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Enhanced fluctuations in small phospholipid bilayer vesicles containing cholesterol

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Abstract. Ultrasonic and calorimetric studies of small homogeneously-sized DMPC unilamellar vesicles showed two thermal transitions at temperatures T_{c1} and T_{c2} ($T_{c2} \geq T_{c1}$); T_{c2} is close to the phase transition temperature, T_c, of large vesicles. The process at T_{c2} is not a fusion of vesicles and is interpreted as characterizing an order-disorder transition essentially similar to that of large vesicles. The temperatures T_{c1} and T_{c2} become increasingly similar as the cholesterol content is increased, while the clusters at T_{c2} ($\simeq 85$ lipid molecules in pure DMPC) increase in size up to approximately 180 lipid molecules at 12 mol% cholesterol. Incorporation of cholesterol thus brings about enhanced fluctuations in this model system of a membrane.

Key words: Phospholipid bilayers, ultrasonics and calorimetry, cluster size, effect of cholesterol, enhanced fluctuations

Introduction

Spherical phospholipid bilayer vesicles have gained wide acceptance as model systems that simulate properties of biological membranes. These bilayers undergo thermally induced transitions from a state in which the aliphatic side chains are relatively rigid and in close van der Waal's contact with their neighbours to a state in which free rotation about carbon-carbon bonds is allowed. In a transition zone the state of a bilayer can be described as a distribution of clusters of phospholipid molecules in the ordered state, and clusters in the disordered state. In large vesicles, at their transition temperature T_c, the ordered and disordered clusters consist of an equal number of phospholipid molecules; for DMPC values of 200 and 330 have been reported.

In view of the involvement of cholesterol in cell function and its widespread occurrence in biological membranes, much attention has been devoted to effects of its incorporation in phospholipid bilayers. Additives are known to reduce the size of the clusters, sometimes quite considerably, for example, as with anesthetics (Mountcastle et al. 1978). For cholesterol, however, this effect is small or non-existent in large bilayers, and herein we describe an increase in the size of clusters for DMPC SUV.

Materials and methods

Synthetic DMPC and cholesterol were purchased from Sigma. Purity was checked by thin layer chromatography. Irradiation with 20 kHz ultrasonic waves at a power level of 150 W (30 min), followed by ultracentrifugation at about 160,000 g (3 h) and gel-filtration on Sepharose colums, produced homogeneously-sized populations of SUV. In one preparation the vesicles had an average diameter of 280 Å, and 73% of them had a diameter between 205 Å and 326 Å. The average value of the diameter remained constant over periods of 24 h in the temperature range 15° to 30 °C, and was reproducible for all preparations to within $\pm$ 30 Å. Homogeneously-sized populations of LUV of average diameter 2,000 Å were obtained by the ether vaporisation procedure (Deamer and Bangham 1976). The lipid concentration was measured as an inorganic phosphate (Bartlett 1959), and cholesterol concentration by colorimetry (Zlatkis et al. 1953).

Ultrasonic absorptions of solutions and solvents, α and α_0 ($\Delta\alpha = \alpha - \alpha_0$), were measured in the frequency range 0.48 to 5.76 MHz using an ultrasonic resonator (Eggers 1967). Calorimetric measurements were made

Abbreviations DMPC: dimyristoylphosphatidylcholine; SUV: small unilamellar vesicles; LUV: large unilamellar vesicles; MLV: multilamellar vesicles

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with the DASM 4 differential scanning microcalorimeter (Privalov 1980), usually at a scan rate of 1° min^{-1}. Particle size and size distribution were measured using a light-scattering Malvern Autosizer II apparatus.

Results and discussion

Lipid concentration was between 0.4 and 2.2 mg/ml in all ultrasonic measurements, and for SUV between 1.3 and 8 mg/ml in calorimetric measurements. Linear behaviour with lipid concentration was observed (not shown). The ultrasonic data are normalized to 1 mg of lipid per ml, and the specific heat values C_p are given in kcal K^{-1} per mole of lipid (Figs. 1 and 2).

