

Domain-Formation in DOPC/SM Bilayers Studied by pfg-NMR: Effect of Sterol Structure

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ABSTRACT Pulsed field gradient (pfg)-NMR spectroscopy was utilized to determine lipid lateral diffusion coefficients in oriented bilayers composed of 25 mol % sterol and equimolar amounts of dioleoylphosphatidylcholine and sphingomyelin. The occurrence of two lipid diffusion coefficients in a bilayer was used as evidence of lateral phase separation into liquid ordered and liquid disordered domains. It was found that cholesterol, ergosterol, sitosterol, and lathosterol induced domains, whereas lanosterol, stigmasterol, and stigmasterol resided in homogeneous membranes in the temperature interval of 24–70°C. Among the domain-forming sterols, differences in the upper miscibility temperature indicated that the stability of the liquid ordered phase could be modified by small changes in the sterol structure. The domain-forming capacity for the different sterols is discussed in terms of the ordering effect of the sterols on the lipids, and it is proposed that the driving force for the lateral phase separation is the reduced solubility of the unsaturated lipid in the highly ordered phase.

INTRODUCTION

Cholesterol (CHOL) is the most abundant sterol in mammalian plasma membranes (1). One of the more demanding questions to be answered in membrane biology is the feature exercised by CHOL in the lipid bilayer. CHOL solubilized in sphingolipids triggers lateral phase separation into domains in the lipid bilayer, often referred to as “rafts” that are believed to be involved in diverse membrane processes such as signal transduction, protein stabilization, protein and lipid sorting, and membrane fusion (2,3). Moreover, CHOL modulates the packing of the phospholipid molecules in the membrane, thereby increasing both bilayer rigidity and mechanical durability as well as reducing passive permeability (4). Obviously, to get a better understanding of the effect of CHOL and other sterols on membrane properties and functions requires investigations of the phase behavior of sterol/lipid systems.

It is well known that increasing the CHOL concentration in appropriate lipid bilayers will cause a phase separation to occur into liquid ordered (l_o) and liquid disordered (l_d) phases (5,6). Hitherto, the number of lipid systems investigated, emphasizing phase behavior, has been quite limited and mainly comprises a couple of phase diagrams for some phosphatidylcholines (PCs) or sphingomyelins (SMs) with CHOL in excess water (6–9) and CHOL solubility in various lipids (10). On the other hand, an almost overwhelming number of studies of various effects of CHOL on both model and cell membranes have been published, in particular after the proposal of the importance of domains for membrane functions in the biological cells at the end of the 1980s.

However, no detailed mechanism underlying the formation of these “rafts”, neither in model membranes nor in living cells, has so far been reached. We have, therefore, undertaken a program, in which systematic studies (11–18) of the dynamics and phase behavior of a variety of lipid/sterol systems are performed. In particular, we utilize a pulsed field gradient (pfg)-NMR method that we have developed to directly determine the lateral diffusion coefficients of lipids in bilayers (17,19). From such measurements, both the dynamics and the phase behavior, such as lateral phase separation, can be obtained.

Recently we proposed that the packing order plays a crucial role in the formation of domains in dioleoylphosphatidylcholine (DOPC)/sphingomyelin (SM)/CHOL systems, in which the distribution of the hydrocarbon chains differed for the natural SMs (14). From these previous studies, we concluded that the lateral phase separation in the systems studied could be rationalized in terms of lipid order and immiscibility of unsaturated lipids, such as DOPC, in the l_o phase. The interpretation of our experimental findings was based on the assumption that the lipid lateral diffusion in bilayers was strongly dependent on the lipid packing order and that no specific interactions between the molecules had to be included (15). Under these assumptions it was concluded that saturated lipids, such as egg yolk SM (eSM), formed more ordered phases than unsaturated lipids and that the addition of CHOL greatly enhanced the ordering, especially for the lipids with saturated chains. We then proposed that the driving force for the lateral phase separation into l_d and l_o phases is the increasing difficulty for the unsaturated lipid to reside in a highly ordered phase. Our findings suggested that the unsaturated lipids had a preference to be located in the l_d phase, whereas eSM preferred the l_o phase. CHOL, on the other hand, seemed to partition into both phases to roughly the same extent, indicating that CHOL had no particular

