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The interaction of cholesterol and cholest-4-en-3-one with dipalmitoylphosphatidylcholine. Comparison based on the use of three fluorophores

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This study compares cholesterol–phospholipid and cholest-4-en-3-one–phospholipid interactions by their effect on thermotropic behavior of dipalmitoylphosphatidylcholine bilayers. This was approached by determining the temperature-dependent steady-state fluorescence anisotropy of three fluorophores; diphenylhexatriene (DPH), hydroxycoumarin (HC) and *trans*-parinaric acid (TPA). The fluorophores monitor different lateral and vertical locations of the lipid bilayers; DPH and HC average laterally the properties of the hydrophobic and headgroup regions of the bilayer, respectively, while TPA distribution is determined by the lateral organization of the bilayer. The data show that the two steroids have similar qualitative but different quantitative effects. Both diminish the pretransition and behave as 'averagers', broadening the main gel to liquid crystalline phase transition through ordering of the acyl chains in the liquid crystalline state and disordering of them in the gel state. However, the mechanisms by which the two molecules operate are different. Cholesterol is more effective particularly on the hydrophobic region of the bilayer, and its effect is not linear with its mole fraction. A sharp increase of the steady-state fluorescence anisotropy occurs around 20 mol% cholesterol. The effect of cholestenone is proportional to its mole fraction. The difference between the effects of the two steroids is explained by the dissimilarity in their lateral distribution. Cholesterol forms cholesterol-rich domains. The size of the boundary regions which surround the cholesterol-rich domains changes drastically at about 20 mol% cholesterol. Cholestenone, on the other hand, is randomly distributed in the bilayer plane and therefore it does not cause the formation of such defined boundary regions. This study as well as reports by others suggests that the important structural differences between the two steroids are the molecular packing parameter and the presence of small polar group at the 3-β position of the steroid.

Introduction

Most biological membranes contain steroids (mainly sterols) which are essential for membrane and cellular functions (Ref. 1, and references listed therein). These are referred to as 'membrane-active steroids'. Cholesterol (cholest-5-en-3β-ol), is the main membrane active steroid of most eukariotic organisms [2,3], a good representative and the most studied member of this group [1,4].

The physical effects of cholesterol on lipid bilayers was studied using almost all physical techniques available for membrane research as reviewed elsewhere [1,4,5]. These studies indicate that cholesterol when present in a bilayer of phospholipid which undergoes gel to liquid crystalline phase transition reduces the number of phospholipid molecules undergoing the transition [6]. At a mole fraction of approx. 0.33, cholesterol causes a complete disappearance of the phase transition (refs. 1 and 4, and references listed therein). Following the degree of order of the phospholipid chains using ^{2}H-NMR, ESR or fluorescence depolarization techniques indicates that cholesterol acts as an 'averager' by reducing the order of the phospholipid molecules in the lipid bilayer in

Abbreviations: CHOL, cholest-5-en-3β-ol (cholesterol); CHON, cholest-4-en-3-one (cholestenone); DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; ESR, electron spin resonance; HC, 4-heptadecyl-7-hydroxycoumarin; MLV, multilamellar large vesicles; NMR, nuclear magnetic resonance; SAE, specific averaging effect; THF, tetrahydrofuran; TPA, *trans*-9,11,13,15-parinaric acid; LD, liquid disordered phase; LO, liquid ordered phase; SO, solid ordered phase.

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their gel state by perturbing the hexagonal chain packing. In the liquid crystalline state cholesterol increases the bilayer order by limiting the probability of *trans-gauche* isomerization in the phospholipid acyl chains. The latter effect is related to the interaction of the rigid cholesterol ring with the acyl chains [1,4,7,8]. Recently, major efforts have been directed to characterize the lateral distribution of cholesterol in the bilayer plane and how it affects the bilayers lateral organization. Snyder and Freire [9] suggested a model for predicting the amount of distinct phases of pure phospholipid, of cholesterol phospholipids mixed domains and other interphases between them. They found a sudden lateral connectivity of the mixed domains at about 20 mol% cholesterol which causes an abrupt decrease in the total interphase area and therefore has major impact on the lateral diffusion of membrane lipids [10]. Presti et al. [11] tried to relate the detailed molecular mechanism for cholesterol phospholipid interaction with the lateral organization of the bilayer. It is also clear that the degree of acyl chain order as well as the lateral organization in lipid bilayer and biological membrane containing cholesterol are strongly influenced by the membrane phospholipid composition [1,4,12–14].