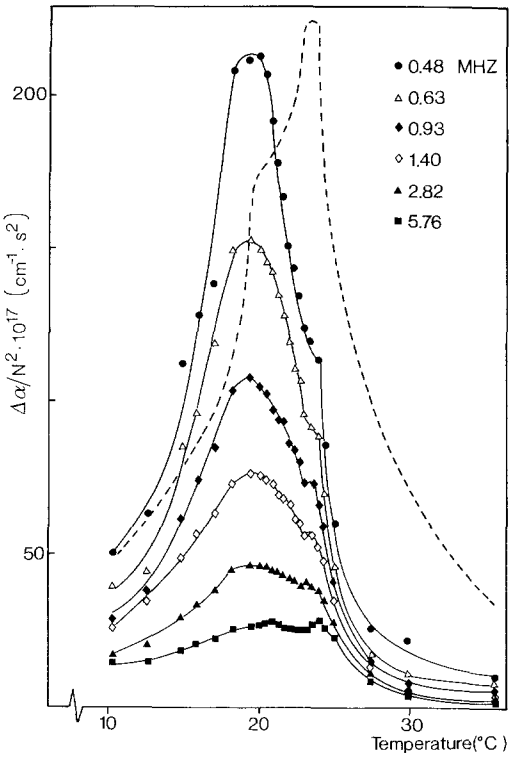

Fig. 1. SUV: 1 mg/ml in 100 m*M* *Tris*-HCl buffer, pH 7.3. Values of $\Delta\alpha/N^2$ versus temperature at different frequencies. *Broken line*: SUV + 10 mol% cholesterol at 0.48 MHz and same conditions otherwise

In pure DMPC two distinct processes occur, the first at $T_{c1} \simeq 21\,^\circ$C, which is lower than the transition temperature T_c for large vesicles, the second at $T_{c2} \simeq 24\,^\circ\text{C} \simeq T_c$. This behaviour has not been reported previously from ultrasonic data. Two well-defined transitions have been described for dipalmitoylphosphatidylcholine SUV from calorimetry studies, and they have been interpreted as reflecting the presence of both SUV and MLV, each having their own order-disorder transition temperature (Suurkuusk et al. 1976). Changing thermotropic behaviour as a function of time has then been interpreted as reflecting a fusion process in which SUV slowly associate to form MLV.

Our DMPC SUV also showed changing thermotropic behaviour when subjected to repeated temperature cycles. However, in our ultrasonic and calorimetric studies we always used fresh solutions, for which no change in size or shape of the vesicles could be detected, neither before nor after ultrasonic spectroscopy measurements. Therefore, the model of a slow fusion process cannot hold in our experiments.

The process at T_{c2} for SUV resembles that at T_c for LUV, as follows from Fig. 3, in which the calorimetric values of T_{c2} and T_c are seen to remain close to each other for all values shown of cholesterol content c_c. Moreover, when c_c is increased, the temperatures T_{c1} and T_{c2} for SUV, and T_c for LUV, as well as the total transition enthalpies ΔH per mole of lipid for LUV and SUV in Fig. 4, become increasingly close to each other. Note that the ultrasonic value of T_{c2} is frequency-dependent.

We now show that at T_{c2} in SUV the clusters increase in size as cholesterol is incorporated up to

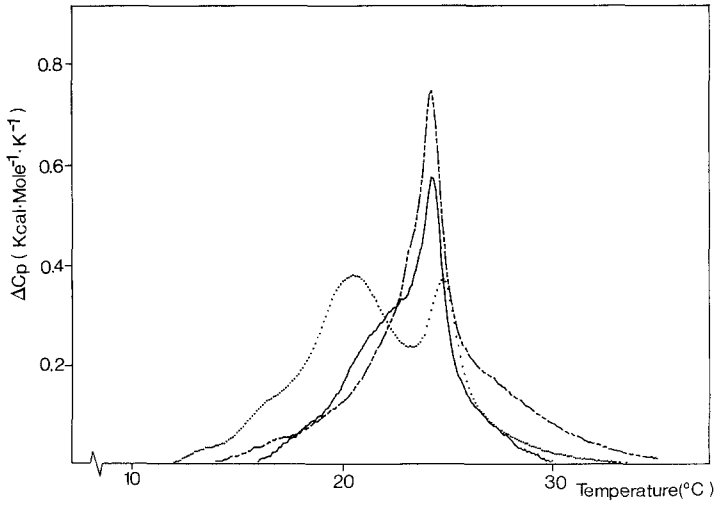

Fig. 2. Calorimetric scans for SUV (····); SUV + 5 mole% cholesterol (——), and +12.5 mol% cholesterol (— — — —)

