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# THE CONFORMATIONS OF NITROXIDE-LABELLED FATTY ACID PROBES OF MEMBRANE STRUCTURE AS STUDIED BY $^2$H-NMR

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**The electron spin resonance (ESR) spectrum of a nitroxide spin probe intercalated in a membrane is influenced by the amplitude of anisotropic motion of the nitroxide group and by the geometry of the oxazolidine ring of the nitroxide. In the analysis of the ESR spectra of nitroxide-labelled fatty acid probes, it is generally assumed that the five-membered oxazolidine ring system is oriented rigidly perpendicular to the long molecular axis of the probe. This assumption is tested in the present study, using $^2$H-NMR of specifically deuterium-labelled nitroxide spin probes. Evidence is presented that the nitroxide does not display the assumed geometry in membranes. The departure from this geometry depends on the position of the nitroxide label on the acyl chain, with a more pronounced departure for position 5 relative to position 12. These and previous data provide an explanation for the discrepancies between spin-probe ESR and $^2$H-NMR order parameters in membranes.**

## Introduction

The reliability of nitroxide spin probes has been tested by comparison of the data derived for the nitroxides and corresponding deuterium NMR probes in phospholipid [1,2] and soap [3] systems. It was observed in both systems that the molecular order parameters derived for fatty acid nitroxide probes were consistently smaller than those derived for the deuterium probes. In addition, poor agreement was found in comparisons of the variation of order parameters with changes in label position [1,3] or cholesterol concentration [1]. These discrepancies were greatest for probes labelled in the upper portion of the chain, i.e., for label positions between C-2 and C-12 of the acyl chain. In contrast, good agreement was found in a similar comparison of nitroxide- and deuterium-labelled steroids [2]. These latter results indicate that the discrepancies between the data derived from the two spectroscopic techniques do not result from a general phenomenon such as the difference in the time-scale sensitivities of the two methods. An examination [2] of the angular dependence of the ESR spectra of fatty acid and steroid nitroxide probes in oriented membrane systems provided further evidence for a severe problem with fatty acid spin probes labelled in the upper portion of the chain.

It is possible to examine these problems in greater detail through the use of specifically deuterium-labelled nitroxides. Preliminary results employing a fatty acid nitroxide, deuterated at positions adjacent to the site of the nitroxide label, provided further evidence of anomalous behaviour

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Abbreviations: PC, phosphatidylcholine; SASL, stearic acid spin label.

of the spin probe [1]. The present studies extend this work using nitroxide probes deuterated on the acyl chain and on the oxazolidine ring system. The probes were incorporated in egg phosphatidylcholine bilayers as fatty acids or as the phosphatidylcholines derived from acylation of lysophosphatidylcholine with a fatty acid spin probe. In addition, the diamagnetic *N*-hydroxy derivatives of the nitroxides were employed to avoid problems associated with obtaining NMR spectra in a paramagnetic system. The data for the deuterated spin probes are combined with the results of the previous studies to provide a unified explanation of the anomalous behaviour noted for the spin probes.

## Materials and Methods

Published procedures were employed for the preparation of methyl-5-oxostearate [4] and the oxidation of 12-hydroxy stearic acid to methyl-12-oxostearate [5]. Methyl-5-oxostearate-2,2,4,4,6,6-$d_6$ was prepared via base-catalyzed exchange in 1% $NaO^2H/^2H_2O$ at 200°C [6]. The following procedure was used for the preparation of 2-amino-2-methyl-1-propanol-1,1-$d_2$. The labile protons of 2-amino-2-methyl-1-propanol (45 g, 0.5 mol) were exchanged by addition of 30 ml $^2H_2O$ followed by removal of water under reduced pressure. This procedure was repeated twice more. Approx. 25 g of Raney nickel in water (W.R. Grace) were exchanged three times with 20 ml $^2H_2O$ to remove $H_2O$. The Raney nickel/$^2H_2O$ suspension was added to the amino alcohol solution and the mixture was refluxed under $N_2$. The exchange was monitored by $^1$H-NMR. After 1.5 h reflux, 50 ml water were distilled off and replaced by fresh $^2H_2O$. After a further 45 min reflux, the suspension was cooled and filtered to remove the catalyst which was subsequently decomposed with 6 M HCl. The filtrate was distilled to remove $^2H_2O$ and the amino alcohol was collected at boiling point 164–166°C, yield 13 g. $^1$H-NMR indicated greater than 90% exchange at the 1-position. The procedure [7] for the preparation of nitroxides was modified to avoid the use of acid catalysis and excess 2-amino-2-methyl-1-propanol. Thus, a slight excess of the amino alcohol was employed with a trace of $I_2$ as catalyst [8]. Spin-labelled phosphatidylcholines were prepared by acylation of egg lysophosphatidylcholine [9] with the acyl imidazolide derivatives [10] of the spin-labelled fatty acids. The acylation was carried out in benzene using NaH as catalyst. The hydroxylamine derivatives of the spin-labelled phosphatidylcholines were prepared by hydrogenation of the nitroxides over platinum oxide [11]. Details of these syntheses have been published elsewhere [12].

