Evidence for a Regular Distribution of Cholesterol in Phospholipid Bilayers from Diphenylhexatriene Fluorescence

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ABSTRACT Cholesterol/dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles were studied by steady-state fluorescence using diphenylhexatriene (DPH) as a probe. A series of dips were found in the plot of DPH fluorescence intensity versus cholesterol concentration at certain specific cholesterol concentrations. This observation indicates that there are dominant domains in which cholesterol molecules are regularly distributed on a hexagonal superlattice in the acyl chain matrix of DMPC at critical cholesterol concentrations. These concentrations can be predicted by an equation or a mathematical series, except the one at 33 mol %. These dips of DPH fluorescence intensity are temperature dependent. The excellent agreement between experimental data and calculated values as well as similar previous findings of dips and/or kinks in the excimer-over-monomer fluorescence in pyrenephosphatidylcholine/phospholipid mixtures confirm our conclusion about lateral organizations of cholesterol and acyl lipid chains in cholesterol/phospholipid multilamellar vesicles. The regular distribution model at critical concentration is consistent with the phase diagram of cholesterol/DMPC. Using the model of regular distribution, the physical origin of the liquid-disordered (L_d) phase, liquid-ordered phase (L_o), and coexistence of liquid-disordered phase and L_o phase (L_o + L_d) is discussed on the molecular level.

INTRODUCTION

Cholesterol is a major lipid component of many biological membranes. Its lateral distribution and organization in membranes has been the subject of much speculation (see review by Yeagle, 1985). The goal of further study of cholesterol-phospholipid interactions is to obtain a deeper understanding of the function of cholesterol and of its distribution in membranes. It is necessary to study model systems such as cholesterol/dimyristoylphosphatidylcholine (DMPC) or cholesterol/dipalmitoylphosphatidylcholine (DPPC), because of the complexity of biological membranes. Even in such relatively simple model systems only containing two components, a complicated phase diagram has been revealed (Lentz et al., 1980; Vist and Davis, 1990; Tamplé et al., 1991; Almeida et al., 1992).

In the studies of cholesterol/phospholipid membranes a variety of stoichiometries have been suggested where physical and dynamic properties exhibit abrupt changes. Such critical concentrations have been found at 30–33 mol % cholesterol in phospholipid using x-ray diffraction (Engelman and Rothman, 1972; Lecuyer and Dervichian, 1969), small angle neutron scattering (Mortensen et al., 1988), lateral diffusion coefficient measurements (Almeida et al., 1992), electron spin resonance (ESR) (Sankaram and Thompson, 1990a), fluorescence (Parasassi et al., 1994), transport experiments (Tsong, 1975), nuclear magnetic resonance (NMR) (Darke et al., 1972), and calorimetry (Melchior et al., 1980; Hinz and Sturtevant, 1972; De Kruyff et al., 1974). The drastic changes around 20 mol % cholesterol have been extensively explored using x-ray diffraction (Hui and He, 1983), differential scanning calorimetry (DSC) (Estep et al., 1984; Mabrey et al., 1978), dilatometry (Melchior et al., 1980), fluorescence (Lentz et al., 1980), spin-label ESR (Recktenwald and McConnell, 1981; Presti and Chan, 1982), freeze-fracture electron microscopy (Copeland and McConnell, 1980; Kleemann and McConnell, 1976; Lentz et al., 1980), and NMR (Opella et al., 1976; Vist and Davis, 1990). Forty and 50 mol % cholesterol also are peculiar points and were studied by means of anisotropy of fluorescence (Lentz et al., 1980) and DSC (Mabrey et al., 1978). In addition, 5, 6, 15, and 25 mol % cholesterol also revealed unusual behavior in spectra of fluorescence (Lentz et al., 1980; Parasassi et al., 1994), NMR (Vist and Davis, 1990), and ESR (Sankaram and Thompson, 1990a).

There is reasonable agreement on the phenomenological description of cholesterol/phospholipid mixtures, but there is no consensus on the interpretation of data in terms of specific structure models. A key question of biological relevance is whether cholesterol in membranes is distributed randomly, regularly or in domains. Many experiments have demonstrated that cholesterol is not distributed homogeneously in cell membranes. In some cases, cholesterol is segregated in cholesterol-rich and -poor domains in the lateral plane of the membranes (Curtain et al., 1988; Schroeder et al., 1987, 1988, and 1991). Recent experimental results have revealed the coexistence of two immiscible fluid phases in phospholipid bilayers containing cholesterol (Vist and Davis, 1990; Sankaram and Thompson, 1990a, 1990b, 1991; Tamplé et al., 1991). From geometrical considerations, Hyslop et al. (1990) proposed an "ordered bimolecular mesomorphic lattice" concept that depicted each sterol with four dioleoylphosphatidylcholine (POPC) molecules as nearest neighbors.

Received for publication 31 October 1994 and in final form 17 February 1995.

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0006-3495/85/05/1944/08 $2.00

Engelman and Rothman (1972) stated that at a molar ratio of 1:2 (cholesterol/phospholipid), each cholesterol molecule is surrounded by seven acyl chains of phospholipid molecules. However, very little evidence is available for a regular distribution of cholesterol in phospholipid bilayers.

