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THE FIDELITY OF RESPONSE BY NITROXIDE SPIN PROBES TO CHANGES IN MEMBRANE ORGANIZATION

THE CONDENSING EFFECT OF CHOLESTEROL

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Summary

The response of doxyl fatty acid spin probes in egg lecithin bilayers to added cholesterol is compared with results from ^{2}H-NMR. Large differences are found between the profiles of order parameter vs. label position and cholesterol concentration.

At constant cholesterol content, the ESR spin probe order parameter decreases continuously as the label position is moved toward the terminal methyl region of the bilayer whereas an order parameter 'plateau' is observed for the upper region of the bilayer by ^{2}H-NMR. In addition, the spin probe order parameters are smaller than those observed by ^{2}H-NMR.

Differences are also observed in the profiles of order parameter vs. cholesterol content for each label position. The spin probes detect a maximal response to added cholesterol for the central portion of the chains with much weaker responses near both ends of the chains. In contrast, the ^{2}H-NMR results indicate a large, approximately constant response for the first ten positions in the chains with a decreasing response toward the terminal methyl group. For all the positions examined, the spin probes show a weaker response than that observed by ^{2}H-NMR.

A direct measure of the perturbing effect of a spin label is made by comparing the deuterium quadrupole splittings in egg lecithin-cholesterol bilayers for stearic acid with and without an attached doxyl moiety. The spin-labelled fatty acid has a much reduced quadrupole splitting and an opposite response to cholesterol addition.

Abbreviation: doxyl, the *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of keto fatty acids.

Introduction

Nitroxide spin probes are widely used in the study of membrane systems, since analysis of the ESR spectra of these probes can yield information concerning both structure and dynamics [1]. A persistent problem in the spin probe technique, and any other method employing extrinsic probes, is the possibility that the probe creates an environment within the membrane which differs significantly from that in the bulk of the membrane. Since the spin probes sense most strongly their immediate environment, data derived from spin probe studies may reflect poorly the true state of membranes.

It is difficult to assess quantitatively the reliability of spin probe data since the nature and magnitude of the nitroxide perturbation are not well understood. Previously, this perturbation has been considered in terms of disruption of chain packing by the bulky doxyl group [2] and the polarity of this group [3]. These effects, and possibly others, may influence the observed ESR spectra of spin probes in membranes. Without a clearer understanding of the perturbation, the relevance of data derived from spin probes remains open to question. A clearer understanding of the problem may also assist in the choice of probes to be used in a particular system and in the design of spin probe experiments. With these aims in mind, we have performed a series of experiments to elucidate the features of this problem.

We present here a comparison of nitroxide spin probe and ^{2}H-NMR data [4] for the system egg lecithin-cholesterol/water. The variation of order parameters derived from both methods is examined with respect to the positions of the labels and changes in cholesterol content. The latter comparison is of particular interest, since spin probes are commonly used in membrane studies as monitors of changes in the membrane resulting from addition of external agents.

Materials and Methods

Stearic acid spin probes with doxyl (dimethyloxazolidinyl nitroxide) labels at positions 5, 12 and 16 were purchased from Syva (Palo Alto, CA). The remaining probes were synthesized by the method of Waggoner et al. [5]. The deuterated spin probe, 5-doxyl [4,4,6,6-^{2}H]stearic acid, was also synthesized by this procedure using methyl-5-keto[4,4,6,6-^{2}H]stearate as starting material. The latter was produced by deuterium exchange of the unlabelled methyl-5-ketostearate in CH_3O^2H under acid catalysis. The other reagents used in the oxazolidine synthesis (*p*-toluenesulfonic acid and 2-amino-2-methyl-1-propanol) were treated with ^2H_2O before use to remove easily exchangeable protons. Egg lecithin was purchased from Lipid Products (Surrey, U.K.). Cholesterol was supplied by Steraloids (Pawling, NY). Double-distilled water was used for the hydration of the lipids.

