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Dimyristoylphosphatidic acid/cholesterol bilayers

Thermodynamic properties and kinetics of the phase transition as studied by the pressure jump relaxation technique*

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Abstract. Lipid bilayers and monolayers composed of dimyristoylphosphatidic acid (DMPA) and cholesterol were characterized by differential scanning calorimetry and film balance measurements. Increasing cholesterol content decreases the bilayer phase transition temperature and enthalpy in a manner similar to that observed before for other lipid/cholesterol systems. In monomolecular films at the air-water interface cholesterol exhibits the well known condensing effect in the liquid-expanded phase, while the liquid-condensed phase is less affected. As with the bilayer phase transition, the transition temperature and change in area at the liquid-condensed to liquid-expanded phase transition, as measured from isobars at 25 dynes/cm, decreases with increasing cholesterol content. The kinetics of the phase transition of DMPA/cholesterol bilayers were measured using the pressure jump relaxation technique with optical detection. Three relaxation times were observed. The relaxation times and amplitudes pass through maximum values at the transition midpoint. With increasing cholesterol content the maximum values of the relaxation times decrease but not in a linear fashion. The time constants display an intermediate maximum at ca. 10% to 12 mol% cholesterol. This observation is discussed in terms of a possible change in the nature of the phase transition from first-order with phase separation to a continuous second-order transition. The dependence of the relaxation amplitudes on cholesterol content gave evidence for nucleation being the rate limiting step for the transition in this particular system.

Key words: Lipid/cholesterol, phase transition, kinetics, second order transition, pressure jump relaxation

Introduction

The interaction of cholesterol with various phospholipids has been studied by numerous techniques in considerable detail (for a review see Demel and de Kruijff 1976). In general it was found that the incorporation of cholesterol into lipid bilayers decreases the enthalpy of the gel to liquid-crystalline phase transition, though the specific shapes of the calorimetric curves, the change in transition temperature, the appearance of overlapping peaks, and the exact dependence of the transition enthalpy on cholesterol content depends on the structure of the phospholipid head groups and the nature of the fatty acyl chains (Ladbrooke et al. 1968; Chapman and Wallach 1968; Mabrey et al. 1978; Estep et al. 1978, 1979; Calhoun and Shipley 1979; Blume 1980). In those systems where complete miscibility of cholesterol with the phospholipids is observed the effect of cholesterol on a molecular level can be described as follows: the lateral interactions of the phospholipid molecules are removed, as cholesterol perturbs the hexagonal packing of lipids in the gel phase. There is fast long axis rotation of the molecules and the acyl chains are in some intermediate state with respect to the gel and liquid-crystalline phase of pure phospholipids (Chapman and Wallach 1968; Wittebort et al. 1982; Blume and Griffin 1982). Macroscopically, this action of cholesterol

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Abbreviations: *DMPA*, dimyristoylphosphatidic acid; *DMPC*, dimyristoylphosphatidylcholine; *DMPE*, dimyristoylphosphatidylethanolamine; *DPPC*, dipalmitoylphosphatidylcholine; *DSC*, differential scanning calorimetry

leads to a broadened phase transition and reduced calorimetric endotherms. Most studies of lipid/cholesterol interactions have focussed on phosphatidylcholine/cholesterol bilayers and very detailed phase diagrams based on the results obtained by different techniques have been proposed (Lentz et al. 1980). Phosphatidylcholine/cholesterol bilayers, however, seem to display a particularly complex behaviour because phosphatidylcholines can adopt three different types of gel phases, namely the $P_{\beta'}$-, the $L_{\beta'}$-, and the L_c-phase, which complicates the interpretation of data. Other phospholipids such as phosphatidylethanolamines and phosphatidic acids have only one type of gel phase over a wide temperature range below the gel to liquid crystalline phase transition and are therefore better suited (Jähnig et al. 1979; McIntosh 1980). For kinetic experiments using the pressure jump relaxation technique with optical detection phosphatidic acids such as dimyristoylphosphatidic acid (DMPA) are particularly advantageous because the vesicles are stabilized by the negative charges of the head groups. We have previously reported on the phase transition kinetics of pure DMPA and have shown that the relaxation times for the phase transition of DMPA are much longer compared to phosphatidylcholines, apparently due to strong head group interactions via hydrogen bonds (Elamrani and Blume 1983; Gruenewald et al. 1980). For relaxation experiments using optical detection it is particularly important that the suspensions remain stable over the time course of the experiments. In experiments with lipid/cholesterol mixtures this problem is even more enhanced because lipid/cholesterol bilayers tend to settle very rapidly. Again DMPA/cholesterol mixtures are more suitable because of their negative surface charge so that a wider range of mixtures can be investigated.

