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Evidence for regular distribution of sterols in liquid crystalline phosphatidylcholine bilayers

(membrane organization/regular lipid distribution/fluorescence)

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ABSTRACT **To investigate the lateral organization of sterols in membranes, the fluorescence intensity of dehydroergosterol at different mole fractions in liquid crystalline dimyristoyl phosphatidylcholine bilayers was examined. A number of intensity drops were observed at specific mole fractions, as predicted from a hexagonal super-lattice model. The fluorescence dips provide compelling evidence that a naturally occurring sterol is regularly distributed at fixed compositional fractions, consistent with the presence of hexagonal superlattices in the fluid membranes. Regularly distributed regions, however, coexist with irregularly distributed regions. The extent of regular distribution varies periodically with sterol mole fraction and, consequently, similar variations take place in the membrane volume and lipid packing. This level of modulation in local membrane structure by minute changes in sterol concentration should have profound implications for the functional role of cholesterol content in cell membranes.**

The lateral organization of cholesterol is an important but not well understood membrane phenomenon (1). Real cell membranes are extremely complex, so that studies of the behavior of cholesterol, or other molecules, in the membrane must be carried out in simple model systems. The simplest system is a membrane composed of two components: the matrix lipid and the guest molecule of interest. In theory, the components of these binary mixtures can be either domain segregated, randomly distributed, or regularly distributed (2), depending on the energy function, defined as $W(r) = E_{12}(r) - [E_{11}(r) + E_{22}(r)]/2$, where $E_{ij}(r)$ refers to the interaction energy between the ith and jth membrane component and r is the distance between the interacting lipids (3). When $W = 0$, lipids are randomly distributed, and when $W > 0$, domain segregation occurs (4). When $W < 0$, and at the same time long-range repulsion between guest molecules exists, guest molecules are regularly distributed. A regular distribution is a lateral organization where the guest molecules are maximally separated in the lipid matrix.

A number of studies on phospholipid model membranes have suggested the existence of cholesterol-rich domains. For example, Lentz *et al.* (5) suggested that cholesterol-rich and cholesterol-poor fluid phases coexist in phospholipid bilayers. Using computer simulations, Snyder and Freire (6) found that cholesterol molecules form many random microdomains and that those small domains link into a network at 22 mol % cholesterol. These random domains are, however, different from the aligned cholesterol domains proposed by Rogers *et al.* (7), who suggested that cholesterol molecules in the phospholipid matrix are aligned along a common axis. The alignment results in macroscopic cholesterol-rich stripe domains, whereas the phospholipids form ribbons between the aligned cholesterol domains.

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In contrast to domain formation, cholestatrienol, a fluorescent cholesterol analogue, was reported to be homogeneously dispersed in 1-palmitoyl-2-oleoyl L-α-phosphatidylcholine vesicles at sterol/phosphatidylcholine ratios of <50 mol % (8). Although differential scanning calorimetric data for cholesterol in dipalmitoyl phosphatidylcholine vesicles showed a negative deviation from ideality ($W < 0$) (9), convincing evidence for regular distribution of sterols in phospholipid model membranes has not been reported. It appears that the question of the lateral distribution of sterols in bilayer membranes is far from answered (for more examples, see refs. 1, 10, and 11).

The present study examines the fractional concentration dependence of dehydroergosterol [$\Delta^{5,7,9(11),22}$-ergostatetraene-3β-ol] fluorescence in the liquid crystalline state of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine [dimyristoyl phosphatidylcholine (DMPC)] multilamellar vesicles. Dehydroergosterol is a naturally occurring fluorescent sterol found in several biological membranes (12, 13) and resembles both cholesterol and ergosterol in its physiological properties (14). The results in this study provide compelling evidence for regular sterol distribution, and a model is proposed for lateral sterol organization in liquid crystalline phosphatidylcholine bilayers.

MATERIALS AND METHODS

Materials. Dehydroergosterol (Sigma) was recrystallized from ethanol and its purity was verified by HPLC. The concentration of dehydroergosterol was determined by using the extinction coefficient at 326 nm in dioxane equal to 10,600 $M^{-1}\cdot cm^{-1}$ (15). The concentration of DMPC (Avanti Polar Lipids) was determined by the method of Bartlett (16).

