THE INFLUENCE OF CHOLESTEROL ON THE MAIN PHASE TRANSITION OF UNILAMELLAR DIPALMYTOYLPHOSPHATIDYLCHOLINE VESICLES

A Differential Scanning Calorimetry and Iodine Laser T-Jump Study

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ABSTRACT The influence of cholesterol (CHOL) on the main phase transition in single shell dipalmytoylphosphatidylcholine (DPPC) vesicles was investigated in equilibrium and kinetic experiments. CHOL increases the optical density and causes a slight hysteresis in turbidity transition curves. Static fluorescence anisotropy measurements showed interesting differences for three probes sensing different parts in the hydrophobic region of the phospholipid bilayer. Differential scanning calorimetry (DSC) peaks can be separated into a narrow and a broad component. The narrow component, which decreases linearly with increasing CHOL content and disappears at 20 mol %, is attributed to the transition of free phospholipid, while the broad component, being associated with the transition of CHOL-lipid units, increases monotoniously from 0 to 20%. Kinetic experiments were performed on our iodine-laser T-jump arrangement with turbidity detection. Three cooperative relaxation signals in the microsecond and millisecond time range were detected for pure DPPC vesicles as well as vesicles containing 7.5 and 16.5 mol % CHOL. All three relaxation processes were changed by CHOL: the superposition of the three relaxation amplitudes can be separated into a narrow and a broad component, as in DSC experiments. A speculative model is presented which assumes an inhomogeneous CHOL distribution fluctuating on a millisecond time scale in the temperature region of the main phase transition.

INTRODUCTION

Cholesterol (CHOL) is an important component of many biological membranes, in which the CHOL fraction may reach as much as 50% of the total lipid content. Therefore phospholipid-CHOL interactions have been of interest for many years (1–3). Different experimental techniques were used to get insight into this system, such as electron spin resonance, nuclear magnetic resonance, and fluorescence anisotropy lifetime measurements (4–14).

It could be shown that CHOL decreases the order of phospholipid bilayers in the crystalline state by perturbing the hexagonal packing of the lipids, while the order of the fluid state is increased because the rigid ring structure of CHOL limits the possibility for *cis-trans* isomerizations of the neighboring lipid chains. In this way CHOL creates lipids in a type of intermediate state of order. This is shown in Fig. 1 on the level of single molecules.

A useful technique to study the influence of CHOL on the phase transition of phospholipids is differential scanning calorimetry (DSC). These experiments showed a broadening and a reduction of cooperativity during the phase transition of different phospholipids which was induced by CHOL. But those experiments gave no information on how CHOL influenced the kinetics of the phase-transitions. Only one kinetic investigation was carried out to solve this interesting question (15), using a pressure jump technique with optical detection to study the dimyristoylphosphatidic acid (DMPA)–CHOL system.

In the work presented here we would like to focus on experiments with dipalmytoylphosphatidylcholine (DPPC) vesicles containing different amounts of CHOL. The system was studied in static experiments using the DSC technique as well as ultraviolet spectrometry (turbidity or fluorescence) to follow the phase transition. The kinetic experiments were performed with the iodine laser temperature jump (ILTJ), in the oscillator version producing pulses of 2.4 μs relaxation time.

The question of interest was how the broadening and reduced cooperativity of the phase transition in the presence of CHOL would influence the three well-separated cooperative relaxation processes which have been observed for pure DPPC vesicles (16; Genz, A., and J. F. Holzwarth, manuscript in preparation) in the microsecond to millisec-

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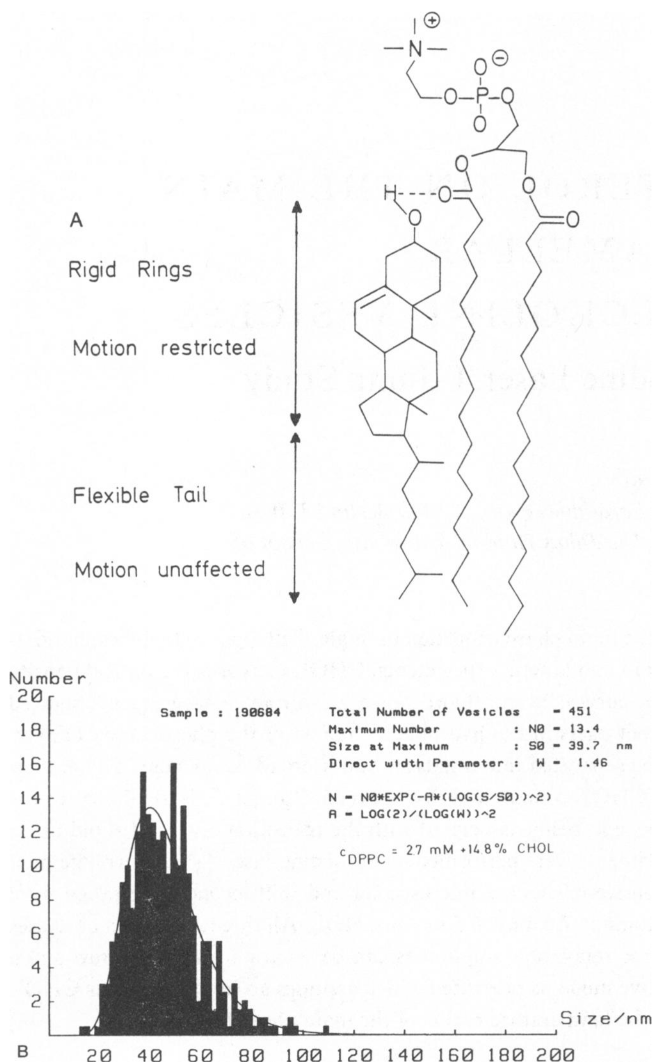