We used differential scanning calorimetry to characterize the thermodynamic behaviour of DMPA/cholesterol mixtures and the monolayer technique to study the condensation effect of cholesterol. By these two methods we could show that DMPA/cholesterol bilayers behave similarly to other lipid/cholesterol systems. The kinetic experiments revealed a discontinuous decrease of the relaxation times with increasing cholesterol content, indicating a change in the properties of the system at ca. 12 mol% cholesterol. Comparison with data derived from X-ray and electron diffraction (Hui and He 1983) and NMR-experiments (Blume and Griffin 1982) together with suggestions put forward by Jähnig (1981 a, b) lead us to believe that at this concentration the phase transition changes its character from first- to second-order. Thus, the observed broad endotherm can no longer be ascribed to the latent heat of a first-order transition but to a change in the heat capacity accompanying the continuous second-order transition of the system.

Materials and methods

DMPA and cholesterol were purchased from FLUKA, Neu-Ulm, W.-Germany and used without further purification. The lipids were pure as tested by thin-layer chromatography on silica gel plates (Merck, Darmstadt, W.-Germany). Mixtures were prepared from stock solutions of the lipids in chloroform/methanol (9 : 1) by mixing the appropriate volumes in glass vials and evaporating the solvent in a stream of nitrogen at elevated temperature (55 °C). Residual organic solvent was removed overnight under high vacuum. The lipid film was then dispersed in bidistilled water at a temperature of 60 °C by vigorous shaking and vortexing for 10 min. After cooling the suspension to room temperature the pH of the dispersion was adjusted to pH 6 with dilute hydrochloric acid using a Radiometer PHM 26 pH-meter with micro glass electrode (Schott, Mainz, W.-Germany). The suspension was again vortexed and then degassed under water aspirator vacuum.

Calorimetry

Differential scanning calorimetry was performed as described before using a Privalov DASM-1 calorimeter (Blume 1983). The lipid concentrations varied between 1 mg/mL for pure DMPA and 4 mg/mL for samples with high cholesterol content. A scan rate of 1 K/min was used for all experiments.

Monolayer experiments

Monolayer experiments were performed as described elsewhere (Blume 1979) using a MGW Lauda film balance with pressure control system. Isobars at 25 dynes/cm were recorded at increasing temperature with a scan rate of ca. 1 K/min.

Pressure jump relaxation experiments

Pressure jump relaxation experiments were performed using a pressure jump autoclave with optical detection (Knoche and Wiese 1976; Gruenewald et al. 1980; Elamrani and Blume 1983). The 0.07 mm brass foils gave a bursting pressure of ca. 100 bar with a dead time of ca. 50 μs. Turbidity ($\lambda = 360$ nm) vs time curves were stored in a Datalab DL 905 transient recorder. Data analysis using semilogarithmic least-squares fits of the experimental curves was done as described before using a HP 9845 A computer (Elamrani and Blume 1983).

