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Reliability of Nitroxide Spin Probes in Reporting Membrane Properties: A Comparison of Nitroxide- and Deuterium-Labeled Steroids†

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ABSTRACT: The reliability for the study of membrane properties of the steroid nitroxide spin probe, 3-doxylcholestane, was tested by comparison of analogous data for the deuterated steroid, cholesterol-3α-*d*. Good agreement between the two probes was found for the dependence of their order parameters on variation of temperature or cholesterol concentration in egg phosphatidylcholine bilayers. This finding is contrasted with the results of a previous study of fatty acid probes where poor agreement was found for the spectral responses of nitroxide- and deuterium-labeled species. The angular dependence of the ESR spectra of nitroxide-labeled probes in oriented multibilayer films was examined to determine if the probes were oriented in a tilted fashion in the bilayer. The 3-doxylcholestane probe and a doxylstearic acid labeled at position 14 orient with their long molecular axes perpendicular to the bilayer plane. In contrast, the stearic acid probe nitroxide labeled at position 5 does not appear to orient in such a fashion. We suggest that the behavior of the latter probe reflects the difficulty of inserting a bulky nitroxide group into a highly ordered region of the bilayer rather than an inherent tilting of the phospholipid acyl chains. On the basis of the comparisons between various types of probes, some suggestions are made concerning the choice of ESR spin probe to obtain reliable information in membrane studies.

Nitroxide spin probes are commonly used in the study of phospholipid membrane organization (Berliner, 1976; Schreier et al., 1978). The reliability of data derived from ESR[1] studies of spin probes may be questioned since the bulky, polar nitroxides may not accurately report on the natural properties of the membrane. We feel that it is important to assess the nature and magnitude of the perturbation induced in the membrane by introduction of the nitroxide. Previously, we

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; doxyl, the 4′,4′-dimethyloxazolidinyl-*N*-oxy derivatives of ketones; CSL, the doxyl derivative of cholestan-3-one; 5-SASL, the doxyl derivative of 5-ketostearic acid; 14-SASL, the doxyl derivative of 14-ketostearic acid; PC, phosphatidylcholine.

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have compared data for fatty acid nitroxide probes and deuterated fatty acids in egg PC bilayers (Taylor & Smith, 1980); several severe shortcomings of the nitroxide-labeled fatty acid probes were noted. In the present study we extend this comparison to include the steroid probes 3-doxylcholestane (CSL) and cholesterol-*3α-d*. Although earlier studies have employed one or the other of these probes, we have chosen to repeat some of the experiments using the same conditions for both probes in order to eliminate potential discrepancies due to differences in samples or experimental conditions. Thus, we have examined the effects of temperature and cholesterol concentration on the ESR spectra of CSL and the deuterium NMR spectra of cholesterol-*3α-d* in egg PC bilayers. We find that CSL is a good qualitative reporter of the effects experienced by cholesterol, in contrast to the low fidelity of response of the spin-labeled fatty acids. Furthermore, in order to detect any effects due to tilting of the phospholipid acyl chains, we have examined the dependence of the spectra of CSL and of the doxylstearic acids labeled at positions 5 and 14 on the angle between the applied magnetic field and the plane of oriented multibilayer films. The data demonstrate that the apparent tilting of the lipid chains reported by 5-doxylstearic acid is due mainly to the difficulty of incorporating the nitroxide-bearing moiety into a highly ordered region of the bilayer.

Materials and Methods

CSL and 5-doxylstearic acid (5-SASL) were purchased from Syva (Palo Alto, CA). The 14-doxylstearic acid probe (14-SASL) was prepared as described previously (Waggoner et al., 1969). Cholesterol-*3α-d* was prepared by $LiAl^2H_4$ reduction of cholest-5-en-3-one (Fieser, 1963) as described previously (Rosenfeld et al., 1954). Egg PC was purified essentially as described previously (Keough & Davis, 1979). The spin probe concentration in lipid was 1%, below the concentration at which spin-exchange effects are appreciable.

