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Orientation and vertical fluctuations of spin-labeled analogues of cholesterol and androstanol in phospholipid bilayers

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We have used ESR and NMR linewidth broadening by spin-labels to determine the overall orientation of spin-labeled analogues of cholesterol and androstanol in egg lecithin bilayers. While the cholesterol analogues were found to have a single orientation in each monolayer, with the acyl chain pointing towards the center of the bilayer, the androstanol analogue appeared, at least in sonicated vesicles, to experience two opposite orientations in the same monolayer, very likely with a rapid reorientation. The possibility of rapid vertical fluctuations of the sterol molecules within the phospholipid bilayer is also discussed.

Spin-labeled analogues of biologically significant steroids are often used to determine the physical properties and location of steroids in lipid bilayers [1–9]. Spin-labeled steroids have also been used to investigate steroid–protein interactions in membranes. For a review, the reader is referred to Ref. 10. Rotational correlation times and wobbling amplitudes have been inferred from the ESR spectral lineshapes of paramagnetic steroids diluted in artificial or natural membranes [7–9]. It is generally assumed a priori that these molecules are intercalated between lipid acyl chains with their long axis perpendicular to the membrane surface and that they experience a single molecular orientation around which small orientational fluctuations are observed. This is a reasonable assumption for naturally occurring cholesterol since the hydroxyl group is the only polar moiety and is likely to anchor these molecules in a single direction. On the other hand, the presence of a nitroxide group may cause a complete reorientation which has to be tested in order to assess the relevance of the spin-labeled analogues. In addition androstanol and its spin-labeled derivative are shorter molecules which are likely to experience large amplitude reorientations within a lipid bilayer as well as vertical fluctuations.

Previous investigators, by assaying the accessibility of the nitroxide probes to ascorbate, have tentatively attributed the orientation of some of these molecules [4,6]. However, the ascorbate technique, while giving valuable information in the case of very slow motions (minute or hour time scale [11]), is relatively ambiguous in the case of rapid molecular reorientation.

In the present study, we used ESR and NMR lineshape analysis to assess the average orientation of spin-labeled analogues of cholesterol and

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androstanol in lipid model systems. The probability of rapid reorientation of these molecules was also investigated. Sonicated vesicles and hand shaken multilayers were used for the ESR experiment, while high resolution ^{1}H-NMR was carried out with sonicated vesicles only.

The molecules utilized are schematically represented in Fig. 1. The fatty acid derivative (I) was synthesized either as a ^{15}N spin label or as a ^{14}N spin label; all other spin labels were ^{14}N. In Fig. 1 the steroid molecules are represented with an arbitrary orientation in a phospholipid monolayer. The ^{15}N derivative of (I) was synthesized according to Ref. 12; the ^{14}N derivative of the same molecule was synthesized according to Ref. 13; II was synthesized according to Refs. 14 and 4; III was synthesized by the procedure of Keana et al. [14] by condensation of 25-oxacholesterol (Sterealoids Company) with amino-2-methyl-2-propanol, followed by oxydation with metachloroperbenzoic acid; IV and V were purchased from Aldrich Company.

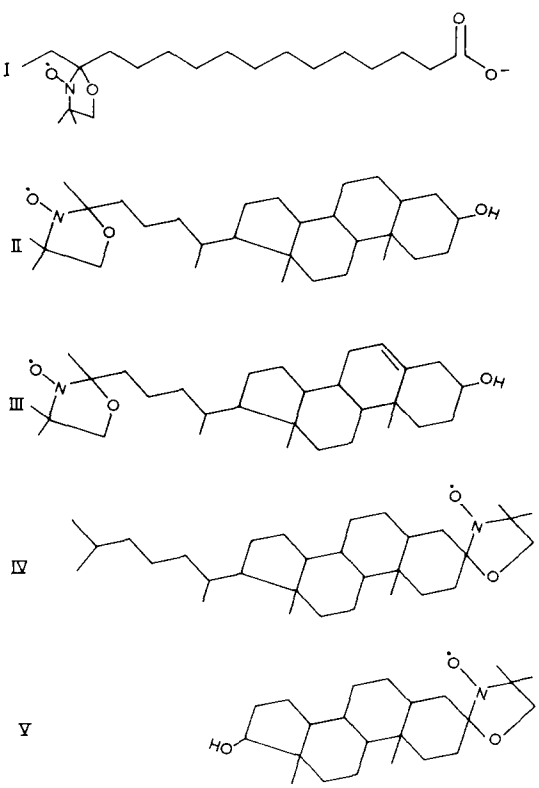

Fig. 1. Spin labels used in this study, with an hypothetical diagrammatic representation of the relative way in which they intercalate into a single phospholipid monolayer.