The lipid composition of the samples was adjusted to match the conditions of the ^{2}H-NMR experiments which employed a predeuterated stearic acid probe [4]. Thus, stearic acid was added to give a spin probe and stearic acid concentration equal to that of the perdeuterated stearic acid in the ^{2}H-NMR study. The spin probe to phosphatidylcholine molar ratio was 1 : 100.

Oriented multibilayer films were prepared as described previously [6]. To

prepare dispersions, $CHCl_3$ solutions of the lipids were evaporated under N_2 and the samples were placed under high vacuum for several hours to remove residual solvent. The dry lipid was then hydrated with water and dispersions were formed with vigorous agitation.

A Varian E-9 X-band spectrometer was used to obtain the ESR spectra. Sample temperature was regulated by the Varian variable temperature regulator for the dispersions and by a home-built (Butler, K.W., unpublished results) regulator for the oriented films. All samples were maintained at 30 ± 1°C and the temperature was monitored continuously by means of a digital thermometer (Newport Laboratories, Santa Ana, CA) with the thermocouple placed directly above the sample portion of the ESR cells. ^{2}H-NMR spectra were obtained at 46.2 MHz on a Bruker CXP-300 spectrometer, at 30°C.

Order parameters were calculated as previously described [7]. No polarity correction was used, and the principal values of the static hyperfine coupling tensor were taken as: A_{xx} = 6.3 G, A_{yy} = 5.8 G, A_{zz} = 33.6 G (Appendix II in Ref. 1).

Results and Discussion

The spin probe experiments were performed using both oriented films and unsonicated lamellar dispersions. The dispersions are less useful than the oriented films due to the difficulty of separating the effects of order and motion in their ESR spectra [8,9], and due to their inability to report order parameters for $S < 0.3$ (beyond the C-9 position of stearic acid). The data derived from the ESR film experiments and the corresponding ^{2}H-NMR results are shown in Fig. 1, where we have plotted the observed order parameters for each probe as a function of cholesterol content.

For the spin probe data, each point represents the average of 2—5 films. The range of S values at each point is 0.03 S units for the worst case, and the average range for the entire set of points is 0.01 S units. The straight lines were fitted by linear regression with correlation coefficients greater than or equal to 0.98 for all lines. For the subsequent data analysis, values of S for 0 and 30 mol% cholesterol were taken from the straight lines.

Order parameters vs. position profiles

The ^{2}H-NMR and spin probe order parameter vs. position profiles are depicted in Fig. 2, where we show the order parameters at zero (circles) and 30 mol% (squares) cholesterol.

The deuterium profile shows the characteristic order parameter plateau [4,10,11] for the upper portion of the bilayer followed by a decrease in S as the terminal methyl group is approached. In contrast, the spin probe order parameters decrease continuously as the label approaches the terminal methyl group of the probe, as noted previously [2,12,13]. In addition, the spin probe order parameters are smaller than the corresponding values of S determined in the ^{2}H-NMR experiment. The ESR order parameters obtained from dispersions are higher than those obtained from multibilayer films, as described earlier [7], but show an essentially linear decrease with position in parallel with the film data. Order parameters could not be measured beyond position C-9 for