Spin probe experiments were performed with lipid films oriented in rectangular ESR cells (Smith & Butler, 1976). For variable-temperature experiments, excess water was left in the cell in order to avoid evaporation of the water at higher temperatures. For room-temperature experiments, excess water was drained from the cell. ESR spectra were obtained with a Varian E-9 spectrometer. Home-built devices were used to regulate temperature (K. W. Butler, unpublished results) and to measure angles between the magnetic field and the ESR cell. Samples for ^2H NMR spectroscopy were prepared by dispersing mixtures of egg PC and cholesterol-*3α–d* in excess water. ^2H NMR spectra were obtained at 46.1 MHz with a Bruker CXP-300 spectrometer and a home-built probe (R. A. Byrd, unpublished results). The quadrupole echo sequence (Davis et al., 1976) was employed, and spectra were folded about the Larmor frequency.

Molecular order parameters for CSL were calculated (Smith & Butler, 1976) by assuming values for the static hyperfine splitting tensor of A_{xx} = 6.3 G, A_{yy} = 5.9 G, and A_{zz} = 32.0 G and axially symmetric motion of the probe about the nitroxide *y* axis. Order parameters for deuterated cholesterol were calculated by assuming a static quadrupole coupling constant equal to 170 kHz and a 90° angle between the C–^2H bond and the axis of motional averaging (Gally et al., 1976; Oldfield et al., 1978).

Results and Discussion

The first comparison between the two steroid probes is made on the basis of response to temperature variation. Figure 1 illustrates the temperature dependence of the order parameters derived from CSL (triangles) and cholesterol-*3α-d* (squares)

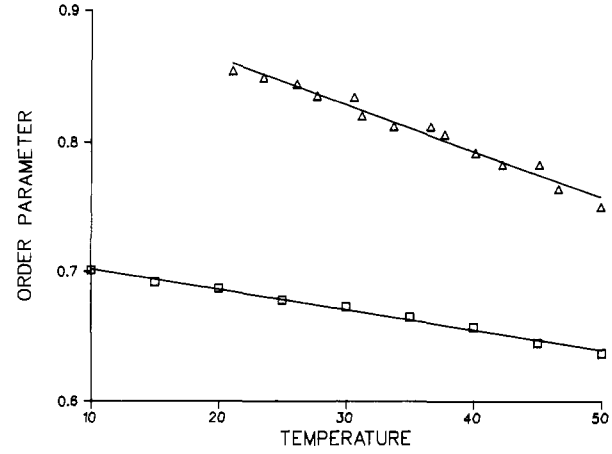

FIGURE 1: Variation of order parameters for CSL (△) and cholesterol-*3α-d* (□) with temperature. Samples consisted of 30 mol % cholesterol in egg PC hydrated with excess water. CSL spectra were obtained with oriented films; random dispersions were employed for the deuterated cholesterol.

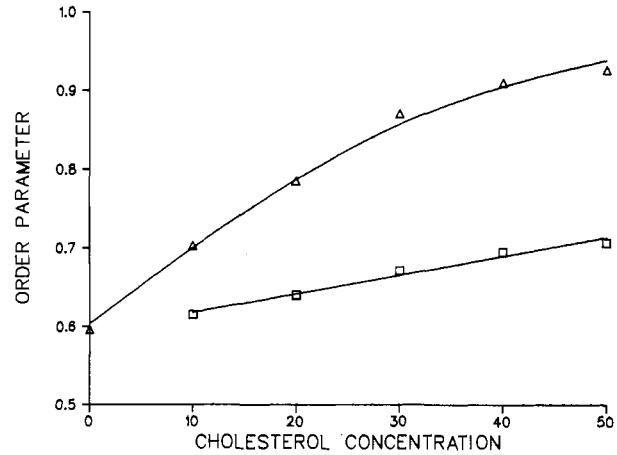

FIGURE 2: Variation of order parameters for CSL (△) and cholesterol-*3α-d* (□) with cholesterol concentration (mol % in egg PC). Sample temperatures: CSL 23 °C, cholesterol-*3α-d* 30 °C.