FIGURE 1 (*A*) Schematic diagram for the DPPC-CHOL interaction on the level of single molecules (after 27). (*B*) Size distribution of unilamellar vesicles of DPPC containing 14.8% CHOL achieved from electron micrographs.

ond time range, and if the sum of the amplitudes from the kinetic experiments reproduce the shape of the equilibrium phase transition dependence on temperature.

MATERIALS AND METHODS

Vesicle Preparation

DPPC and CHOL were purchased from Fluka Chem. Corp., Ronkonkoma, NY. For the DSC measurements CHOL was delivered by Calbiochem-Behring Corp., LaJolla, CA. The buffer, pH 7.4, consisted of 0.1 M NaCl (E. Merck, Darnstadt, Federal Republic of Germany) and 0.01 M Tris (hydroxy-methyl) aminomethane (Fluka Chem. Corp.) in triple-distilled water. All chemicals were of the highest commercially available grade and used without further purification.

The vesicles were prepared by the modified injection method (23). 15 μM of the DPPC-CHOL mixture were dissolved in 0.5 ml ethanol and slowly (10–15 min) injected into 5 ml of Tris-buffer at 52°C, and dialyzed against pure buffer for at least 10 h. With this technique it is not possible to prepare vesicles with more than 20 mol % CHOL because otherwise the preparation tends to aggregate during the injection process. The incorporation of fluorescence probes into the vesicles has been described elsewhere (17). The vesicles were checked by electron microscopy using the negative staining technique. Vesicles with 15% CHOL showed slightly bigger size at the maximum of the distribution (40 nm) (see Fig. 1 *B*) than vesicles of pure DPPC (35 nm), but the shape of the size distribution remained unchanged by the CHOL incorporation; both size distributions were monomodal. Dynamic light scattering measurements showed significantly bigger values for the vesicle diameter (Servuss, unpublished results) in agreement with the data published (18).

Equilibrium Measurements

The phase transition was investigated in static experiments to characterize the system and to allow a comparison between static and kinetic results; the sum of the kinetic data over the whole time window should reproduce the thermodynamic measurements. Turbidity transition curves were measured on a spectrometer (model Lamda 5, Perkin-Elmer Corp., Norwalk, CT) at 300 nm. The temperature inside the cuvette (Hellma Cell Inc., Jamaica, NY) was scanned using a rate of 18°C/h on a Haake thermostat equipped with PG11 temperature control unit and determined in control experiments with a digital thermometer. Details for the fluorescence anisotrophy measurements are explained elsewhere (16). DSC measurements were performed on a MC-1 microcalorimeter (Microcal Inc., Amherst, MA) using 0.9 ml of solution for reference as well as the sample cell and a scan rate of 21.7°C/h. The ΔH values were determined referring the transition curves to a calibration pulse and calculating the area under the curves by the paper weighting method. The electron micrographs to produce the size distribution were taken from an EM 400 T (Philips Electronic Instruments, Inc., Mahwah, NJ) and plotted using a computer (model 9816, Hewlett-Packard Co., Palo Alto, CA).

Kinetic Measurements

Our ILTJ with turbidity detection is described elsewhere (19). The laser was used in the oscillator mode (2.4 μs pulse relaxation time). The detection wavelength was 360 nm. The signal recording has been modified, so that relaxation processes in three different time ranges could be measured during one single temperature jump.

An AD (model 7912, Tektronix, Inc., Beaverton, OR) equipped with a 7A22 amplifier plug-in (Tektronix, Inc.) was used for signal recording in the microsecond range and a waveform recorder (model 1010, Biomation, Inc., Cupertino, CA) in the dual time base mode for the two millisecond relaxation signals. The computer program (model 9845B, Hewlett-Packard Co.) was modified to allow the sampling of all three signals simultaneously, and to calculate the corresponding relaxation times. The relaxation process around 1 ms (signal 4) was superimposed by a very weak faster signal, which was too small to be computer-fitted. All data were averaged for at least two different vesicle preparations.