Results

Differential scanning calorimetry

Figure 1 shows calorimetric scans of DMPA/cholesterol mixtures with increasing cholesterol concentration. Incorporation of cholesterol into the lipid bilayers produces the well known broadening of the phase transition with a decrease in transition enthalpy. The transition temperature, T_m, decreases with increasing cholesterol content and the transition becomes asymmetric (see Fig. 1). The decrease of ΔH is almost linear as shown in Fig. 2a. This behaviour is very similar to that observed before for the dimyristoylphosphatidylethanolamine/cholesterol system (Blume 1980) but different from dimyristoylphosphatidylcholine/cholesterol mixtures (Mabrey et al. 1978) where an increase in T_m is observed with increasing cholesterol content. A plot of the peak maximum in the DSC curves vs cholesterol content is shown in Fig. 2b. $C_{P_{max}}$ passes through an intermediate maximum at ca. 10 mol% cholesterol. A careful inspection of Fig. 2a shows that this discontinuity may also be present in the ΔH-plot.

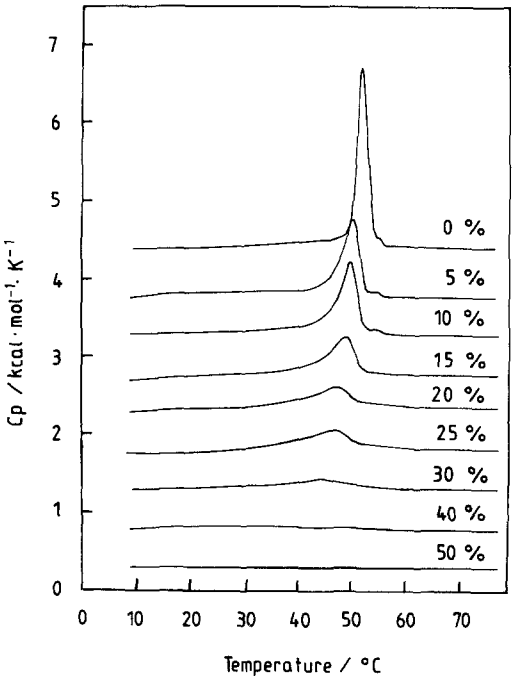

Fig. 1. Differential scanning calorimetric curves of DMPA/cholesterol mixtures. Numbers designate cholesterol content in mol%

Monolayer experiments

Figure 3 shows isobars of monomolecular films of DMPA/cholesterol mixtures recorded at a film pressure of 25 dynes/cm. As observed in the corresponding DSC curves of DMPA/cholesterol bilayers, the transition from the liquid-condensed to the liquid-expanded state is broadened and the midpoint of the transition is shifted to lower temperature upon addition of cholesterol. The condensing effect of cholesterol on the liquid expanded phase is more evident when the molecular area at 25 dynes/cm is plotted vs the mole fraction of cholesterol (Fig. 4a). The change in molecular area, Δf, between 15 and 50 °C decreases in much the same way as ΔH decreases in the bilayer system (Fig. 4b). The condensing effect in the liquid-expanded phase amounts to ca. 6 Å^2/molecule at 50 mol% cholesterol. In the liquid-condensed phase only a slight condensing effect is observed, probably due to a change in tilt of the DMPA molecules induced by cholesterol.

Pressure jump relaxation experiments

As reported before (Elamrani and Blume 1983) three relaxation processes could be resolved for the transi-

