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ESR STUDIES ON THE ORIENTATION OF CHOLESTERYL ESTER IN PHOSPHATIDYLCHOLINE MULTILAYERS

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Summary

The alignment of cholesteryl esters in multilayer phosphatidylcholine membranes was investigated using two spin-labelled cholesteryl esters: 10 : 3 ester (I) and 1 : 14 ester (II). The nitroxide label of I is aligned in the membrane with a very large angle of tilt (47° ± 1.5°) with respect to the normal to the membrane surface; II does not show such a tilt. I gives spectra corresponding to immobilized label while II gives nearly isotropic spectra. Ascorbate treatment of the multilayers shows that the labels in I and II are not present at the phosphatidylcholine-water interphase.

The data supports a 'horseshoe' configuration for the cholesteryl ester in the bilayer, with both the fatty acid chain and the cholesteryl moiety extending deep into the hydrophobic region of the membrane and with the ester linkage near the surface.

Introduction

Cholesteryl esters can be incorporated into phosphatidylcholine model membranes in small amounts. Recently, using ^{14}C-labelled cholesteryl palmitate, it has been demonstrated that this ester can be incorporated into egg phosphatidylcholine unilamellar liposomes (vesicles) in concentrations as high as 5 mol % [1]. The cholesteryl palmitate-containing vesicles were shown to be more permeable to Pr^{3+} and to ethylenediaminetetraacetate than pure egg phosphatidylcholine vesicles [2]. Janiak et al. [3] presented some evidence that, depending on the degree of hydration, 2—5 mol % cholesteryl linolenate was intercalated

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Abbreviations: CDI: 1,1′-carbonyl-diimidazole; 3-doxylcholestane, 3-spiro-[2′-*N*-oxyl-4′,4′-dimethyloxazolidine]-5α-cholestane; 10 : 3 acid, 5-doxylpalmitic acid; 1 : 14 acid, 16-doxylstearic acid; 10 : 3 ester, cholesteryl ester of 10 : 3 acid; 1 : 14 ester, cholesteryl ester of 1 : 14 acid.

into the lamellar crystalline structure formed by hydrated phosphatidylcholine. The authors established the lamellar structure of the membrane using X-ray diffraction and polarizing light microscopy methods [3], and proposed several possible structures and orientations for cholesteryl esters in the unilamellar liquid crystalline phase of the phosphatidylcholine-water system. Below ~13% water the ester is proposed to reside entirely within the hydrocarbon region; above 13% water the ester is proposed to adopt a 'horseshoe' conformation, the carbonyl group of the ester present at the phosphatidylcholine-water interphase and the cholesterol moiety as well as the aliphatic chain of the acid extending into the membrane [3].

ESR studies using multilayers prepared from synthetic lipids have proven useful in determining the structural components of the various membranes [4—14]. For instance, the orientation of the aliphatic acids in membranes has been studied using nitroxide-labelled fatty acids like III and IV (Fig. 1) [7—10]. Similarly, 3-doxylcholestane (V) has been studied to establish the alignment of cholesterol in the phospholipid multilayer membranes [11—14]. Such past studies have yielded information on the orientation of the labels in the liquid crystals, and the degree of freedom available to the label. The present work focuses on the orientation and mobility of cholesteryl esters incorporated into egg phosphatidylcholine multilayers. The two esters studied, 10 : 3 ester (I) and 1 : 14 ester (II), are derivatives of III and IV, respectively. The present study also includes examination of the various ESR parameters of I and II incorporated into oriented phosphatidylcholine multilayers, and into random phosphatidylcholine multilayers, as well as the accessibility of the two probes to L-ascorbate diffusing from the aqueous phase.

