*ORIENTATION OF LIPID SPIN LABELS IN LECITHIN MULTILAYERS**

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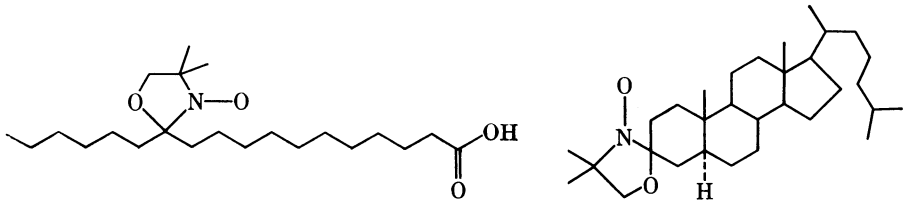
Abstract.—Nitroxide-labeled stearic acid and cholestane have been incorporated into lecithin multilayers. The pronounced anisotropy of the preparations reflects a nonrandom structure of the lecithin film and demonstrates that these lipid probes can report structural information from lipid regions resembling those thought to occur in membranes. These two probes differ with respect to the orientation of the NO bond to the long axis, but both orient with their long axes perpendicular to the phospholipid film. Simulated spectra, calculated on the basis of a Gaussian distribution of orientations, are in agreement with the experimental results.

Nitroxide spin labeling is currently receiving wide acceptance as a technique for studying membrane model systems and biological membranes.[1, 2] The approach is similar to the familiar practice of introducing an organic dye into a biological system and observing the dye by optical methods. The electron spin resonance (ESR) spectrum is sensitive to the rotational mobility of the spin labels, the polarity of the solvent surrounding the spin labels, and the orientation of the spin labels with respect to the laboratory magnetic field. The first two effects have been used to advantage in numerous studies of membranes and membrane models.[1] To take full advantage of the third effect, the membrane should be oriented with respect to the laboratory frame of reference. Although this is a troublesome matter, the information that could be gained makes this approach attractive. With a suitable probe it may be possible, for example, to distinguish between the unit membrane model and other proposed models for biological membranes. In the unit membrane structure originally proposed by Danielli and Davson,[3] the phospholipids are aligned in a bimolecular leaflet. The ESR spectrum of lipid spin labels oriented in such structures would differ significantly from the spectrum of the same labels dispersed in a randomly oriented phase. Thus, lipid spin-labeled probes can, in principle, be used to test membrane models and to compare the structures of various biological membranes. Similarly, changes in membrane properties that alter the structure of the lipid regions might be detected as relative changes in anisotropy. An important question to be answered is whether existing lipid spin labels will faithfully report the orientations of the surrounding molecules or whether the nitroxide moiety is a sufficiently large perturbation to cause local disorder. In the present study we approached this problem by studying the ESR spectra of the two nitroxide-labeled lipids I and II in lecithin multilayers.



I II

Experimental.—Chemicals: The stearic acid nitroxide I and the cholestane nitroxide II were prepared from ketones by the general method of Keana, Keana, and Beetham.[4] The detailed procedures for the synthesis of I[5] and II[4] are given elsewhere. Both nitroxides were purified by thin-layer chromatography with diethyl ether as the eluent. L-α-lecithin from egg yolk (Type IV-E, Sigma Chemical Co.), obtained in sealed ampules, was used without further purification.

Preparation of lecithin multilayers: The procedure followed was essentially that of Bangham and Horne.[6] A solution of phospholipid and nitroxide, made by adding 10 μl of 10^{-2} *M* solution of the nitroxide in $CHCl_3$ to 10 mg of phospholipid in chloroform in a vial, was evaporated to dryness under nitrogen. After adding 2 ml of distilled water, the mixture was mechanically agitated for several minutes and then sonicated for 1 min. A small aliquot of the opalescent nitroxide-containing phospholipid dispersion was evaporated on a 10 × 50 × 0.1-mm Corning glass cover slip (hereafter referred to as a glass slide) at 39°C on a warming table until no visible water remained. The ridge that surrounded the phospholipid film was trimmed with a razor blade prior to making the ESR measurements.

ESR measurements: The glass slide was inserted into a slotted Teflon rod. A no. AX-4-D drum dial from the PIC Design Corporation was attached to the other end of the Teflon rod so that the orientation of the glass slide in the ESR cavity could be easily determined. All ESR spectra were recorded on a Varian E-3 X-band ESR spectrometer. The majority of spectra were recorded at 23°C with the glass slide parallel to the magnetic field and again after a 90° rotation of the glass slide. Temperature studies were performed with a Varian V-4540 variable-temperature controller and slides only 5 mm wide so that they could be inserted into the Dewar. The theoretical ESR spectra were generated with an IBM 360/50 computer equipped with a Calcomp plotter.