Vesicle Preparation. Dehydroergosterol in dioxane was dried under nitrogen, and then DMPC in chloroform was added. After drying under nitrogen, the mixtures were dried under vacuum overnight and then suspended in 22.5 mM phosphate buffer at pH 7.1. The dispersion was vortexed for 3 min at about 40°C, well above the main phase-transition temperature of DMPC (T_m = 24°C). The samples were cooled to 4°C for 30 min and then incubated at 40°C for 30 min. This cooling/heating cycle was repeated two more times. Finally, the samples were stored under nitrogen at about 22°C for at least 4 days prior to fluorescence measurements.

Fluorescence Measurements. Fluorescence intensity measurements were made with an SLM DMX-1000 fluorometer (SLM Instruments, Urbana, IL). Samples were excited at 324 nm with a 1-nm bandpath. The emission was observed

Abbreviations: DMPC, dimyristoyl phosphatidylcholine; PyrPC, 1-palmitoyl-2-(10-pyrenyl)decanoyl phosphatidylcholine; X_{DHE}, mole fraction of dehydroergosterol; Y_{DHE} and Y_{DHE}, critical values of X_{DHE} at which the dehydroergosterol molecules and the DMPC acyl chains, respectively, form regularly distributed hexagonal super lattices.

through a monochromator with a 4-nm bandpath. The total fluorescence intensity, F, was determined as the intensity between 340 and 460 nm. Blank readings from vesicles in the absence of dehydroergosterol (F_0) were subtracted from the sample readings. The amount of DMPC in the samples was fixed at 15 μM.

RESULTS AND DISCUSSION

The plot of the normalized dehydroergosterol fluorescence (total fluorescence intensity per μmol of dehydroergosterol) vs. the mole fraction of dehydroergosterol (X_{DHE}) in DMPC at 35°C shows a number of intensity drops (e.g., at 51, 33, 25, 20.2, 15.4, 14.3, 11.8, 10.0, 9.3, 8.0, 6.8, and 5.8 mol %) (Fig. 1). These intensity drops are hereafter referred to as dehydroergosterol dips (DHE dips). It is important to stress that the reproducibility of the DHE dips should be judged by the dip position, not by the depth of the dips. The depth of the dip varies (e.g., 20%) from experiment to experiment due to differences in thermal history; however, the dip positions are highly reproducible at 15.4, 20.2, 25, and 33 mol %.

The appearance of DHE dips at specific mole fractions below 30 mol % can be understood in terms of the hexagonal super-lattice model (17, 18). It is assumed that (*i*) the acyl chains of the DMPC molecules form a hexagonal host lattice (19), (*ii*) dehydroergosterol fits into the hexagonal lattice, and (*iii*) at certain mole fractions, the dehydroergosterol molecules maximally separate in order to minimize the total energy; as a result, they form hexagonal superlattices in the hexagonal lattice matrix (Fig. 2). For a given dehydroergosterol molecule, its position in the hexagonal lattice can be described by two integer coordinates, n_a and n_b, once the origin and the principal axes are specified, where n_a and n_b are the number of translational steps in the lattice along the two principal axes a and b, respectively (17). Then Y_{DHE}, the critical dehydroergosterol mole fraction at which the dehydroergosterol molecules form regularly distributed hexagonal superlattices, can be calculated by the equation (17)