RESULTS

Equilibrium Measurements

The degree of order inside the phospholipid bilayer changes during the main phase transition. Fluorescence anisotropy measurements can monitor these changes. We used three fluorescence probes, which are localized in different parts of the hydrocarbon chain region; details are explained in (16). The first probe, diphenylhexatriene (DPH), is not specifically located in the hydrophobic part of the bilayer; it is considered to be distributed along the full length of the hydrocarbon chains and may even lie between the two monolayers perpendicular to the chains if the bilayer membrane is in the fluid state (16). The

trimethylamino group of the chemical analogue 1-[4-(trimethyl[amino])phenyl]-6-phenyl-hexa-1,3,5 triene (TMADPH) is anchored due to its positive charge in the headgroup region of the bilayer and is therefore a probe for the glycerol backbone as well as the adjacent hydrocarbon chain region. The fluorescent lipid 1{3[p-(6-phenyl(1,3,5-hexatrienyl)-phenyl]-propionyl}2-palmitoyl-3-phosphatidyl-choline (DPHPC) is a probe for the deep hydrophobic interior of the bilayer because DPH is replacing 13 C-atoms at the lower part of one palmitoyl fatty acid chain. We measured the steady state anisotropy transition curves of pure DPPC vesicles and vesicles with 15 mol % CHOL using all three probes (Fig. 2, *A–C*). The anisotropy changes for vesicles without cholesterol have been explained previously (16). In the crystalline phase TMADPH showed the most pronounced effect when CHOL is incorporated, indicating a disordering of the phospholipid arrangement in the upper fatty acid chain region caused by the steroid ring system, while here DPHPC showed nearly no effect. In the fluid phase DPH, the probe with the highest mobility is most affected, but the anisotropy for DPH is still lower than that of the other two probes.

The optical density of the phospholipid bilayer membrane changes during the phase transition. This causes a change in the amount of light scattered by the samples. For a detailed theoretical discussion see reference 20. This effect was used to study the phase transition of DPPC vesicles with different amounts of CHOL (Fig. 3). The optical density of the bilayer increased linearly with increasing CHOL content. The absolute change occurring at the phase transition was reduced and the transition was broadened.

The temperature, T_m, at which the transition is observed was slightly increased and the temperature/turbidity dependence, showed growing hysteresis effects with increasing CHOL content. Samples containing 19.5% CHOL showed an extreme hysteresis in their temperature-turbidity dependences: this concentration of CHOL is the highest possible content for vesicles prepared by the injection technique. Here irreversible changes in the vesicle structure during multiple heating-cooling cycles through the phase transition could not be excluded.

Besides fluorescence anisotropy and turbidity measurements a third technique was used to study the main-phase transition of unilamellar DPPC vesicles. DSC measurements were performed for vesicles of different CHOL contents (Fig. 4). We found a broadening of the phase transition similar to the optical studies. The reason for this broadening is obvious from the DSC results. Besides a sharp melting peak a second broad component appeared at higher CHOL concentrations. The sharp component, which is attributed to the melting of free phospholipids, was decreasing linearly with increasing CHOL content and disappeared at 20 mol % CHOL, while the broad component, associated with the transition of CHOL-lipid units, increased monotoniously from 0 to 20 mol % (Fig. 5). The transition temperature for the sharp component, T_m (narrow), decreased with increasing CHOL content, while T_m (broad), which occurs at higher temperatures, was slightly increasing. The ratio of the van't Hoff enthalpy