Results.—The spectra recorded from thin films of lecithin containing nitroxides I and II prepared as described above are shown in Figure 1. The magnetic field in each case was either parallel or perpendicular to the surface of the glass slide which supported the lecithin film. The two nitroxide-labeled lipids exhibited

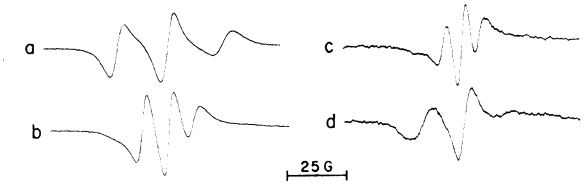


FIG. 1.—Room-temperature ESR spectra of nitroxide-labeled lipids in lecithin films, showing the changes in spectra which result when the lecithin films are rotated in the magnetic field. Spectra (*a*) and (*b*) are of nitroxide I with the magnetic field perpendicular and parallel, respectively, to the plane of the glass slide supporting the lecithin film. Spectra (*c*) and (*d*) are of nitroxide II with the magnetic field perpendicular and parallel, respectively, to the plane of the glass slide. The mole ratio of nitroxides I or II to lecithin is 5×10^{-3}.

three line spectra. It is convenient in describing these spectra to define a splitting, a', equal to the distance in gauss between the center line and the low field line (left of the center line). As the slide supporting the lecithin film is rotated in the magnetic field, a smooth change in a' is observed.

The value of a' for nitroxide I is maximum when the glass slide is perpendicular to the magnetic field, and minimum when the slide is parallel (Fig. 1, spectra *a* and *b*, respectively). The difference between the maximum and minimum splittings for a sample is referred to as $\Delta a'$. At 23°C, $\Delta a'$ usually fell between 8 and 10 gauss for nitroxide I. An occasional slide gave a lower value of $\Delta a'$ (6–8 gauss), but this was accompanied by visible defects in the otherwise smooth transparent film. It is of interest to note that films produced by laying down the lecithin and nitroxide I out of chloroform-methanol solutions, both with and without subsequent hydration, orient essentially as well. This procedure is somewhat similar to that used by Reeves and Dowben[7] to produce phospholipid vesicles.

With films containing nitroxide II, the orientation of the slide for maximum splitting is just the opposite. From Figure 1, spectra *d* and *c*, it can be seen that with this probe the maximum value of a' is obtained with the slide parallel to the magnetic field, and the minimum splitting is seen when the slide is perpendicular. For nitroxide II at 23°, $\Delta a'$ ranged between 9 and 11 gauss, with one preparation giving a value of 12 gauss. Since trimming the edges of the film and removing obviously defective areas in the film increased the value of $\Delta a'$ as much as 2–3 gauss, the variability in the values obtained for both probes is clearly due to drying defects in the films.

As the temperature of the films was raised above room temperature, the values of $\Delta a'$ for both probes decreased. At 50°, the films containing nitroxide II showed $\Delta a'$ values approaching 0. Films containing nitroxide I still showed $\Delta a'$ of 2–3.5 gauss.

As the temperature of the films was lowered below room temperature, the values of $\Delta a'$ increased. However, as 0° was approached, the spectra began to show the characteristics of rigid glass spectra (see the bottom simulated spectra in Fig. 2). This rigid glass character was seen in spectra from both orientations of the slide supporting a film containing nitroxide I, but it was only seen with the slide containing nitroxide II when it was parallel to the magnetic field. Decreasing the temperature further increased this rigid glass character. The spectrum of nitroxide II with the slide perpendicular to the magnetic field showed only a broadening of the lines with no rigid glass character.

An interesting feature of these spectra is the complete reversibility of the changes, in the temperature range tested (from −60° to +50°). When the sample is returned to room temperature, in each case the original spectrum is regained. Dehydration of the films (*in vacuo* with P_2O_5 for several hours) does not alter these results; hence, it is unlikely that trapped free water contributes to the spectral changes seen with low temperature.

