*MOTION OF STEROID SPIN LABELS IN MEMBRANES**

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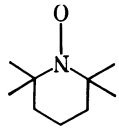
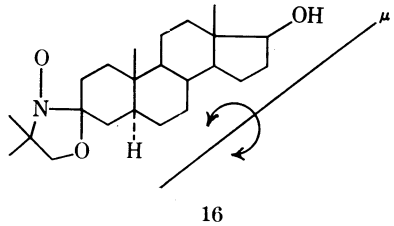
Abstract.—Steroid spin labels have been found to undergo rapid and sometimes anisotropic rotational motion in membranes and phospholipid vesicle preparations. N-oxyl-4′,4′-dimethyloxazolidine derivatives of 5α-androstan-3-one-17β-ol, 5β-androstan-3-one-17β-ol, and 5α-androstan-3-one were prepared according to the procedure described by Keana. The paramagnetic five-membered ring is rigidly attached to the steroid nucleus at the 3-position, and thus the resonance spectrum of the nitroxide group reflects the motion of the entire steroid nucleus. This rapid motion, with rotational diffusion frequencies of the order of 10^7 to 10^8 second^{-1} is considered to lend plausibility to models of membrane transport involving rotations and/or translations of carriers within the hydrophobic region of the membrane. The steroid spin labels also form a convenient class of probes for studying conformational changes in membranes, since (*a*) their resonance spectra often fall in the category of "intermediate immobilization," which is most sensitive to small changes, and (*b*) these labels are quite soluble in a wide variety of membranes.

Spin labels[1] have recently been used to show the existence of fluid hydrophobic regions in a number of excitable membranes.[2] A comparison of spectra of labeled membranes and spectra of the same labels in phospholipid suspensions indicates that these fluid hydrophobic regions in membranes are associated with the long hydrocarbon chains of phospholipids.[2] In this previous work we used the simple label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl).



In recent work other investigators have also used spin labels to study membranes;[3–5] the work of Keith *et al.*[5] also provides evidence for fluidity of the long-chain hydrocarbon region of mitochondrial membranes. We have now prepared a number of other labels that bind to fluid hydrophobic regions more strongly than does TEMPO, and also bind strongly to a number of other membrane systems where TEMPO showed no detectable binding (e.g., mitochondrial and erythrocyte membranes). Of these labels, steroid derivatives such as



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have been found to be particularly useful. The preparation and interesting resonance spectra of a number of steroid labels are described here; our preparation of these labels follows closely the method described by Keana *et al.*[6]

Materials and Methods.—The N-oxyl-4′,4′-dimethyloxazolidine derivatives were prepared from the corresponding 3-keto-steroids (Steraloids, Inc.). The two epimeric nitroxide compounds, if formed, were not separated.

(*i*) N-oxyl-4′,4′-dimethyloxazolidine of 5α-androstan-3-one-17β-ol (5α, 17-OH). Analysis after crystallization from methanol-water gave C: 70.37, H: 10.20, N: 3.76%. The calculated composition for $C_{23}H_{38}NO_3 \cdot CH_3OH$ is C: 70.57, H: 10.36, N: 3.42%. Melting point (uncorrected) was 172–174°C.

(*ii*) N-oxyl-4′,4′-dimethyloxazolidine of 5β-androstan-3-one-17β-ol (5β, 17-OH). Analysis after purification by preparative thin-layer chromatography gave C: 73.68, H: 10.14, N: 3.57%. The calculated composition for $C_{23}H_{38}NO_3$ is C: 73.36, H: 10.17, N: 3.71%. Melting point (uncorrected) was 171–173°C.

(*iii*) N-oxyl-4′,4′-dimethyloxazolidine of 5α-androstan-3-one (5α, 17-H). Analysis after recrystallization from acetone-water gave C: 76.72, H: 10.60, N: 3.84%. The calculated composition for $C_{23}H_{38}NO_2$ is C: 76.61, H: 10.62, N: 3.88%. Melting point (uncorrected) was 159–160°C.

Unmyelinated nerve fibers were removed from the walking legs of the Maine lobster, *Homarus americanus*, and bathed in artificial seawater of the following composition (in mM): NaCl, 450; KCl, 15; $CaCl_2$, 24; $MgCl_2$, 4; $MgSO_4$, 3; N-Tris(hydroxymethyl)-methyl-2 amino-ethane sulfonic acid, 2.