In this work we have utilized the fluorophore diphenylhexatriene (DPH), which has a marked environmental sensitivity and a geometrical similarity with acyl chains as well as cholesterol. The purpose of our studies was to investigate the lateral distribution of cholesterol molecules among the phospholipid acyl chains and the cholesterol-phospholipid interactions. In the cholesterol-containing vesicles, DPH probe properties roughly reflect the average properties in the coexisting environments (Lentz et al., 1980). Moreover, the rigid rod-shaped DPH also facilitates adopting a lateral behavior in a way similar to that of cholesterol in the bilayer (D. Tang, unpublished results), so that reliable information on the lateral distribution of cholesterol can be obtained with this probe.

Our experiments showed that there is a series of dips in the plot of DPH fluorescence intensity versus cholesterol concentration at specific cholesterol concentrations. By analyzing geometric patterns, we discovered that the cholesterol molecules are regularly distributed within the acyl chain matrix of DMPC on a hexagonal superlattice above the DMPC phase transition temperature at critical concentrations below 50 mol % cholesterol. These critical concentrations of cholesterol can be accurately predicted by an equation or a mathematical series. These dips in DPH fluorescence intensity are sensitive to temperature, which reflects the dependence of the lateral organization of cholesterol and phospholipid on temperature. Moreover, the understanding of regular distribution of cholesterol gives us insight into the physical origin of the liquid-disordered (L_d), liquid-ordered (L_o), and coexistence ($L_o + L_d$) phases.

MATERIALS AND METHODS

Materials

DPH was purchased from Molecular Probes (Eugene, OR) and was purified by a recrystallization method. DMPC was purchased from Avanti Polar Lipids (Alabaster, AL) and was used without further purification. Cholesterol was obtained from Fisher Scientific (Cincinnati, OH) and further purified by recrystallization. Cholesterol was carefully weighed, and then dissolved in chloroform to produce a stock solution. Chloroform solutions of DPH, cholesterol, and DMPC were prepared and stored at −20°C. The concentration of DPH was determined using an extinction coefficient of 91,000 M^{-1} cm^{-1} at 350 nm in methanol. The phospholipid concentration was determined as inorganic phosphate by the method used by Bartlett (1959).

Preparation of liposomes

Appropriate proportional amounts of DMPC, cholesterol, and DPH dissolved in chloroform were homogeneously mixed in Eppendorf vials, in which the amounts of DMPC and DPH were kept constant and the amount of cholesterol was varied only by the desired mixing ratio of cholesterol and DMPC. The mixed solution was dried first under nitrogen, and then under vacuum overnight. The dried mixtures were suspended in 0.05 M KCl solution at pH 7.5. The dispersion was vortexed for 2 min at 38°C to form multilamellar vesicles (MLVs). The vesicles were cooled to 4°C for 30 min and then placed at 38°C for 30 min. This cooling/heating cycle was repeated three times. After the samples were incubated at 4°C under nitrogen for about 12 h, the samples were stored at room temperature at least 8 h before fluorescence measurements were made.

Fluorescence measurements

Fluorescence intensity measurements were made with an SLM 8000 fluorometer (SLM Instruments, Urbana, IL). Samples were excited at 350 nm with 4-nm bandpath. The emission was observed at 450 nm through a monochrometer with 8-nm bandpath. The concentration of lipids used for fluorescence measurements was about 2–3 × 10^{-5} M. The ratio of DPH to DMPC was 1:500. To strengthen the comparability of data from different samples with variable concentrations, we prepared many samples with a range of cholesterol concentrations at the same time covering at least two dips in a certain concentration region. For example, a group of samples was prepared in which the concentrations of cholesterol ran from 26 to 54 mol % exhibiting three dips (at 33, 40, and 50 mol % cholesterol). This procedure ensured that all samples in one group had exactly the same thermal history, so that the reliability of comparison of relative intensities was increased.

RESULTS

Fluorescence intensity curves of DPH were measured in cholesterol/DMPC MLVs with cholesterol concentrations varying from 3 to 54 mol %. Fig. 1 shows the plots of fluorescence intensity of DPH versus cholesterol concentration at 24°C (*A*), 35°C (*B*), 45°C (*C*), and 55°C (*D*). All experiments were repeated at least three times, and the typical error bars are shown in Fig. 3. The data of fluorescence intensities were normalized to the fluorescence intensity of a sample containing 18 mol % cholesterol in DMPC MLV at 24°C.

The contour of DPH fluorescence intensity versus cholesterol concentration

Fig. 1 *A* clearly shows that the fluorescence intensity of DPH deviated significantly from a simple monatomic behavior. At 24°C, the contour of the intensity can be divided into three parts: between 3 and 10 mol % cholesterol (the low cholesterol region) the intensity is low; from 10 to 28 mol % (the intermediate cholesterol region) the intensity rapidly increases and reaches a maximum; beyond 28 mol % (the high cholesterol region) the intensity decreases again.

