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PHASE TRANSITIONS AND CHOLESTEROL EFFECTS IN PHOSPHOLIPID LIPOSOMES

A NEW METHOD EMPLOYING THE ENHANCEMENT OF THE O-O VIBRONIC TRANSITION OF PYRENE

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The enhancement of the weakly allowed 0-0 vibronic transition in the fluorescence spectrum of the probe pyrene, which we previously showed to result from ground-state complexation with polar groups, has been shown in the present study to offer a new method for determining phase transition temperatures of liposomes and for studying the effects of cholesterol on the structure of their semipolar glycerol backbone. For dipalmitoylphosphatidylcholine it is found that small cholesterol contents (~9 mol%) induce an increase in the polarity of the microenvironment of the probe, whereas contents ≳13 mol% induce a decrease in the polarity. The results are discussed in terms of cholesterol effects on the frequency and extent of thermally-induced structural fluctuations which, in turn, affect the penetration of the probe into the bilayer.

We previously employed [1] the enhancement of the weakly allowed 0-0 vibronic transition in the fluorescence spectrum of the probe pyrene, which is the result of the reduction of the molecular symmetry brought about by ground-state complex formation with neighboring polar groups [2,3], to obtain information on the approximate location of the probe in phospholipid bilayer liposomes. It was inferred [1] that the probe is located in the vicinity of the semipolar glycerol backbone. The effective polarity of its microenvironment was found to depend on the nature as well as on the state of the liposomes: the probe was found to penetrate further into the bilayer above its phase transition temperature.

The present study demonstrates that the enhancement can be employed as a new method for determining phase transition temperatures of bilayer liposomes as well as for studying the effects of cholesterol on the structure of their glycerol backbone.

The enhancement ratio, R, for pyrene in dipalmitoylphosphatidylcholine liposomes was measured as the ratio of the intensity of the 0-0 vibronic band at ~376 nm to that at ~387 nm in its fluorescence spectrum (see Fig. 1). The former band is very weak in inert solvents becoming enhanced considerably when the probe interacts with neighboring polar groups, whereas the latter band is the strongest in inert solvents [3,4]. Thus, R is a measure of the effective polarity of the microenvironment of the fluorescent probe.

Fig. 2 shows R as a function of temperature. It is seen that there is a significant decrease in R at the phase transition, implying further penetration of the probe into the bilayer. A value of 36.0 ± 0.4°C was obtained for the transition temperature, T_t, from the melting profile, which is in very good agreement with the following values obtained for sonicated liposomes by employing the methods of differential scanning calorimetry [5], fluorescence depolarization [6] and Raman spectroscopy [7]: 36.9, 36.4 and 37°C,

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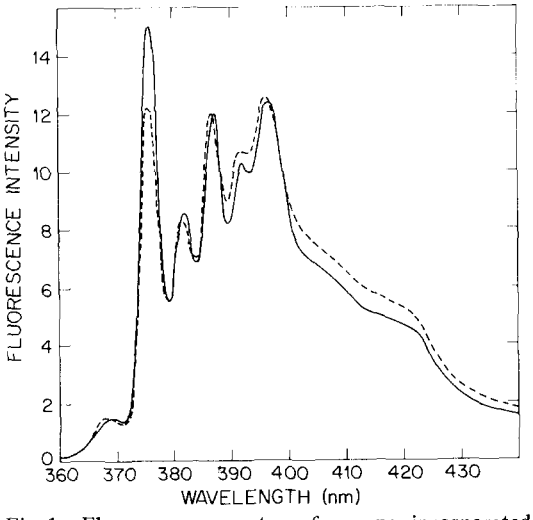

Fig. 1. Fluorescence spectra of pyrene incorporated into dipalmitoylphosphatidylcholine liposomes at 25°C: ———, in the absence of cholesterol; - - - - - -, in the presence of 50 mol% cholesterol. Excitation was at 334 nm. The excitation and emission bandwidths were 7 nm and 0.5 nm, respectively. The spectra were normalized at the vibronic band at ~387 nm, which is the strongest band in the inert solvent heptane, as was done previously in other enhancement studies [1,3,4]. Further experimental details are given in the caption to Fig. 2.