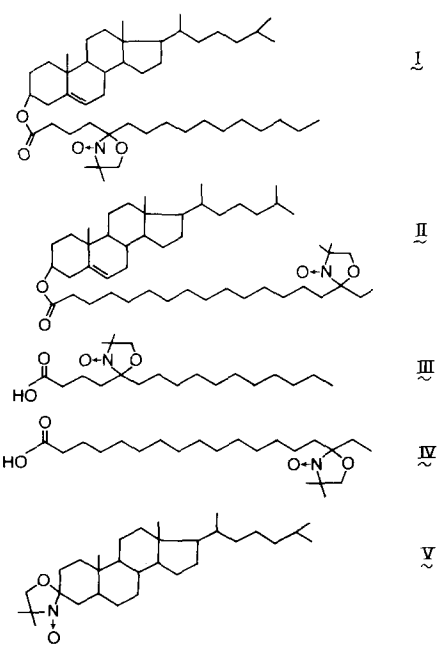

Fig. 1.

Experimental Methods

Materials. Cholesteryl palmitate, dipalmitoyl phosphatidylcholine and L-ascorbic acid-sodium salt were purchased from Sigma Chemical Co. Cholesterol was purchased from Fischer Chemical Co. The spin label, 1 : 14 acid, was supplied by Syva, Palo Alto, Calif. The spin probes, 10 : 3 acid and 3-doxylcholestane, were generous gifts from Dr. A.C. Oehlschlager, Department of Chemistry, Simon Fraser University, Burnaby, B.C., Canada. The cholesteryl esters (I and II) were synthesized as described previously [15]. Egg phosphatidylcholine was purified as described previously [2] and gas chromatography indicated a fatty acid composition of 42.7% palmitic acid, 16.9% stearic acid, 27.7% oleic acid, 11% linoleic acid and 1.6% palmitoleic acid.

Preparation and ESR spectroscopy of oriented multilayers. The method of Schreier-Muccillo et al. [16] was used for preparation of oriented lipid multilayers in a flat-walled quartz aqueous ESR cell. The spin label concentration was 1 mol % of the total lipids constituting the multilayers. The multilayers were dried under vacuum for at least 2 h and then hydrated by adding a buffer containing 0.05 M Tris-HCl, pH 7.4, and draining off the buffer. The ESR spectra were determined on a Varian EPR-4 spectrometer. The spectra were recorded at several field directions, the field direction (θ) being the angle between the magnetic field and the normal to the flat section of the ESR cell. The g-value was measured as the point where the central line of the spectrum crosses the baseline and was uncalibrated. The T value was measured as the separation between the low-field and the central line. The values of $g_\parallel$ and $T_\parallel$ were obtained from the spectra recorded at $\theta = 0°$, and $g_\perp$ and $T_\perp$ from those recorded at $\theta = 90°$. The parameter $2T'_{zz}$ was also obtained from the spectra recorded at several values of θ. $2T'_{zz}$ corresponds to the separation between the first maxima and the last minima of the three line ESR spectrum in the dispersion mode.

Preparation and ESR spectroscopy of random multilayers. Random multilayers were prepared by scraping hydrated planar oriented multilayers onto the edge of a coverglass. A chloroform solution (20—30 μl) containing 10 μmol egg phosphatidylcholine and 0.1 μmol of the specified spin label was sandwiched between two microscope coverglass pieces (50 × 4 mm) and dried under vacuum. The samples were hydrated in 0.05 M Tris-HCl, pH 7.4, for at least 15 min. The coverglass pieces were placed in a 5 mm NMR tube with a wet glass-wool plug in the top of the tube. These multilayers gave spectra identical to those obtained using the flat-walled ESR quartz cell. The multilayers were then scraped onto the edge of a 15 × 4 mm coverglass and immediately placed in the NMR tube as before. Spectra of the scrapings were recorded and the values of T_{max}, the separation of low-field maximum and high-field minimum, and T_{min}, the separation of the low-field minimum and high-field maximum, were measured. From the T_{max} and T_{min}, the value of $\overline{T}_\perp$ was determined according to Eqn. 1 [6]:

$$\overline{T}_\perp = T_{min} + 1.4(1 - (T_{max} - T_{min})/(T_{zz} - \tfrac{1}{2}(T_{xx} + T_{yy}))) \quad (1)$$

The values of the principal components of the T-tensor were assumed to be $T_{xx} = T_{yy} = 6$ G, and $T_{zz} = 32$ G. These are essentially the crystal parameters of the

acids from which the labels have been derived [5]. The value of $\overline{T}_{\parallel}$ was assumed to be the same as that of T_{max} [6].