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preference for any of these phases in a ternary system with saturated and unsaturated lipids, probably because of the rigid sterol structure, making it rather insensitive to the molecular order of the environment. Therefore, the role of CHOL in the phase separation process is to increase the ordering of the (homogeneous) lipid membrane to such an extent that the system finally favors a phase separation, where most of the unsaturated lipid is squeezed out from the l_o phase into the l_d phase (15). Note that the situation is slightly different for a binary system with only a saturated lipid, where there is a fast chemical exchange of molecules between the l_o and l_d phases, indicating that the domains are small (12). To investigate the mechanism(s) behind the formation of domains in bilayers, utilizing the pfg-NMR method, this simple order and packing model of the lipid bilayer has also been applied in this investigation, where we have studied the effect of the structure of the sterols (Fig. 1) on the lipid lateral diffusion in DOPC/eSM bilayers. As will be seen, just moving a double bond one step in the sterol skeleton, seemingly a small alteration, can have a rather strong effect on the phase behavior.

MATERIALS AND METHODS

Materials

DOPC was purchased from Avanti Polar Lipids (Alabaster, AL); eSM ~99%, CHOL grade 99+%, lanosterol 50–60% (LAN) with the major impurity being dihydrolanosterol, stigmasterol 95% (StiSte), stigmasterol 95% (StiSta), ergosterol 95% (ERG), and lathosterol 98% (LATH) were all from Sigma Aldrich (St. Louis, MO); β -sitosterol SIT 78% was from Seraloids (Newport, Rhode Island). Deuterium oxide, 99.9% in ^2H , was obtained from Larodan Fine Chemicals AB (Malmö, Sweden).

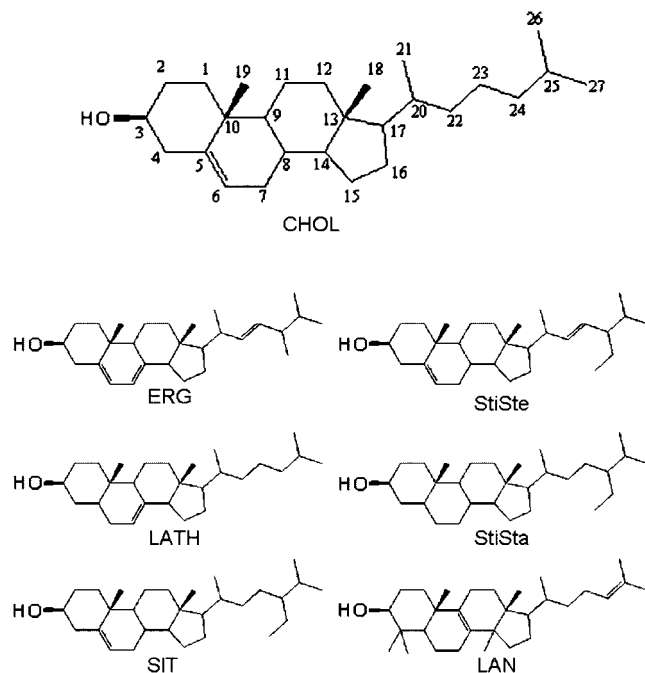


FIGURE 1 Structures of the sterols utilized in this study.

Preparation of oriented samples

The macroscopically aligned samples were prepared with 25 mol % sterol and equimolar amounts of the phospholipids DOPC and eSM. The appropriate amount of each compound was weighed and dissolved in a 1:4 volume ratio of methanol/propanol solvent to a concentration of 15 mg/mL of total lipid; 25 μL of the solution was then put on each of ~30 glass plates (5 \times 14 mm, Marienfeldt, Germany). The solvent was evaporated at atmospheric pressure, and the sample was then kept under vacuum at room temperature for at least 6 h.

The glass plates were stacked in a special sample holder and put in a $^2\text{H}_2\text{O}$ atmosphere at 45°C for 5 days. After the formation of hydrated, oriented bilayers, excess deuterated water was added to a filter paper at each end of the sample to ensure maximum hydration during the experiments. The sample tube was sealed and left to equilibrate for an additional 2 days before the measurements were performed.