Despite the large amount of information and proposed models it is still not clear how structural features determine whether a steroid is membrane active or not. This important problem can be best approached by comparing steroids which clearly are classified as membrane active and inactive and assess their effect on lipid bilayers. The aim of this study is to compare the lateral organization and degree of order of the hydrocarbon and the head group regions of bilayers containing cholesterol and cholest-4-en-3-one.

It is well accepted that the cholesterol and cholest-4-en-3-one are good representatives of the membrane active and inactive steroids, respectively. The main chemical differences between these steroids are: (i) the β-OH group of cholesterol is replaced by a keto group. (ii) the double bond shifted from the B ring Δ^5 to the A ring at position Δ^4 of the steroid molecule. This results in larger minimal molecular area at the air/water interface of cholestenone (52 Å^2), than for cholesterol (32 Å^2) [34], possibly due to the β-OH at position 3 which enables better packing. The β-OH group has been suggested as a hydrogen donor and the phospholipid acyl carbonyl group [35–37] or the glycerol oxygen as acceptors [11].

This model was challenged by the finding of Lai et al. [38], who demonstrated that the presence of a small polar group at the β position rather than direct the appropriate alignment and the β-OH itself is essential to the cholesterol and DPPC interaction. The polar region of the cholestenone, which lacks the hydrogen-bonding capability is smaller than of the cholesterol, due to the differences between the keto and the hydroxyl groups at position 3 of the two steroids, respectively. The loss of stereo specificity in the cholestenone and the shift of the double bond from ring B (Δ^5) of the cholesterol to ring A (Δ^4) in the cholestenone causes a change in the conformation of these two rings. Recent data [39] indicate that the position of the double bond has major effect on the condensing effect of the steroids in fluid PC bilayers which also affects bilayer permeability. These studies indicate that Δ^5 is most effective in this respect [39].

The comparisons were done using steady-state fluorescence anisotropy of three well-characterized fluorophores. The main advantage of this method is its simplicity and its applicability to biological membranes.

Materials and Methods

Materials

Lipids. L-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CH-s) (CHOL) were obtained from Sigma (St. Louis, MO). Cholest-4-en-3-one (cholestenone, CHON) was a gift of Dr. M. Shinitzky (the Weizmann Institute, Rehovot, Israel). Based on thin-layer chromatography, loaded with 0.5–1 μmol lipid per 2 cm strip all lipids were better than 99% pure [15].

Fluorophores. All-*trans* 1,6-diphenyl-1,3,5-hexatriene (DPH), 4-heptadecyl-7-hydroxycoumarin (HC); and all-*trans* 9,11,13,15-parinaric acid (TPA) were purchased from Molecular Probes (Junction City, OR). All fluorophores were stored in dark at $-20\,^\circ$C either in dry state or in tetrahydrofuran (THF). All other reagents and solvents were of analytical grade or better.

Vesicle preparation

Multilamellar large vesicles (MLV) were prepared in 50 mM KCl, containing 15% sucrose [15]. The presence of sucrose prevents MLV precipitation [15]. DPPC concentration was determined according to Bartlett [16]. Cholesterol concentration was determined using cholesterol oxidase as described elsewhere [17], sodium taurocholate (Sigma) was used to solubilize the liposomes for the cholesterol assay. Cholestenone was determined from its absorbance at 235 nm in the heptane upper phase of the Dole biphasic system used for cholesterol determination [17].

Labeling of the vesicles with the fluorophores

A fluorophore at the desired concentration in tetrahydrofuran, was injected into the MLV dispersion. Using a probe/lipid mole ratio of 1:1000–1:2000 for DPH and 1:250 for HC and TPA. The final volume of THF was always below 0.1% of the aqueous solution. The dispersion was vortexed and incubated at $50\,^\circ$C (above the DPPC gel to liquid crystalline phase transition) till fluorescence intensity reached plateau (usually not more than 1 h) [15].