Permeability to L-ascorbate. Kornberg and McConnell [17] treated vesicles containing phosphatidylcholine, spin-labelled on the headgroup, with L-ascorbate at 0°C. The resonance due to the spin label on the outside of the vesicles decayed immediately, while that portion due to nitroxide on the inside of the vesicles had a lifetime of several hours. L-Ascorbate-induced decay of ESR signals has also been used as a spectroscopic ruler by previous workers to measure the distance between the aqueous region of a bilayer and a doxyl group of a spin probe [18]. In the present study, the L-ascorbate solution contained 0.15 M NaCl and was titrated to pH 6.83 ± 0.03 with NaOH or HCl. The L-ascorbate concentration, where not otherwise specified, was 10^{-2} M. The ascorbate solution was added to the aqueous tube containing vacuum dried multilayers. The tube was then placed in the spectrometer with the multilayer surface perpendicular to the magnetic field, and the low-field component of the ESR spectrum recorded. Subsequent spectra were then recorded at specified intervals of time (typically 1.25 or 2.25) min at identical spectrometer settings. The spectral amplitude decreased with time. However, the decay, measured as peak height vs. time, was not exponential. Analysis of finite differences [19] revealed that a quadratic fit between ln (peak height) and time was necessary and sufficient to describe the data. The relationship ln (peak height) = $a - bt + ct^2$ was fitted to the data by the method of least squares [20]. Here t designates time, and a, b and c are the parameters which describe the relationship. For a given data set, the values of a, b and c were thus obtained. The value of the first half-life ($^1t_{1/2}$) was then obtained from these parameters by solving the equation:

$$\ln 2 - b(^1t_{1/2}) + c(^1t_{1/2})^2 = 0 \qquad (2)$$

The parameter, $^1t_{1/2}$, corresponds to the time required for the amplitude to decrease to 50% of its extrapolated zero time value. A quadratic fit was thus used to obtain a value of the first half-life of the spin probe.

Results

ESR spectra of cholesteryl esters in phosphatidylcholine multilayers

Fig. 2 depicts the ESR spectra of the spin-labelled cholesteryl esters, incorporated into hydrated egg phosphatidylcholine multilayers, at 23°C. The spectra of the two esters (I and II) differ significantly in line-shape. The spectra of I show broad low-field lines and very broad high-field lines (Fig. 2A). This is typical of spectra obtained with the labels immobilized on the ESR time scale ($\tau_c \leqslant 10^{-8}$ s) [4]. From the $\theta = 0°$ and 90° spectra the parameters listed in Table I were computed. The spectra at $0° < \theta < 90°$, however, do not permit the evaluation of the hyperfine splitting because the low-field line is distorted in these spectra. The spectra using the 1 : 14 ester (II) incorporated into the oriented multilayers, on the other hand, gave narrow three line spectra at all angles (Fig. 2B), indicative of spectra one obtains when the motion of the label is rapid and very nearly isotropic [4—6]. Thus, the nitroxide group of II is placed in a very fluid environment. From the values of $T_{\parallel}$ and $T_{\perp}$ given