Phosphatidyl serine was obtained from Nutritional Biochemical Co. Vesicles of phosphatidyl serine were prepared by agitation or sonication of the solid in 0.05 *M* Tris-HCl, pH = 7.5. The vesicle preparations were 5% by weight of phospholipid.

Steroid labels were incorporated in phospholipid vesicles by first dissolving the labels in ethanol or dioxane, and then evaporating the solvent to produce a thin film of the label. The aqueous solution of phospholipid vesicles was added, and the mixture was agitated on a vortex mixer until the label was taken up. The final label concentration usually corresponded to a mole ratio of 500:1 phospholipid:steroid label. For the walking-leg nerve fiber, labeling was accomplished by exchange of the label from a 5% solution of bovine serum albumin (fatty acid poor, Mann Research Laboratories) to the nerve fiber membranes. The serum was labeled in the manner described above for the phospholipid vesicles. Typically, the mole ratio of serum albumin to steroid label was about 2 to 1. Exchange was continued until a usable signal-to-noise ratio was obtained. The nerve was then rinsed well in fresh seawater. The spectra of the steroid labels bound to bovine serum albumin are "strongly immobilized" and are thus qualitatively different from the spectra of the labels bound to (fresh) membranes.

Results and Discussion.—Representative resonance spectra of steroid spin labels in the neural membranes and sonicated phospholipid suspensions are shown in Figures 1 and 2. The spectral line shapes do not depend on label concentration in the range of concentrations used here. All spectra show one interesting general feature. Qualitatively, the spectra show a high degree of motion of the nitroxide group, with correlation times in the order-of-magnitude range 10^{-7} to 10^{-8} second. Since the five-membered nitroxide ring is rigidly linked to the steroid nucleus, the observed rapid rotational motion then indicates that the entire molecule undergoes rapid rotational motion in a hydrophobic region of the nerve fiber membranes. We have observed a similar rapid motion of these labels in other biological membranes. Some of the spectra show evidence of anisotropic motion. A semi-quantitative discussion of this anisotropic motion in relation to the resonance spectra is given below.

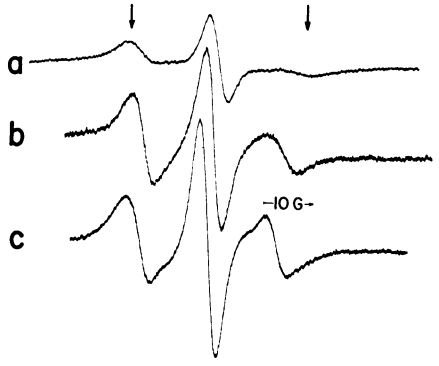


FIG. 1.—Resonance spectra of the steroid spin labels incorporated in the Homarus walking-leg nerve fiber by exchange from bovine serum albumin. N-oxyl-4',4'-dimethyloxazolidine derivatives of (*a*) 5α-androstan-3-one-17β-ol, (*b*) 5β-androstan-3-one-17β-ol, and (*c*) 5α-androstan-3-one.

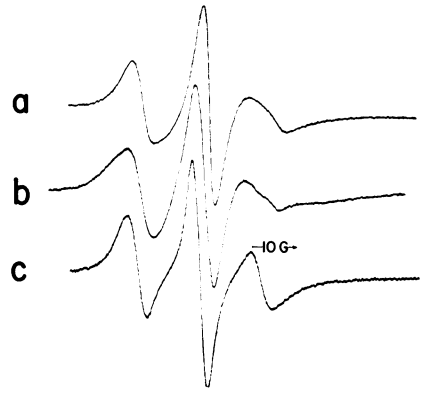


FIG. 2.—Resonance spectra of the steroid spin labels incorporated in phosphatidyl serine vesicles. N-oxyl-4',4'-dimethyloxazolidine derivatives of (*a*) 5α-androstan-3-one-17β-ol, (*b*) 5β-androstan-3-one-17β-ol, and (*c*) 5α-androstan-3-one.

Anisotropic motion: The resonance spectrum in Figure 1*a* for the steroid spin label I in the walking-leg nerve fiber is quite unusual, and suggests anisotropic motion of the steroid nucleus about its long molecular axis, $\mathbf{y}$. This molecule has two possible epimeric structures, corresponding to the two possible orientations of the N-oxyl-4',4'-dimethyloxazolidine ring about the optically active carbon atom C3 of the steroid nucleus. Pauling-Cory-Koltum models of either epimer show the axis of large N^{14} nuclear hyperfine splitting—the π-orbital axis—to be perpendicular to the long molecular axis of the steroid. As will be evident from the following calculation, this near perpendicularity is the crucial structural feature that gives a sensitive relation between the anisotropic motion and the resonance spectra. To simplify the discussion, however, we consider only the epimer for which the nitroxide group is *cis* to the axial methyl groups. This will be referred to as the *cis* epimer of I. A model of the *cis* epimer of I is shown in Figure 3*a*.