Lipid samples were prepared by evaporating chloroform solutions of the appropriate composition to dryness and removing the residual solvent under high vacuum overnight. The lipids were then dispersed in deuterium-depleted water (Aldrich) with vigorous agitation. The lipid dispersions were then taken through several freeze-thaw cycles to improve homogeneity. $^2$H-NMR spectra were obtained at 46.1 MHz on a Bruker CXP-300 spectrometer using a home-built probe.

## Results and Discussion

The first studies dealt with a fatty acid probe bearing a nitroxide label at position 5 and deuterium labels at positions 2, 4 and 6 (5-SASL-$d_6$). The samples studied consisted of 25 mg spin probe in 250 mg egg phosphatidylcholine with variable amounts of cholesterol. The $^2$H-NMR spectra for the probe are shown in Fig. 1. Three sets of quadrupole splittings are observed for the probe. On the basis of a previous study [1] of a related deuterated spin probe, the smaller splittings can be assigned to the deuterons adjacent to the nitroxide and the larger splitting to the deuterons at position 2. Note that the high signal-to-noise ratio of Fig. 1 enables the resolution of separate splittings for positions 4 and 6, which were not resolvable in the earlier study [1]. These results may be compared with data for deuterated stearic acid intercalated in egg phosphatidylcholine bilayers [14]. In Fig. 2, the quadrupole splittings observed for different cholesterol concentrations are plotted for the 2–10 positions of stearic acid (squares); the 2-position of 5-SASL-$d_6$ (triangles); and the 4,6-positions of 5-SASL-$d_6$ (diamonds). For the stearic acid probe, a sharp increase is noted in the quadrupole splittings as the cholesterol concentration is increased, reflecting the increase in order produced by addition of cholesterol.

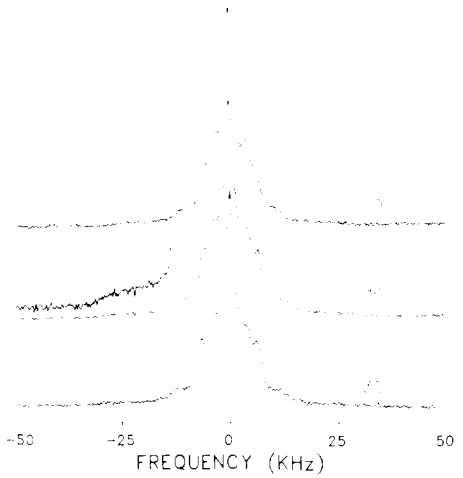


Fig. 1. $^2$H-NMR spectra (46 MHz) of 5-SASL-2,2,4,4,6,6-$d_6$ in aqueous dispersions of egg phosphatidylcholine of indicated cholesterol concentration, 30°C. Spectra were acquired with a recycle time of 0.1 s, and 72000 acquisitions. A vertical expansion is given in the one case to show the limit of detectable spectral intensity. Figures on right are mol% cholesterol.

Rather anomalous behaviour is noted for the 5-SASL-$d_6$ probe. Firstly, the quadrupole splittings for the probe are much smaller than those of the normal fatty acid, particularly for the positions adjacent to the nitroxide. Secondly, an unusual response to the addition of cholesterol is noted. For the 2-position, the quadrupole splitting increases slightly upon addition of cholesterol,