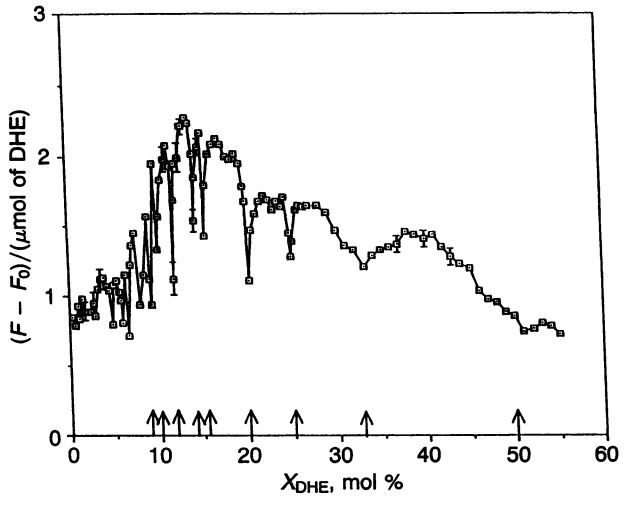

FIG. 1. Concentration dependence of dehydroergosterol fluorescence intensity in DMPC multilamellar vesicles at 35°C. Typical error bars are shown which represent the standard errors of fluorescence resulting from three different samples. The y axis shows the value of the total fluorescence intensity (F, arbitrary unit) minus the blank reading from vesicles without dehydroergosterol (F_0), divided by the amount of dehydroergosterol (DHE, μmol) in the cuvette. The errors of fractional composition resulting from lipid concentration determinations are estimated to be ±0.1 mol %. Arrows indicate the places expected for the dips as calculated from Eqs. 1 and 2 and as discussed in the text.

$$Y_{DHE} = 2/(n_a^2 + n_a n_b + n_b^2 + 1). \quad [1]$$

The calculated Y_{DHE} values (<30 mol %) are listed in Table 1, along with the concentrations where the DHE dips are actually observed. For the purpose of comparison between the theoretical and the observed values, the DHE dips in the low concentration region turn out to be less useful than those in the high concentration region because the DHE dips in the low concentration region (<8 mol) are closely packed and their positions cannot be unambiguously determined. For the DHE dips at >8 mol %, we found good agreement between the positions of the observed DHE dips and the calculated Y_{DHE} values. This good agreement provides strong evidence that dehydroergosterol molecules can be regularly distributed into hexagonal super lattices at critical mole fractions.

The dips at 33 and 51 mol % (Fig. 1) are broad and shallow when compared with the dips at <33 mol %, and the dip at 33 mol % is not predicted from Eq. 1. Still, these two dips can be understood in terms of lipid regular distribution. As modeled earlier, at low sterol concentrations (<30 mol %), a DMPC molecule is assumed to occupy two hexagonal lattice points, and a dehydroergosterol molecule occupies one (Fig. 2 *A* and *B*). In the liquid crystalline state, sterols cause an ordering effect on phospholipids (22, 23). As a result, the cross-sectional area of DMPC decreases as the sterol content increases. According to the calculation made by Almeida *et al.* (24), the average effective area per DMPC acyl chain at 35°C is reduced from 29.5 Å^2 at 0 mol % cholesterol to 26.7 Å^2 at 30 mol % cholesterol, with the assumptions that cholesterol is not compressible and that the area for cholesterol (26.6 Å^2) does not change. It has been calculated that, at 35°C and 30 mol % cholesterol, the area per DMPC acyl chain is about equal to the area of one cholesterol molecule (24). At 30 mol % or higher, the cross-sectional area per DMPC acyl chain should be further reduced, since the ordering effect of cholesterol on phospholipids persists over a wide range of cholesterol concentrations. Thus, 30 mol % is a breakpoint in terms of the relative cross-sectional area of cholesterol with respect to the acyl chains of DMPC at 35°C. Below and at 30 mol % cholesterol, each cholesterol molecule occupies one hexagonal lattice point. Above 30 mol % cholesterol, each cholesterol molecule may need to occupy two hexagonal lattice points in order to fit into the DMPC matrix where each DMPC acyl chain occupies one lattice point (Fig. 2*C*). It is reasonable to assume that the lateral distribution of dehydroergosterol behaves similarly to that of cholesterol because dehydroergosterol so closely resembles cholesterol in its physical and physiological properties (14). As such, Y'_{DHE}, the critical concentration of dehydroergosterol at which the DMPC acyl chains form hexagonal superlattices, is given by (18)

$$Y'_{DHE} = 1 - [2/(m_a^2 + m_a m_b + m_b^2)], \quad [2]$$

where m_a and m_b are the projections along the a and b axes, respectively, for an acyl chain of DMPC.