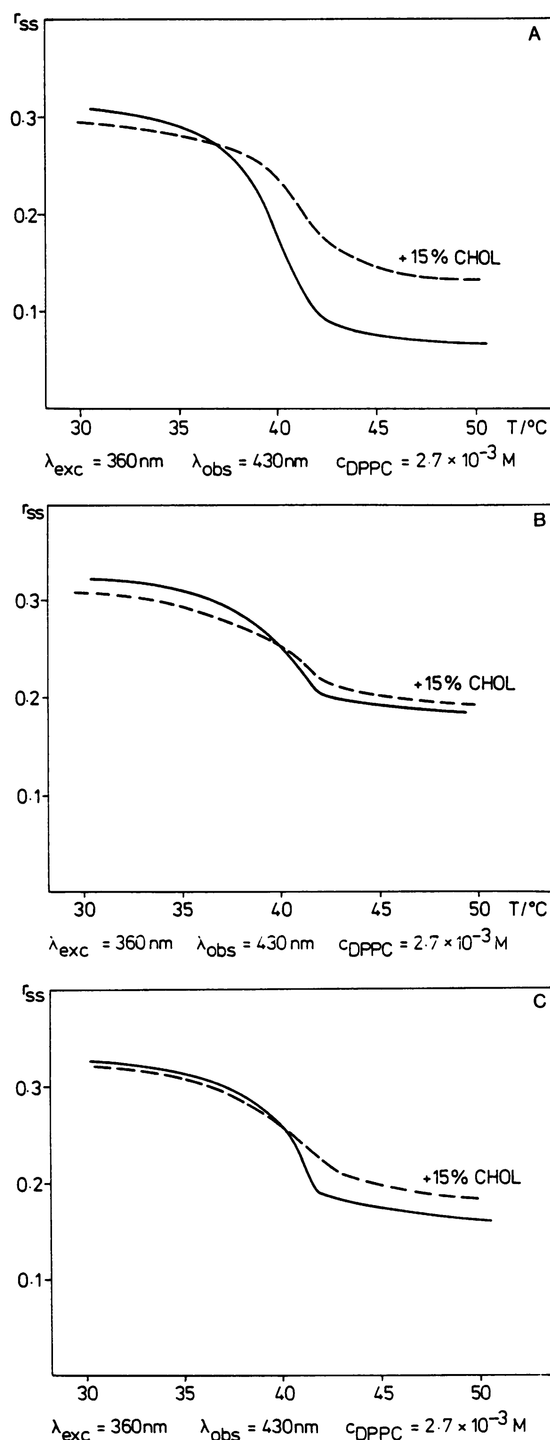

FIGURE 2 Steady state anisotropy temperature dependences for vesicles of DPPC and DPPC + 15 mol % CHOL, using the probes DPH (400:1) (*A*), TMADPH (400:1) (*B*), and DPHPC (400:1) (*C*). The values were averaged for increasing and decreasing temperature scans.

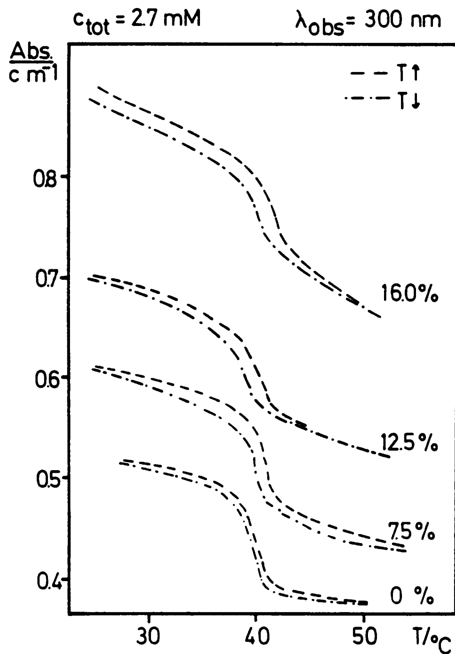

FIGURE 3 Turbidity-temperature dependences around the equilibrium phase transition for DPPC vesicles containing different amounts of CHOL. Scans for increasing and decreasing temperatures are shown.

(ΔH_{vH}) and the enthalpy measured in the DSC experiment (ΔH_{cal}) is commonly used as a measure for cooperativity. The results are shown in Fig. 6. The data should not be taken too seriously, because the calculation is based on an oversimplification of the kinetic process.

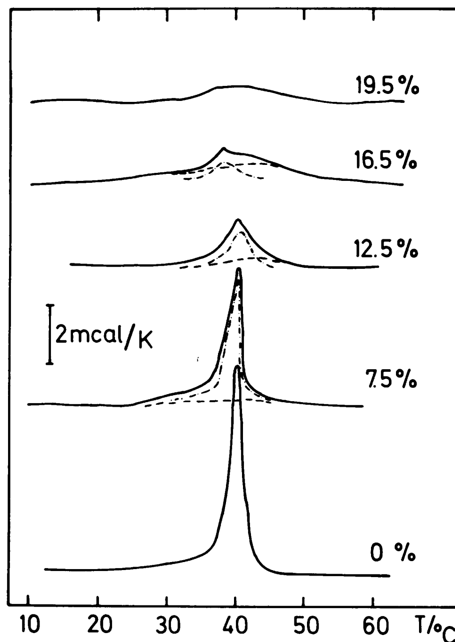

FIGURE 4 Differential scanning calorimetric results of DPPC vesicles containing different amounts of CHOL. Resolution into sharp and broad component is shown for 7.5%, 12.5% and 16.5% CHOL. Concentration of DPPC + CHOL is 2.7 mM. Scan rate is 21.7°C/h.