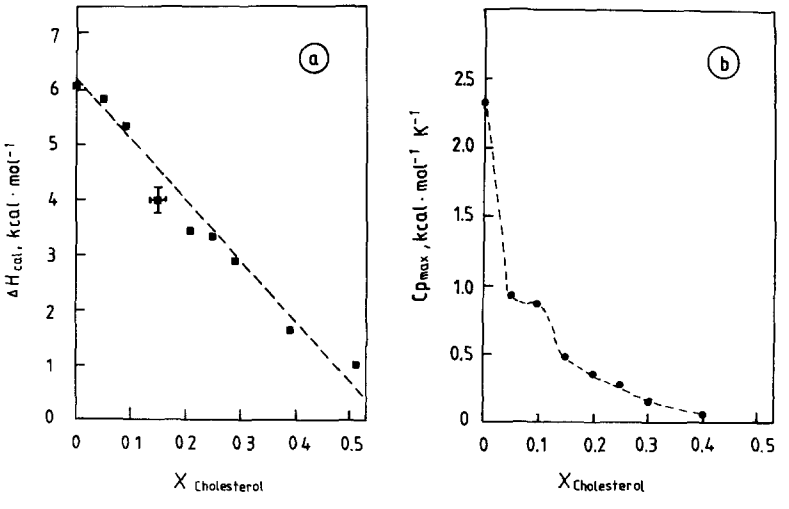

Fig. 2. a Transition enthalpy of DMPA/cholesterol mixtures as a function of cholesterol content. **b** Maximum height of the calorimetric peaks $C_{P_{max}}$ taken from Fig. 1 as a function of cholesterol content

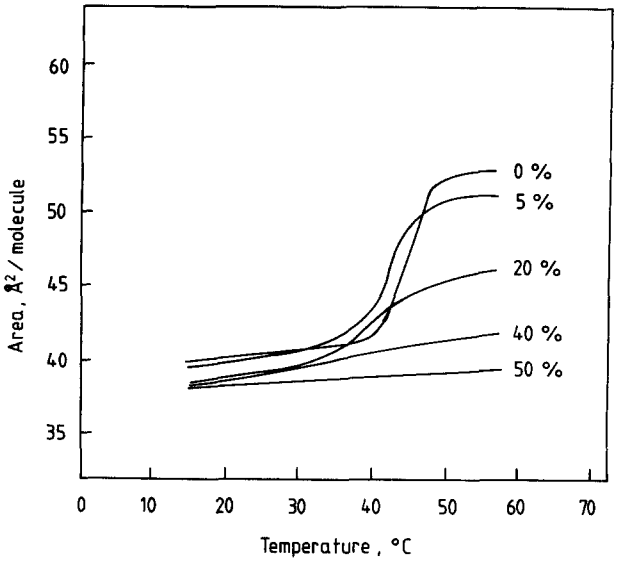

Fig. 3. Monolayer isobars of DMPA/cholesterol mixtures at a lateral pressure of 25 dynes/cm. Numbers designate cholesterol content in mol%

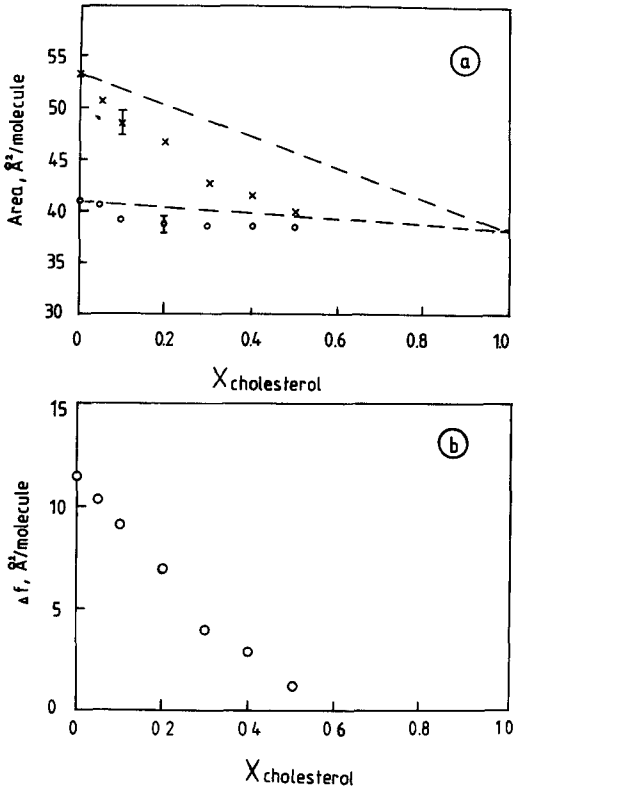

Fig. 4. a Molecular area of monolayers of DMPA/cholesterol mixtures at 25 dynes/cm in the liquid condensed phase at 15 °C (○) and in the liquid-expanded phase at 50 °C (×). **b** Change in molecular area Δf between 15° and 50 °C as taken from data in **a**