Discussion.—It is immediately apparent from Figure 1 that the features of the ESR spectra change as the samples are rotated in the magnetic field. This anisotropy is direct evidence that nitroxides I and II are not randomly oriented on the glass slide. However, to determine the degree of orientation it is necessary

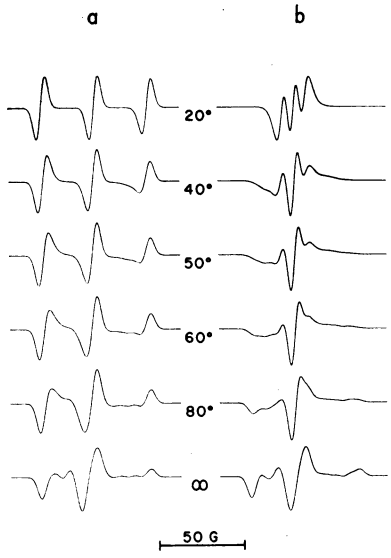


FIG. 2.—Computer-simulated ESR spectra for various degrees of alignment of a collection of nitroxide free radicals, assuming an absence of molecular motion. In all spectra the magnetic field is perpendicular to the plane of the glass slide. Series (*a*) assumes that the nitroxide free radicals tend to orient with their *z* axes perpendicular to the glass slide. In series (*b*), the nitroxide free radicals tend to align with their *y* axes perpendicular to the glass slide. The distribution of orientations is taken to be Gaussian, and the angle given is the Gaussian line width parameter (θ_0). The degree of orientation decreases from the top spectrum to the bottom spectrum in both the (*a*) and (*b*) series.

to refer to the anisotropy observed in the spectra of precisely oriented nitroxides. Dilute concentrations of nitroxides oriented in single diamagnetic crystals have been studied.[8] The spectra consist of three lines, and the anisotropy is reflected in the splitting between the lines (the coupling constant, *a*) and the absolute position of the center of the spectrum (described by the *g*-value). The principal values of *a* and *g* occur with the magnetic field along three directions, the *x*, *y*, and *z* molecular axes. The *x* axis is along the N—O bond, the *z* axis is parallel to the nitrogen 2*p* orbital associated with the unpaired electron, and the *y* axis is perpendicular to the *x* and *z* axes. The principal values of the *a* and *g* parameters reported for di-*t*-butyl nitroxide[8] are $a_{xx} = 7.1$ *G*, $a_{yy} = 5.6$ *G*, $a_{zz} = 32$ *G*, $g_{xx} = 2.0089$, $g_{yy} = 2.0061$, and $g_{zz} = 2.0027$. Closely related nitroxides such as I and II almost certainly have parameters similar to these values.[9] The most important observation is simply that the largest splitting occurs when the magnetic field is parallel to the nitroxide *z* axis, and the minimum splitting occurs with the magnetic field perpendicular to the *z* axis. When these data are compared to those of Figure 1, it is clear that the *z* axes of I tend to align at right angles to the glass slide, whereas the *z* axes of II tend to lie in a plane parallel to the glass slide. Molecular models of I indicate that the nitroxide *z* axis is parallel to the long hydrocarbon chain of the stearic acid nitroxide. In contrast, models of II suggest that the *z* axis is more nearly perpendicular to the long axis of the cholestane group. Thus, the ESR data provide strong evidence that both I and II preferentially orient with their long axes perpendicular to the glass slide.

Further refinement of these potentially important observations is not entirely straightforward. Comparison of the data with single crystal results reveals that the nitroxides are not all aligned perpendicular to the glass slide. This may result in part from defective regions or domains in the multilayers. It is equally likely that a broad Gaussian distribution of orientations exists throughout the multilayers. Considerable molecular motion is evident from the temperature studies

and this provides an additional complication. Nevertheless, it is of interest to compare spectra calculated for a simple model with the experimental results. The starting point is the familiar spin Hamiltonian[10]

$$\mathcal{H} = \beta_e H \cdot g \cdot \hat{S} + g_N \beta_N H \cdot \hat{I} + \hat{S} \cdot A \cdot \hat{I},$$

where g and A are tensors derived from the principal values of the g-values and splittings. Exact calculations require diagonalization of 12 × 12 matrices. However, several approximations have been developed [11, 12] and checked against the exact solutions and against the experimental results for di-*t*-butyl nitroxide oriented in the host 2,2,4,4-tetramethyl-cyclobutanedione.[12] A good approximation for a and g at a given orientation is

$$a = (a_{xx}^2 l_x^2 + a_{yy}^2 l_y^2 + a_{zz}^2 l_z^2)^{1/2} \quad (1)$$

$$g = (g_{xx}^2 l_x^2 + g_{yy}^2 l_y^2 + g_{zz}^2 l_z^2)^{1/2}, \quad (2)$$

where l_x, l_y, and l_z are the direction cosines of the field direction with respect to the molecular axes. From the parameters reported for di-*t*-butyl nitroxide and the above equations, one may calculate the splitting and g-value when the magnetic field is at any orientation with respect to the molecular axes.