The regular drops of fluorescence intensity of DPH at specific cholesterol concentrations

The most interesting feature of Fig. 1 is that a number of sharp minima in fluorescence intensity–we call them "DPH dips"–were found at specific concentrations of cholesterol. The positions of these dips (except 33 mol % dip) are summarized in Table 1. The shape of the dips varies notably with cholesterol concentration. At 3–10 mol % cholesterol the dips are sharp but shallow (see Fig. 2); between 10 and 28 mol %, the dips are very sharp and deep; above 28 mol % the dips become broad and shallow (see Fig. 1 *A*).

In Fig. 3, we show a typical dip around 20 mol % cholesterol with standard deviations representing the errors resulting from three independent groups of samples. What

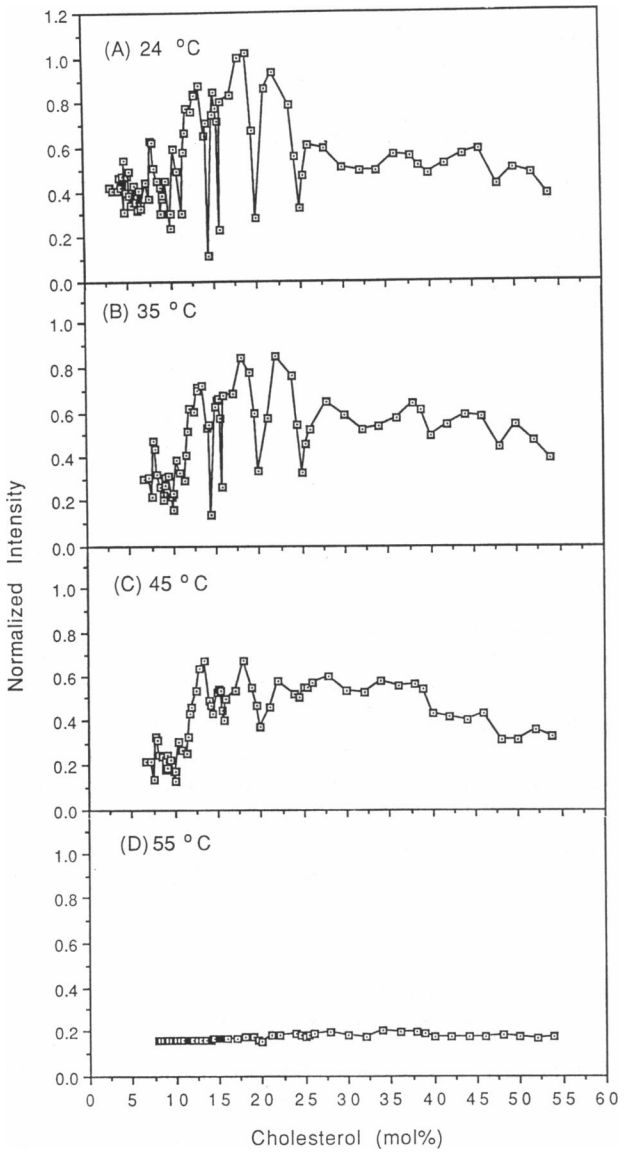

FIGURE 1 DPH fluorescence intensity as a function of cholesterol concentration in cholesterol/DMPC MLVs at (*A*) 24°C, (*B*) 35°C, (*C*) 45°C, and (*D*) 55°C. All intensity data were normalized to the value of the sample containing 18 mol % cholesterol at 24°C. Error bars are similar to the ones shown in Fig. 3.

should be emphasized is that the absolute values of the fluorescence intensity for each experimental point (or individual cholesterol concentration) is not the most important in our experiment, because the fluorescence intensity of DPH in the liposome is affected by oxygen and thermal history. Crucial is that the dips are reproducible, i.e., the unusual drops of fluorescence intensity at critical cholesterol concentrations can be observed in different groups of independently prepared samples by comparing the intensities of neighboring concentrations.

The effect of temperature on DPH dips

The dependence of the depth of dips on temperature was studied. The definition of the depth of a dip can be found in a previous paper (Chong et al., 1994). The dips are discernible above the phase transition temperature of DMPC. The trend of the dip depth versus temperature is qualitatively presented in Fig. 4, in which the depth of all dips were normalized to the depth of the dip at 20 mol % cholesterol at 24°C. With increasing temperature, the depth of the dips below 28 mol % cholesterol becomes smaller, and most of the dips disappear at 55°C. It should also be noted that the three dips above 28 mol % cholesterol (33, 40, and 50 mol %) are clearly broadening with increasing temperature, as shown in Fig. 1.

TABLE 1 Comparison between the observed DPH dips (within parentheses) and theoretically predicted critical cholesterol mole fractions for 10 cases where the cholesterol molecules are regularly distributed on hexagonal superlattices

	n_a				
n_b	1	2	3	4	5
0		40.0 (40.0)	20.0 (20.0)	11.8 (11.4)	7.7 (7.5)
1	50.0 (48.0)	25.0 (25.0)	14.3 (14.5)	9.1 (9.0)	
2		15.4 (15.4)	10.0 (10.0)		

The two integer coordinates, n_a and n_b, describe the position of a cholesterol molecule in the hexagonal lattice where a and b are the two principal axes (Fig. 5).