tion kinetics of pure DMPA bilayers, the fastest in the ms, the second in the 100 ms, and the slowest in the s time range. This is shown in Fig. 5. Each of these three relaxation processes revealed a distinct maximum of the relaxation amplitude and relaxation time at the midpoint of the transition. The total amplitude observed after a pressure jump was always larger than the sum of the three individual relaxation amplitudes indicating the presence of additional processes shorter than the dead time of our instrument (ca. 50 μs). Addition of cholesterol to DMPA bilayers produces a general decrease of the total relaxation amplitude and a decrease in the relaxation times as well. Our experiments covered the concentration range from 0 to 25 mol% cholesterol. Dispersions with higher cholesterol content tend to settle during the time course of the experiments. In addition the relaxation amplitudes become very small with a concomitant increase in base line noise. At a percentage of 20 to 25 mol% cholesterol a distinct maximum of the relaxation times and amplitudes can no longer be observed due to the broadening of the phase transition as evident from the calorimetric scans in Fig. 1. Figure 6 shows relaxation times and amplitudes of the slowest process for pure DMPA and bilayers with 10 and 20 mol% cholesterol. As mentioned above the total relaxation amplitude decreases with increasing cholesterol content (Fig. 7a). The three individual relaxation amplitudes however, show a different behaviour. The relative amplitude of the slowest process first increases upon cholesterol incorporation before decreasing again (see Fig. 7b). The amplitudes for the other two observed processes display a more or less continuous decrease with cholesterol content (see Fig. 7c and Fig. 7d). The relative amplitude of the fast process not resolvable by the pressure jump technique first decreases and then increases again (not shown). The relaxation times show a very characteristic dependence on cholesterol content. Figure 8 shows the maximum values of the relaxation times τ_1 and τ_3 observed at the transition midpoint as a function of cholesterol content. A general decrease of τ_1 and τ_3 is observed, but between 10 and 15 mol% cholesterol the relaxation times pass through an intermediate maximum, which is more distinct for τ_1 than for τ_3. For the relaxation time τ_2 no characteristic dependence on cholesterol content could be observed due to the relatively large scatter of the data, because this process has a comparatively small relative amplitude (see Fig. 7c). It is evident from Fig. 8 that τ_3 decreases initially much faster than τ_1. At 20 mol% cholesterol τ_1 has decreased by a factor of ca. 2, τ_2 by a factor of ca. 4–5 (not shown) and τ_3 by a factor of 3.5.