for bilayers formed from 30 mol % cholesterol in egg PC. Below 20 °C, broadening of the resonances for CSL precludes accurate measurement of its order parameter. Each probe shows a rather weak linear response to temperature variation which is more pronounced for the CSL probe. It is interesting to note that the order parameters for CSL are greater than those for the deuterated cholesterol. This is in sharp contrast to our previous comparison of nitroxide- and deuterium-labeled fatty acids where the discrepancies in order parameter were much greater, and the order parameters for the nitroxide-labeled fatty acids were always less than those of their deuterium-labeled analogues (Taylor & Smith, 1980). The difference in magnitudes of the order parameters will be discussed in more detail below. In general, it can be seen that the qualitative and quantitative features of the responses of both probes are quite similar.

A second comparison can be made on the basis of the response of each probe to variation of cholesterol concentration in the membrane. Figure 2 shows the dependence of order parameters on cholesterol concentration for CSL (triangles) and cholesterol-*3α-d* (squares). The CSL probe shows a stronger, and less linear, response to cholesterol addition than the deuterated cholesterol, particularly at lower cholesterol concentrations. The two probes exhibit essentially parallel responses at higher cholesterol concentrations. The results for CSL show reasonable agreement with a previous study of CSL in egg PC (Schreier-Muccillo et al., 1973).

The present data indicate that the behavior of the two probes is quite similar. The greatest discrepancy observed between the probes is in the magnitudes of the calculated molecular order parameters. When the values of the order parameters for the two types of probes are compared, it must be kept in mind that their magnitudes are sensitive to the assumed values for the static hyperfine or quadrupole couplings and the orientation of the axis about which the probe undergoes anisotropic motion. It has been shown (Seelig, 1970; Hemminga & Berendsen, 1972) that to a good approximation, the motion of the CSL probe is axially symmetric about the nitroxide y axis, as was assumed here. The rotation axis of cholesterol has not been determined, but is probably located at 90° to the C–^{2}H bond at position 3 of cholesterol (Gally et al., 1976; Oldfield et al., 1978). It should be noted, however, that small deviations from this assumed geometry would result in an increase in the calculated values of molecular order parameters.

The larger order parameters observed for CSL may also be due to inherent differences in the properties of the two probes. It can be seen in Figures 1 and 2 that the correspondence between the magnitudes of the order parameters for the two probes is best in situations of low inherent order (high temperature or low cholesterol concentration) and diverges as the membrane is rigidified. This may be due to a changing balance in the factors which influence the motions of the probes in the membrane. Each probe is amphiphilic with a polar region which anchors the probe at the aqueous interface and a large, rigid steroid framework embedded in the phospholipid acyl chain region. The behavior of both probes is influenced by interactions between the lipid acyl chains and the apolar region of the steroid and by the strength of polar interactions in the interface region. In an environment of low order (high temperature or low cholesterol concentration) the acyl chains undergo large amplitude motions and the apolar interactions may dominate the motion of the steroid probes. In this case, similar behavior can be expected for both types of probes. As the membrane is rigidified, polar interactions at the anchoring site of the probes may influence the motional behavior of the probes more strongly. In this case, higher order parameters for the CSL probe may be expected due to the high polarity of the nitroxide group (Vasserman et al., 1965) relative to the hydroxyl function of cholesterol.

In any case, it would be unreasonable to expect exact agreement for the order parameters of the two probes. The observation of large order parameters for the CSL probe indicates that this particular nitroxide does not seriously disrupt the organization of the membrane. In assessing the reliability of CSL as a membrane probe, it is more important to consider the order parameter trends observed with changes in the membrane rather than their absolute magnitudes. Satisfactory agreement is found between the responses of CSL and cholesterol-*3α-d* for temperature and cholesterol concentration variation, which indicates that CSL can yield reliable information regarding changes in the membrane organization.