DISCUSSION

Virtanen et al. (1988) showed that the formation of regular two-dimensional crystal-like structures by the embedded guest molecules in phospholipid membranes appears to be possible at certain molar proportions of the guest.

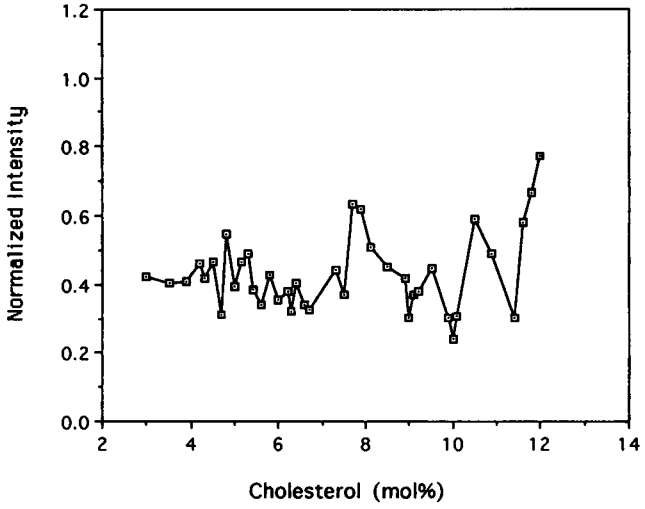

FIGURE 2 Enlarged plot of DPH fluorescence intensity versus cholesterol concentration from 3 to 12 mol % at 24°C. The normalization of the data is the same as in Fig. 1. Error bars are similar to the ones shown in Fig. 3.

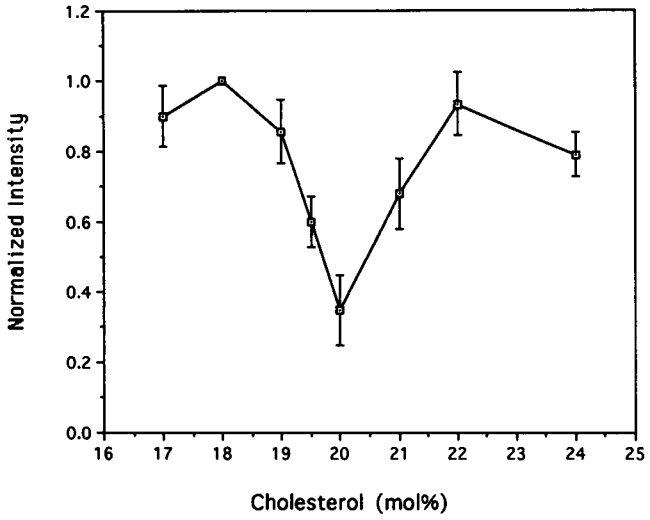

FIGURE 3 Typical plot of the standard deviation around 20 mol % cholesterol dip at 24°C, which results from three independent groups of samples. All data were normalized to the value at 18 mol % cholesterol.

For the phospholipid molecules with two acyl chains the concentrations, Y_d, at which the labeled acyl chains form hexagonal lattice can be predicted by Eq. 1 (Virtanen et al., 1988).

$$Y_d = 2/(n_a^2 + n_a n_b + n_b^2) \quad (1)$$

where n_a and n_b are non-negative integers, and (n_a,n_b) represents the position of a labeled acyl chain in a two-dimensional plane with respect to another labeled chain at the origin. The first integer, n_a, is the coordinate along the x axis or a axis; the second integer, n_b, is the coordinate along the b axis, which makes a 60° angle with the a axis (see Fig. 5, below).

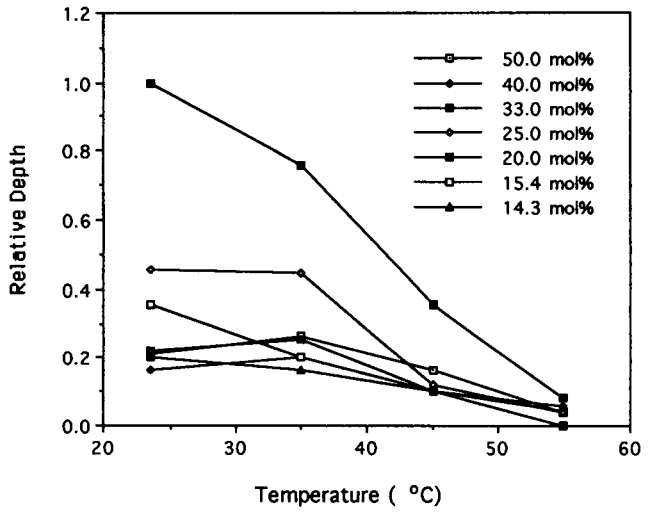

FIGURE 4 Effect of temperature on the depth of dips in cholesterol/DMPC MLVs at the following mol %: 50.0 (*open squares with dark points*), 40.0 (♦), 30.0–33.0 (*filled squares with open central points*), 25.0 (◇), 20.0 (■), 15.4 (□), and 14.3 (▲). All data are normalized to the depth of dip at 20 mol % at 24°C.