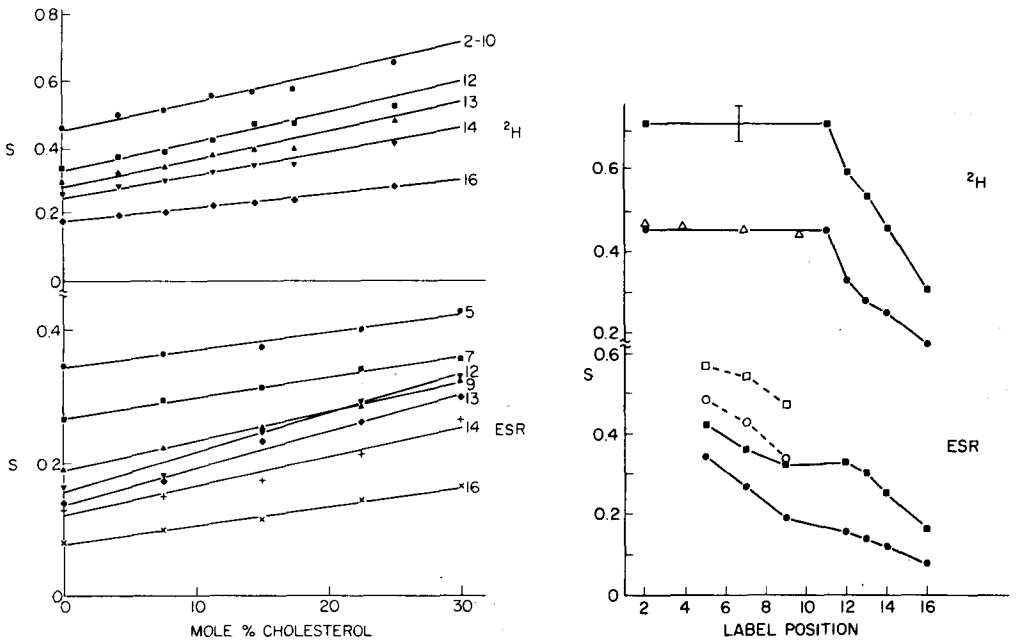

Fig. 1. Variation in order parameters (S) with cholesterol content in egg lecithin. The individual lines are for the various positions of stearic acid. The upper plot for ^{2}H-NMR was constructed with data for dispersions from Ref. 4. The lower plot shows ESR spin probe data from the present work. Note changes in scales of ordinates.

Fig. 2. Variation of order parameter (S) as a function of label position for egg lecithin. Circles are for cholesterol-free bilayers and squares for bilayers containing 30 mol% cholesterol. ------, the ESR data for dispersions. In the ^{2}H-NMR data, the triangles represent order parameters for specifically deuterated positions and the bar indicates the estimated uncertainty in the measurement.

dispersions due to the absence of resolved spectral features.

A possible explanation for the difference between the ESR spin probe and ^{2}H-NMR order vs. position profiles is tilting of the phospholipid acyl chains [12]. If the lifetime of the tilt is short on the ^{2}H-NMR time scale, but long on the spin probe time scale, the deuterium quadrupole splittings would reflect an additional averaging not observed for the spin probes. If only the upper portion of the bilayer is tilted, this additional averaging could be responsible for the detection of an order parameter plateau by ^{2}H-NMR but not by the spin probes. In this case, however, the ^{2}H-NMR order parameters are expected to be smaller than the corresponding spin probe order parameters [11], in contrast to the present and earlier [2] data.

A similar comparison of the two methods employing a different type of liquid-crystalline system has been previously published [2]. The observed behaviour of the two types of probe was similar in that a continuous dependence of the spin probe order parameter on position was found, whereas a plateau of S values was observed in the ^{2}H-NMR experiment. The difference in behaviour was attributed to the larger radius of the spin probes. Disruption of the packing of the alkyl chains by the bulky doxyl group may create a defect volume at the label site allowing a higher proportion of *gauche* rotamers, and

hence a lower order parameter. As was noted [2], the spin probe order parameter vs. position profiles have been accounted for by models which consider only *trans-gauche* isomerizations and neglect interactions between chains (Ref. 2 and references cited therein). In addition, Belle and Bothorel [14] were able to reproduce a spin probe order vs. position profile using a model which treated both intra- and intermolecular interactions. However, it was necessary to assume an unusually large interchain distance. With smaller interchain distances, the profiles were similar to the ^{2}H-NMR results. The agreement of the spin probe data with models which neglect interactions between chains suggests that the spin probes are somewhat isolated from their surrounding lipid matrix. From this point of view, spin probes are not expected to yield an accurate picture of the absolute magnitudes of order parameters in membranes. In the next section we discuss this problem in terms of changes in order determined by spin probes.