The reasonable agreement found for CSL and cholesterol-*3α-d* can be contrasted with the results of our previous comparison of spin-labeled and deuterium-labeled fatty acids (Taylor & Smith, 1980). In that study, particularly poor agreement was noted in order parameter trends for label positions in the upper (carbon atoms 2–10 of stearic acid) portion of the acyl chains. It is interesting to note the difference between the behavior of CSL and these particular doxyl fatty acids. Similar behavior could be expected for a steroid and the upper region of the acyl chains since the rigid framework of the steroid lies in this region. Such similarities are, in fact, observed by deuterium NMR of deuterated cholesterol and acyl chains deuterated in this region. Essentially equal quadrupole splittings were observed for cholesterol-*3α-d* and the 5 position of the palmitoyl chains in an equimolar mixture of cholesterol and dipalmitoyl-PC (Gally et al., 1976). The same observation can be made by comparing the order parameters for cholesterol-*3α-d* (this study) and the 2–10 positions of stearic acid (Stockton & Smith, 1976) in egg PC–cholesterol (30 mol %).

The different responses of steroid and acyl chain spin probes may be due to special conformational features of fatty acid spin probes labeled in the upper chain. Previous ESR spin probe studies have provided evidence for tilting of the phospholipid chains (McConnell & McFarland, 1972; Gaffney & McConnell, 1974; Birrell & Griffith, 1976). In the models derived from these studies, the acyl chains are tilted away from the normal to the bilayer plane with a greater tilt angle for the upper portion of the chain. We have examined the rotational behavior of the spectra of spin probes in oriented films of egg PC–cholesterol (30 mol %) to determine if such a tilted structure exists in this lipid system. We have plotted in Figure 3 the variation in the observed hyperfine splittings for CSL, 5-SASL, and 14-SASL with the angle θ between the field and the normal to the flat cell surface. The hyperfine splittings were measured as one-half the separation between the zero crossing points of the low- and high-field resonances. For all orientations, the spectra for CSL and 14-SASL consisted of three well-resolved symmetric lines. In contrast, the 5-SASL probe gave such a spectra only for angles near 0 or 90°. Such a position-dependent behavior has been noted for phospholipids spin-labeled at positions 5 and 16 (Gaffney & McConnell, 1974, Figure 9A,B) and was attributed to a position-dependent tilt of the fatty acyl chains. The solid lines of Figure 3 represent the equation

$$A(\theta) = (A_{\parallel}^2 \cos^2 \theta + A_{\perp}^2 \sin^2 \theta)^{1/2}$$

where $A(\theta)$ is the observed hyperfine splitting at angle θ and $A_{\parallel}$ and $A_{\perp}$ are the splittings observed for the field parallel and perpendicular to the normal to the bilayer, respectively. This relationship should be obeyed if the axis of averaging is oriented along the normal to the bilayer plane rather than tilted away from it. In addition, if the acyl chains orient perpendicular to the bilayer plane, the splitting observed at the "magic angle" ($\theta \simeq 54.7°$) should be equal to the isotropic splitting. Good agreement with theory is observed for the CSL and 14-SASL probes, indicating that these probes orient perpendicular to the membrane plane. The angular dependence of the spectra for 5-SASL indicates that this probe is not oriented in such a fashion. It might be possible to simulate the rotational behavior of the 5-SASL spectra by using models with tilted acyl chains, but it is difficult to explain the presence of a tilt of the fatty acid chains in this region in view of the result for CSL. As stated above, the steroid probe should display behavior similar to that of the upper portion of the acyl chains, and, in particular, it could be expected to orient in a tilted fashion if the phospholipids were tilted. It is important to note that CSL and the doxyl fatty acids are sensitive to motions with similar time scales, so that both types of probes should sense a tilt if it is present. This is not necessarily the case with deuterium NMR where a tilt may not be observable due to the lower time resolution of deuterium NMR (Gaffney & McConnell, 1974). The discrepancy between CSL and the doxyl fatty acids may be due to difficulties associated with the insertion of the doxyl group into the upper region of the bilayer. The results of deuterium NMR studies of a number of phospholipids (Stockton et al., 1977; Oldfield et al., 1978;