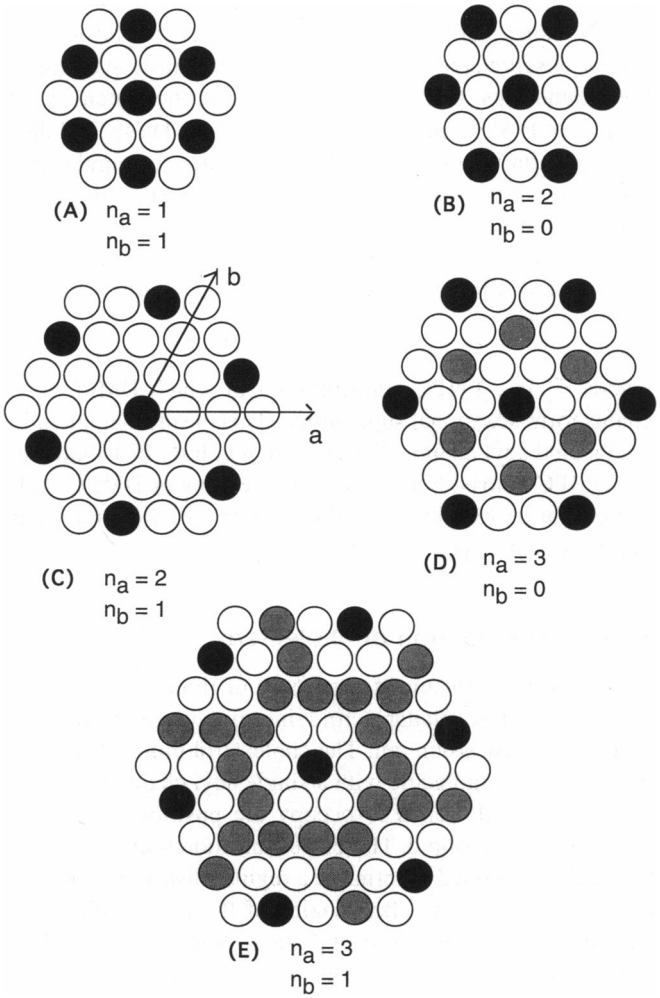

FIGURE 5 Schematic diagrams for some regular distribution patterns of cholesterol in DMPC. The black circles represent the cholesterol molecules, and the other circles represent acyl chains of DMPC molecules: the white and gray circles represent acyl chains having nearest and next-nearest neighboring cholesterol molecules, respectively. The regular patterns of cholesterol:DMPC are (*A*) 50:50 ($n_a = 1$, $n_b = 1$), (*B*) 40:60 ($n_a = 2$, $n_b = 0$), (*C*) 25:75 ($n_a = 2$, $n_b = 1$), (*D*) 20:80 ($n_a = 3$, $n_b = 0$), and (*E*) 14.3:85.7 ($n_a = 3$, $n_b = 1$). Here a and b are the principal coordinate axes, and n_a and n_b are the a and b coordinates of one of the six cholesterol molecules with respect to the center, which is also occupied by a cholesterol molecule. The unit is the distance between two neighboring chains.

For the phospholipid molecules with only one labeled chain the critical concentrations, Y_d, can be predicted by Eq. 2 (Virtanen et al, 1988).

$$Y_s = 2/(n_a^2 + n_a n_b + n_b^2 + 1) \quad (2)$$

Somerharju et al. (1985) found that pyrene-labeled acyl chains of phospholipid molecules are regularly distributed in DPPC and egg-phosphatidylcholine (egg-PC) vesicles. Kinnunen et al. (1987) observed the formation of regular distribution patterns of 1-palmitoyl-2-(10-pyrenyl)decanoyl-*sn*-glycerol-3-phosphatidylcholine (PyrPC) in Langmuir-Blodgett films of DPPC and PyrPC. In their experiments, they determined the critical concentrations by the discontinuity (kinks) in the plot of excimer-over-monomer fluores-

cence (E/M) versus PyrPC concentration. Tang and Chong (1992), using a slightly different method of preparing liposomes, observed two new phenomena. First, instead of kinks, they found dips, sharp minima of fluorescence intensity, in the plot of E/M versus PyrPC concentration. These E/M dips are more convincing than kinks as evidence for regular distribution of host or guest chains on a hexagonal lattice. Second, in addition to the E/M dips predicted by Eq. 1, they also found some E/M dips, and the concentration locations of these dips can be expressed by another equation:

$$Y'_d = 1 - 2/(n_a^2 + n_a n_b + n_b^2) \quad (3)$$

where Y'_d is a PyrPC concentration in which DMPC acyl chains form hexagonal superlattice. This means that not only the labeled acyl chains of PyrPC are regularly distributed in the DMPC matrix, but also that acyl chains of DMPC molecules can be regularly distributed in the PyrPC molecular matrix at some specific concentrations.