Fluorescence measurements

Steady-state fluorescence anisotropy was measured using the L format MPF-44 Perkin Elmer Spectrofluorimeter which was modified as described by Lentz et al. [15]. Correction for G factor and light scattering were performed as described by Litman and Barenholz [18]. Fluorescence intensity life time was measured using SLM 4800 spectrofluorimeter. Limiting anisotropy (r_0) was measured for the fluorophores in propylene glycol at $-50\,^{\circ}C$ using the same instrument described elsewhere [20].

Temperature scans of the fluorescence anisotropy were performed as described elsewhere [15,20]. The data was plotted by describing r as function of T or $1/T$. Standard deviations of r values were low, not exceeding 2.5%. For example, for TPA in pure DPPC, low temperature range, $r = 0.273 \pm 0.002$ ($n = 9$) for HC in 20.2 mol% cholestenone, at high temperature $r = 0.101 \pm 0.002$ ($n = 7$) and for DPH in 25 mol% cholesterol at high temperature, $r = 0.175 \pm 0.002$ ($n = 5$). For systems in which gel to liquid crystalline transition occurred, the transition is characterized by its boundaries, t_s and t_l which describe the lower (solidus) and the upper (liquidus) boundaries, respectively [21]. t_{sf} and t_{lf} are the boundaries which obtained by extrapolating of the temperature range in the phase transition region in which the slope of the curve is maximal (Table I and Fig. 1). $T_{1/2}$ is defined as the midtemperature of the range of the maximal slope (t_{sf}–t_{sl}). T_m is the temperature at which the largest derivative (dr/dt) was obtained (Table I); r is the steady-state fluorescence anisotropy and t is the temperature in °C).

Results

Fig. 1 describes representative scans of temperature dependent steady-state fluorescence anisotropy of the three fluorophores (DPH, HC and TPA) in MLV made of DPPC-cholesterol, and DPPC-cholestenone mixtures. For the MLV composed of pure DPPC all the three fluorophores monitor the main gel to liquid crystalline phase transition characterized by T_m at about 41° C (see Table I) and a pretransition at about 32° C. This agrees well with the well-known values obtained using various physical methods [22]. However, looking at the fine details of the curves (Figs. 1 and 2) it is clear that the three fluorophores do not report identically. To compare the data obtained through the use of the three fluorophores, a parameter of Δr is introduced. Δr is defined as the difference in steady-state fluorescence anisotropy at 25° C ($r_{\circ,25\,^{\circ}C}$) and at 50° C ($r_{\circ,50\,^{\circ}C}$).

$$\Delta r = r_{\circ,25\,^{\circ}C} - r_{\circ,50\,^{\circ}C}$$

These two temperatures were selected for DPPC MLV because they cover the full range of thermotropic changes of the bilayer from below the pretransition through the $P_\beta \rightarrow L_\beta$ transition and the $L_\beta \rightarrow L_\alpha$ transition (main gel to liquid crystalline transition) to the liquid crystalline phase [23]. Δr when corrected for r_0 values is a good measure for the so-called 'averaging' effect of the steroid. In plain DPPC MLV Δr for the fluorophores are in the following order: $\Delta r_{DPH} > \Delta r_{HC} = \Delta r_{TPA}$ (0.215 > 0.132 = 0.132, respectively). For the fluorophores used in this study, the differences cannot be a result of their intrinsic fluorescent properties. Differences in the limited anisotropy (r_0) of the three fluorophores can be ruled out, since r_0 values obtained in propylene glycol at $-50\,^{\circ}C$ were almost identical: 0.368 ± 0.005, 0.364 ± 0.005, 0.368 ± 0.005 for DPH, HC and TPA, respectively, suggesting that for all of them the dipole moments of excitation and emission are parallel [25].