Profiles of the effect of cholesterol on order parameters

Nitroxide-induced perturbations which result in inaccurate values of order parameters do not necessarily rule out the use of spin probes in biological studies. Often it is only necessary to have a reliable monitor of the relative values of membrane properties under different conditions. In this section we compare the effect of cholesterol addition on the order parameters observed by the two techniques. The response to cholesterol is measured as the slope of the dependence of the order parameter on cholesterol content for each label position.

The results for both methods are depected in Fig. 3. The delta (Δ) parameter

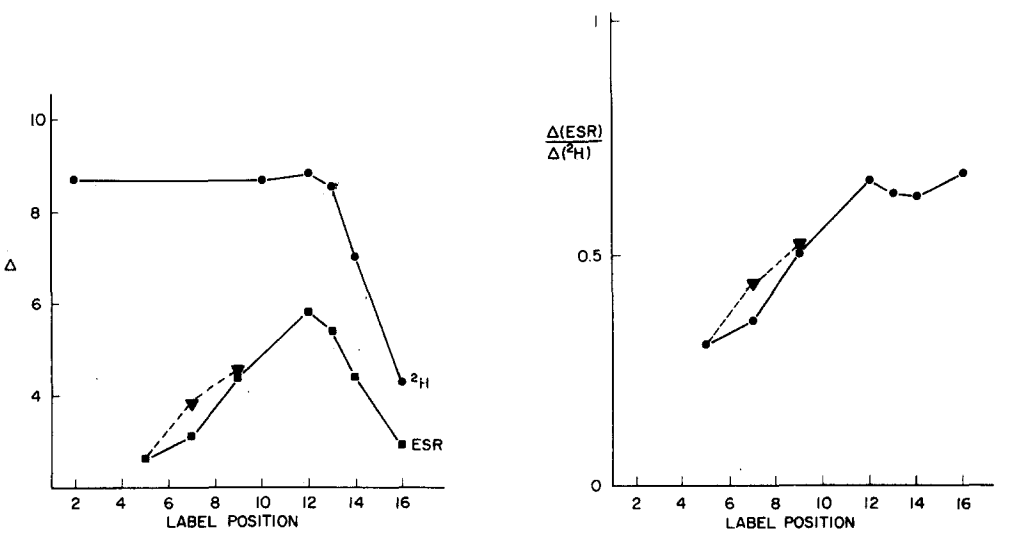

Fig. 3. Measure of the response of S in egg lecithin to added cholesterol. The Δ parameter for each position is the slope (X1000) of the order parameter vs. cholesterol content line in Fig. 1: ●, ^{2}H-NMR data; ■ and ———, ESR spin probe-oriented film data; ▼ and - - - - -, ESR dispersion data.

Fig. 4. Ratio of the ESR and ^{2}H-NMR responses for each label position: ● and ———, using ESR data for oriented films; ▼ and - - - - -, using ESR data for dispersions.

is the slope (X1000) of the order parameter vs. cholesterol content line (Fig. 1) for each label position; it provides a measure of the response of the bilayer to added cholesterol. For the ESR profile, the solid line is derived from oriented film experiments and the broken line from dispersions (vide infra).

Two differences between the techniques are readily apparent. Firstly, the spin probes show a much weaker response than the corresponding deuterium probes. Secondly, the shapes of the two profiles are distinctly different. In Fig. 4, the ratio of the slopes observed for both techniques at each label position is plotted versus position. A qualitatively similar response is seen for both ^{2}H-NMR and ESR probes with labels in positions 12—16. For positions 5—9 there is a much larger discrepancy, since the ESR probe response decreases continuously toward the carboxyl group whereas the ^{2}H data show a constant response in this region. Plots of Δ/S vs. position, for fixed cholesterol concentrations, show similar large discrepancies between the ^{2}H and ESR results for positions 5—10.