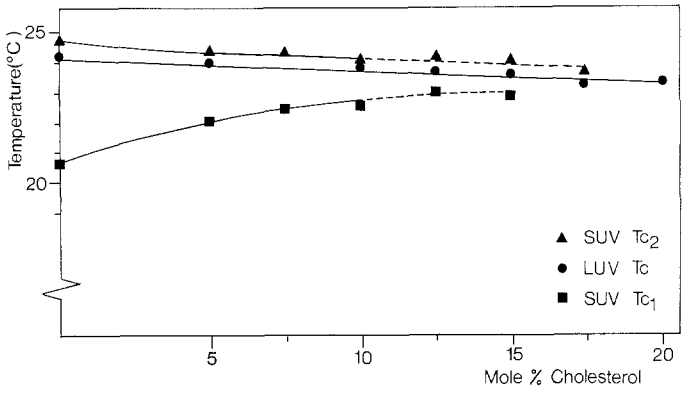

Fig. 3. Critical temperatures for cholesterol containing LUV and SUV. *Broken lines* indicate that the determination of critical temperature values requires the deconvolution of thermograms

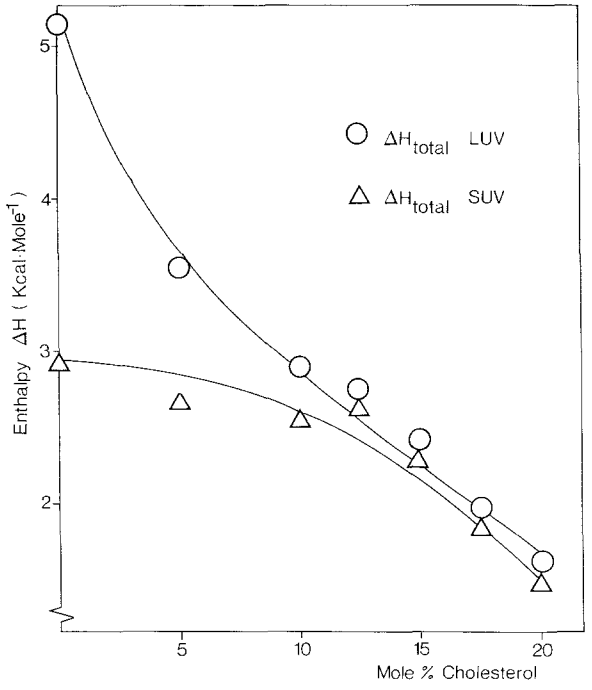

Fig. 4. Total transition enthalpies for cholesterol containing LUV and SUV

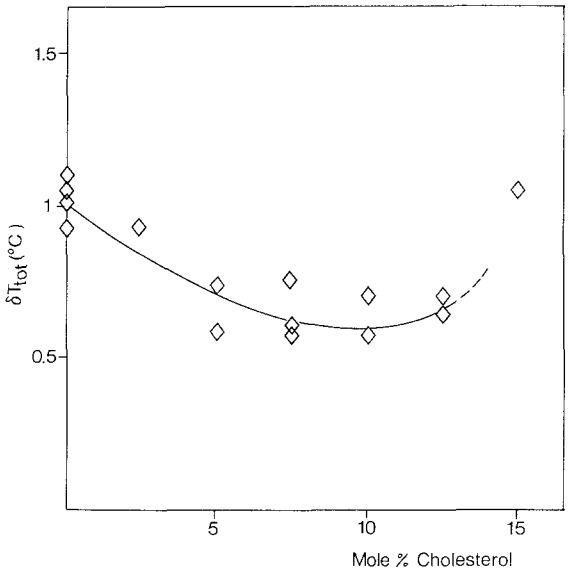

Fig. 5. Measured half-width at half-height, δT_{tot}, for the transition at T_{c2} in cholesterol containing SUV

about 12 mol%. Above this value the peak at T_{c2} broadens. When cholesterol is incorporated, a thermogram contains a third contribution at $T_{c3} > T_{c2}$, and deconvolution into elementary transitions depends on arbitrary assumptions. Therefore, we first use a qualitative argument as follows.