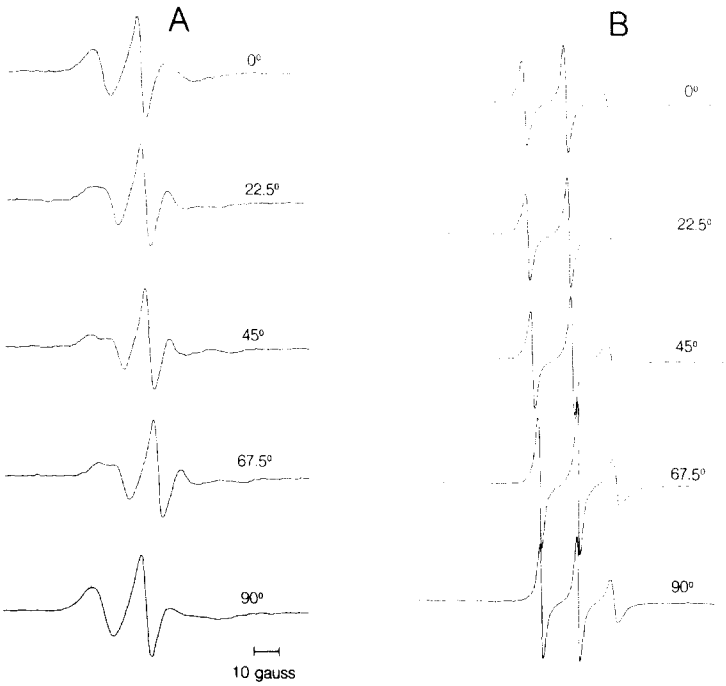

Fig. 2. ESR spectra of cholesteryl esters incorporated in egg phosphatidylcholine multilayers. A, 10 : 3 ester (I) and B, 1 : 14 ester (II). The numbers along the spectra indicated the values of θ at which these spectra were recorded.

for II in Table I, one can compute an order parameter (S) according to the following equation:

$$S = \frac{T_{\parallel} - T_{\perp}}{T_{zz} - \frac{1}{2}(T_{xx} + T_{yy})} \cdot \frac{T_{xx} + T_{yy} + T_{zz}}{T_{\parallel} + 2T_{\perp}} \tag{3}$$

This equation is very similar to the one used for the corresponding acids [11] and assumes a very rapid rotation in the xy-plane, thereby averaging the T_{xx} and T_{yy} components of the T tensor. The value of the order parameter so obtained is 0.029 ± 0.004. This small value of the order parameter is consistent with unrestricted motion of the spin label near the terminal CH_3 of the fatty acid chain. Because the nature of the spectra for ester I (Fig. 2A) indicate

TABLE I

SPECTRAL PARAMETERS OF THE CHOLESTERYL ESTERS IN ORIENTED EGG PHOSPHATIDYLCHOLINE MULTILAYERS AT 23°C

Spin probe	$g_{\parallel}$ *	$g_{\perp}$	$T_{\parallel}$ **	$T_{\perp}$
10 : 3 ester (I)	2.0092	2.0086	14.4	15.7
1 : 14 ester (II)	2.0086	2.0085	15.2	14.2

* The error limit for the g-values is ±0.0025.

** The values of T in Gauss, are averages of 10 experiments for I and 4 for II.

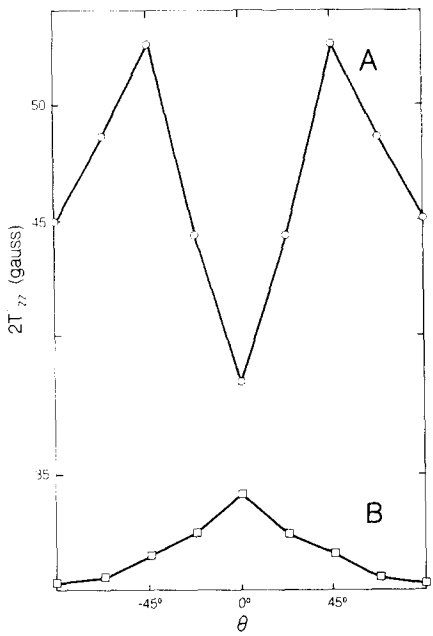

Fig. 3. Plots of $2T'_{zz}$ vs. θ. A, 10 : 3 ester (I) and B, 1 : 14 ester (II).

motions at the slow limit of τ_c application of Eqn. 3 to ester I system is precluded.

The spin probes (III—V) were also studied. The order parameters, S, observed for these probes in EYL multilayers were: III, 0.535 ± 0.04; IV, 0.055 ± 0.004; and V 0.68 ± 0.03.