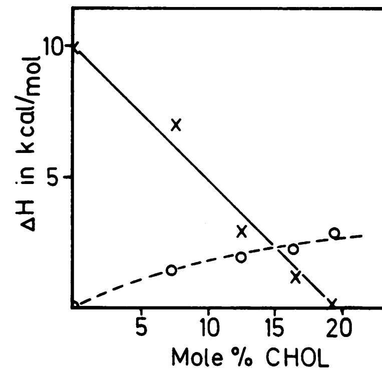

FIGURE 5 The variation with CHOL content of the enthalpies for the sharp (x) and broad (o) component of the phase transition in DPPC vesicles.

The effects explained above had been observed earlier for multilamellar DPPC-CHOL aggregates (21, 22), but to our knowledge this is the first DSC study of the DPPC-CHOL system using unilamellar well characterized vesicles.

Kinetic Measurements

ILTJ measurements were performed for pure DPPC vesicles and vesicles with 7.5 and 16.5% CHOL (Fig. 7, *A–C*). T is the temperature at which the T-jump ($1.3\ K \leq \Delta T \geq 1.4\ K$) started. For pure DPPC vesicles a lower laser energy ($\Delta T = 0.9\ K$) was used, but the amplitudes were normalized to $\Delta T = 1.3\ K$. The three relaxation signals for pure DPPC vesicles showed strong maxima in their relaxation times as well as their corresponding amplitudes; this is indicative for cooperative processes. The slowest signal, τ_5, showed the strongest cooperativity. This was also observed in kinetic experiments applying fluorescence anisotropy as the detection parameter using the three probes DPH, TMADPH, and DPHPC (16), and when an absorption probe Acridinorange lecithin (Genz, A., and J. F. Holz-

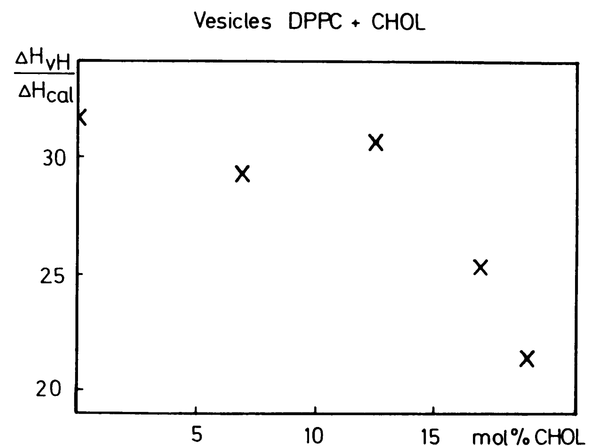

FIGURE 6 The ratio $\Delta H_{v.H.}/\Delta H_{cal}$ as a function of CHOL content used as the value for the cooperative unit. ($\Delta H_{v.H.} = 4R[T_m^2/\Delta T_{1/2}]$) (see reference 28).

warth, manuscript in preparation) was incorporated into the bilayer.

From these experiments it was clear that the signals reported here are no artifacts caused by the turbidity detection arrangement. We preferred turbidity as the detection parameter for the kinetic experiments because of its very good signal to noise ratio and the possibility of avoiding specific interactions between any probes and the system. How did CHOL influence the relaxation signals τ_3, τ_4, and τ_5 observed in the pure DPPC system? The overall amplitude ΣA was reduced. The amplitudes of the two slower processes A_4 and A_5 decreased, while A_3 slightly increased. The broadening of the transition observed in equilibrium measurements could also be seen to different extents in the relaxation amplitudes of the three signals in the microsecond to millisecond range. Signal 4 showed little broadening, while signal 5 showed a markable effect and signal 3 became extremely broad at high CHOL contents. A superposition of the three relaxation amplitudes ΣA (Fig. 8) could be separated into a broad and a narrow component like the DSC transition curves. Extrapolation showed that the narrow component vanished at 25 mol % CHOL. The ratio between the area under the two peaks was in good agreement with the DSC measurements, while the absolute values could not be compared. This is no surprise because no simple proportionality between ΔH and ΣA exists. The three relaxation times τ_3, τ_4, and τ_5 showed a behavior similar to their corresponding amplitudes A_3, A_4, and A_5. While the profile for τ_4 showed only a slight broadening, the relaxation time distribution for τ_5 became much wider and τ_3 lost its maximum at high CHOL concentrations (16.5%). The absolute values are nearly constant for τ_5, become greater for τ_4, and smaller for τ_3. In the following discussion section we will try to give some interpretations for this rather complex behavior.

DISCUSSION

The fluorescence anisotropy measurements showed that CHOL lowers the order of the crystalline state (decreased anisotropy) and increases the order of the fluid state (increased anisotropy). This results in an intermediate state of order of the lipids. The CHOL influence on the fluid state is more pronounced, which is in agreement with monolayer studies (16, 23). The DSC measurements indicate a phase boundary for the DPPC-CHOL system around 20 mol % CHOL, which is in agreement with phase diagrams published in the literature (1, 24). This phase boundary should be the reason why vesicles with higher CHOL contents could not be prepared by the injection technique.