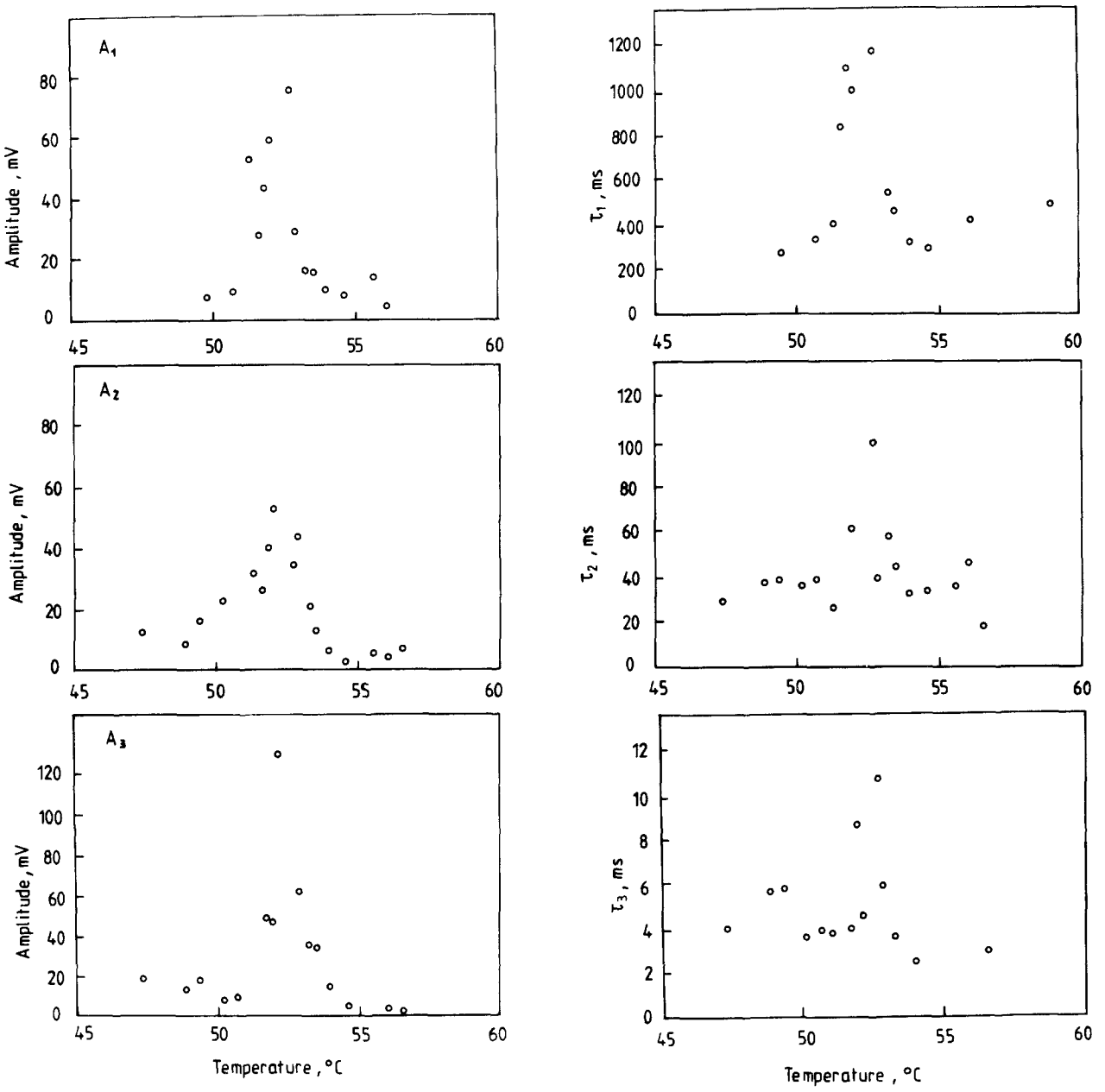

Fig. 5. Amplitudes and relaxation times of the three processes observed by the pressure jump relaxation method for the transition of pure DMPA bilayers

Discussion

The effect of cholesterol incorporation on the thermotropic behaviour of phospholipid bilayers has been an area of intensive research. In general, cholesterol incorporation leads to an increase in the width of the phase transition and a decrease in the transition enthalpy (Ladbrooke et al. 1968; Chapman and Wallach 1968). In most cases the transition peaks can be decomposed into sharp and broad component peaks, the sharper disappearing between 15 and 25 mol% cholesterol (Mabrey et al. 1978; Estep et al. 1978, 1979; Calhoun and Shipley 1979; Blume 1980). This is also observed for the DMPA/cholesterol system, though in this case the superposition is not as evident as in DMPC/cholesterol or DPPC/cholesterol bilayers. In this respect DMPA/cholesterol resembles DMPE/cholesterol bilayers where the peaks are also asymmetric but a decomposition into component peaks seems somewhat arbitrary (Blume 1980). The strong interaction between PA and PE head groups, which are responsible for their higher transition temperatures as compared to PC, are probably the reason for this phenomenon. Conse-