The spin Hamiltonian for a nitroxide free radical can be represented as follows.[1]

$$\mathcal{H} = |\beta|\mathbf{S}\cdot\mathbf{g}\cdot\mathbf{H}_0 + h\mathbf{S}\cdot\mathbf{T}\cdot\mathbf{I} - g_N\beta_N\mathbf{I}\cdot\mathbf{H}_0 \qquad (1)$$

Here $\mathbf{g}$ and $\mathbf{T}$ are the tensors for the spectroscopic splitting factor and hyperfine interaction, respectively. The electron and N^{14} nuclear spin operators in units of $\hbar$ are designated $\mathbf{S}$ and $\mathbf{I}$, $|\beta|$ is the absolute value of the Bohr magneton, and $\mathbf{H}_0$ is the applied magnetic field vector. It has previously[7] been pointed out that if a spin label undergoes a sufficiently rapid anisotropic rotational diffusion, its resonance spectrum may be approximated using a new effective Hamiltonian, $\mathcal{H}'$.

$$\mathcal{H}' = |\beta|\mathbf{S}\cdot\mathbf{g}'\cdot\mathbf{H}_0 + h\mathbf{S}\cdot\mathbf{T}'\cdot\mathbf{I} - g_N\beta_N\mathbf{I}\cdot\mathbf{H}_0 \qquad (2)$$

Here the elements of $\mathbf{g}'$ and $\mathbf{T}'$ are derived from suitable averages of the elements

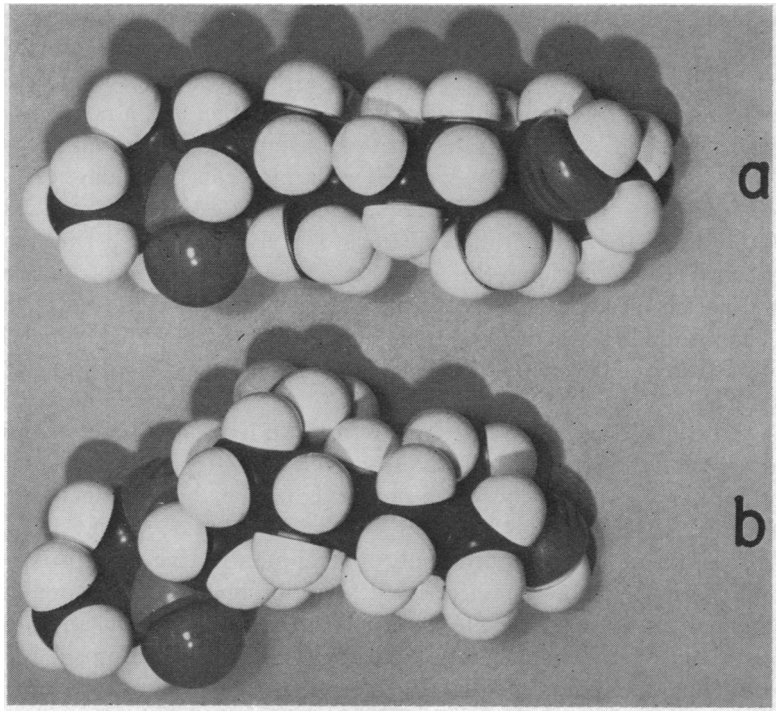


FIG. 3.—Pauling-Corey-Koltum models of N-oxyl-4',4'-dimethyloxazolidine derivatives of (*a*) 5α-androstan-3-one-17β-ol, (*b*) 5β-androstan-3-one-17β-ol. Note that the presence of the nitroxide moiety does not significantly alter the approximate cylindrical character of the steroid nucleus.