Angle of tilt

Birrell and Griffith [21] have shown that from a plot of $2T'_{zz}$ vs. θ one can determine a tilt angle (δ), the angle between the z-axis of the nitroxide label and the normal to the membrane surface. Plots of $2T'_{zz}$ vs. θ for the two esters (I and II) intercalated into hydrated egg phosphatidylcholine oriented multilayers are presented in Fig. 3. Compound II shows only a small variation in $2T'_{zz}$ and the maxima in this instance occurs at $\theta = 0°$ (Fig. 3B). The nitroxide label of the 1 : 14 ester is thus not tilted with respect to the membrane normal. The 10 : 3 ester (I), on the other hand, shows minimum values of $2T'_{zz}$ at $\theta = 0°$ and local minima at $\theta = \pm 90°$. There are two maxima and these occur at $\theta = \pm 45°$ (Fig. 3A). The presence of two maxima symmetrically spaced about $\theta = 0°$ indicate that the z-axis of the spin label (I) is tilted at the angle equal to one-half the separation distance ($\sim 45°$) with respect to the normal to the membrane surface.

ESR parameters in random multilayers

Hydrated egg phosphatidylcholine multilayers were scraped onto the edge of a coverglass to obtain a sample of randomly oriented, hydrated multilayers, as described in the Experimental Methods. Scrapings containing 1 : 14 ester (II) gave sharp three line spectra while those containing 10 : 3 ester (I) gave powder

TABLE II

SPECTRAL PARAMETERS OF CHOLESTERYL ESTERS IN RANDOMLY ORIENTED EGG PHOSPHATIDYLCHOLINE MULTILAYERS AT 23°C (GAUSS)

Spin label	$2T_{max}$	$2T_{min}$	$\overline{T}_{\parallel}$	$\overline{T}_{\perp}$	$\overline{T}_{\parallel} + \overline{T}_{\perp}$
10 : 3 ester (I)	52.85	17.7	26.42	9.3	45.05 ± 0.2
1 : 14 ester (II)	30.7	25.5	15.35	14.01	43.37 ± 0.2

TABLE III

FIRST HALF-LIVES ($^1t_{1/2}$) OF REACTION OF SPIN PROBES IN EGG PHOSPHATIDYLCHOLINE MULTILAYERS TO ASCORBATE AT 23°C

Spin probe	$^1t_{1/2}$ (min) *
10 : 3 ester (I)	14.0 ± 4.0
1 : 14 ester (II)	16.2 ± 4.0
10 : 3 acid (III)	16.5 ± 4.0
1 : 14 acid (IV)	16.5 ± 4.0
Cholestane (V)	4.1 ± 1.7

* L-Ascorbate concentration = 10^{-2} M.

spectra. The value of $2T_{max}$, $2T_{min}$, $\overline{T}_{\parallel}$, $\overline{T}_{\perp}$ and $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$ are given in Table II. Compound II shows a very small difference between $\overline{T}_{\parallel}$ and $\overline{T}_{\perp}$, thereby indicating that there is little order in the system, i.e. the nitroxide group in II is mobile on the ESR time scale. Spectra of randomly oriented multilayers containing I, however, show a value of $\overline{T}_{\parallel}$ almost three time as large as the value of $\overline{T}_{\perp}$, thus indicating the anisotropy in the hyperfine tensor is not lost in the nitroxide label of the 10 : 3 ester incorporated in these randomly oriented hydrated multilayer samples. This further confirms that label I is present in a very rigid environment.

Permeability to L-ascorbate

L-Ascorbate-induced decay of various spin labels incorporated into egg phosphatidylcholine multilayers was studied as described in the Experimental Methods. The first half-lives ($^1t_{1/2}$) of the various probes are given in Table III. The two cholesteryl esters (I and II) and the corresponding acids (III and IV) decayed with $^1t_{1/2}$ of 15 ± 5 min. The spin-labelled cholestane (V), however, had a $^1t_{1/2}$ of only 4.1 ± 1.7 min. Thus, the accessibility of the two esters (I and II), in egg phosphatidylcholine multilayers, to L-ascorbate diffusing from the aqueous phase was similar to that of the corresponding acids (III and IV), and differed significantly from that of the spin-labelled cholestane (V) under the same conditions.