It follows from Eq. 2 that 33 mol % and 50 mol % are two of the Y'_{DHE} values. Thus, the dips observed at 33 mol % and 51 mol % (Fig. 1) can also be understood in terms of lipid regular distribution into a hexagonal superlattice. A square lattice model fails to explain the dips at 33 and 51 mol %. The only model which explains the dips at 33 and 51 mol % requires the assumption that dehydroergosterol occupy two hexagonal lattice points. In this case, the sterol molecule does not fill all the space of two lattice points. Rather, some significant void space is associated with the sterol (Fig. 2*C*). Hence, above 30 mol %, a perfect super lattice is not warranted in the regular regions. This may explain why the DHE dip at >30 mol % is broad and shallow. Above 30 mol %, the increased void space with increasing sterol content

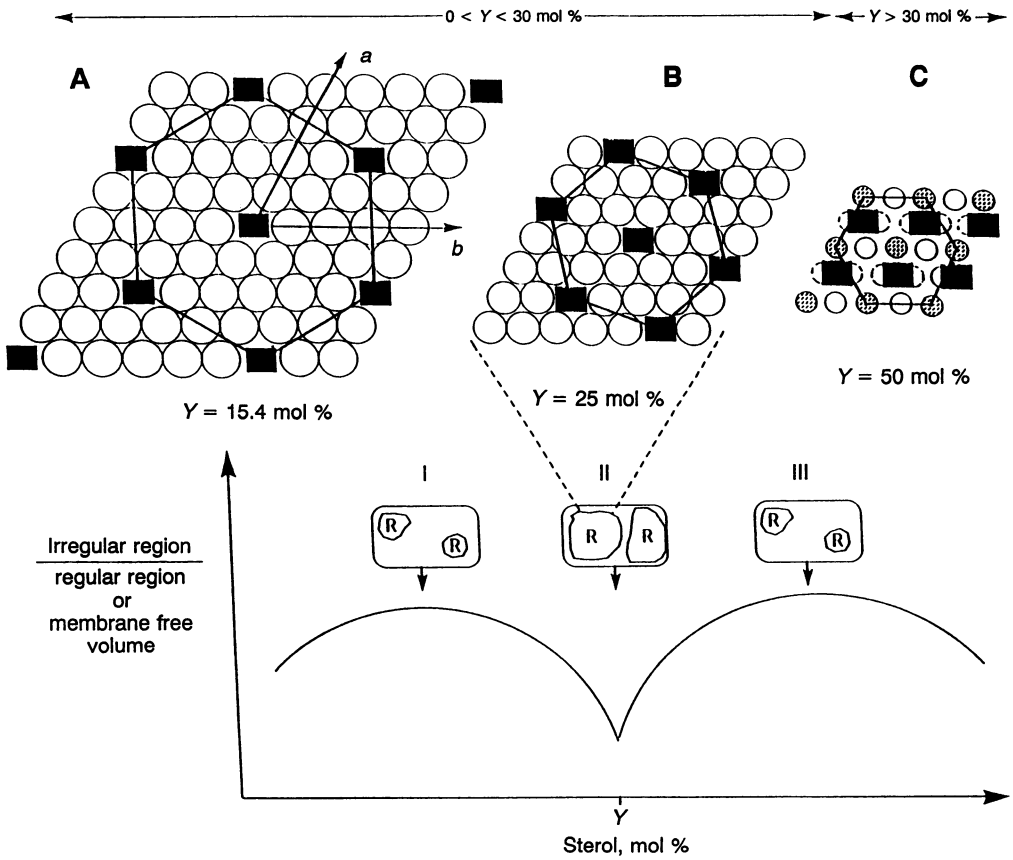

FIG. 2. (*Lower*) Schematic diagrams (I–III) showing the coexistence of regular (labeled as R) and irregular (the blank areas between the regular areas) regions in the liquid crystalline state of sterol/phosphatidylcholine bilayers in the vicinity of a critical sterol mole fraction *Y*. The curve and the diagrams I–III indicate schematically that, at *Y*, the size of the irregular regions relative to the regular regions (20), as well as the membrane free volume (21), reaches a local minimum. (*Upper*) The lateral organization within the regular region at a critical mole fraction *Y* is illustrated by 15.4 (*A*), 25.0 (*B*) and 50.0 (*C*) mol % sterol in DMPC. Open circles represent DMPC acyl chains. The size variation of open circles reflects the change in the cross-sectional area due to the condensing effect of sterols on the lipid acyl chains. Dark blocks represent sterol molecules, which presumably have a constant cross-sectional area over the concentration range examined. At critical mole fractions below 30 mol % (*A* and *B*), sterol molecules are distributed into hexagonal superlattices (such lattices are shown by the solid lines). Above 30 mol % (*C*), each sterol occupies two lattice points, and the critical mole fractions are described by Eq. 2; here the stippled circles are the DMPC acyl chains participating in the super lattice, whereas the open circles are the acyl chains not participating. The dashed outlines indicate the void space created by a sterol molecule.

destabilizes vesicles, which explains why a lamellar structure is hard to maintain when the sterol content in phosphatidylcholine bilayers approaches 50 mol % (ref. 25 and references therein).