The simple model chosen is essentially a Gaussian distribution of rigid nitroxides. The more important assumptions implicit in the calculations are the following: (1) The parameters reported for di-*t*-butyl nitroxide are applicable for I and II. (2) The z axis of nitroxide I is parallel to the long axis of the molecule, and the z axis of nitroxide II is perpendicular to the long axis of this cholestane derivative. (3) Molecular motion is neglected. (4) The long axes of the nitroxide lipids tend to align perpendicular to the slide. (5) The distribution of orientations about this direction can be expressed as a Gaussian, $\exp(-2\theta^2/\theta_0^2)$, where θ is the angle of the long axis from the normal to the slide and θ_0 is the usual Gaussian line width parameter. For convenience, the number of nitroxides included in the calculation was limited to 2000. With the aid of a computer program designed to generate random numbers, all 2000 nitroxides were assigned random orientations. An ESR spectrum of each nitroxide was calculated from equations (1) and (2), assuming that each individual first-derivative ESR line had a Gaussian line shape and was five gauss wide (peak to peak). Instead of these spectra being summed directly as in a rigid glass calculation, the amplitude of each spectrum was weighted by the distribution function $[\exp(-2\theta^2/\theta_0^2)]\sin\theta$ ($\sin\theta$ arises from the area element in polar coordinates). The resulting 2000 spectra were summed to produce each final spectrum. A summary of the results is given in Figure 2.

In all spectra of Figure 2, the magnetic field is oriented along $\theta = 0$, corresponding to a direction normal to the glass slide. In series (*a*) the hypothetical nitroxide free radicals tend to orient with their z axes perpendicular to the glass slide, and in series (*b*) they tend to orient with their z axes parallel to the glass slide. The uppermost spectra in the two series correspond to relatively narrow distributions of orientations, and these spectra do in fact resemble experimental single crystal spectra. The bottom spectra in the two series closely approximate the familiar rigid glass spectrum of a randomly oriented sample.

In view of the qualitative arguments discussed above, series (*a*) and (*b*) of Figure 2 should correspond to collections of nitroxides I and II, respectively. The comparison, then, is between Figure 1*a* and series (*a*), and between Figure 1*c* and series (*b*). The most striking agreement is between Figure 1*c* and the $\theta_0 = 40°$ spectrum of series (*b*). The agreement is also favorable between Figure 1*a* and the $\theta_0 = 40°$ spectrum of series (*a*), except that the splittings of Figure 1*a* are somewhat smaller. This difference is due, in part, to molecular motion. Calculations were also performed with the magnetic field perpendicular to the glass slide. For these spectra, agreement was much poorer with II than with I. This is because the experimental ESR results are more sensitive to molecular motion about the long axis of nitroxide II than motion about the long axis of I. Consequently, there is strong evidence for molecular motion about the long axis of the cholestane nitroxide in the lecithin multilayers, and little can be concluded at this point regarding molecular motion about the long axis of the stearic acid nitroxide. In any case, the model based on a distribution of orientations about a direction normal to the glass slide[13] appears to account for the prominent features of the ESR data.

The essential points of this paper are summarized schematically in Figure 3. The stearic acid nitroxide and the cholestane nitroxide tend to orient with their long axes perpendicular to the glass slide. The preferential alignment of these two probes clearly indicates the presence of many regions of ordered structure, oriented as shown in Figure 3. These ordered areas probably represent either flattened-out regions of large phospholipid spherulites or vesicles or perhaps a more homogeneous multilamellar structure.[14]

In any case, the nitroxide lipids do orient in the lecithin films and must be

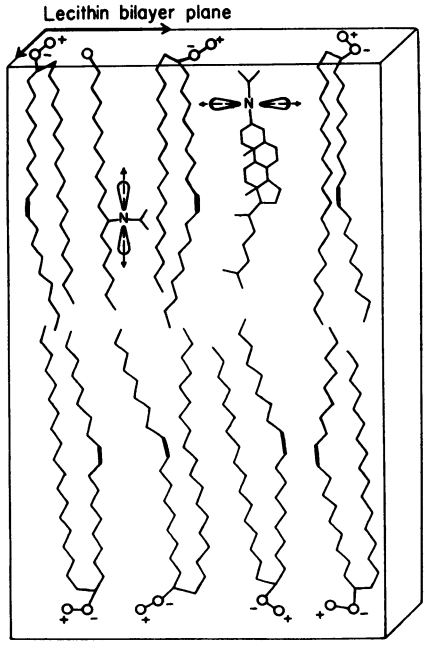


FIG. 3—Schematic drawing of a lecithin bilayer region containing both the stearic acid nitroxide and the cholestane nitroxide. The direction of the stearic acid nitroxide *z* axis is perpendicular to the plane of the lecithin bilayer, and the *z* axis of the cholestane nitroxide is parallel to the bilayer plane.

reporting over-all orientation of lipid structure. Thus, orientable membranes that include these probes in the lipid regions should be useful in obtaining structural information about natural membranes under nearly physiological conditions.

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