Discussion

The ESR spectra of the 1 : 14 ester (II) incorporated into hydrated multilayers, whether planar or randomly oriented, are nearly isotropic, and the lines in these spectra are very narrow. Thus, label II undergoes rapid fluctuations causing an averaging of the anisotropic g and T tensors.

The 10 : 3 ester (I) gave ESR spectra with very broad high field lines indicating that the label must be immobilized on the ESR time scale. The large difference between $\overline{T}_{\parallel}$ and $\overline{T}_{\perp}$ in the spectra of the randomly oriented multilayers (Table II), confirms this finding. This difference is comparable to the difference between the corresponding values obtained using 10 : 3 acid (III) which is known to be severely restricted [4—10]. However, unlike the corresponding acid, the hyperfine splittings in oriented multilayers are essentially equal (Table I). This discrepancy is explained by observing the behavior of the maximum splitting $2T'_{zz}$ with θ, the angle between the nitroxide z-axis and the field, B_0. Birrell and Griffith [21] have shown that, for $\theta = 0$, the field direction is along the normal to the bilayer plane. The separation of the two maxima, found for ester I in egg phosphatidylcholine (Fig. 3A) yields twice the angle of tilt the nitroxide moiety makes with the normal to the bilayer surface. The separation of the two maxima (Fig. 3A) yield an angle of tilt, $\delta \sim 45°$. The following relationships between $\overline{T}_{\parallel}$, $\overline{T}_{\perp}$, $T_{\parallel}$, and $T_{\perp}$ can be employed to determine the value of the angle of tilt (δ) more accurately:

$$T_{\parallel}^2 = \overline{T}_{\parallel}^2 \cos^2\delta + \overline{T}_{\perp}^2 \sin^2\delta \tag{4}$$

$$T_{\perp}^2 = \overline{T}_{\parallel}^2 \sin^2\delta + \overline{T}_{\perp}^2 \cos^2\delta \tag{5}$$

Using the values of $T_{\parallel}$ and $T_{\perp}$ from Table I and those of $\overline{T}_{\parallel}$ and $\overline{T}_{\perp}$ from Table II, the above relationships yield a value of $\delta = 47° \pm 1.5°$. Thus, the z-axis of the nitroxide label of I is tilted at an angle of 47° with respect to the normal to the membrane surface, and the slow motion of the label does not permit averaging of this anisotropy in the hyperfine tensor.

Permeability studies indicate that the reducing agent, L-ascorbate, reacts much more slowly with esters I and II than with cholestane spin-label (V). At 23°C, the half-lives for I and II were the same within experimental error. Since it has been shown that the label of V resides near the aqueous interphase, the permeation results place the labels of I and II in the aliphatic region of the membrane. Further experiments, using dipalmitoyl phosphatidylcholine multilayers, where the half-lives become several times longer due to closer packing of the phosphatidylcholine molecules, confirmed this finding. In a set of experiments using dipalmitoyl phosphatidylcholine multilayers the accessibility of the probes to L-ascorbate was determined to be in the order III > I > IV > II ($^1t_{1/2} = 17 \pm 4$, 42 ± 8, 50 ± 9, and 71 ± 12 min, respectively). These experiments suggest that the nitroxide group of I is closer to the membrane surface than that of II. Furthermore, the value of $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$, obtained using I in egg phosphatidylcholine multilayer scrapings (Table II), indicates that the label of I is present in a slightly more polar environment than that of II.