In order to explain this phase behavior, it has been suggested that associates of 1:1 and 1:2 stoichiometry between CHOL and phospholipids are formed (17, 25). According to this model one phospholipid is tightly bound to one CHOL molecule through a hydrogen bond between the β-OH groups of CHOL and the glycerol ester oxygen of the phospholipid (Fig. 1). A second lipid is associated to this complex by van der Waals interactions. Above 33 mol % CHOL, 1:1 and 1:2 complexes coexist, and above 50%, the maximum CHOL content in biological membranes, e.g., in erythrocytes, a phase of pure CHOL would exist, making the bilayer unstable. Assuming a 2:1 lipid-CHOL complex, Monte-Carlo simulations based on interaction energies deduced from calorimetric results showed a coexistence of CHOL-rich and CHOL-free domains. At ~20 mol % the CHOL-rich areas suddenly become connected, forming a network that extends over the entire bilayer (25). Because of experimental limitations using unilamellar vesicles, we just reached a CHOL content of ~20%, and Figs. 4 and 5 show the expected behavior: the disappearance of the narrow component which is clearly present at lower CHOL concentrations.

Our model of the main phase transition in pure DPPC vesicles (19, 16; Genz, A., and J. F. Holzwarth, manuscript in preparation) assumes the coexistence of clusters of different order in the temperature region of the phase transition. Complex rotational isomers are formed in single lipid chains and their nearest neighbours with a characteristic time of 10 μs (τ_3). Thereby fluid microdomains are created. Their growth (τ_4) and fusion (τ_5) occurs on the millisecond timescale. Clusters of all *trans* chains are disappearing (τ_5). These processes are associated with a lateral expansion of the bilayer and can be seen as a reduction of turbidity in the kinetic experiments.

Incorporation of CHOL reduces the amount of free lipid and limits the clusters to a smaller size, while a CHOL-rich buffer zone of intermediate lipids is created (Fig. 9). Therefore the amplitudes of the processes associated with the variation of cluster sizes A_4 and the disappearance of crystalline clusters A_5 are reduced. The reduction of the cluster size should also reduce the corresponding relaxation times, but the decoupling of rigid and fluid regions through the increased intermediate buffer zone has the opposite effect on the relaxation times τ_4 and τ_5. As a result, τ_5 is constant and τ_4 increases at high CHOL concentrations. Another observation of the kinetic measurements is that with increasing CHOL content an increasing part of the total relaxation amplitude is shifted to the microsecond time range (τ_3) and that at high CHOL concentrations the main part of the observed broad component in ΣA is caused by A_3.

This shift of relaxation amplitude to shorter times backs the idea of the intermediate lipid order in the buffer zone because in such an intermediate state we expect a high mobility of the lipid molecules.

The interpretations given above are in agreement with detailed computer simulations on the phase transition in pure lipid, lipid-CHOL and lipid-polypeptide systems (26). These studies clearly showed the coexistence of microdomains of different lipid order near T_m and indicated an influence of CHOL and polypeptides on the domain walls.

The DSC and pressure jump results of Blume and Hillmann (15) on DMPA/CHOL are in good agreement with our data if we accept the following. Their τ_1, on which they concentrated, mainly corresponds to our τ_5; they both broaden with increasing CHOL content in their amplitude and time dependence on temperature. Their relaxation 2 showed almost no change with CHOL; our corresponding τ_4 also showed little changes at different CHOL concentrations but is stronger in pure DPPC. A difference exists in τ_3, the fastest process, wherein we measured a strong broadening of the relaxation but an almost constant amplitude maximum. We assume that they had difficulties with the pressure jump technique which do not exist with our ILTJ. The explanation of the two slower processes are in perfect agreement (cluster formation and fusion). For the fastest process our interpretations of being caused by lipids in an intermediate state of order is not discussed in Blume's paper.

The interpretation for the kinetic results is still speculative but we believe that there is good evidence from our