For the ESR experiments, vesicles containing the spin labels (one molecule of spin label for one hundred phospholipids unless otherwise mentioned) were made with egg yolk phosphatidylcholine purified according to Singleton et al. [15], and resuspended by vortex agitation in Hepes buffer (100 mM Hepes (pH 8), carefully degassed). Sonicated vesicles were obtained by 15 min sonication of resuspended liposomes, at 0°C under nitrogen (Ultra Son Annemasse, large tip), followed by 1 h centrifugation at $60\,000 \times g$ to remove the titanium particles and multilammellar vesicles. For the NMR experiments, the phospholipids containing the spin labels were lyophilized twice from ^2H_2O containing 0.1 mM EDTA (p^2H adjusted to 7.0 with NaO^2H) at a concentration of 3 mg/ml. This suspension was introduced into NMR tubes, degassed and sealed. Small unilamellar vesicles were formed by 1 h bath sonication (Branson sonicator).

^{15}N–^{14}N spin–spin interactions. Heisenberg spin exchange can be used to measure collision frequencies between spin labels. When the local concentration of nitroxide is sufficient electron–electron spin interactions create line broadening. Mixing spin levels with different nitrogen nuclear spins (hetero-isotopic mixing) allows one to estimate the collision frequencies between unlike molecules. For example, if a ^{15}N-nitroxide is linked to a fatty acid, at the 16th carbon position, one can evaluate how deep a ^{14}N-nitroxide linked to a different compound penetrates within the bilayer by studying the ^{15}N-nitroxide linewidth as a function of ^{14}N-nitroxide concentration [16]. Alternatively the ELDOR technique employed by Hyde and collaborators allows the measurement of spin exchange frequencies between unlike spins [17]. We have presently applied the former technique (i.e. line broadening) to measure the penetration of the probes associated with the various steroids analogues. The broadening is measured from the peak height of the low-field ^{15}N line, according to the relation:

$$\Delta(\mathrm{LW}) = \mathrm{LW}_0\left(\sqrt{H_0/H} - 1\right)$$

where LW_0 is the intrinsic linewidth of the low-field ^{15}N-nitroxide peak, H_0 its height in the

absence of ^{14}N-nitroxide and H its height in the presence of ^{14}N-nitroxide.

Experiments were carried out at a relatively high temperature (45°C) in order to minimize the long distance dipole–dipole interaction. As a result, the broadening mechanism is dominated by Heisenberg spin exchange, a process which requires close contact between interacting spins (see the discussion in Ref. 16). Fig. 2 shows typical ESR spectra obtained at a fixed low concentration of ^{15}N-labeled fatty acid I and variable amounts of ^{14}N spin-label IV. Fig. 3 shows the results of a series of experiments carried out by mixing the various spin labels in non-sonicated liposomes. Fig. 3A was obtained with liposomes without cholesterol. Very similar results were obtained with the same sonicated mixtures. Fig. 3B corresponds to non-sonicated egg phosphatidylcholine/cholesterol (10 : 8, molar ratio). The broadening of ^{15}N-(I) by ^{14}N-(I) serves as a reference: by comparison its appears that the probes on II and III have frequent encounters with a probe at the 16th position of a long fatty acid chain (I), while the probe on IV has only rare collisions with the same fatty acid probe. These results indicate that the orientations of molecules II, III and IV are correctly selected in Fig. 1. Finally the probe on molecule V has rather frequent collisions with the probe on molecule I particularly in the samples devoided of cholesterol (Fig. 3A). It suggests that molecule V should be drawn with nitroxide moiety pointing towards the center of the bilayer, unlike sketched in Fig. 1. The same conclusion was put forward by Seelig [1] from the isotropic hyperfine splitting a_N determined in oriented multilayers and by Schreier-Mucillo et al. [4], from ascorbate experiments carried out also on oriented multilayers. However, NMR experiments will show that the situation might be more complicated.