The value of $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$ has been shown to decrease with the decreasing polarity of the solvent [6,22]. When 10 : 3 ester (I) was dissolved in hexane a value of 42.6 ± 0.5 G for $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$ was obtained, and in a chloroform solution this value was 45.5 ± 0.5 G. Thus, label II, which shows the smaller value of $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$, must be located in a more hydrophobic environment in the membrane. The middle region of the membrane being the most non-polar as well as the most fluid part of the bilayer, would thus be the site for the nitroxide label of the 1 : 14 ester (II).

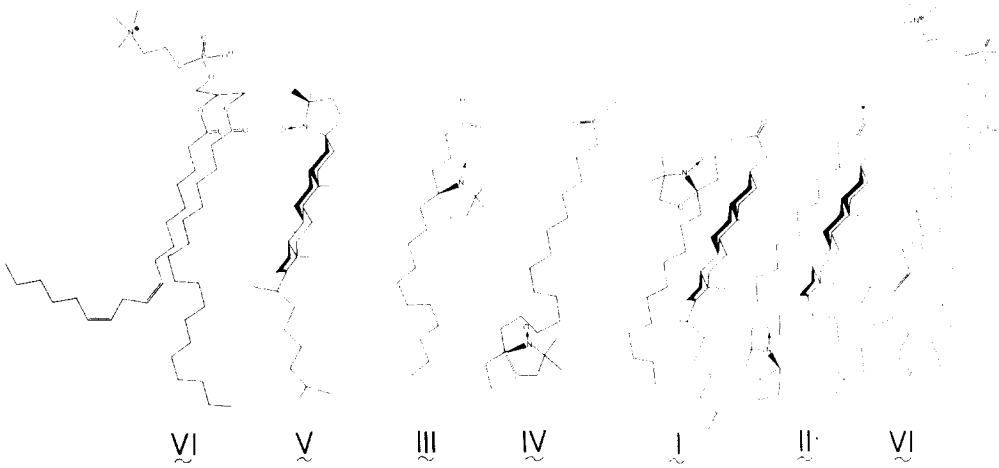

Fig. 4. Orientation of spin probes in multilayers. Structures I—V are shown in Fig. 1. Structures VI represent egg phosphatidylcholines containing saturated and unsaturated fatty acid residues.

Janiak et al. [3] proposed that in hydrated multilayers (>13% water content) cholesteryl linolenate molecules, intercalated between phosphatidylcholine molecules, are in 'horseshoe' type of configurations, i.e. the carbonyl group of the ester is present near the head-group of the phosphatidylcholine molecules, and the cholesterol moiety and the hydrophobic tail of the ester extend into the membrane. This model is consistent with the data presented here. The model predicts that the nitroxide group attached to carbon 16 of the aliphatic chain would be buried in the middle of the membrane. The label would thus be expected to: (a) give narrow three line spectra with very small angular dependence of the T tensor; (b) give a low value for $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$; and (c) not be easily accessible to L-ascorbate diffusing from the aqueous phase. The 10 : 3 ester (I) data are also consistent with the 'horseshoe' conformation. If the carbonyl group is present near the aqueous phase, the aliphatic chain near C1 would be in a rigid environment and the large tilt angle (δ) for the nitroxide at position 5 is explained by the nature of the 'horseshoe' since this would place C5 at the highly curved portion of the molecule. The broad lines in the ESR spectra and $\delta = 47°$ confirm this prediction. The larger value of $(\overline{T}_{\parallel} + 2\overline{T}_{\perp})$ observed for II compared to I indicates that I is present nearer the aqueous interphase.

Fig. 4 illustrates the proposed conformations of esters I and II in an egg phosphatidylcholine bilayer (VI = phosphatidylcholine). Also included are compounds III—V, based on their known structures [17—22].

The model of Janiak et al. [3] further predicts that the spin label of V would be more accessible than that of I, to L-ascorbate diffusing into the membrane from the aqueous phase as is seen. Thus, the 'horseshoe' model for the orientation of cholesteryl esters in hydrated multilayers phosphatidylcholine membranes is well in accord with the polarizing light microscopy, calorimetry and X-ray diffractions data [3] as well as the ESR and the permeability data presented in this paper.

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