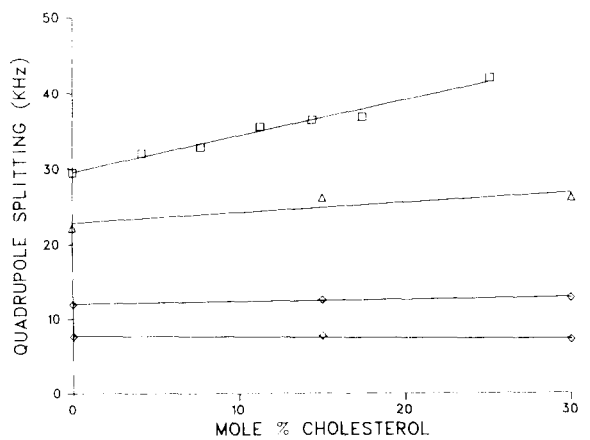


Fig. 2. Variation of the observed quadrupole splittings with cholesterol concentration for: □, the 2–10-positions of stearic acid (from Ref. 1), △, the 2 position of 5-SASL-$d_6$; and ◇, the 4 and 6 positions of 5-SASL-$d_6$. Data for 30°C.

whereas the splittings for the deuterons adjacent to the nitroxide show a much weaker response with both a slight increase and decrease in the magnitudes of the resolved splittings. These data can be compared with the behaviour of the stearic acid probe.

For the 2-position of 5-SASL-$d_6$, the quadrupole splitting is distinctly smaller than that of the normal stearic acid probe. This is probably due to an increase in motional freedom at this position due to the presence of the bulky nitroxide group at position 5. At the site of the nitroxide label, the cross-sectional area of the probe is much greater than that of a normal fatty acid. Because of this, the acyl chains of the surrounding phospholipid matrix may not pack as efficiently near the label site. As a result, the methylene groups of the spin probe near the label site would have a greater volume available in which to move. An increase in the amplitude of motion for the methylene group at position 2 would result in smaller quadrupole splittings, relative to the normal fatty acid, as noted here.

The weak response of the quadrupole splittings for position 2 observed for addition of cholesterol may be associated with the difficulty in packing the sterol adjacent to the bulky spin probe. As discussed previously [1], the concentration of cholesterol in the local region surrounding the spin probe may not be as great as in the bulk of the membrane. This would lead to an apparently weaker effect of cholesterol on the probe.

The behaviour of the deuterons adjacent to the nitroxide is much more complex. The magnitudes of the observed splittings are much smaller than those of the normal fatty acid and also than those of the 2-position of the probe. Also, the splittings diverge slightly as cholesterol is added, with differences between the splittings of 4.6 kHz and 6.0 kHz at 0 and 30 mol% cholesterol, respectively. The origin of the reduced magnitude of the splittings might be assigned to the same effects postulated for the 2-position of the probe. The response to cholesterol addition, however, indicates that such a simple explanation is not sufficient to describe the behaviour of the probe close to the nitroxide label site.

The factors which regulate the motional averaging of magnetic resonance parameters must be

considered individually. The degree of averaging of the hyperfine or quadrupole coupling tensors is affected by both the amplitude and geometry of the motion of the probes in anisotropic media [15]. The present data for the effect of cholesterol indicate that it is necessary to consider the effects of probe geometry more closely. It is known from other experimental techniques [16] that cholesterol decreases the amplitude of motion in fluid bilayer systems such as egg phosphatidylcholine. This increase in molecular order is expected to result in an increase in the net anisotropy of the hyperfine tensors of the spin probes. The anomalous behaviour for the 4,6 deuterons of 5-SASL-$d_6$ suggests that local geometric factors strongly influence the spectroscopic properties of the spin probes. This possibility was investigated in greater detail using spin probes bearing deuterons on the methylene carbon of the nitroxide oxazolidine ring system. The $^2$H-NMR behaviour of such probes should be most informative in comparison with the EPR behaviour of the spin probes since, in both cases, the magnetic resonance label is attached to the ring system of the nitroxide. Care was taken in these experiments to minimize extraneous perturbations resulting from the use of high concentrations of fatty acid spin probes in the membrane. Thus, the probes used were the phosphatidylcholine probes derived from the acylation of lysophosphatidylcholine with a deuterated fatty acid spin probe. The probe concentration was reduced to 5 mol%, which more closely approximates the concentration employed in EPR studies (typically 1–2%). $^2$H-NMR spectra were also obtained for the diamagnetic hydroxylamine derivatives of the nitroxides to eliminate paramagnetic effects on the $^2$H-NMR spectra. These derivatives, produced by catalytic hydrogenation of the nitroxides, differ from the nitroxides only by replacement of the N-O bond by an N-OH group.

Fig. 3 illustrates the $^2$H-NMR spectra for the phosphatidylcholine spin probes bearing ring-labelled deuterated nitroxide groups at positions 5 and 12, in egg phosphatidylcholine bilayers at 5 mol% concentration. In each spectrum, two sets of quadrupole patterns are observed. For the 5-position, a component with splitting of approx. 4 kHz is found as well as a second, broad component with splitting of approx. 25 kHz.