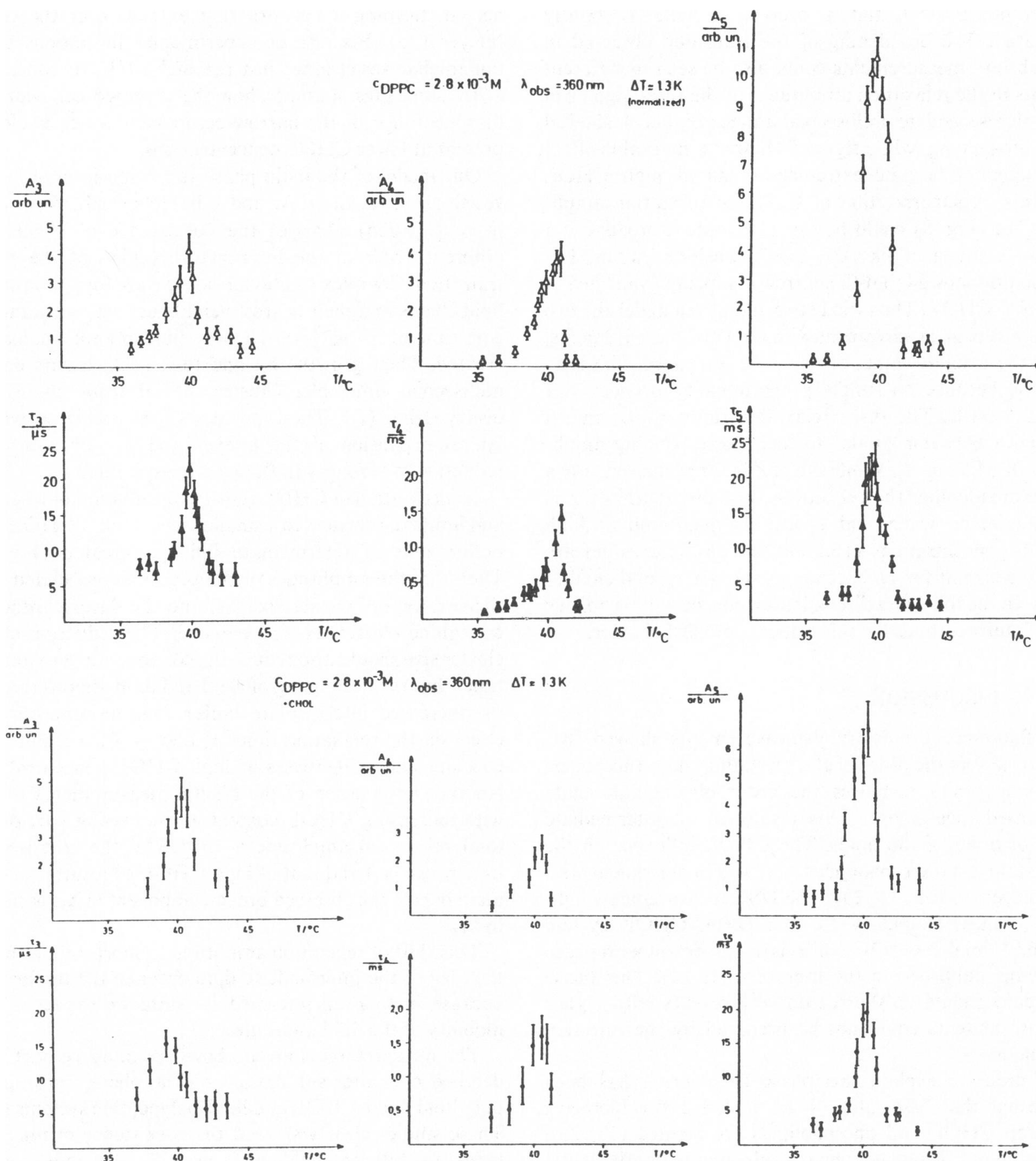

FIGURE 7 (continued)