Diffusion measurements

Pfg-NMR measurements were performed mainly on a 100-MHz Chemagnetics Infinity (Varian, Fort Collins, CO) NMR spectrometer. Some measurements were also made on a 400-MHz Chemagnetics Infinity (Varian) NMR spectrometer. The macroscopically aligned samples were oriented at the magic angle with respect to the main magnetic field in a goniometer probe. Details of the pfg-NMR method can be found elsewhere (17).

The stimulated echo pulse sequence (20) was used for all measurements; in this procedure, the attenuation of the amplitude of the echo, A , and its dependence on translational diffusion are described by a sum over all the different diffusion components present in the sample

$$A = \sum_i A_{oi} \exp \left(-\gamma^2 \times \delta^2 \times g^2 \times D_i \times \left(\Delta - \frac{\delta}{3} \right) \right), \quad (1)$$

where A_{oi} is the initial amplitude of component i without applied magnetic field gradients, γ is the gyromagnetic ratio, δ and g represent the duration and amplitude of the pulsed field gradients, Δ is the time interval between the two gradient pulses, and D_i is the self-diffusion coefficient of component i (17,21).

The experimental settings were as follows: the time between the first two 90° pulses, τ , and the time interval between the second and the third 90° pulses, τ_1 , were kept constant in each experiment but varied for different experiments between 10–14 ms and 90–288 ms, respectively. The majority of the experiments were performed with $\tau = 12$ ms, and $\tau_1 = 188$ ms. Δ was typically 200 ms, and δ varied in each experiment between 1 and 11 ms. The number of accumulations ranged from 16 to 512 to achieve an acceptable signal/noise ratio. Measurements were performed between 24 and 60°C in steps of 3° with a waiting time of 20 min before each measurement. The obtained diffusion coefficients did not depend on the thermal history of the sample.

The data was Fourier transformed, and the total integral and/or peak intensities were used in a nonlinear fit to Eq. 1. For each sample and temperature, one or two diffusion coefficients corresponding to the lipid translational diffusion (1–20 $\mu\text{m}^2/\text{s}$) were obtained. At low temperatures the fast diffusion of water (100–300 $\mu\text{m}^2/\text{s}$) was also sometimes observed. Fig. 2 gives an example of the results obtained for LATH at 30°C. The relative errors as reported from the nonlinear fits were generally below 5% for the monoexponential data and between 5% and 20% for the biexponential decays. The choice of Δ did not affect D , and it was thus independent of the diffusion time. Because the bilayer normal and the magnetic field gradient form an angle of 54.7°, the lateral diffusion coefficient, D_L , is obtained by multiplying the measured diffusion coefficient D by 1.5 (22).

RESULTS AND DISCUSSION

Fig. 1 shows the sterol molecules utilized in this investigation. It can be inferred from this figure that the structural

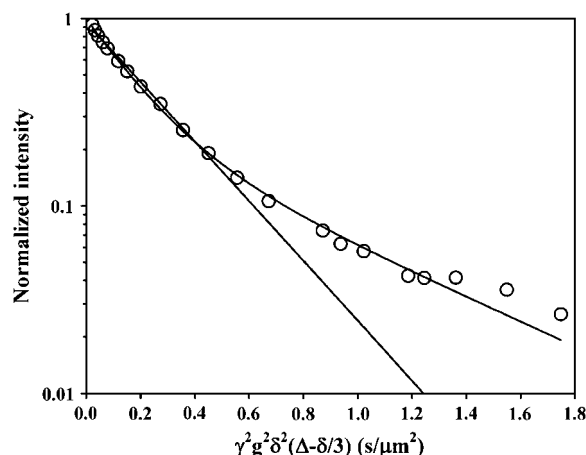


FIGURE 2 Obtained decay of the lipid signal intensity for DOPC/eSM bilayers containing LATH at 30°C. The lines in the plot show the best fits to one and two exponential decays, respectively.

differences, compared to CHOL, are rather small. LATH has a Δ^7 double bond instead of a Δ^5 , SIT and StiSte are ethylated at carbon 24, and StiSte also possesses a Δ^{22} double bond. StiSta is the saturated form of StiSte. ERG has two extra double bonds (Δ^7 and Δ^{22}) and is methylated at carbon 24. Finally, LAN has a $\Delta^{8(9)}$ double bond instead of a Δ^5 and is also 4,4,14-trimethylated.