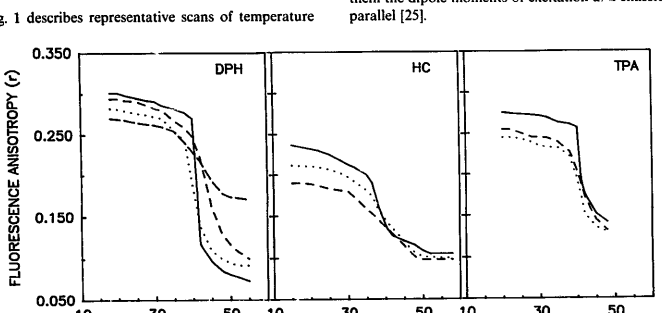

Fig. 1. Temperature-dependent fluorescence anisotropy of the various probes in MLV made of DPPC (———), cholesterol/DPPC 16.4 mol% (– – – –), cholesterol/DPPC 25 mol% (— —), and cholestenone/DPPC 20.2 mol% (·····).

TABLE I

Characterization of MLV main phase transition by three fluorophores

Mole fraction and probe	Transition limits (°C)		$T_{1/2}$ [a] (°C)	T_m [a] (°C)	Δr	SAE
	$t_s - t_i$ [a]	$t_{sf} - t_{tf}$ [a]				
DPPC						
+ DPH	36.4–46.0	39.7–41.8	40.75	41.55	0.215	
+ HC	32.8–44.6	38.5–41.9	40.20	– [c]	0.132	
+ TPA	34.6–43.8	40.6–42.0	41.30	41.45	0.132	
DPPC/CHON 20.2 mol%						
+ DPH	34.2–46.2	37.2–43.3	40.25	40.7	0.187	0.139
+ HC	29.3–50.4	32.8–46.2	39.50	– [c]	0.108	0.119
+ TPA	35.8–46.7	38.2–43.0	40.60	– [c]	0.112	0.099
DPPC/CHON 32.5 mol%						
+ DPH	29.0–49.6	33.3–46.2	39.75	41.05	0.151	0.199
+ HC	– [b]	– [b]			0.063	0.214
+ TPA	29.0–44.8	38.1–44.0	41.05	41.25	0.085	0.145
DPPC/CHON 46.2 mol%						
+ DPH	– [b]	– [b]			0.085	0.280
+ HC					0.045	0.188
+ TPA					0.048	0.182
DPPC/CHOL 7.2 mol%						
+ DPH	32.2–46.6	37.8–42.9	40.35	39.8	0.172	0.597
+ HC	26.8–47.0	35.1–45.4	40.25	– [c]	0.084	0.667
+ TPA	35.8–47.2	38.4–45.5	41.95	– [c]	0.129	0.042
DPPC/CHOL 16.4 mol%						
+ DPH	37.6–49.0	39.7–44.3	42.00	42.5	0.156	0.360
+ HC	31.3–49.4	38.2–46.4	42.30	– [c]	0.086	0.280
+ TPA	36.2–47.0	37.4–46.3	41.85	41.5	0.125	0.043
DPPC/CHOL 25 mol%						
+ DPH	34.4–49.0	35.9–48.0	41.95	40.0	0.092	0.492
+ HC	– [b]	– [b]			0.061	0.284
+ TPA	– [b]	– [b]		41.5	0.070	0.248

[a] Defined in Materials and Methods. For more details see Ref. 21.

[b] Too broad to determine.

[c] The signal-to-noise ratio is too high.

Effect of temperature on fluorophore life time may also introduce artifacts [24,25]. This is not the case here, since for all three fluorophores used in this study the change in r_s is related mainly to changes in system order as expressed by the residual anisotropy [19,25,26].

The presence of either the membrane active cholesterol or the membrane inactive steroid cholestenone in the DPPC bilayer seem to have a similar gross effect on the properties of the bilayer (Fig. 1): diminishing the pretransition at a low mol% of both steroids, broadening of the temperature range of the main gel to liquid crystalline phase transition and suppressing the expression of the phase transition. These effects are related to the 'averaging' effect on the membrane order as assessed from the effect on Δr (see above) (Fig. 2 and Table I). Careful analysis of the data shows clearly the differences in the effect of cholesterol and cholestenone. For both steroids, the change in three features of the thermotropic behavior (T_m, transition width, and transition suppression) as detected by the three probes is concentration dependent, but in a different way. In general for all three fluorophores cholesterol is more effective than cholestenone above its 'connectivity' concentration (25 mol%) [9,10]. For DPH and HC this is true throughout all the phase diagram.