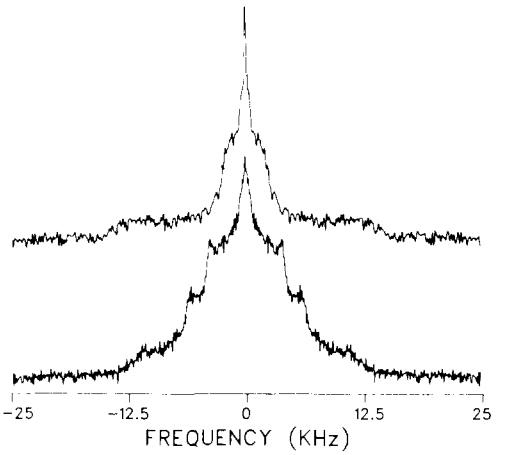


Fig. 3. $^2$H-NMR spectra (46 MHz) of the phospholipid spin probes (PC-SASL-$d_2$) deuterated at the methylene carbon of the oxazolidine ring at position 5 (upper trace) or 12 (lower trace) of the stearoyl chain. The probes were 5 mol% in aqueous dispersions of egg phosphatidylcholine at 25°C. Spectra were accumulated with a recycle time of 0.1 s and accumulation times of 8–10 h.

The poor signal-to-noise ratio for the spectrum results partially from the low probe concentration and partially from paramagnetic broadening due to the nitroxide group. The effect of the paramagnetic nitroxide is more severe for these samples than for the previous samples due to the low concentration of the spin probe in the present case. At high concentration, the broadening is reduced because spin exchange between paramagnetic probes tends to uncouple the effect of the free electron on the NMR spectra [17]. Since spin exchange requires collisions between nitroxides, it is less efficient at low probe concentrations, resulting in the large degree of broadening observed.

For the 12-position, two components with splittings of approx. 8 and 12 kHz are readily observed.

In order to verify that the two component spectra observed for these probes did not arise as a result of the paramagnetic nature of the nitroxides, these experiments were repeated employing the diamagnetic hydroxylamine analogues. The $^2$H-NMR spectra are shown in Fig. 4. For the 5-position, two components are observed. The first component, with splitting of approx. 4 kHz, is assigned to the presence of residual nitroxide in the sample.

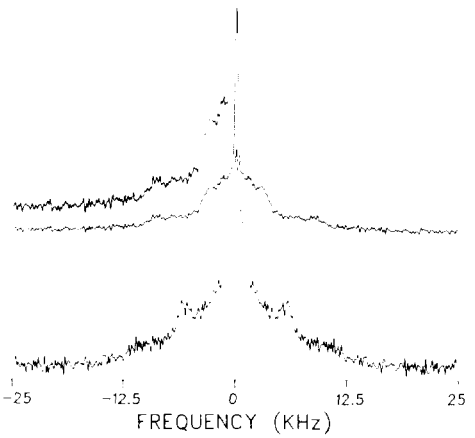


Fig. 4. $^2$H-NMR spectra (46 MHz) of the hydroxylamine derivatives of the phospholipid spin probes (PC-SASL-$d_2$) deuterated at the methylene carbon of the oxazolidine ring at position 5 (upper trace) or 12 (lower trace) of the stearoyl chain. Conditions and composition as in Fig. 3.

The hydroxylamine derivatives are rather unstable [11] and, during the course of the experiment, may regenerate the parent nitroxide. The other components, with splittings of approx. 6 and 19 kHz are assigned to the deuterons of the hydroxylamine analogue. For the 12-position, a splitting is clearly observed of approx. 12 kHz, as well as a broad, unresolved central component.

The differences in magnitudes of the splittings observed for the nitroxides and their hydroxylamine derivatives may be due to an alteration of the conformation of the five-membered oxazolidine ring system. In addition, severe line broadening in the paramagnetic systems may distort the $^2$H-NMR lineshapes. In either case, the important point is that the deuterons of the ring system give rise to inequivalent quadrupole splittings in both the paramagnetic and diamagnetic systems.

The observation of inequivalent deuterons on the oxazolidine ring system provides direct evidence for an unexpected geometry for the nitroxide group. In the analysis of the ESR spectra of fatty acid spin probes, it is generally assumed that the oxazolidine ring is oriented rigidly perpendicular to the axis of motional averaging of the probe. If this were the case, both methylene deuterons of the ring system would be oriented at the same angle to the averaging axis. For this geometry, equal quadrupole splittings are expected in the $^2$H-NMR spectra. The indication of a deviation from the assumed geometry has serious consequences for the interpretation of the spin-probe ESR spectra.