Fig. 3 summarizes the distribution of the predicted DHE dips over the concentration range of 0–50 mol %. Below 30 mol %, the dips appear according to Eq. 1. Above 30 mol %, the dips follow Eq. 2.

Similar peculiar fluorescence properties have been reported in phosphatidylcholine bilayers containing 1-palmitoyl-2-(10-pyrenyl)decanoyl phosphatidylcholine (PyrPC)

Table 1. Comparison between the observed DHE dips (within parentheses) and theoretically predicted critical sterol mole fractions, Y_{DHE}, where the sterol molecules are regularly distributed in a hexagonal super lattice

	Y_{DHE}, mol %				
n_b	$n_a = 1$	$n_a = 2$	$n_a = 3$	$n_a = 4$	$n_a = 5$
0			20.0 (20.2)	11.8 (11.8)	7.7 (8.0)
1		25.0 (25.0)	14.3 (14.3)	9.1 (9.3)	
2		15.4 (15.4)	10.0 (10.0)		

Dips at <8 mol % or >30 mol % are not considered here. The two integer coordinates n_a and n_b describe the position of a dehydroergosterol molecule in the hexagonal lattice where *a* and *b* are the two principal axes (Fig. 2).

(18, 21). In a previous study (18), many dips were detected in the plot of *E/M* (the ratio of excimer to monomer fluorescence intensity) vs. the mole fraction of PyrPC in DMPC. The appearance of *E/M* dips was interpreted as the result of lipid regular distribution (18, 26). Both the pyrene moiety of PyrPC and the tetracyclic ring of dehydroergosterol are bulky and rigid. Thus, the similarity in the behavior of lipid lateral organization between PyrPC/DMPC and dehydroergosterol/DMPC seems to suggest a general phenomenon. That is, guest lipid molecules with a cross-sectional area significantly larger (or smaller) than the cross-sectional area of the acyl chain in the matrix lipid tend to maximally separate and form regularly distributed patterns.

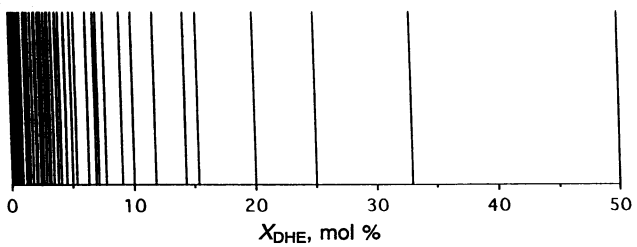

FIG. 3. Distribution of the predicted DHE dips in dehydroergosterol/DMPC mixtures over the concentration range 0–50 mol %.

It should be stressed that in the liquid crystalline state of dehydroergosterol/DMPC or PyrPC/DMPC mixtures, regular and irregular distributions always coexist (diagrams I–III in Fig. 2; refs. 18 and 20). Irregular distributions are the lateral organizations where a regular pattern cannot be recognized (20). A perfect superlattice arrangement through the entire membrane is unlikely to occur under ambient conditions, because of thermal fluctuations, the presence of impurities, and/or variations in membrane curvature. The coexistence of regular regions with irregular regions explains why E/M does not go to zero at critical PyrPC mole fractions (18). Several other physical principles underlying lipid regular distribution have been revealed via the studies of E/M dips in the PyrPC/DMPC system as a function of temperature, pressure, and vesicle diameter (21). It was found that membrane free volume is less abundant at critical PyrPC mole fractions than at noncritical mole fractions (ref. 21; bottom curve in Fig. 2). This result is in parallel with results obtained from computer simulations (20) which indicate that the ratio of regular region to irregular region reaches a local maximum at critical PyrPC mole fractions and a local minimum between two neighboring critical mole fractions (Fig. 2 *Lower*). Moreover, the existence of a regular pattern relies on the balance between the energy minimization due to the maximal separation of the bulky sterol rings and the entropy-driven randomization. The balance breaks down at high temperature, where the entropy-driven randomization prevails (21).