The weaker response of the spin probes may be due to the same perturbation which results in the smaller values of the spin probe order parameters. As stated above, the spin probe data appear to be consistent with models in which interactions between chains are weak. If the spin probes are not well coupled to the lipid matrix, they may be expected to be less sensitive to changes in the surrounding lipid.

The difference in the shapes of the response profiles is more difficult to interpret. The approximately constant response for the first twelve carbons seen by ^{2}H-NMR can be understood on the basis of the structure of cholesterol [4]. This upper portion of the bilayer interacts with the steroid nucleus of cholesterol [15]. Since this portion of the molecule is rigid, it exerts a constant effect on the surrounding lipids. For the remaining chain positions, the effect of cholesterol diminishes toward the terminal methyl group. This portion of the bilayer interacts with the flexible alkyl side-chain of cholesterol, so a lesser effect is expected.

For the upper portion of the bilayer, the spin probe data indicate a decreasing response as the carboxyl terminal is approached. This may result from a disturbance of chain packing which lowers the cholesterol concentration in the lipid pool surrounding the spin probe. If cholesterol does not readily enter this pool, an erroneously small response will be seen, since the spin probes reflect most strongly their immediate environment. For the upper portion of the bilayer, the packing disturbance would be most severe in terms of excluding cholesterol, since this region corresponds to the rigid steroid framework of cholesterol. One might expect that this perturbation would be worst for spin probes with labels near the lipid/water interface, since this is the anchoring site for cholesterol [15]. For positions 12—16, the spin probes and deuterium probes exhibit a similar response to cholesterol. Chain packing disturbances would not have as severe an effect on local cholesterol concentrations in this region, since the flexible side-chain could more readily adapt to the perturbation.

^{2}H-NMR of spin-labelled probes

As a direct measure of the perturbing effect of a doxyl moiety we have ob-

tained ^{2}H-NMR data for the 5-doxyl stearic acid probe with ^{2}H at positions 4 and 6, in dispersions of egg lecithin. Fig. 5 shows the NMR spectra and Fig. 6 the dependence of the quadrupole splitting on cholesterol concentration. Also included in Fig. 6 is the cholesterol-dependence of the quadrupole splitting for the non-spin-labelled stearic acid probe under similar conditions [4]. The disordering effect of the doxyl moiety on the acyl chains is considerable, and the response to cholesterol addition is opposite in direction for the two probes. These data confirm our conclusions stated earlier, based on comparison of ^{2}H-NMR data for dispersions with those for ESR spin probes in multibilayer films and dispersions.

Comparison of results for dispersions and oriented films

Some of the comparisons presented here between ^{2}H and spin probe order parameters have been made using data derived from oriented films for spin probes and unsonicated dispersions for ^{2}H-NMR. In order to exclude the possibility that the discrepancies noted resulted from structural differences in the two systems or imperfections in the oriented films, parallel studies were performed using spin probes in dispersions. Only spin probes labelled at positions 5, 7 and 9 were used, since these probes yield spectra with well-resolved hyperfine splittings.