The extent of motional averaging of a tensor property, such as the hyperfine splitting of a nitroxide, depends simultaneously on the amplitude of anisotropic motion of the probe and on the geometry of the probe relative to the motional averaging axis. For the nitroxide spin probes, the effects of probe motion and geometry on the ESR spectra cannot be separated using only the measurement of the residual hyperfine splittings. In order to allow interpretation of the ESR spectra in terms of the amplitude of motion of the probes, i.e., in terms of order parameters, it is necessary to assume a particular geometry for the nitroxide group. In the analysis of ESR spectra of nitroxide spin probes, it is generally assumed that the ESR-active N-O function of the nitroxide is oriented with the principal component of the hyperfine coupling tensor along the axis of motional averaging. This geometry leads to the maximum possible hyperfine anisotropy for a probe located in a fluid anisotropic medium. Any deviation from the assumed geometry will lead to a reduction in the observed hyperfine anisotropy. Thus, for a constant amplitude of anisotropic motion, the observed hyperfine anisotropy of a spin probe may be reduced simply by alteration of its geometry. This clearly complicates the interpretation of the ESR spectra since an observed reduction in the hyperfine anisotropy may be due to geometric factors, to an increase in the amplitude of probe motion, or even to a combination of these.

A further complication arises from the interpretation of the ESR spectra in terms of molecular order parameters for the probes. The ESR spectra yield directly only the order parameter for the $Z$-axis of the N-O fragment of the nitroxide. In order to convert this order parameter to the molecular order parameter, it is again necessary to assume a geometry for the N-O function. Thus, the reliability of the derived order parameters depends directly on the validity of the assumptions concerning the probe geometry. Geometric problems with the interpretation of spin probe data in terms of molecular order parameters have been discussed [15].

An analogous situation is found in the interpretation of the $^2$H-NMR spectra of phospholipids bearing deuterated oleic acid (*cis*-9,10-octadecenoic acid) acyl chains [18,19]. In the region of the *cis* double bond, the observed quadrupole splittings are much smaller than those of positions far removed from the double bond, or those of the same label positions on fully saturated acyl chains. In that case, it was possible to demonstrate that the smaller magnitude of the splittings in the double-bond region was due to the geometry about the *cis* double bond rather than to an increase in the amplitude of motion of the acyl chain [18]. It was possible to separate the effects of geometry and amplitude in that study using the known geometry of the deuterons on the rigid double bond and additional evidence from infrared dichroism [20]. A more recent study employing fatty acids with *cis* cyclopropyl groups has confirmed the validity of these conclusions [21]. In the present study using deuterated spin probes, this separation cannot be performed since the angular relationships between the various deuterons are unknown and the probes have an unknown degree of internal freedom of motion.

The results of the previous comparisons of nitroxide- and deuterium-labelled probes can be rationalized in terms of the geometry at the nitroxide label site. As discussed above, any deviation from the standard, assumed geometry for the nitroxide group would result in a reduction of the hyperfine anisotropy and erroneously small derived molecular order parameters. In the comparison of the fatty acid nitroxide and deuterium probes [1], it was noted that the ESR order parameter were less than those derived from the corresponding deuterated analogues, which is consistent with the effect expected for alteration of the nitroxide geometry.

It is possible to speculate on the physical origin of this perturbation based on the evidence discussed above. The probe geometry may be altered as an adaptation by the probe to allow its insertion into the ordered matrix of the membrane. In the assumed geometry, the cross-sectional area of the probe is twice that of an unlabelled chain. It is clear that such a probe would not pack easily into the ordered structure of the membrane. The cross-sectional area of the probe could be reduced by twisting the acyl chain in the region of the nitroxide group such that the oxazolidine ring system would no longer be perpendicular to the long axis of the probe. This possibility is supported by the effects observed with addition of cholesterol. As cholesterol is added, the acyl chains of the membrane tend to pack more efficiently, which should increase the stress on the nitroxide group. Evidence for this effect is seen in the $^2$H-NMR behaviour of the deuterons adjacent to the nitroxide group of 5-SASL-$d_6$. For these positions, the quadrupole splittings diverged as the cholesterol concentration was increased. This effect would not be noted if the probe maintained a constant geometry as the cholesterol concentration was increased.