These physical principles should apply to sterol/DMPC mixtures, since sterols also have a bulky ring structure. Like the E/M dips of PyrPC, the DHE dips appear favorably in the liquid crystalline state of the matrix lipid (unpublished results). The occurrence of regular distributions in the liquid crystalline state is of biological significance, as most biomembrane lipids are in the fluid state at physiological conditions.

Unlike PyrPC, dehydroergosterol is not known to form excimers at the concentration levels used in this study. Then why does the dehydroergosterol fluorescence intensity decrease at critical mole fractions? It can be postulated that the vertical position of sterols in the regular region is different than in the irregular region. In the regular region, sterol molecules may be embedded somewhat less deep into the bilayer because the regular region contains less membrane free volume. As a result, sterol molecules in the regular region experience a higher dielectric constant than sterol molecules in the irregular region. Since dehydroergosterol fluorescence decreases with increasing dielectric constant of the medium (27), the fluorescence intensity of dehydroergosterol drops at critical mole fractions where the size of the regular region reaches a local maximum (ref. 20; Fig. 2 *Lower*).

To summarize, I propose a model for the lateral organization of sterols in liquid crystalline phosphatidylcholine bilayers. This model includes the following essential elements. (*i*) Sterol molecules tend to be maximally separated and regularly distributed in a hexagonal lipid matrix. The physical origin for maximal separation is believed to be the long-range repulsive interaction between the bulky, rigid ring structure of sterol molecules (17, 20, 21). (*ii*) Due to thermal fluctuation, changes in vesicle curvature and the presence of impurities, irregular distributions always coexist with regular distributions (Fig. 2) (7, 20, 21). (*iii*) The ratio of regular distributions to irregular distributions reaches a local maximum at critical sterol mole fractions and reaches a local minimum between two consecutive critical sterol mole fractions (20) (Fig. 2). The critical sterol mole fractions can be predicted from Eqs. 1 and 2 on the basis of the extended hexagonal superlattice model (18). (*iv*) The sterol disposition in the regular region is different from the sterol disposition in the irregular region. (*v*) Membrane defect is more abundant in irregular regions than in regular regions (25). Since there are many critical mole fractions over a wide range of sterol concentrations, membrane defect varies with sterol mole fraction in a periodic manner, with a local minimum in membrane defect at critical sterol mole fractions. This behavior is consistent with the direct volume measurement made by Melchior *et al.* (28), who showed several local minima and maxima in the plots of partial specific volume vs. mole fraction of cholesterol in the liquid crystalline state of dipalmitoyl phosphatidylcholine.

The above model is not incompatible with the models of aligned sterol domains (7) or homogeneous dispersions (8). In my model, both regular and irregular regions coexist. In the regular regions, sterols are aligned (Fig. 2 *A–C*) and may be homogeneously distributed (<30 mol %; Fig. 2 *A* and *B*). Although my model contains regular distributions, it does not preclude the presence of randomly distributed domains or randomly distributed individual sterol molecules, which are allowed in the irregular regions.

This study provides evidence that a naturally occurring sterol can be regularly distributed into hexagonal superlattices in the liquid crystalline state of phosphatidylcholine bilayers. The above model proposes that the lateral organization of sterol/phosphatidylcholine mixtures is modulated by minute changes in lipid composition (e.g., 1–3 mol %) on either side of a critical mole fraction. Slight concentration deviations on either side of Y_{DHE} or Y'_{DHE} cause an increase in the ratio of irregular to regular areas (20) and an increase in membrane free volume (21). As such, certain membrane functions, especially those requiring free volume for their normal activities, should be modulated by slight variations of sterol content on either side of a critical mole fraction. This level of modulation represents a "local" cholesterol concentration effect which may explain why some cellular activities can be significantly altered by small amounts of cholesterol, though the effects cannot be interpreted in terms of gross changes of membrane fluidity (29).

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