently closed so as to immobilize the labeling molecule. *Since*

an exact fit of the spin label to a pre-existing protein structure is out of the question, we can only conclude that the protein structure has an elastic character. This is illustrated schematically in Figure 2. Figure 2*a* shows a native protein structure in which the SH group is contained in a protected crevice. Figure 2*b* shows a label attached to the SH group so as to produce an immobilized spin. (For contrast, a mobile spin label attached to an ϵ-NH_2 group of lysine is sketched in Fig. 2*c*.)[18] Much of the restoring force holding the jaws of the crevice closed in Figure 2*b* may be attributed to "hydrophobic bonding," since this force should not decrease rapidly with the small or modest deformations of protein structure that arise from attachment of the spin label.

We are most gratefully indebted to our associates for permission to cite their experiments that show the wide applicability of the spin label III. We are also indebted to Professors W. S. Johnson, L. S. Stryer, and E. van Tamelen for helpful discussions.

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[9] Krause, S., and C. T. O'Konski, *J. Am. Chem. Soc.*, **81**, 5082 (1959).

[10] A similar immobilization of free radicals attached to biomolecules has been reported in refs. 1 and 2 above, and also in Ehrenberg, A., *Electronic Aspects of Biochemistry*, ed. A. Pullman (New York: Academic Press, 1964), p. 379. In the latter case, this immobilization is to be expected, since the flavin molecule functions as a coenzyme bound to a protein.

[11] A precise description of this immobilization requires a detailed line shape analysis. For the nitroxide spin label in BSA, a rough preliminary statement can be made by noting that the outermost peaks in Fig. 1*a* come from radicals with the odd-electron in a π-orbital whose axis is parallel to the applied magnetic field (see ref. 2). If this axis were to undergo small high-frequency cylindrically symmetric oscillations about the field direction with a root-mean square angular deviation of δ, then the peak-to-peak separation (62 gauss in Fig. 1*a*) would be reduced by a factor of $1-2\ \delta^2$. The peak-to-peak separation in Fig. 1*a* differs from the low-temperature rigid glass separation by less than 4 gauss, and corresponds to root-mean square angular deviations of less than 15°. S. Ogawa (private communication) has pointed out that the small variations in peak-to-peak separation considered here may not be due to low-amplitude oscillations at all, but rather to environmental differences (e.g., polar vs. hydrophobic) that have a small effect on the hyperfine interaction. In that case, the root-mean square angular deviation may be even less than estimated above.

[12] Ogawa, S., and H. M. McConnell, unpublished.

[13] Hamilton, C., and H. M. McConnell, unpublished.

[14] Berliner, L., and H. M. McConnell, unpublished.

[15] Boeyens, J., P. Becker, and H. M. McConnell, unpublished.

[16] Boeyens, J., and H. M. McConnell, unpublished.

[17] Nielsen, W., R. Young, and H. M. McConnell, unpublished.

[18] The sketch in Fig. 2*c* does not necessarily imply that the mode of amine attachment is across the maleimide double bond. See, e.g., Smyth, D. G., A. Nagamatsu, and J. S. Fruton, *J. Am. Chem. Soc.*, **82**, 4600 (1960).