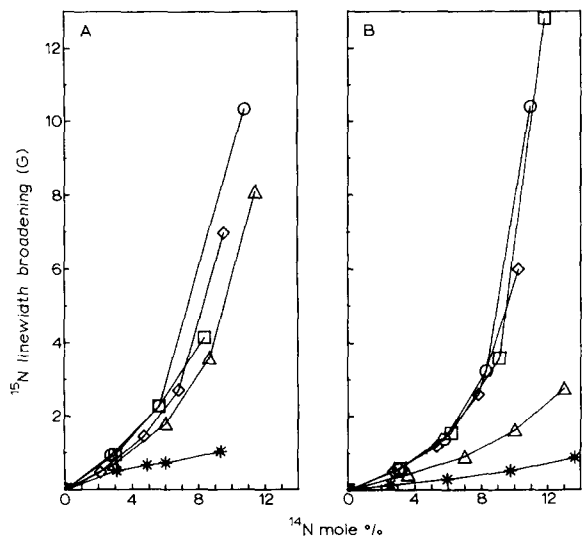

Fig. 3. Broadening of the linewidths of a ^{15}N-nitroxide spin label located at the 16th carbon position of a fatty acid chain (molecule I) due to increasing concentrations of fatty acid or steroid ^{14}N spin labels. Temperature 45°C. The low-field ^{15}N-line broadening in gauss (G) is determined from peak heights as explained in the text for: ◇, I; ○, II; □, III: *, IV; △, V. A was obtained without cholesterol; B with phosphatidylcholine/cholesterol (10 : 8, molar ratio).

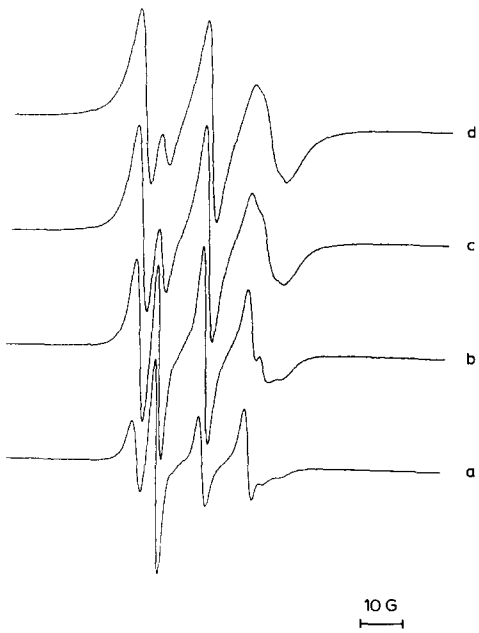

Fig. 2. ESR spectra of various mixtures of ^{15}N-I and ^{14}N-II in sonicated egg phosphatidylcholine. No cholesterol present. Temperature 45°C. The ratio of spin-labeled molecules per phospholipid is 1% for the ^{15}N spin-label I, and for spin-label II, respectively (a): 0.7%, (b): 1.7%, (c): 4.3% and (d): 6%.

High resolution ^{1}H-NMR experiments. The addition of a low concentration of spin-labeled phospholipids into egg phosphatidylcholine sonicated vesicles enables the selective broadening of the NMR lines associated with the protons in close contact with a nitroxide [18]. Similarly spin-labeled steroids, diluted in egg yolk phosphatidylcholine sonicated vesicles, produce selective broadenings.

The NMR spectra were recorded at different temperatures on a Bruker WM 500 (500 MHz) spectrometer. The sensitivity to the paramagnetic probes was found optimum around 25°C. At higher temperatures, the lines were narrow but the broadening not very specific. At 4°C, on the other hand, the NMR lines were broad and overlaped even in the absence of paramagnetic molecules. Fig. 4 presents the broadening of four specific lines corresponding, respectively, to the headgroup region (α, γ), to the glycerol backbone (c) and to the methyl terminal (7) of a phosphatidylcholine molecule. This figure reveals that the androstanol analogue (V) has a comparable interaction with the headgroup region and the methyl terminal. On the contrary the probes on the cholesterol analogues interact either with the headgroup region