When r_s of DPH are compared for the MLV of the various compositions it became clear that cholesterol is more efficacious than cholestenone in its 'ordering'

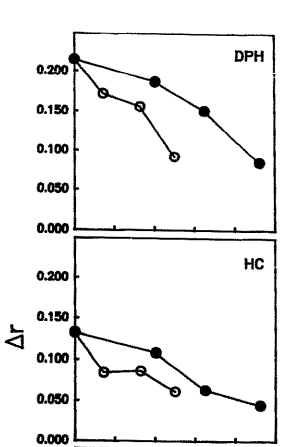

Fig. 2. Δr, the difference between the fluorescence anisotropy above and below the phase transition ($r_{s,25°C} - r_{s,50°C}$), of the various probes in DPPC/steroid MLV as a function of the mol% of cholesterol (○) and cholestenone (●).

effect measured at the liquid crystalline state ($r_{s,50°C}$) (Fig. 1). This effect is not linear with cholesterol mol%, having the major changes in $r_{s,50°C}$ occur between 16.4–25.0 mol%. This effect is clearly expressed in Δr values (Fig. 2).

The effect of both steroids is more pronounced on the hydrophobic region than on the headgroup region as the changes observed for Δr_{DPH} are larger than those monitored by HC (Δr_{HC}). Quantitatively, the absolute steroid dependent reduction in Δr (relative to Δr in MLV of pure DPPC) is larger for DPH than for HC, suggesting that the absolute effect on the order of hydrophobic region of the bilayer is larger than for its headgroup. However, the relative reduction in Δr is similar (Table I and Fig. 2).

The averaging effect of any molecules on the lipid bilayer can be evaluated and ranked by determining a specific averaging effect defined as SAE

$$SAE = \frac{\Delta r - \Delta r_E}{X_E}$$

where Δr and Δr_E are obtained in the absence and the presence of the effector (E), respectively. X_E is the mole fraction of the effector in the bilayer.

Generally the larger the SAE, the more effective is the steroid as an averager of membrane order and dynamics. Table I shows that the specific averaging effect of cholesterol is much higher than this of the cholestenone, especially at low mol% of the steroid. Analyzing temperature-dependent r_s profiles below the pretransition and above the main transition for DPH and HC demonstrates that the cholesterol affects the hydrophobic and the headgroup regions in an opposite way. For the hydrophobic region cholesterol increases $r_{s,DPH}$ in the liquid crystalline phase to a much larger extent than cholestenone while both disorder the gel phase similarly. For the headgroup region of both steroids, the major effect is disordering of the gel phase, that is decreased $r_{s,25°C}$ of HC. However, the disordering effect of cholesterol is larger than of the cholestenone.

The results for TPA which monitors lateral phase separation, are more complex. Fig. 1 shows that the main effect of both steroids is to disorder the gel phase with almost no effect on the liquid crystalline phase. Fig. 2 which describes the effect of the steroid on Δr is rather peculiar. At low mol% of cholesterol, Δr remains unaffected (Table I) and then there is a sharp reduction at about 16 mol%. For cholestenone the same reduction occurs but at a much higher mol% (between 32–46 mol%).

Discussion

This study compares the interaction of DPPC with the membrane-active steroid cholesterol and the membrane-inactive steroid cholest-3-en-4-one. The criteria of these definitions are reviewed elsewhere [27–30]. We compared the lateral organization and order of the headgroup and the hydrophobic regions of the bilayers. This was approached using steady-state fluorescence anisotropy of three well-established probes, DPH, HC and TPA. Diphenylhexatriene (DPH) monitors the hydrophobic region of the bilayer. Being equally distributed between gel and liquid crystalline domains, DPH averages information throughout all the system [24,25]. Heptadecyl hydroxycoumarin (HC) contains a nega-

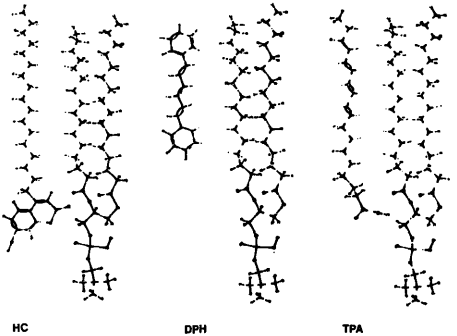

Fig. 3. The proposed location of the fluorescent probes in a half bilayer plane in relation to dipalmitoylphosphatidylcholine molecules (molecular models).