of **g** and **T**. Now consider a rapid rotational diffusion of I about the long axis of the molecule, μ. The model of the *cis* epimer of I indicates that the principal nuclear hyperfine axes z, x, and y are oriented such that the axis of largest nuclear hyperfine splitting (z) is perpendicular to μ, and lies in the "plane" of the steroid nucleus, and x (the N—O bond direction) is perpendicular to the plane of the steroid and is also perpendicular to μ. A rapid rotational diffusion about the long molecular axis, which is very nearly parallel to y in the model of the *cis* epimer, leads to the following principal values of **T'**, providing the inverse of the correlation time for this motion is large compared to $|T_z - T_x|$, and $h^{-1}|\Delta g\,\beta|H_0$, where $|\Delta g|$ is the maximum anisotropy of the g-tensor.

$$T'_\perp = 1/2(T_x + T_z)$$
$$T'_\parallel = T_y \qquad (3)$$

The principal axes of **T'** are parallel ($\parallel$) and perpendicular ($\perp$) to μ. To within the experimental errors, the principal axes of **g** for several spin labels have been found to be parallel to the principal axes of the nuclear hyperfine interaction,[1, 8, 9] so that for this molecular motion similar equations hold for the elements of **g** and **g'**:

$$g'_\perp = 1/2(g_z + g_x);\ g'_\parallel = g_y \qquad (4)$$

The observed spectrum in Figure 1*a* can be interpreted in terms of the reduced Hamiltonian of equation (2), assuming no motion of the axis $\mathfrak{y}$. In this case one expects outer peaks with separation $2T'_{\perp}$ having an unsymmetrical appearance (e.g., a positive deflection of the low-field signal and a negative deflection of the high-field signal). The observed separation of the outer lines indicated by $\downarrow$ in Figure 1*a*, 37 gauss, is well within the range of values of $T_x + T_z$ estimated from single crystal studies (e.g., 35 gauss is estimated from values of $T_x + T_z$ obtained from hyperfine splittings of a five-membered ring nitroxide label attached to hemoglobin in single crystals).[9] The anisotropic motion considered here is also consistent with the observed spectrum in that both outer pairs of signals are symmetrically spaced about the central signal, which has an observed g-factor of 2.006 ± 0.001. This is expected since g-factors for nitroxide radicals are such that $g_x \neq g_y \neq g_z$, but $1/2\ (g_x + g_z) \backsimeq g_y = 2.006$. Calculations for the trans epimer of I are similar to those for the *cis* epimer discussed above, except for the g-factor terms. However, the g-tensor is not sufficiently anisotropic to enable us to say which epimer is present on the basis of the observed spectra.

This discussion of course affords only a semiquantitative description of the anisotropic motion; a more quantitative description requires not only a detailed line-shape analysis but also knowledge of which epimer is present and quantitative information on the spin Hamiltonian for the nitroxide group in I. However, one qualitative feature of this calculation needs to be emphasized: anisotropic motion is most easily detected with nitroxide spin labels when the axis of rapid motion is perpendicular to the π-orbital axis. In other cases, the anisotropy of a rapid motion may not be so easily detected. For example, if the axis about which rapid rotation takes place makes angles of $\cos^{-1}\alpha$, $\cos^{-1}\beta$ and $\cos^{-1}\gamma$ with the principal axes x, y, z, then the principal hyperfine interactions parallel and perpendicular to this axis are

$$T_{\parallel} = \alpha^2 T_x + \beta^2 T_y + \gamma^2 T_z$$

$$T_{\perp} = 1/2(1 - \alpha^2)T_x + 1/2(1 - \beta^2)T_y - 1/2(1 - \alpha^2)T_z$$

Thus, for example, if the axis of rapid anisotropic motion makes angles of 54° 44′ with x, y, and z, a spectrum similar to that arising from rapid isotropic motion can result.

We now consider the molecular origin of the anisotropic motion of I. First, note that an anisotropic molecule must always exhibit some anisotropic motion, even in a hypothetical continuous isotropic fluid. Our failure to observe spectra similar to that in Figure 1*a* for I in water-glycerol mixtures of varying viscosities, and in phospholipid vesicles (see below), indicates that such systems do not provide molecular environments that bring about sufficiently strong anisotropic motion. We suggest that the anisotropic motion of I arises from three basic structural features of the membrane-steroid label system. *First*, as can be seen from Figure 3, label I is rather cylindrical in shape, so there should be relatively little resistance to rotation about the long molecular axis. *Second*, amphiphilic steroids from which I is derived concentrate at oil-water interfaces, with the hydrophobic steroid nucleus in the oil phase and the hydrophilic —OH

group in the aqueous phase. Thus, concentrations of I in excess of 1 mM may be included in aqueous suspensions of phospholipid vesicles with no precipitation, whereas the water solubility of I is of the order of $10^{-5} - 10^{-6}$ M. The steroid nucleus can then be pictured as intercalated between the hydrocarbon chains of the phospholipids according to the usual scheme, with the $-$OH group "anchored" in the aqueous phase,[10, 11] or complexed with the base of a phospholipid.[12, 13] *Third*, in the neural membrane, it may be assumed that the polar head group region of the lipids forms a relatively rigid "floor" to which the $-$OH group of the steroid is anchored. The existence of such a rigid "floor" is indicated by the obvious long range order of the surface of the neural membrane.