It turns out that although the changes in structure among the different sterols can be considered as quite small, the phase behavior differs in that some of them are able to induce domains in DOPC/SM bilayers, whereas some of them are not. The goal with our investigation is thus to try to make clear what kind of physicochemical properties of these sterols are determining such a diverse phase behavior.

Sterols in homogeneous lipid bilayers

The NMR diffusion data for StiSte and StiSta were well described by one lipid diffusion coefficient, and D_L increased monotonically with increasing temperature (Fig. 3). The apparent activation energies obtained for the lipid membranes containing StiSte (33 kJ/mol) and StiSta (28 kJ/mol) were typical for an l_d phase in accordance with previous investigations (12). The D_L values were close to those for a DOPC/eSM membrane with no sterol (11).

The diffusion data for the LAN system were also well described by one lipid diffusion coefficient (Fig. 3), with an activation energy of 47 kJ/mol. This was higher than for StiSte and StiSta, but it is still within the values obtained in previous studies of other l_d phases (11,12).

The observation of a single diffusion coefficient does not in itself rule out the possibility of microscopic phase separation with domain sizes much smaller than 1 μm because then only a weighted mean value of the D_L s would be observed due to a fast exchange between the l_o and l_d phases (23).

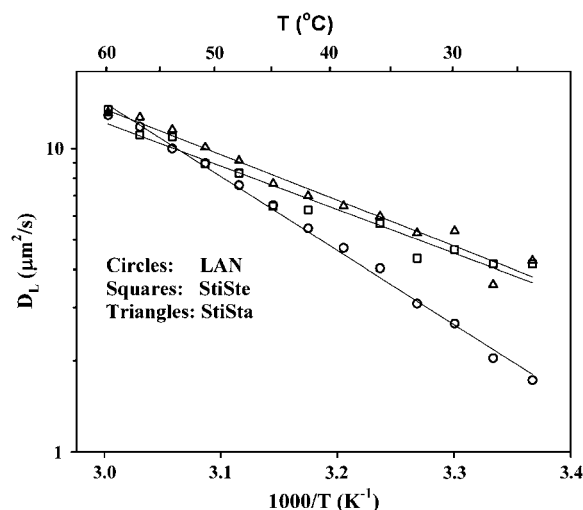


FIGURE 3 Temperature dependence of D_L for the homogeneous DOPC/eSM/sterol bilayers containing 25 mol % StiSte (squares), StiSta (triangles), and LAN (circles). The lines are linear least-square fits to the points.

In earlier studies of binary systems of saturated lipids and CHOL, the plot of the temperature dependence showed curved features that were attributed to the presence of small domains in the bilayers (12). No such effect was observed in Fig. 3, and it could be concluded that the sterols resided in an l_d phase and that no domains were present in the lipid bilayers containing StiSte, StiSta, or LAN.

Domain formation in bilayers

The results from the remaining sterols in this study are summarized in Fig. 4 A. At high temperatures, all systems exhibited a single diffusion coefficient, but at some point all the systems reached an upper miscibility temperature (T_M) at which the data were better described by two diffusion coefficients. This temperature was highest for LATH, followed by ERG, CHOL, and SIT. Even though the obtained NMR signal contains overlapping contributions from the two phospholipids, the occurrence of two D_L s cannot simply be attributed to the motions of each of the two lipid species. Rather, previous investigations using isotopically labeled molecules have shown that all lipids (phospholipids as well as sterols) have the same D_L as long as they reside in the same phase (or domain) in the bilayer (18,24). Instead, two lipid D_L s indicate a lateral phase separation into the l_o and l_d phases in the bilayer. This conclusion is well supported from other studies in several raft-forming systems (11,14), and the observation of a diffusion decay with two components in pfg-NMR therefore provides a convenient method for investigation of the lateral phase separation process.