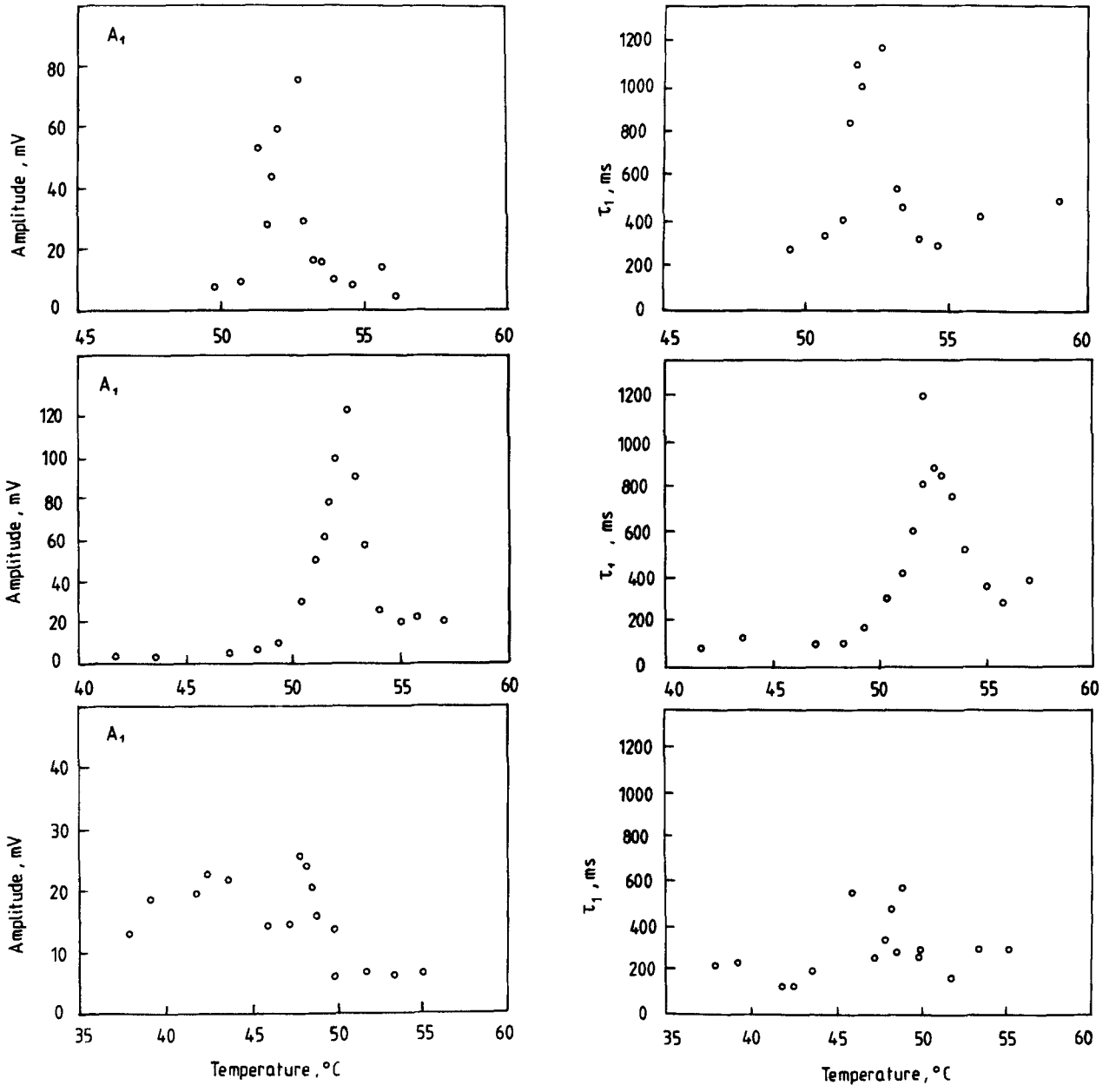

Fig. 6. Amplitude A_1 and relaxation time τ_1 for the slowest process at different cholesterol concentrations. *Top:* 0 mol% cholesterol, *middle:* 10 mol% cholesterol, *bottom:* 20 mol% cholesterol