Secondly, the response to addition of cholesterol noted in the ESR behaviour of the spin probes was weaker than that observed for the $^2$H-NMR behaviour of the deuterated stearic acid probes [1]. This weak response could represent a balance between the effects of cholesterol on the amplitude of motion of the probes and their geometry. The addition of cholesterol should increase the hyperfine anisotropy for the probes by decreasing the amplitude of their motion. The expected effect would be attenuated, however, since the increased degree of order of the phospholipid matrix would induce a greater deviation from the assumed orientation of the oxazolidine ring, resulting in reduction of the hyperfine anisotropy.

Further evidence for the importance of chain packing for the orientation of the nitroxides is obtained from the label position-dependence of the spectral behaviour of the probes. The upper region of the membrane is characterized by a high and essentially constant degree of order [22]. Consequently, packing-induced effects on the nitroxide orientation should be more pronounced for this region than for the less ordered lower region of the membrane. This suggestion is supported by the orientation dependence of the EPR spectra of the 5-SASL and 14-SASL probes in oriented lipid film samples. As discussed previously [2], the 14-SASL probe displays the behaviour expected for the assumed probe geometry, whereas the 5-SASL probe shows a marked deviation from the expected behaviour. The results of the $^2$H-NMR study of the phosphatidylcholine spin probes deuterated on the

oxazolidine ring may also be interpreted in terms of a position-dependent orientational effect. As was stated above, the methylene deuterons would exhibit equal quadrupole splittings if the oxazolidine ring system were oriented perpendicular to the motional averaging axis. As seen in Fig. 3, the inequivalence of the splittings for these deuterons is greater for the 5-position probe, which is to be expected if the higher efficiency of chain packing in this region induces a larger alteration in the nitroxide geometry.

As a final observation, it is interesting to note the effects of paramagnetism on the $^2$N-NMR spectra of the deuterium-labelled nitroxides. As stated above, line broadening may occur in the $^2$H spectra, particularly at low nitroxide concentration. In addition, it is possible to observe effects on the chemical shift behaviour of the $^2$H resonances. This contact shift results from the strong, local magnetic field produced by the unpaired electron. The effect of the unpaired electron is transmitted through the contact hyperfine interaction, so that the magnitude and sign of the contact shift is dependent upon the nature of the hyperfine coupling constant between the $^2$H nucleus and the unpaired electron [17]. Close examination of the $^2$H-NMR spectra for 5-SASL-$d_6$ (Fig. 1), reveals that the quadrupole patterns for the deuterons adjacent to the nitroxide label site are not symmetric about the zero frequency, but rather are shifted to higher frequency. In the case of the nitroxide probes with deuterons at the methylene carbon of the oxazolidine ring, no contact shift was evident. These observations are consistent with $^1$H-NMR studies of related oxazolidine nitroxides where shifts to higher frequency were noted for protons at corresponding positions (Ref. 17 and references cited therein). The signs and magnitudes of the shifts noted here indicate a much larger, negative hyperfine coupling constant for the deuterons on the acyl chain than for those of the methylene carbon of the oxazolidine ring. Quantitative values of the shifts and derived hyperfine coupling constants would best be obtained through high-resolution solution $^2$H-NMR of the nitroxides and their diamagnetic hydroxylamine derivatives.

## Conclusions

The $^2$H-NMR data for the deuterated spin probes presented here give evidence for unexpected geometrical properties for the nitroxide spin probes. These geometric effects are important for the interpretation of the ESR spectra of spin probes due to their influence on the spectral properties of probes inserted in anisotropic media. It is seen that several of the findings discussed previously may be explained in terms of alteration of the nitroxide geometry. This discussion is necessarily speculative, since the influence of geometry on the observed spectra cannot be separated from the influence of the degree of motional freedom of a probe. The inability to separate these factors presents a serious problem in the interpretation of the ESR spectra of spin probes in membranes. Generally, the spin probe spectra are interpreted only in terms of order parameters based on an assumed geometry. The data presented here, for the dependence on label position and cholesterol concentration, indicate that ignoring the geometric effects may result in serious misinterpretation of the spin-probe data.

The departure from the assumed nitroxide geometry appears greater for nitroxides in highly ordered regions of the bilayer (position 5, high cholesterol concentration). This suggests that the nitroxide alters its geometry in order to pack more efficiently in regions of high molecular order. This is consistent with the previous comparison of nitroxide- and deuterium-labelled fatty acids [1], where the greatest discrepancies were noted for label positions in the highly ordered region of the bilayer.

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