In dispersions, the values of the measured order parameters are higher than

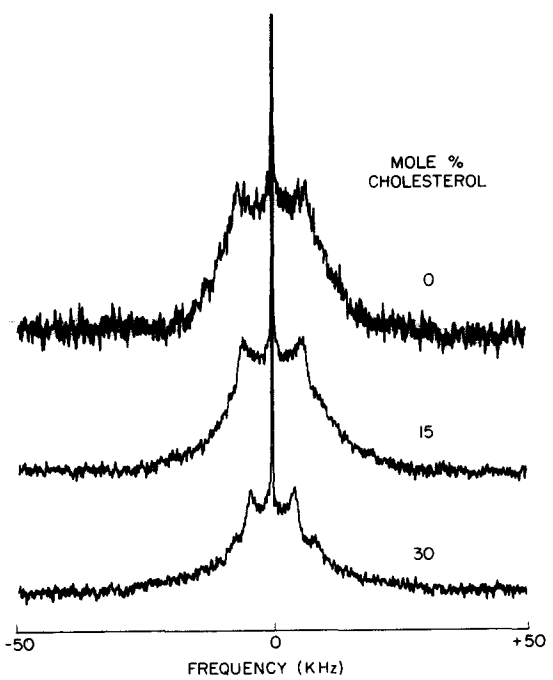

Fig. 5. ^{2}H-NMR spectra (46.2 MHz) of 5-doxyl [4,4,6,6-^{2}H]stearic acid (50 mg) in dispersions of egg lecithin (500 mg in 1 ml H_2O) containing differing amounts of cholesterol. The spectra were obtained using the quadrupole echo sequence (90°_x—τ—90°_y—echo) with a recycle time of 0.1 s, 36 000 accumulations, at 30°C.

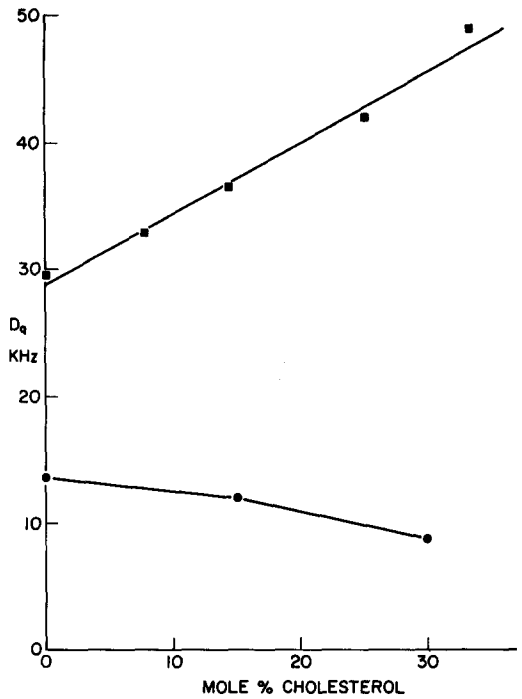

Fig. 6. The dependence of the quadrupole splittings (D_Q) on cholesterol concentration for stearic acid (■) and 5-doxyl stearic acid (●) labelled at the 4- and 6-positions with ^2H. Separate quadrupole splittings are not observed for positions 4 and 6; the values indicated are the resolved quadrupole splittings.

those found for the corresponding oriented films (Fig. 2). For the 5-doxyl stearate probe, the ESR order parameter measured in dispersions was equivalent to the ^2H-NMR order parameter. However, the order parameter decreases for positions 7 and 9, as in the oriented film experiments, to values below those in the corresponding ^2H-NMR experiments.

Differences in ESR order parameters obtained for dispersions and oriented films have been discussed previously [9]. Schreier-Muccillo et al. [7] found a strong dependence of S on lipid concentration, as vesicles proceeded from unilamellar to multilamellar. Marsh et al. [16] reported that the ESR order parameter was lower in isolated unilamellar vesicles than in large multilamellar vesicles. It is apparent that the rate of probe motion within the bilayer affects the ESR lineshapes of vesicular systems in a manner such that overlap of the component subspectra can preclude the accurate measurement of order parameters [8,9]. This is much less of a problem with the oriented bilayer system [6], where spectra due to particular angles between the axis of motional averaging and the applied magnetic field can be measured separately. Thus, the oriented bilayer system provides the least equivocal measurement of the ESR order parameter. One must consider the possibility that the degree of molecular organization within oriented multibilayers and vesicles is different. To date this has not been demonstrated experimentally. ^2H-NMR measurements on both oriented films and dispersions could resolve this point; these have not as yet been performed due to the low signal intensity available from oriented films.

