Transport of 8-Anilino-1-naphthalenesulfonate as a Probe of the Effect of Cholesterol on the Phospholipid Bilayer Structures†

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ABSTRACT: The transport of 8-anilino-1-naphthalenesulfonate in dimyristoyl-L-α-lecithin bilayers has been found to be extremely sensitive to the crystalline state of the phospholipid dispersions. Thus this reaction may be used for probing the membrane structures. In binary mixtures of cholesterol and phospholipid the fluorescence enhancement of the dye completely disappears when the mole fraction of cholesterol reaches 33%. At temperatures below and above the phase transition of the lipid bilayers, the rate of the probe transport increases significantly in the binary mixtures. It reaches a maximum at 17 mol % of cholesterol. The rate at this cholesterol content approaches the maximum value obtained for the probe transport in pure phospholipid, i.e., the rate at the midpoint of the phase transition. These observations indicate that the effect of cholesterol in the phospholipid dispersion is to maintain the bilayer structure close to the melting temperature of the lipid phase transition. In other words, cholesterol may be an effective buffer for membrane crystalline state when its concentration is near 17 mol %.

Although cholesterol is an important component of biological membranes its precise function in the membranes is still unknown. Many of the recent studies of its effect on membranes have focused on the physical interactions of the cholesterol molecule with the phospholipid constituents (Chapman, 1973; Hinz and Sturtevant, 1972; Engelman and Rothman, 1972; Shimshick and McConnell, 1973; Huang et al., 1974; Oldfield and Chapman, 1971; Ladbrook et al., 1968; Lippert and Peticolas, 1971; Papahadjopoulos et al., 1973). Some important observations of these studies can be summarized as follows. Cholesterol seems to promote the liquid crystalline state of phospholipid bilayers even at temperatures well below the phase transition of the pure lipids (Chapman, 1973; Ladbrook et al., 1968). The endothermic transition of the phospholipid bilayers also completely disappears as the concentration of cholesterol reaches 33 mol % in the binary mixtures (Hinz and Sturtevant, 1972). That there is a critical change in the mixed bilayer structures at 33 mol % cholesterol has also been reported in the x-ray analysis (Engelman and Rothman, 1972; Lecuyer and Dervichian, 1971) and the nuclear magnetic resonance (NMR) studies of the binary dispersions (Chapman, 1973; Oldfield and Chapman, 1971).

In the preceding paper (Tsong, 1975) we have reported that a stopped-flow mixing of ANS with DML or DPL bilayers, in the phase transition regions of the lipid dispersions, has allowed us to detect a dye transport reaction which is especially sensitive to the crystalline state of the lipid structures. This reaction will now be used to probe the physical state of cholesterol–lipid binary mixtures. It will be shown that additions of cholesterol into the lipid bilayers tend to shift the crystalline state of the binary mixtures to the melting point (T_m) of the lipid phase transition, judged by its enhancement of the rate of the ANS transport reaction.

Materials and Methods

Cholesterol–DML Mixture. Two different methods were employed to prepare cholesterol–DML binary dispersions. In the first method suitable amounts of cholesterol were mixed with DML in a 0.05 *M* phosphate buffer (pH 7.0) containing 0.1 *N* NaCl. The suspension was thoroughly degassed and sonicated at 100-W power level, in a Biosonic IV sonicator, at 30° under constant nitrogen stream for 15 min. The solution was then incubated at 25° for at least 1 hr before use. This incubation period was found to be important for obtaining consistent kinetic result. In the second method suitable amounts of cholesterol and DML were dissolved in a milliliter of chloroform. After removing the solvent with a stream of nitrogen the residual chloroform was evaporated under oil pump vacuum. The dried mixture was then dispersed in the phosphate buffer and sonicated as described in the first method for 15 min. The samples prepared by the two different methods gave similar results. However, most data reported here were obtained with samples prepared by using the second method.

Other Procedures. All other experimental procedures and sources of chemicals are given elsewhere (Tsong, 1975).

Experimental Results

Kinetics of ANS Transport in the Binary Mixture of Cholesterol and DML. Addition of cholesterol in DML dispersions did not change the transition temperature of the DML bilayers when the 90° light scattering was monitored. However, the magnitude of the light-scattering changes was found to gradually decrease and completely disappear at 33 mol % cholesterol. The fluorescence enhancement of ANS in the lipid bilayers also reduced to zero as the cholesterol concentration reached the same level. These results confirm the observations of many authors that the endothermic phase transition of phospholipid disappears at this choles-

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[1] Abbreviations used are: DML, dimyristoyl-L-α-lecithin; DPL, dipalmitoyl-L-α-lecithin; ANS, 8-anilino-1-naphthalenesulfonate.