As the temperature was lowered further, the ERG and CHOL systems returned to a single diffusion component, whereas the two-phase behavior for the LATH and SIT systems remained to the lowest temperature studied. The

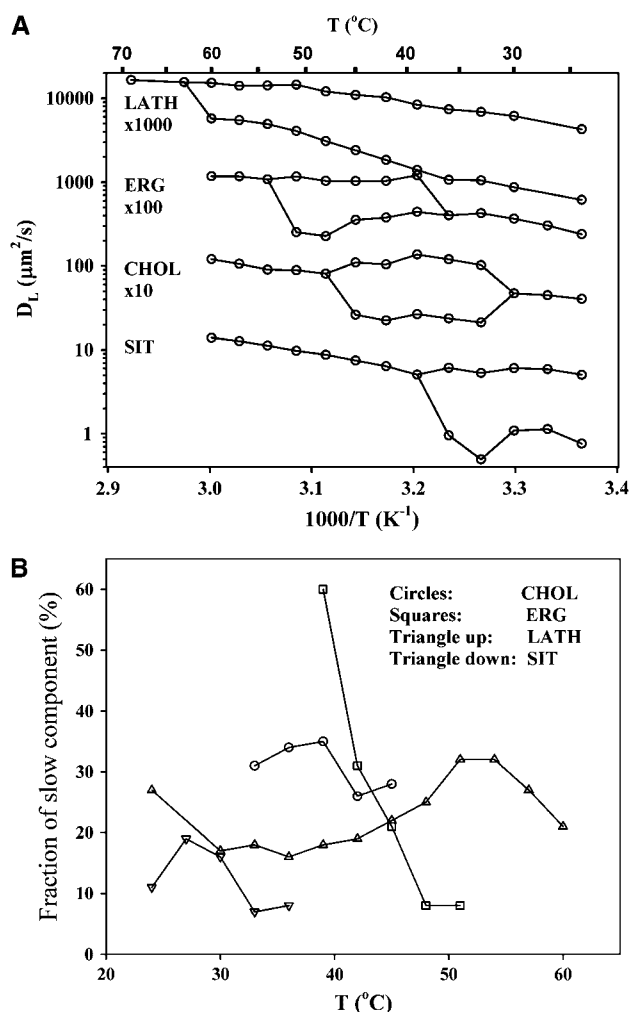


FIGURE 4 (A) Temperature dependence of D_L for the DOPC/eSM/sterol bilayers forming domains. The curves for CHOL, ERG, and LATH have been shifted upward for clarity. (B) The fraction of the slow diffusion component obtained from the fits to Eq. 1 as a function of temperature.

results for the CHOL system were in good agreement with earlier reports (11).

Within the two-phase region, the relative amplitude of the slow diffusion coefficient varied only slightly within ~20–30% for CHOL, SIT, and LATH, whereas it decreased monotonically from 60% to 10% for ERG (Fig. 4 B). This relative amplitude is not necessarily a measure of the actual amount of the l_o phase because it is weighted with the transverse and longitudinal proton relaxation in the phases, but it can be used as a rough estimate of the relative quantities of l_d and l_o phases. At lower temperatures we cannot rule out the presence of a solid ordered (s_o , gel) phase because the very fast relaxation in this phase makes it effectively invisible in the pfg-NMR experiment.

The ratio between the fast and slow diffusion coefficients was found to vary between 2 and 7 for all systems. For CHOL and SIT, it was more or less constant throughout the

temperature range at a value of 5 and 6, respectively. For LATH it decreased with increasing temperature from 7 at 20 $^{\circ}\text{C}$ to 3 at 60 $^{\circ}\text{C}$. The opposite trend was observed for ERG, in which the ratio increased from 3 at 42 $^{\circ}\text{C}$ to 5 at 54 $^{\circ}\text{C}$.