Regardless of any possible differences between the systems, the important comparisons made here are the variation of S with position, and the response of S to cholesterol addition. The relative values of these parameters should be relatively insensitive to any differences between oriented films and dispersions. Wherever ESR data for both films and liposomes are available (Fig. 2—4), the spin probes respond in a qualitatively similar fashion. We note that in an earlier study of spin probes in dispersions (Schreier-Muccillo et al. [7]), the 12-doxyl stearic acid probe responded more steeply to cholesterol addition than did the 5-doxyl stearic acid probe, in agreement with the present results.

The qualitative agreement between dispersions and oriented films for the position and response profiles indicates that the discrepancies between the results reported by ESR spin probes and ^{2}H-NMR are not due to differences in the structures of dispersions and planar films.

The validity of data for fatty acid probes

Recently Hauser and Guyer [17] have suggested that deuterated fatty acids may cluster in lipids so as to invalidate conclusions reached by this type of probe. This suggestion was based on the apparent additional charge contributed to egg lecithin, as measured by electrophoretic mobility. In contrast, spin-labelled fatty acids appeared to confer the expected incremental charge.

The fidelity of response of the deuterated fatty acid probes is attested to by a variety of comparisons. Firstly, the order parameter vs. position profiles obtained for fatty acid probes in egg lecithin [4,18], are virtually the same as those found for the deuterated palmitoyl chains of dipalmitoyl phosphatidylcholine [19], 1-palmitoyl-2-oleoyl phosphatidylcholine [19], dipalmitoyl phosphatidylethanolamine [19] and the membrane lipids of *Acholeplasma laidlawii* [10, 20]. Secondly, the response to cholesterol addition of the deuterated fatty acid probes is similar to that observed for the 5-position of the palmitoyl chains in dipalmitoyl phosphatidylcholine [21], several positions in the myristoyl chains of dimyristoyl phosphatidylcholine [22], and all positions of the palmitoyl chains in the membrane lipids of *A. laidlawii* [10]. We feel, therefore, that the fatty acid probes used in the present comparison provide a reasonable view of the behaviour of egg lecithin and its response to cholesterol addition.

Conclusions

Using ^{2}H-NMR as a reference, some insight can be gained into the nature of the perturbation introduced by the nitroxide label. The view that the spin probes are partially isolated from the bilayer matrix is supported by the profile of order parameter as a function of label position, the weak response of the spin probes to added cholesterol, and the low quadrupole splittings observed for the deuterated spin probes.

The magnitude of the discrepancy between the results obtained by the ESR and deuterium probes depends strongly on the position of the labels. For positions 12—16, both techniques give qualitatively similar results for the effects of position and cholesterol content on the observed order parameters. Presumably, this reflects the greater ease with which this region of lower inherent order can adapt to a steric perturbation. However, measurements of order

parameters are difficult for these probes, especially in dispersions or biological membranes, due to the lower degree of ordering at these positions.

The 5-doxyl stearate probe gives spectra characteristic of a high degree of anisotropy and the magnitude of the order parameter is the most similar to that of the corresponding deuterium probe examined. However, it shows the greatest discrepancy with respect to the more accurate deuterium probes for the ordering effect of cholesterol. Thus, it may be the least attractive spin probe for studies examining the effects of external agents.

The type of study described above can be extended by comparing the effects observed by ESR and ^{2}H-NMR probes under the influence of different membrane agents or changes in the environment of the membranes (temperature, pH, etc.). This has been done recently in the membranes of *A. laidlawii*, where several discrepancies between the two methods were noted [23].

Finally, the comparison of quadrupole splittings obtained from ^{2}H-NMR of specifically deuterated probes, with and without a doxyl moiety, provides an unequivocal demonstration of the large perturbation produced by the spin label and the inaccurate response of spin labels to cholesterol addition.

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