tively charged fluorophore protruding into the aqueous environment in the same plane as the phospholipid head groups while its paraffinic chain is aligned parallel to the phospholipid acyl chains [19]. HC is also evenly distributed between the gel and liquid crystalline domains [19]. *Trans*-parinaric acid (TPA) is a polyunsaturated conjugated fatty acid which aligns parallel to the phospholipid acyl chains with its fluorophore close to the bilayer center and its carboxyl polar group located at the interface between the polar and apolar regions of the bilayer [31,32]. Unlike DPH or HC, TPA is preferentially distributed into the more ordered gel domains in which its fluorescent quantum yield is enhanced [33]. This amplification enables detection of gel domains even when they compose only a small fraction of the lipid bilayer. The preferred location of the probes along the DPPC molecules based on molecular models is described in Fig. 3.

As mentioned before (see Introduction), the packing parameter of cholestenone is larger than that of cholesterol, due to structural differences between them.

The use of molecular models (data not shown) suggests that the A ring of cholestenone is more planar than in cholesterol, while for the B ring the opposite occur. Molecular models also show that the keto group of cholestenone is closer to the ring than the β-OH group, thus the packing parameter [40] of cholestenone is larger.

Another important aspect of cholesterol–phospholipid interaction is the various critical values of cholesterol mol% observed in the cholesterol DPPC phase diagram [1,11]. These different critical values are related to various parts of the steroid molecule. A low mol% of the steroid causes disappearance of the DPPC pretransition (Fig. 1), as was demonstrated previously through the use of other physical methods [1]. This effect is not specific and was contributed mainly by the hydrophobic planar fused ring system of the steroids. It was monitored by all three fluorophores, suggesting that both the hydrophobic and the head group regions of the bilayer are affected.

It can also be related to the large packing parameter of the steroids [40], which increases the average packing parameter per molecule to a degree sufficient to abolish the need for the DPPC acyl chain to tilt. This tilt is related to the pretransition [11]. This effect may be related to the increase of the dielectric constant observed at low mol% of sterols [42]. Similar observations were made recently for DMPC-cholesterol MLV, using Raman spectroscopy of selectively denaturated DMPC [41].

Cholesterol and cholest-3-en-4-one both diminish the pretransition and behave as 'averagers' by having a disordering effect on the gel phase and an ordering effect on the liquid crystalline phase. The stronger effect of cholesterol expressed as the specific averaging effect (SAE) is due to its more pronounced effect on the hydrophobic region in the liquid crystalline state (monitored by DPH) and on the head group region in the gel state (monitored by HC). The absolute change in the order of the hydrophobic region is larger than that of the head group in agreement with previously described NMR data [43].

It is worth noting that the effect of cholesterol is not linear with cholesterol mol% and the SAE changes more drastically between 16–25 mol%. These results are simi-

lar to those obtained by other methods which suggest the region of 20 mol% cholesterol as a critical point [44]. Above it, an abrupt change in the lateral diffusion was observed [10]. The level of 20 mol% cholesterol was interpreted as being the result of having the maximal number of DPPC molecules present in the boundary regions between cholesterol-rich domains and DPPC-pure domains [9,44]. These boundary regions are very disordered. Therefore, DPH molecules present in these regions have had a very low r_s value which reduced the average r_s of DPH. The abrupt reduction in these regions which occur between 16–25 mol% cholesterol causes a parallel increase in $r_{s,50\,^\circ C}$. The 'connectivity' phenomenon is not sensed by HC which has its reporter group in the head group region [19]. For cholestenone-DPPC the changes in Δr are smaller and more graduate than for cholesterol-DPPC (Fig. 2). This suggests that the lateral arrangements of DPPC-cholesterol and DPPC-cholestenone bilayers are different. While the distribution of cholesterol molecules in the DPPC bilayer plane is not a random one [1,11], we propose that cholestenone is evenly distributed in the membrane plane and therefore there are no boundary regions and the 'connectivity' phenomenon [9] does not exist. At the gel state, cholestenone, being radomly distributed, has a larger disordering effect on the hydrophobic region. Cholesterol is concentrated mainly in cholesterol-rich domains, and therefore it has a smaller and limited overall disordering effect. The larger packing parameter of cholestenone together with its more random lateral distribution may explain why in the gel phase it has a larger disordering effect than cholesterol (compare data obtained with DPH and TPA, Fig. 1). It may also explain why in the liquid crystalline phase cholestenone increases the order, in the polar region of the bilayer while the cholesterol does not (data shown).