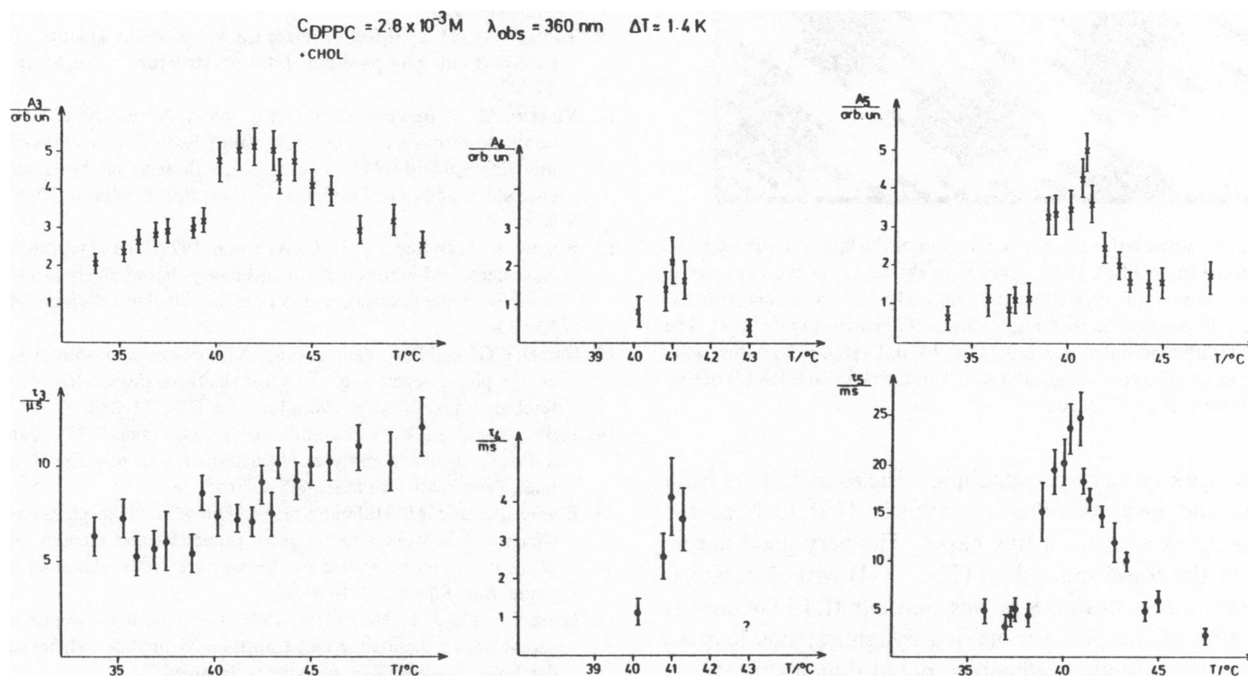

FIGURE 7 Relaxation times and their corresponding amplitudes as a function of the temperature where the T-jump started: (*A*) pure DPPC vesicles, (*B*) vesicles with 7.5 mol % CHOL, and (*C*) vesicles with 16.5 mole % CHOL.

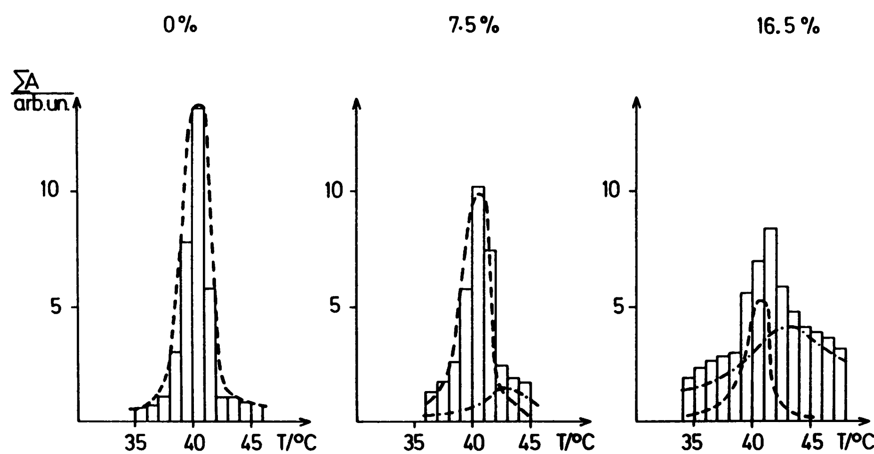

FIGURE 8 Superposition of the relaxation amplitudes for the three cooperative processes in vesicles of DPPC + CHOL in the micro-to-millisecond range separated into a broad and a narrow component. $\Sigma A = A_3 + A_4 + A_5$ normalized to $\Delta T = 1K$.

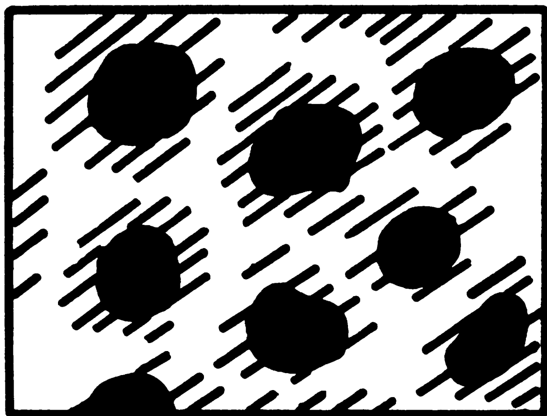

FIGURE 9 Speculative schematic diagram of the lateral DPPC-CHOL distribution in DPPC/CHOL vesicles. In the region of the main phase transition clusters of crystalline (all *trans*, shown here as black areas) lipids are in coexistence with regions in the fluid state (white area). The areas of different order are separated by a CHOL-rich buffer zone (intermediate; shown as diagonal lines). This distribution is fluctuating on the time scale of milliseconds.

earlier work on pure phospholipid vesicles as well as from results and ideas of other researchers (15) to back the interpretations given in this paper. The very good agreement of the equilibrium data (Figs. 2–4) with the relaxation amplitudes in Fig. 8 proves that our ILTJ covered at least 80% of the phase-transition dynamics; this justifies our hope for a further refinement of our dynamic model.

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