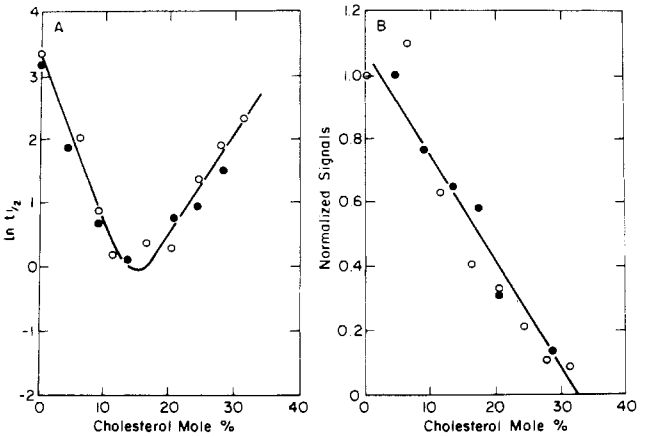

FIGURE 1: Binding of ANS to binary mixtures of cholesterol–DML dispersions at 21° ($<T_m$ = 24.1°). (A) The half-time of the ANS transport reaction as a function of the cholesterol contents in the binary mixtures. (B) The stopped-flow signals vs. the cholesterol contents. The signals measure the ANS fluorescence enhancements due to its binding to the lipid dispersions. Final conditions: 20 μM DML, 4 μM ANS, and appropriate amount of cholesterol in a 0.05 M phosphate buffer containing 0.1 N NaCl, at pH 7.0. (●) Data from samples prepared by the first method described under Materials and Methods. (○) Data from samples prepared by the second method.

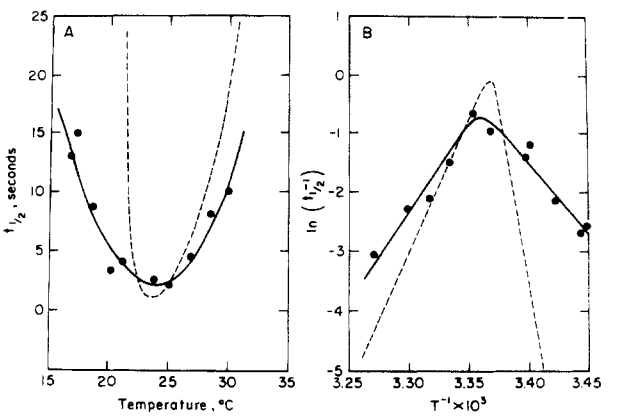

FIGURE 2: Effect of temperature on the transport of ANS in a binary mixture of cholesterol–DML. The mixture contains 20 mol % cholesterol. The experimental points are given in the filled circles. The dashed curves represent the results obtained for the pure DML dispersions, and are shown here for comparison. Final conditions: 20 μM DML, 5 μM cholesterol, and 4 μM ANS in a 0.05 M phosphate buffer containing 0.1 N NaCl, at pH 7.0.

terol concentration, and that there is a critical change of the bilayer structures at this point (Hinz and Sturtevant, 1972; Engelman and Rothman, 1972; Huang et al., 1974; Lippert and Peticolas, 1971).

In the stopped-flow experiments we have limited our study to the slow kinetic phase of ANS binding reactions (Tsong, 1975). This reaction is unimolecular and may represent a transport of the probe molecule across the bilayer structures. The stopped-flow signals of this reaction disappear at 33 mol % cholesterol both at 21° ($T < T_m$ = 24.1°, Figure 1B) and at 29° ($T > T_m$, data resembles Figure 1). The half-time of the reaction, $t_{1/2}$, also shows a strong dependence on the cholesterol contents (Figure 1A).

Instead of a linear dependence of $t_{1/2}$ on the cholesterol concentration of the binary mixtures up to 33%, as is seen in other physical properties, the value of $t_{1/2}$ decreases until 17 mol % and increases again beyond that point. The minimum $t_{1/2}$ occurs at about a half-way between 0 and 33%. An equally interesting observation in this experiment is that at 17% of cholesterol the rate of the ANS transport is comparable to that of the transport in pure DML bilayers at the phase transition temperature (24.1°). The increase in the rate is about 20-fold under these conditions.

The effect of cholesterol on the membrane structures may be different for temperatures above and below the phase transition point (Engelman and Rothman, 1972; Träuble, 1972). However, our data indicate that the effect of cholesterol on the transport of ANS in the binary mixtures is quantitatively similar at the temperatures both below and above the phase transition.

Effects of Temperature. The effect of temperature on the ANS transport in the pure DML dispersions was described in the preceding paper. The same type of experiment was carried out for a sample of cholesterol–DML mixture with a cholesterol concentration of 20 mol %. The result is given in Figure 2 in the filled circles. In Figure 2, the data obtained for the pure DML dispersion are also shown in the dashed lines for comparison. In both cases the minima in $t_{1/2}$ of the transport reaction fall at the same temperature, namely the T_m of the lipid phase transition (Figure 2A).