Regular distribution of cholesterol

A cholesterol molecule is composed of three parts: a polar head group, a rigid planar steroid ring structure, and a flexible nonpolar hydrocarbon tail. The cholesterol molecule can be thought of as a molecule with a single acyl chain; each cholesterol molecule tends to occupy the position of an acyl chain of a phospholipid. Therefore, our fluorescence data can be readily explained in terms of a regular distribution model proposing that 1) the alkyl chains of the phospholipid are situated on a triangular host lattice (Rucco and Shipley, 1982), and 2) the guest elements (cholesterol molecules) tend to be maximally separated to minimize the total free energy. Based on these two conditions, a geometry model can be built, in which cholesterol molecules form hexagonal superlattices in the matrix of DMPC acyl chains. For a given cholesterol molecule, its position in the hexagonal lattices can be described by two integer coordinates (n_a,n_b), once the origin and the principal axes are specified, where n_a and n_b are the numbers of translation steps in the lattice along the two principal axes a and b, respectively. Then, the critical cholesterol mole fraction, $Y_{cholesterol}$, at which the cholesterol molecules are regularly distributed into hexagonal super lattices, can be calculated from Eq. 2 or can be read from the triangular series for Y_s (see Appendix). The calculated critical values are listed in Table 1.

By comparing calculated values with experimental data (between parentheses in Table 1), it was found that from 5 to 54 mol % cholesterol, these calculated values from Eq. 2 are surprisingly consistent with experimental data. Below 5 mol % cholesterol, the calculated values are too dense to be identified experimentally.

In Fig. 5, some typical patterns of regular distribution are shown. In this kind of lateral distribution pattern, the cholesterol molecules are maximally separated by acyl chains of phospholipid, and each cholesterol molecule is surrounded by six acyl chains of phospholipid. At the same time, the regular distribution pattern also reveals that there is a long-range order phenomenon of arrangement of cholesterol molecules in liposomes containing two components.

Of course, all of the above models are based on ideal construction. In reality, deviations will occur. Even in a solid crystal, in which all atoms vibrate around their equilibrium positions, the spatial lattice patterns could not be exactly like the ones depicted in Fig. 5. In fluid phases, all molecules are in lateral motion, and the existence of the thermal fluctuation, impurities, and defects also should not be ignored. Therefore, the models in Fig. 5 only represent time-averaged arrangements of cholesterol and phospholipid molecules in the domains of the membranes.

Based on the above considerations, a coexistence of irregular domains at critical concentrations and regular domains at critical concentrations is pronounced. The coexistence of different types of regular and irregular distributions was found by Monte Carlo simulations (Sugar et al., 1994) in a binary mixture system. However, the areas occupied by regular arrangements are maximal at critical concentrations, and irregular areas are maximal at noncritical concentrations. Therefore, we propose that in our experimental system there are two kind of domains of regular and irregular distributions, and the proportion of the area occupied by regular and irregular domains is altered with the cholesterol concentration. Some irregular domains described in this paper are either cholesterol-rich or cholesterol-poor domains that also were found in experiments and theoretical studies (Curtain et al., 1988; Cruzeiro-Hansson et al., 1989; Schroeder et al., 1987, 1988, and 1991).

The hexagonal superlattice originates from long-range repulsive interaction between guest molecules (Sugar et al., 1994); it is the result of an optimized molecular organization, where the free energy of the membrane is minimized exhibiting the most stable structure.

It should be noted that the 33 mol % cholesterol concentration where a peculiar fluorescence quenching was observed is not included in the Table 1. However, many experiments have found discontinuities in physical properties at this critical point (see Introduction). According to a virtually complete experimental phase diagram for the cholesterol/DMPC system (Tamplé et al., 1991; Almeida et al., 1992) and for cholesterol/DPPC mixtures (Vist and Davis, 1990; Sankaram and Thompson, 1990a,b, 1991) obtained using NMR, DSC, and ESR techniques, above the melting temperature of DMPC, this system can exist in one of two possible liquid phases: an L_d phase, at low cholesterol concentrations; and an L_o phase, at high cholesterol concentrations; or these two phases can coexist at the same temperature and pressure (L_d + L_o). The boundary of the L_d + L_o coexistent phase and the L_o phase lies around 29–33 mol % cholesterol (Almeida et al., 1992). Therefore, it is supposed that the dip near 30 mol % dip could be attributed to boundary effects of phases, because quenching behavior can be influenced by partitioning of a fluorophore between phases giving different quantum yields (Yeager and Feigenson, 1990). The molecular organization in the membranes at this specific concentration still remains uncertain.

In this paper, we extended the experimental results of Lentz et al. (1980). They also observed an unusual drop of DPH fluorescence intensity around 22 mol % cholesterol in cholesterol/DPPC and cholesterol/egg-PC MLV labeled with DPH. They did not observe more dips in their experiments, probably because they employed large concentration intervals of cholesterol (about 5–10 mol %). In addition, they observed several noticeable inflections in their anisotropy data. They determined phase boundaries by means of these inflection points.