In simple models the width of a transition is inversely proportional to the van t'Hoff enhalpy: $\Delta h_{VH} = m\,\Delta h$, where m is the number of cooperative units (molecules) and Δh is the molar transition enthalpy per unit. We define δT_2 as the half-width of the peak for the sole transition at T_{c2}; δT_2 is taken, at half-height, from the vertical of the top to the right flank of the peak. The effect of the transitions at T_{c1} and T_{c3} is to increase the measured total half-width δT_{tot} at T_{c2}, and to raise δT_{tot} further as c_c is increased. By allowing δT_2 to equal δT_{tot}, shown in Fig. 5, we always underestimate the decrease of δT_2 for increasing c_c. Thus, Δh_{VH} for the transition occuring at T_{c2} rises as c_c is increased.

Furthermore, in SUV the process at $T_{c2} \simeq T_c$ is closer to the process at T_c in LUV than is the process at T_{c1} in SUV. Therefore, the dependence of Δh upon c_c must be intermediate between those of the total transition enthalpies ΔH for SUV and for LUV. Consequently, for SUV Δh must decrease more than ΔH does, as c_c is increased (see Fig. 4). Because Δh_{HV} increases and Δh decreases, the number m of cooperative units rises as c_c is increased. We observed no such effect when 3-doxyl-cholestane was incroporated into the vesicles.

Calorimetry measures enthalpy fluctuations, as is known, and, in an aqueous medium, ultrasonics measures volume fluctuations (Cerf 1985). Furthermore, it has already been suggested that volume fluctuations among ordered and disordered clusters could be of importance in the functioning of membrane systems

(Mountcastle et al. 1978), but the occurrence of ordered domains of the size encountered in lipid phase transitions in real systems (see Introduction) is still under debate.

The number m of cooperative units, i.e., the size of a cluster, can be obtained from the maximum value $C_{p,\mathrm{MAX}}$ of C_p. At T_{c2} this is found to be about 85 phospholipid molecules in pure DMPC SUV. It is likely that the higher curvature of lipid layers in SUV prevents the formation of ordered clusters of a size comparable to that found for LUV. The constraints that limit the size of a cluster in SUV would then partially be lifted when cholesterol is incorporated into the vesicles, until c_c approaches about 12 mole%, when m can be estimated as approximately 180 lipid molecules, i.e., about twice the value for pure DMPC. This estimate of m at T_{c2} in 12% cholesterol rests on deconvolution of the thermogram.

Because different NMR signals are produced by SUV when cholesterol is incorporated into the outer and inner layer, respectively (Sillerud and Barnett 1982), it is probable that the clusters melting in SUV at T_{c2}, close to T_c in LUV, are located in the outer layer of lower curvature. However, to produce the larger enthalpy change at T_{c1} in pure DMPC (Fig. 2), clusters melting at this temperature must be found in both the inner and outer layers. Moreover, from the width of the transition at T_{c1}, it follows that in pure DMPC at T_{c1} clusters must be of smaller average size ($\simeq$ 50 lipid molecules) than at T_{c2}.

Because m defines the size of the fluctuating unit, the fluctuations at T_{c2} are *enhanced* by incorporating cholesterol into SUV up to about 12 mol%. Furthermore, this result suggests that when defects due to either heterogeneity in lipid composition, or proximity of protein, are present, cholesterol might increase the size of clusters, and therefore enhance the fluctuations. That enhanced fluctuations may play a part in biological processes has been proposed previously (Cerf 1985) on the basis of data obtained from viral capsids and from viruses (Cerf et al. 1979). Here, small phospholipid bilayers containing cholesterol provide an instance when enhanced fluctuations appear in a model system of a membrane.

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