Sterol properties influence domain formation

To get a better understanding of why a certain sterol will induce domains in lipid bilayers, we need to look into what properties of the bilayer are changed by the incorporation of the sterol and the roles played by its chemical structure. Because raft formation seems to be intimately coupled to the packing and ordering of the molecules in the bilayer, it is appropriate to first briefly summarize some relevant data from the literature on the influence that sterols may have on these properties. Here, many methods have been employed, such as order parameter determinations, fluorescence anisotropy and quenching, membrane permeability, and detergent insolubility. Methods that probe bilayer properties such as bending stiffness and area compressibility are also related to the packing properties, and so is the lipid lateral diffusion. See Table 1 for a compilation of some methods used for various systems containing the sterols in our study. In the following we have tried to rank the sterols studied with respect to their ability to promote domain formation by influencing the packing properties in the bilayer. In such a

TABLE 1 Summary of experimental methods used to characterize domain formation and some physicochemical properties in bilayers

Experimental method and measured characteristic	References
Deuterium quadrupole splittings: order parameters	29,31,36,38,39,49
Vesicle deformation and orientation in a magnetic field: bending energy	29
Micropipet aspiration: area expansion modulus	29,31,50
Fluorescence microscopy: lateral organization	44,45
Fluorescence correlation spectroscopy: lateral diffusion	45
Permeability of water, small molecules, and ions	33,34,36,51,52
Fluorescence quenching: domain stability	25,40,43,53
Triton X100 solubility: domain packing	25,35,40,43
Fluorescence anisotropy: domain packing	28,40,52,53
Dynamic light scattering: lateral tension, surface viscosity	32
Differential scanning calorimetry: transition temperatures and enthalpies	26,35,38,49
Resonance energy transfer: domain formation	35
Pulsed field gradient NMR: lateral diffusion	11–15,18,37
Electron paramagnetic resonance: order parameters	39

The l_o phase is characterized by a high degree of ordering of the lipids, which results in large order parameters, stiffer and less compressible membranes, low lateral diffusion, low permeability, resistance to Triton X100 solubilization, and high fluorescence anisotropy. The effectiveness of different sterols in changing these characteristics is utilized to rank their domain-forming ability.

comparison, the nature of the lipid also has to be taken into account because the ordering effect of the sterols depends on the degree of unsaturation of the lipid chains.

Binary systems

In systems with a saturated lipid, LATH generated about the same ordering as CHOL, but it produces more stable domains (25,26). For ERG, the ordering seemed to depend on the degree of lipid unsaturation. Thus, for saturated lipids, such as DMPC and DPPC, ERG is more potent than CHOL in creating a highly ordered bilayer (27–30), whereas in systems of varying degree of unsaturation (palmitoyl-oleoyl-phosphatidylcholine, dielaidoylphosphatidylcholine, and egg phosphatidylcholine), the opposite is true (31–33). However, on lipid extracts from *Acholeplasma laidlawii* grown on both saturated and unsaturated fatty acids, no difference could be seen between ERG and CHOL (34). It should also be mentioned that the ordering obtained from the ^2H NMR quadrupole splitting of the chain methyl showed a large temperature dependence for the ERG system and that the induced ordering, at least in this position, could be larger or smaller than for CHOL, depending on the temperature (29). A similar but opposite difference between saturated and unsaturated lipids was found for SIT; i.e., the ordering capability was smaller than for CHOL in saturated systems (28,35), whereas it was larger for unsaturated systems (36).

A majority of studies showed that LAN was clearly inferior to CHOL in the capacity to pack both saturated and unsaturated systems (27–29,31,32,37–39), although in one study LAN was found to increase ordering in bilayers to the same extent as CHOL (40). One simulation study (41) reported no domain formation for LAN, whereas another found only small differences between LAN and CHOL (42). Also StiSte seemed to be less prone to form domains than CHOL in both saturated and unsaturated systems (27,28,33–36).