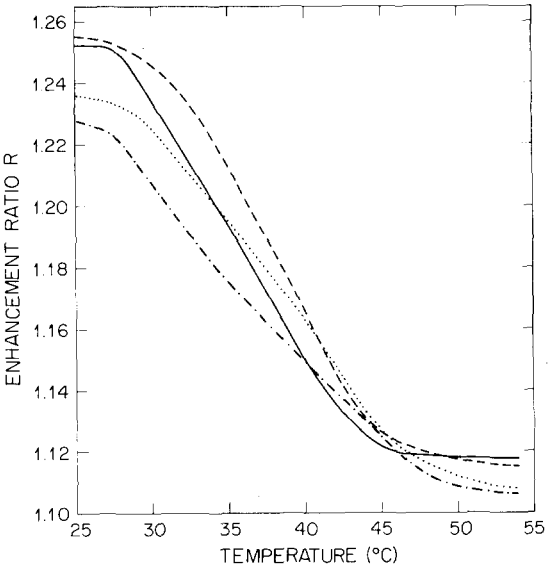

Fig. 2. Plot of the enhancement ratio, R, defined as the ratio of the 0-0 vibronic band intensity at ~376 nm to that of the band at ~387 nm in the fluorescence spectrum of pyrene, as a function of temperature for different cholesterol contents. At every temperature the emission monochromator was carefully reset successively at the two fluorescence spectral maxima in order to take into account very small temperature-induced spectral shifts. The cholesterol mole fractions were as follows: ———, 0 mol%; - - - - - -, 9.3 mol%; · · · · · ·, 11.1 mol%; and · – · – ·, 13.1 mol%. Fluorescence measurements were made with a spectrofluorometer previously described [18]. Excitation was at 334 nm. The emission bandwidth was 0.5 nm. Chromatographically pure L-α-dipalmitoylphosphatidylcholine was obtained from Sigma. Zone-refined pyrene was obtained from P. Bauric, Philadelphia. Cholesterol, primary standard grade, was obtained from P.L. Biochemicals. The preparation of the liposomes was done according to published procedures [1,19,20]. A mixture of dipalmitoylphosphatidylcholine (2 mg/ml), pyrene (0.05 ml of 1 mM dioxane solution in total 10 ml solution), and cholesterol, in 0.01 M phosphate buffer pH 7 (in triply distilled water) was stirred for 30 min. It was then sonicated for 20 min using a Heat Systems-Ultrasonics 20 kHz model W-375 sonifier equipped with 0.5 inch tip at 50% full power. Finally, it was centrifuged at 65 000 × g for 30 min. Sonication was done at 45°C, above the transition temperature of the liposomes. All procedures were carried out under nitrogen. No excimer formation was detected at the low incorporated concentration of pyrene. Steady-state and nanosecond fluorometric techniques did not detect any fluorescence from liposomes unlabelled with pyrene or containing only cholesterol.

respectively. This then provides a new and accurate method for determining T_t.

The effect of cholesterol on the structure of the liposomes was next studied. It is seen from Fig. 2 that for a cholesterol content of 9.3 mol% the profile is higher than that in the absence of cholesterol. Thus, the probe finds itself in a more polar environment. This finding implies that cholesterol forces the probe to some extent from the glycerol region toward the polar head groups. A cholesterol content of 13.1 mol%, on the other hand, reverses the trend and yields a profile which is predominantly lower than that in the absence of cholesterol, implying a further penetration of the probe into the interior of the bilayer. A cholesterol content of 11.1 mol% is seen to induce effects which are intermediate between those induced by 9.3 and 13.1 mol%. Fig. 3 shows that the trend in the lowering of the profile continues for 17 mol%, 23.2 mol%, 27.3 mol%, 42.5 mol% and up to the maximum content of 50 mol%. The high-temperature plateau in the profile is seen to be practically abolished above 17 mol%. Interestingly, calorimetric studies [8–10] reported that a sharp phase transition occurs only if the cholesterol content is less than ~15 mol%. For 50 mol% the enhancement ratio

is seen from Fig. 3 to be only slightly dependent on temperature. The reduction in the enhancement of the 0-0 band (at ~376 nm) for that case relative to that in the absence of cholesterol is seen in Fig. 1.

For interpreting the present data, it is important to have information on the approximate location of the probe in the bilayer. That the probe does not penetrate deep into the hydrophobic core upon increasing the temperature or the mole fraction of cholesterol can be deduced from the fact that the observed R values, ranging from ~1.26 to 1 (Figs. 2 and 3) are much higher than the value of 0.64 obtained in the inert solvent heptane. In fact, it takes ~8 M of butanol in heptane solution to yield an R value of 1, the value representing the most hydrophobic environment of the probe in the phospholipid-cholesterol mixtures employed in the present study.