TPA, being a more sensitive probe to monitor lateral phase separation is of special interest. For DPPC MLV the range of t_{sf}–t_{lf} as measured by DPH or HC is in the middle of t_s–t_l range (Table I). For TPA, the t_{sf}–t_{lf} range is shifted towards the upper boundary of the t_s–t_l range, supporting the previous indications that TPA is concentrated in the gel phase [45]. The main effect of both steroids on DPPC, as monitored by TPA is a disordering effect in the gel phase. From the comparison between the two steroids it is clear that from the view point of TPA cholestenone is more efficient than cholesterol as an 'averager'. Increasing the mol% of cholestenone causes a gradual increase in the SAE, suggesting that TPA is affected by cholestenone throughout the entire DPPC-cholestenone phase diagram (Fig. 1, Table I). This suggests that the two steroids differ in their effect on distribution of the TPA in the bilayer plane. The most simple explanation would be that due to steric problems TPA is rejected from the cholesterol-rich domain, as demonstrated by the unaltered Δr and SAE for 7.2 and 16.4 mol% cholesterol. Therefore more TPA will partition into DPPC-rich domains, regardless of its physical state. This indicates that other factors rather than the simple distribution between gel to liquid crystalline phases affect the lateral partition and distribution of TPA.

This assumption is supported by a recent more detailed theoretical model which describe phase equilibria in DPPC-cholesterol MLV [46]. According to this model, the authors propose that the phase diagram of PC and cholesterol has three possible phases: (1) liquid disordered (LD), which resembles that of pure PC above its T_m; (2) solid ordered (SO), which is similar to the gel phase of pure PC; and (3) liquid ordered (LO), an additional phase enriched with cholesterol. Above the T_m of the PC, either LD, LO or their mixtures exist, while below the PC T_m SO, LO or their mixtures exist. For a given PC the exact composition of each phase is dependent on the cholesterol mol% and the temperature. For example going along the temperature axis under our experimental conditions for 7.2 mol% cholesterol, the phases change from a mixture of SO/LO below the DPPC T_m into pure LD phase above it. For 16.4 mol% cholesterol, the phases change from SO/LO mixture below DPPC T_m into LD above it. For 25 mol% cholesterol, the system is almost completely LO phase throughout all the temperature range.

The fact that the Δr and SAE values for TPA are so different from those monitored by DPH and HC may be related to the differences in distribution of the fluorophores between these two phases (LD + LO). DPH and HC equally partition between these two phases and the ratio between the two phases is affected by the temperature; therefore, Δr and SAE will be affected too. This situation resembles the distribution between gel and liquid crystalline phases [15,19]. TPA on the other hand prefers the LD phase more than the LO phase and therefore when these two phases co-exist, TPA is concentrated in the LD phase. However, when only the LO phase exists, TPA will stay there. This induces a large reduction in Δr (compare Δr of 9.070 at 25 mol% cholesterol with Δr values of 0.129 and 0.125 for 7.2 and 16.4 mol% cholesterol, respectively (Fig. 2 and Table I)).

To sum up, this study demonstrates that important information on membrane organization and on interaction between bilayer components can be obtained through the use of steady-state fluorescence anisotropy. More than one probe is required for such analysis and the selection of appropriate fluorophores is important.

This technique is attractive because it is simple and widely available to many investigators. The study supports the conclusion that the small polar group in the 3-β position of the steroid and Δ^5 bonds are essential in order to have a membrane-active steroid. However, the extent of contribution of hydrogen bonding and the

alignment of the steroid ring with the phospholipid acyl chains remains an open question.

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