Ternary systems

In systems of saturated lipid/unsaturated lipid/sterol, LATH was shown to induce the most stable domains (25), but ERG, StiSte, and SIT also gave results that indicated that these sterols promoted domain formation (43). According to the latter study, all of these three sterols induced more stable domains than CHOL in the corresponding system, although the domains induced by SIT seemed to be less packed than for CHOL. LAN gave somewhat ambiguous results, in which fluorescence microscopy methods indicated that no domains formed in DOPC/DPPC/LAN systems (44), whereas domains were detected in DOPC/stearoyl-SM/LAN systems (45). Other studies indicated that LAN induced only small changes in domain formation compared to the sterol-free membranes (40).

Further indications that CHOL could be replaced by other sterols of similar structure came from in vivo studies showing that SIT could support cell growth, provided that

small amounts of CHOL were present. The results suggested that SIT can replace CHOL for bulk membrane functions but that small amounts of CHOL were required for other cellular functions (46). Similar results were obtained with desmosterol, which could replace cholesterol with regard to membrane functions (47).

From this summary, it is clear that our results are quite consistent with the general trends published in the literature for these sterols. Let us now discuss our results obtained for each one of the sterols with the basic understanding that the packing capability of the sterol is the main reason for the domain formation.

It seems reasonable that the extra bulkiness obtained by introducing a side group in the hydrocarbon chain of CHOL will impair the packing in the bilayer, thereby destabilizing the l_o phase. SIT shows exactly this effect, where T_M is substantially lowered compared to CHOL. Adding a double bond to the hydrocarbon chain, as in StiSte, would further impair the ability to produce tightly packed bilayers (cf. the effect of lipid unsaturation in bilayers), and therefore, StiSte does not induce domain formation. A comparison of SIT and StiSta shows that the double bond of the ring system is essential for domain formation for the chain ethylated compounds. This is rather surprising because dihydrocholesterol, i.e., CHOL lacking the double bond, has very similar properties to CHOL (48).

For ERG it seems that the destabilizing effect of having both a double bond and a methyl group in the end chain is more than compensated for by the Δ^7 double bond. Also, the high T_M for LATH reflects a tendency for this sterol to form highly stable domains. Thus, changing the Δ^5 double bond to a Δ^7 acts to stabilize domains. It is rather remarkable that the position of double bonds in the sterol skeleton can have such a large effect. Perhaps this position affects the molecular shape, leading to a different packing of the sterol in the lipid bilayer. Further investigations are needed to clarify this. Finally, the reason why LAN does not induce raft formation is probably related to the bulky methyl groups on the sterol skeleton that will reduce its ability to increase the necessary lipid ordering, or, again, the position of the double bond in the ring and in the chain might play a role.

Let us now try to rationalize the results in terms of the order and packing model mentioned in the introduction. The decrease in the Gibbs free energy on lateral phase separation originates from several sources. The ordering of the sterol in the l_o phase leads to a closer packing between the molecules, resulting in an increase in the van der Waals interaction (enthalpy). At the same time the entropy decreases, most for the unsaturated lipid in the ordered phase, because it will lose configurational entropy in the ordering process. However, when the unsaturated lipid leaves the l_o phase for the l_d phase, a rather large increase in the configurational entropy can be expected. This may be the driving force for domain formation in these kinds of ternary systems, and the phase separation occurs because of a large difference in ordering

between the two phases. This reasoning can be used to explain the observed increasing trend in T_M for SIT, CHOL, and ERG (Fig. 4 A). The induced order in the mainly saturated l_o phase will be largest for ERG and smallest for SIT, whereas in the mainly unsaturated l_d phase, the ordering will be largest for SIT and smallest for ERG. Therefore, the difference in ordering between the two phases will increase in the order $SIT < CHOL < ERG$, and we would expect the same order for T_M for these systems, at least as long as the partitioning of the membrane components is similar to that found in the CHOL system.

It should be noted that for the ternary system the tielines in the two-phase region with the l_o and l_d phases are not very far from being parallel with the base of the triangular phase diagram, CHOL being at the top (8). This means that CHOL has no preference for either of the two phases, as stated in the introduction and also observed previously for our systems (15,18). Thus, the CHOL content is similar in the l_o and l_d phases in equilibrium. It can be concluded that there is a rather subtle balance between the enthalpic and entropic forces for a domain to form in these systems. This shows why small changes in the sterol structure play such an important role in the ability to induce domains.

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