The above picture is strongly supported by the observation that the steroid spin label 5α,17-H, which has the same shape as I but no polar $-$OH group, shows no comparable anisotropic motion, as seen from the spectrum in Figure 1*c*. Our discussion of the significance of the cylindrical shape of I is also consistent with the observation that the "bent" steroid label 5β,17-OH shows no evidence for anisotropic motion in the nerve fiber (Fig. 1*b*).

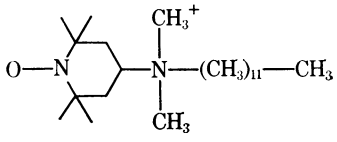
Figure 2(*a* and *c*) shows the corresponding spectra for these labels in sonicated dispersions of phosphatidyl-L-serine. None of the labels, including I, shows evidence for highly anisotropic motion. In the case of I, we attribute this absence of apparent anisotropic motion to the absence of a rigid floor (polar head group region) in these phospholipid vesicles. In the acidic phosphatides, divalent metal ions decrease the rotational rate of I and the spectra show some evidence of anisotropic motion. On the other hand, the addition of small hydrophobic or amphiphilic molecules, including local anesthetics, tends to increase the rotational freedom of the label. The latter effect has also been observed for I in the walking-leg nerve fiber membranes. Under no condition, however, has a high degree of rotational anisotropy been detected in the phospholipid systems.

The resonance spectrum of I in the walking-leg axonal membranes is apparently quite sensitive to the physiological state of the axon. Thus, when a nerve fiber is allowed to stand for 1–2 days at 4°C and is then labeled, a different line shape is observed, indicating that a great deal of the radical has become "strongly immobilized." This new line shape is very similar to that observed for the radical bound to BSA. This same effect can be observed if a toxic dose of a local anesthetic is given to the preparation. This phenomenon is observed in other membranes as well. Thus, when a hemolytic amount of a local anesthetic is added to an erythrocyte ghost suspension labeled with I, the appearance of strongly immobilized radicals is observed.

Localization of spin labels: The following experiment provides strong evidence that the nitroxide portion of label I is localized in the (fluid) hydrophobic region of the membrane. Sodium ascorbate at 20 mM rapidly reduces the nitroxide function of I at room temperature in artificial seawater at pH = 7.5. If a walking-leg axon labeled with I is treated with artificial seawater in which 20 mM of the sodium chloride has been replaced by sodium ascorbate, only very slow reduction is observed. Assuming that the radical is located in the membrane, this means either that the nitroxide function is buried in the hydrophobic

interior of the membrane (ascorbate is insoluble in hydrocarbon solvents) or that the membrane is impermeable to the reducing agent and the nitroxide function is on the interior interface of the membrane. To test the latter hypothesis, a walking-leg axon labeled with I was homogenized, thus allowing the ascorbate to reach the interior of the axon. The reduction rate of the label was the same as that for the intact axon; that is, it was very slow compared to the reduction rate of a nitroxide radical in aqueous solution. Thus, the nitroxide function of I must be located within the hydrophobic interior of the membrane. We attribute the slow but finite reduction of the nitroxide in the membrane to the finite membrane/water distribution coefficient of I.

By contrast, the label II[14] possesses the same amphiphilic character as I and is therefore expected to orient itself at an oil-water interface. An important



II

difference between labels I and II bound to membranes or phospholipids is that the nitroxide function of II is expected to be located in an aqueous environment since the nitroxide group is located on the hydrophilic portion of the molecule, contrary to that of label I. Thus a rapid reduction of this label by ascorbate is expected, and is observed under the same conditions as described above for label I. Additional evidence for these configurations of the labels in phospholipid vesicles has recently been obtained from unpublished proton resonance studies by J. Metcalfe.[15] Label I produces a broadening of the long-chain methylene proton signals, and label II produces a broadening of choline proton resonance signals, in sonicated dispersions of lecithin.

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