Explanation of the cholesterol/DMPC phase diagram on the molecular level

Above the DMPC phase transition temperature, there are three main phase regions in cholesterol/DMPC mixtures (Tamplé et al., 1991; Almeida et al., 1992): an L_d phase, at low cholesterol concentrations; an L_o phase, at high cholesterol concentrations; and a coexistence phase (L_d + L_o), at intermediate concentrations. By the use of hexagonal patterns of cholesterol distribution, an explanation of the phase diagram of cholesterol/DMPC mixtures can be obtained on the molecular level. The interaction between hydrocarbon chains is inversely proportional to the fifth power of the interchain distance (Nagle and Wilkinson, 1978). This is a short-range attractive interaction (Van der Waals interaction). The Van der Waals interaction between a hydrocarbon chain and a cholesterol molecule should follow the same power law, but with a greater proportionality constant. Considering the comparable size of cholesterol and a DMPC chain, it is reasonable that only nearest and next-nearest neighboring cholesterol molecules are considered in the following discussion on the "ordering effect" of cholesterol.

In the case of the liquid ordered phase (L_o), the strong lipid-cholesterol interaction can be experienced by each lipid hydrocarbon chain. For example, at 50 mol % cholesterol (n_a = 1, n_b = 1; see Fig. 5 *A*) and at 40 mol % cholesterol (n_a = 2, n_b = 0; see Fig. 5 *B*), each DMPC acyl chain has three and two nearest neighboring cholesterol molecules, respectively. This is the reason why all phospholipid acyl chains are in ordered state at high cholesterol concentrations. In the intermediate concentration region, i.e., L_d + L_o phase, several main critical concentrations can be found. These are 25, 20, 15.4, 14.3, 11.8, etc. mol % cholesterol. For the pattern of 25 mol % (n_a = 2, n_b = 1; see Fig. 5 *C*), each acyl chain has one nearest neighboring cholesterol molecule; but for 20 mol % (n_a = 3, n_b = 0; see Fig. 5 *D*) and 14.3 mol % (n_a = 3, n_b = 1; see Fig. 5 *E*), some acyl chains (the gray circles in Fig. 5) do not have any cholesterol molecules as nearest neighbors. Obviously, the order of these acyl chains is less than those directly surrounded by at least one nearest neighboring cholesterol molecule. In the pattern of other critical concentrations some acyl chains have neither nearest nor next-nearest neighboring cholesterol molecules. The ordering effect of cholesterol on them will be even weaker. Therefore, a coexistence of L_o and L_d phases is expected.

The situation is different in the low cholesterol concentration region. In this region, most acyl chains are located far away from cholesterol molecules; they are therefore beyond the scope of the ordering effect of cholesterol. For instance, in the hexagonal pattern of 5 mol % cholesterol (n_a = 5, n_b = 1), the ratio of acyl chains having some nearest neighboring cholesterol molecules to those having none is about 1:5. Even if the next nearest is considered, the ratio still is 1:2. This means that the majority of DMPC acyl chains is in the disordered state and the membrane is in the (L_d) phase above the phase transition temperature of DMPC.

The temperature dependence of dips

The shape of dips is sensitive to temperature. This suggests that the lateral distribution and organization of cholesterol and DMPC molecules are entropy driven. The different behaviors of the depth of dips with different temperatures in low, intermediate, and high cholesterol regions are probably attributable to different phase regions and reflects the interactions between cholesterol and phospholipid in different phase regions. Above 55°C most dips are less noticeable. This is the result of molecular lateral diffusion at high temperature. It implies that highly rapid movement of molecules can destroy the regular organization of cholesterol molecules in membranes.

The temperature dependence of the fluorescence contour

Another interesting phenomenon is the temperature dependence of the outline of fluorescence intensity of DPH versus cholesterol. At 24°C (Fig. 1 *A*) the contour of fluorescence intensity in the range of 3–55 mol % cholesterol is very similar to the results for the cholesterol/DPPC system labeled with DPH (Lentz et al., 1980) and for the dehydroergosterol (DHE)/phospholipid system (Schroeder et al., 1987; D. Tang and P. L.-G. Chong, unpublished results; Chong, 1994). The common characteristic is that the relative fluorescence intensity first increases with increasing mol % cholesterol or DHE until 10 mol % (our experiment) or 6 mol % (Schroeder et al., 1987). Then the fluorescence intensity exhibits a rapid increase and reaches a maximum. Above 28 mol %, this parameter gradually decreases until 54 mol % is reached. Therefore, three distinct regions of fluorescence intensity were found and roughly correspond to the three different phases: L_d, coexistence (L_d + L_o), and L_o phases. However, the three regions of fluorescence intensity became gradually less distinct with increasing temperature. Above 55°C, the contour of the fluorescence intensity is flattened, and the three regions can no longer be distinguished. This trend is in agreement with the cholesterol/DMPC phase diagram where the boundaries of L_d/L_d + L_o and L_d + L_o/L_o tend to be ambiguous (Almeida et al., 1992) or even disappear (Tamplé et al., 1991) above 55°C.

Schroeder et al. (1987) explained that the decrease in fluorescence intensity in the high DHE concentration region (L_o

phase) is the result of self-quenching. But this explanation cannot be used for the similar phenomenon we observed in cholesterol/DMPC mixtures labeled with DPH, because the DPH concentration was too low to lead to self-quenching. The quenching of fluorescence should have another source.