The Arrhenius plots (Figure 2B) indicate that the apparent activation energy of the transport reaction in the binary mixture is much smaller than that in the pure lipid system. In the first half of the phase transition ($T < T_m$ = 24.1°) E_a = 48 kcal/mol for the transport in the mixture compared to 240 kcal/mol in the pure dispersion. In the second half ($T > T_m$), E_a = −62 kcal/mol for the ANS transport in the mixture compared to −96 kcal/mol in the pure lipid system. These results suggest that molecular transport in a cholesterol-containing membrane may require much less activation energy than is required for the same transport in a non-cholesterol membrane system.

Discussion

The belief that the ANS transport reaction can be used for probing the bilayer structures of the cholesterol–lipid binary mixtures is based on the following facts. (1) The reaction is unimolecular and represents probably a transport of the probe molecule across the bilayer after its initial adsorption on the bilayer surface (Tsong, 1975). Hence the rate depends only on the physical state of the bilayer but not on the concentration of the available binding site. (2) No fluorescence enhancement of ANS was observed in a cholesterol suspension. Neither was the enhancement observed in the 1:2 binary mixture of cholesterol–phospholipid. Since no contribution to the kinetics of the probe transport would be expected from these domains, any change in rate must reflect a change in the overall structure of the binary mixture. These arguments can be best explained by the illustrations shown in Figure 3.

Consider a model given in Figure 3a (Träuble, 1972). If the cholesterol molecules cluster in the binary mixture the fluorescence enhancement of ANS would result only from its interaction with the pure lipid phase. In such a case kinetics of the probe transport would not be distinguishable from the kinetics of the transport in the pure lipid suspension. On the other hand, if the cholesterol molecules disperse into the entire mixture one would expect an overall change in the bilayer structures and hence an altered kinetic pattern for the probe transport reaction.

One may argue that the introduction of cholesterol clusters in the lipid bilayer may create new interfaces between the cluster phase and the lipid phase, and that ANS trans-

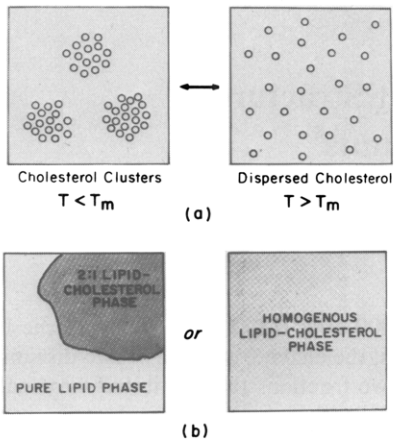

FIGURE 3: Some models proposed for the interactions of cholesterol in phospholipid bilayers. (a) A model which suggests that at temperatures below the critical point of the lipid phase transition, cholesterol clusters in the binary mixtures. At temperatures above the phase transition cholesterol disperses in the bilayers. (b) The model on the left suggests that for the binary mixtures containing less than 33 mol % cholesterol, there is a separation of phospholipid and cholesterol into two phases, one composed of pure lipid and the other composed of a cholesterol–lipid mixture, with a cholesterol content of 33 mol %. The model on the right is an alternative, which considers lipid and cholesterol totally miscible in the binary mixtures. These models are discussed, in connection with the observed kinetics of the ANS transport in the binary mixtures, in the text.

port may be faster in the newly created interfaces. In such a case, the rate of the probe transport would increase as the cholesterol content increases. Our kinetic data do not agree with such contentions (Figure 1A). Instead, the rate increases up to 17% cholesterol and decreases beyond that point. It appears that our ANS transport experiment favors the dispersed model (the right figure of Figure 3a) for the binary mixtures of cholesterol–DML dispersions over the entire temperature range studied (15–30°) (see Added in Proof).

The other model given in Figure 3b has been proposed by Engelman and Rothman (1972) to account for their x-ray diffraction study of the binary mixture. The model in essence predicts that in the binary mixture which contains less than 33 mol % of cholesterol there will be a separation of cholesterol and lipid into two phases, one composed of pure phospholipid and the other composed of both lipid and cholesterol with a molar ratio of 2:1 (the left figure of Figure 3b). The homogeneous model given here is indistinguishable from the dispersed model given in Figure 3a.

Although we mentioned that the ANS transport data favored the dispersed model in Figure 3a, the Engelman–Rothman model can also account for our observation of a maximum rate at 17 mol % of cholesterol if indeed the interfaces between the two phases can mediate the ANS transport. At this mole % of cholesterol the Engelman–Rothman model would predict a maximum area of interfaces but not the cluster model.

In summary we have employed a transport reaction of a fluorescence dye ANS to study the effect of cholesterol on the bilayer structures of phospholipid dispersions. Our data indicate that the rate of ANS transport reaches a maximum at 17 mol % cholesterol. At this composition the rate approaches the maximum value obtained for the transport of the probe in pure phospholipid dispersion, i.e., the transport at the midpoint of the phase transition. Our data also suggest that molecular transport in biological membranes may be regulated by their cholesterol contents.

Added in Proof

Hui and Parsons have reported that at 11°C, which is 30° below the T_m, domain structures of a binary mixture of cholesterol and DPL could be imaged directly by selected reflection dark-field electron microscopy. The ANS transport reaction reported here is too slow to be measured at that temperature.

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