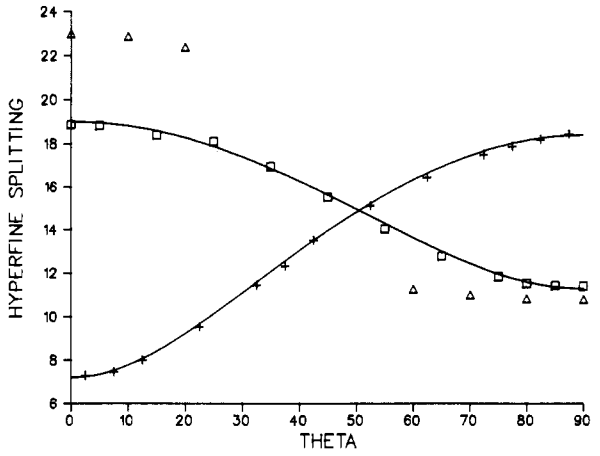

FIGURE 3: Angular dependence of the hyperfine splittings for spin probes 5-SASL (△), 14-SASL (□), and CSL (+). The angle θ was measured between the magnetic field and the normal to the flat surface of the rectangular ESR cell. Samples were 30 mol % cholesterol in egg PC at 23 °C.

Seelig & Browning, 1978; Davis, 1979) indicate that this region of the bilayer shows a high and essentially constant degree of order. A tilting of the nitroxide moiety of the 5-SASL probe may represent a conformational adaptation by the probe to allow insertion of the bulky nitroxide group into this efficiently packed region. Such an effect may be responsible for the anomalous behavior seen in the ^2H NMR of positions adjacent to the site of the nitroxide label (Taylor & Smith, 1980). Such a perturbation diminishes the utility of this probe in biological studies since it is difficult to distinguish between effects due to inherent membrane properties and those resulting from difficulties in the incorporation of the probe.

Based on these and previous comparative studies of nitroxide spin probes and deuterated probes, we feel that the most reliable spin probe experiments for membranes can be performed by using the cholestane spin probe or doxyl fatty acids labeled in the lower portion of the acyl chains. The behavior of these probes is at least qualitatively similar to that of the corresponding deuterium probes in terms of their response to variation of cholesterol concentration, temperature, or label position. The CSL probe is suitable for studies of bulk effects in membrane systems due to its large, rigid structure which penetrates deeply into the bilayer (Smith & Butler, 1976). For studies requiring information on effects in a particular region of the bilayer, doxyl fatty acids labeled in the lower portion of the chain appear to yield data which are at least qualitatively reliable. Clearly, however, either type of study is best performed with a nonperturbing technique such as deuterium NMR.

The use of either type of nitroxide spin probe discussed above is somewhat limited by the nature of the ESR spectra observed for these probes in membranes. To calculate order parameters for these probes, it is necessary to determine the parallel and perpendicular components of the hyperfine splitting. For CSL and some of the doxyl fatty acids, the difference between these components is too small to allow accurate determination of the order parameter for random dispersion (liposome) samples. In egg PC, for example, it is not possible to extract the splittings for random dispersion samples for label positions beyond carbon 12 of the stearic acid chain (Taylor & Smith, 1980). Thus, reliable spin probe studies for these probes are restricted to oriented lipid film systems. This limits the usefulness of these probes, since many systems, in particular natural membranes, are difficult to orient. In addition, the interpretation of spectra for oriented systems can be complicated if the lipids adopt nonbilayer phases (Taylor & Smith, 1981).

Added in Proof

We have recently determined the orientation of the axis of motional averaging of cholesterol (M. G. Taylor, T. Akiyama, and I. C. P. Smith, unpublished results). These results indicate that the 3α-^2H bond is oriented at $79 \pm 2°$ to the rotation axis. Molecular order parameters for cholesterol calculated by using this orientation are approximately 10% greater than those calculated by using the assumed 90° orientation for the C–^2H bond. Thus, the quantitative agreement between order parameters calculated for CSL and cholesterol-*3α-d* is even better than indicated in the present work.

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