Because the quenching only reflects the hexagonal superlattice structure at certain cholesterol concentrations, we call it "geometry quenching" to distinguish it from other quenching phenomena caused by molecular collision and resonance energy transfer. In a previous paper (Tang and Chong, 1992), we explained the E/M dip as a maximum separation of PyrPC molecules, which leads to a difficulty of excimer formation. However, our results indicate that geometry quenching at critical concentrations is perhaps a much more common phenomenon for fluorophores in membranes. The principles behind this geometry quenching are not yet completely clear. One possibility is that the high order at critical cholesterol fractions could force the DPH molecules to segregate into domains, which would lead to self-quenching due to probe-probe interactions. To verify this idea an experiment was designed in which the ratio of DPH to DMPC was changed from 1/500 to 6/500 around 20 mol % cholesterol. If the above idea were true the depth of 20 mol % dip with 6/500 DPH in DMPC should be deeper than that with 1/500 DPH in DMPC. Nevertheless, the predicted result was not observed. We believe that the regular geometrical organization could have changed the microenvironment or vertical location of DPH in bilayers, and therefore the quenching of fluorescence was observed.

The regular distribution of cholesterol molecules at critical concentrations in DMPC MLVs is a very important finding. Cholesterol molecules form an essential component in biological membranes. The understanding of lateral distribution and organization of cholesterol molecules in membranes will supply a new clue to study the biological function of cholesterol molecules in membranes, although the real biomembranes are more complex than model membranes. It is possible that the discontinuity of structure will reveal a discontinuity in biological function with altering cholesterol content. The biological consequences of a possible long-range order in the lateral organization of cholesterol molecules on a superlattice requires further study as well.

APPENDIX

It is interesting to note that the critical mole fractions predicted by the hexagonal superlattice model (Eq. 1) form an interesting pattern that obeys the following triangular series:

$$Y_d:$$

$$\frac{2}{4}\quad \frac{2}{9}\quad \frac{2}{16}\quad \frac{2}{25}\quad \frac{2}{36}\quad \frac{2}{49}\quad \frac{2}{64}\quad \frac{2}{81}$$

$$\frac{2}{(4-1)}\quad \frac{2}{(9-2)}\quad \frac{2}{(16-3)}\quad \frac{2}{(25-4)}\quad \frac{2}{(36-5)}\quad \frac{2}{(49-6)}\quad \frac{2}{(64-7)}\quad \frac{2}{(81-8)}$$

$$\frac{2}{(16-3-1)}\quad \frac{2}{(25-4-2)}\quad \frac{2}{(36-5-3)}\quad \frac{2}{(49-6-4)}\quad \frac{2}{(64-7-5)}\quad \frac{2}{(81-8-6)}$$

$$\frac{2}{(36-5-3-1)}\quad \frac{2}{(49-6-4-2)}\quad \frac{2}{(64-7-5-3)}\quad \frac{2}{(81-8-6-4)}$$

$$\frac{2}{(64-7-5-3-1)}\quad \frac{2}{(81-8-6-4-2)}$$

$$Y_s:$$

$$\frac{2}{5}\quad \frac{2}{10}\quad \frac{2}{17}\quad \frac{2}{26}\quad \frac{2}{37}\quad \frac{2}{50}\quad \frac{2}{65}\quad \frac{2}{82}$$

$$\frac{2}{(5-1)}\quad \frac{2}{(10-2)}\quad \frac{2}{(17-3)}\quad \frac{2}{(26-4)}\quad \frac{2}{(37-5)}\quad \frac{2}{(50-6)}\quad \frac{2}{(65-7)}\quad \frac{2}{(82-8)}$$

$$\frac{2}{(17-3-1)}\quad \frac{2}{(26-4-2)}\quad \frac{2}{(37-5-3)}\quad \frac{2}{(50-6-4)}\quad \frac{2}{(65-7-5)}\quad \frac{2}{(82-8-6)}$$

$$\frac{2}{(37-5-3-1)}\quad \frac{2}{(50-6-4-2)}\quad \frac{2}{(65-7-5-3)}\quad \frac{2}{(82-8-6-4)}$$

$$\frac{2}{(65-7-5-3-1)}\quad \frac{2}{(82-8-6-4-2)}$$

Note that the top diagonal has the form $2/n^2$, where $n = 2, 3, 4$, etc., and that the numbers on the next diagonal fit $2/(n^2 - m)$, where n^2 is the numerator immediately above it and $m = 1, 2, 3, 4$, etc. The next diagonal is created similarly. This triangular series can be used as an alternative to Eq. 1.

By adding 1 to all the numerators in the triangular series above, we can create a similar series for Y^s. Note that the top diagonal has the form $2/(n^2 + 1)$, where $n = 2, 3, 4$, etc., and that the numbers on the next diagonal fit $2/(n^2 + 1 - m)$, where $n^2 + 1$ is the numerator immediately above it and $m = 1, 2, 3, 4$, etc. The next diagonal is created similarly. This triangular series can be used as an alternative to Eq. 2.

This work is supported by the National Science Foundation EPSCoR program (EHR-9108764).

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