Cholesterol is known to affect the different regions of the bilayer to a varying degree. Whereas it liquifies the fatty acyl chains below T_t and solidifies

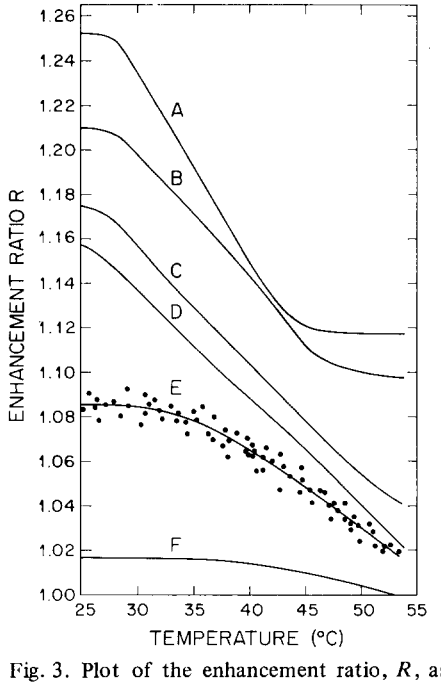

Fig. 3. Plot of the enhancement ratio, R, as a function of temperature for the following cholesterol mole fractions: (A) 0 mol%; (B) 17 mol%; (C) 23.2 mol%; (D) 27.3 mol%; (E) 42.5 mol%; and (F) 50 mol%. For clarity, experimental points are shown only for curve E. Experimental details are given in the caption to Fig. 2.

them above T_t [11], it does not affect significantly the mobility of the polar head groups [12]. The only available information regarding the effect on the glycerol backbone is for 50 mol% cholesterol for which the phase transition is abolished [8] and the fatty acyl chains attain an intermediate mobility [11]. An NMR study [13] for that cholesterol mole fraction reported pronounced enhancement of the mobility of the backbone which exceeds its mobility even in the liquid-crystalline state of the pure phospholipid. On the basis of the model put forward by Träuble [14], according to which the transport of molecules is accomplished through lipid conformational changes, it would then be expected that thermally-induced structural fluctuations would be facilitated giving rise to enhanced penetration of the probe into the bilayer. The present data for 50 mol% cholesterol are in line with such an interpretation as are those for mole fractions down to 13.1%. A mole fraction of 9.3%, on the other hand, is found to diminish the penetration of the probe and to force it toward the polar head groups. Thus, apparently small cholesterol contents tend to reduce the frequency and/or extent of lipid structural fluctuations. The different effects of cholesterol in that case cannot be readily explained, as the detailed mode of the cholesterol-lipid interaction has not yet been established [15]. It is of interest to note in this regard that the distribution of low cholesterol concentrations in the bilayer has been reported to be heterogeneous [9,10]. Moreover, an X-ray diffraction study [8] reported for dipalmitoylphosphatidylcholine differences between the effects of low and high cholesterol contents. At 7.5 mol% cholesterol the long spacing reaches a maximum value increasing from 65 to 81 Å (6.5 to 8.1 nm). That effect was attributed to a change in the tilt of the hydrocarbon chains to a configuration normal to the lipid-water surface and to an increase in the thickness of the bound water layer by about 9 Å. The long spacing was found to decrease upon further addition of cholesterol reaching a value of 64 Å at 50 mol%. A difference in the behavior of small cholesterol contents was also observed in the effect on the water permeability of egg phosphatidylcholine liposomes [16]. The permeability increases for small cholesterol concentrations reaching a maximum value at ~10 mol%, whereas it decreases upon addition of more cholesterol.

It could be argued that an increase in the water permeability of dipalmitoylphosphatidylcholine liposomes at low cholesterol mole fractions could explain the observed increase in the enhancement ratio relative to that in the absence of cholesterol. There is strong evidence, however, against such an interpretation. As we pointed out previously [3], at least 1 M of polar solvent in binary heptane solutions is needed for a barely observable enhancement of the 0-0 vibronic band. Thus, unusually large amounts of water incorporated into the bilayer would be needed to explain the pronounced enhancements observed in the lipid-cholesterol systems studied in the present work.

Work in progress in our laboratory extends the present approach to include other phospholipids as well as the fluorescent probe 1,12-benzoperylene which we have shown to exhibit pronounced enhancement of its 0-0 vibronic band when interacting with polar groups in solution [17] and when incorporated into bilayer liposomes [1].

It is of interest to compare the enhancement method for obtaining information on the polarity of the microenvironment of a fluorescent probe with that utilizing fluorescence spectral shifts [probes of the 1-anilinonaphthalene-8-sulfonate (ANS) type have been used extensively in the literature in such studies]. The accuracy of polarity determinations by the latter method can be adversely affected by restricted relaxation of neighboring polar groups during the lifetime of the excited state of the probe [21, 22]. The enhancement method, on the other hand, does not suffer from such an uncertainty, as the emission monitored is from a probe-polar group molecular complex, formed in the ground electronic state of the probe [2,3], whose association constant does not change appreciably upon excitation; that inference is reached on the basis of the very small shifts of the 0-0 vibronic band in polar environments relative to that in inert solvents [3,17,23].

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