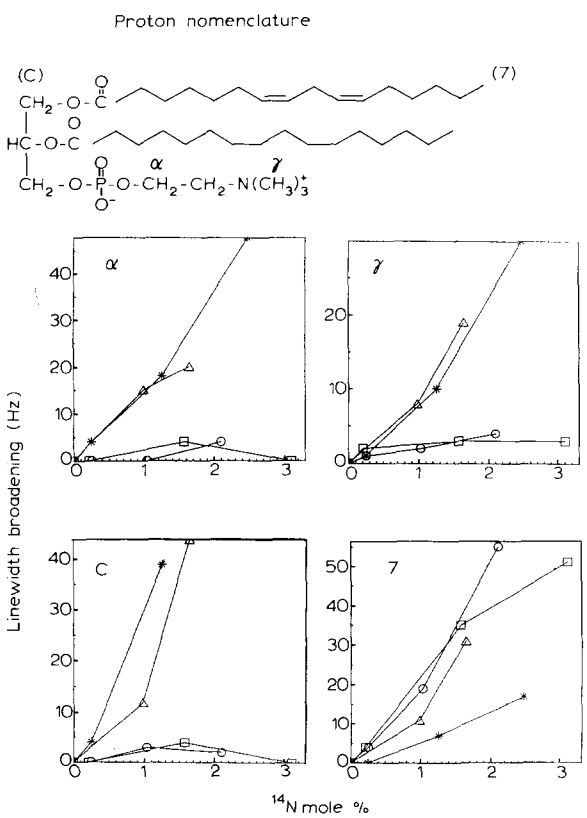

Fig. 4. Broadening in Hz of specific ^{1}H-NMR lines due to increasing concentration of spin labels in phosphatidylcholine sonicated vesicles. ○, II; □, III; *, IV; △, V. The proton nomenclature is that of Ref. 18, α and γ correspond to the headgroup; c to the glycerol backbone; 7 to the methyl terminal of the acyl chains.

(IV) or with methyl terminal (II and III) but not with both. However, addition of a high concentration of IV has a small effect on the peak associated with the methyl terminal.

This study demonstrates the differences between the physical properties of cholesterol and androstanol derivatives in a lipid bilayer. The cholesterol analogues seem to be present with a single orientation in each monolayer. On the other hand, our data show that the androstanol molecule V is present in at least two opposite orientations within the same monolayer, alternatively it may undergo rapid reorientation within each monolayer. This conclusion follows from the NMR observation that the probe interacts with equal efficiency with the headgroup region and with the methyl terminal of the acyl chains. However, the latter experiment has to be performed with sonicated vesicles. Consequently an influence of the surface curvature can be invoked to explain the difference between our conclusion and that of Seelig [1] and Schreier-Mucillo et al. [4]. Indeed it has been shown that the physical properties of the lipids in small unilamellar vesicles are slightly different from those of the lipids in large size vesicles, in particular under favorable circumstances, the two monolayers of sonicated vesicles can be resolved by ESR [11] or by NMR [19].

What would be the time scale of the flip-flop of V within a lipid monolayer? The ^{1}H-NMR experiments show that V broadens the NMR lines associated with the headgroup region or the hydrophobic region with the same efficiency than probes present in a single orientation. This is only possible if V flips very rapidely from one orientation to the other. Typically the flipping rate must be comparable to the lateral diffusion rate, i.e. approx. $10^7\ s^{-1}$. A rigorous quantitative treatment of the broadening resulting from the two-dimensional diffusion of an electron spin is a very difficult problem [20]. Here, we only attempt to give a crude figure. Note that the rapid rotation within a single monolayer does not give any direct information on the rate of transverse diffusion from one monolayer to the other. However, it seems quite likely that androstanol diffuses between both layer at a rate much faster than reported for cholesterol flip-flop in sonicated lipid

vesicles (i.e., several hours) [21,22]. In fact an alternative explanation for the androstanol derivative ubiquitous behavior could be large scale vertical fluctuations. This would imply that the molecule spans rapidly the whole bilayer, having equally well both orientations. The behavior of molecules II and III, which during the course of all this investigation gave identical results, is quite in contrast to that of V but also of IV. Indeed molecule IV has a moderate but measurable effect on the peaks associated with the methyl terminals. This can be due to a wobbling of the extremity of the fatty acid chain bearing the ^{15}N-nitroxide and/or to vertical fluctuations of the cholestane spin label.

In conclusion this investigation shows that specific linebroadening produced by spin-labels can be used to provide information on the orientation and mobility of spin-labeled molecules in lipid bilayers. The draw-back of the ESR technique is the requirement of ^{15}N-nitroxide synthesis, on the other hand ESR can be carried out with non sonicated vesicles. The use of small radius vesicles which is a requisite for the NMR experiments, is indeed a limitation.

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