quently the transition shifts to lower temperatures when these head group interactions are perturbed by cholesterol molecules acting as spacers. In DMPC/cholesterol bilayers, however, the transition is shifted to higher temperature (Mabrey et al. 1978). The similarity between DMPE and DMPA as opposed to DMPC is also reflected in the monolayer behaviour. The condensing effect of cholesterol on lipid monolayers in the liquid-expanded state as observed in the monolayer isotherms has been known for a long time (see Demel and de Kruijff 1976). We have shown in the past that the measurement of isobars offers certain advantages as the change in molecular area at the transition can be directly observed when the temperature is increased and then compared to the bilayer behaviour (Blume 1979). The question of what the internal lateral pressure of a bilayer should be or at what lateral pressure of the monolayer these two different systems should be compared has been addressed by a number of studies (Nagle 1976; Albrecht et al. 1978; Pink and Chapman 1979; Marcelja 1974). We have suggested before that a lateral pressure of 30 dynes/cm is a good estimate and that at this pressure the molecular areas of different

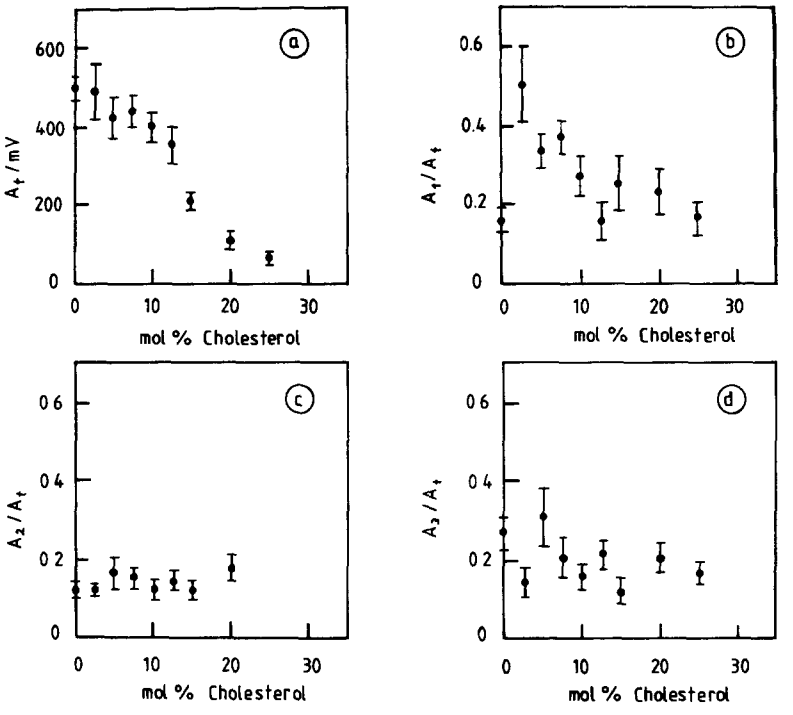

Fig. 7. Amplitudes observed in the pressure jump experiments as a function of cholesterol content. **a** Total observed amplitude in mV (10 mV = 0.001 OD); **b** Relative amplitude A_1/A_t for the slowest process; **c** Relative amplitude A_2/A_t for the middle process; **d** Relative amplitude A_3/A_t for the fast process

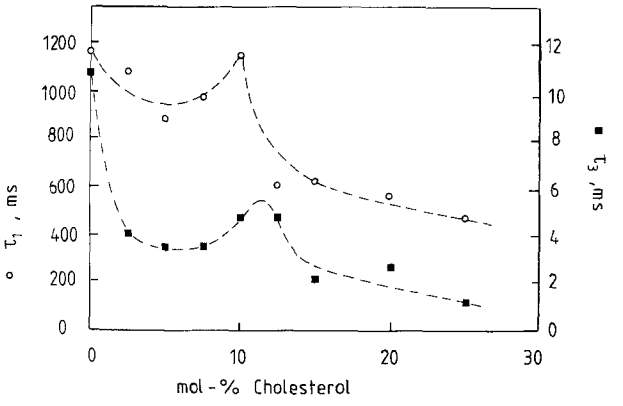

Fig. 8. Maximum values of the relaxation times τ_1 (○) and τ_3 (■) as a function of cholesterol content

phospholipids and the relative changes of the transition temperature with head group structure compare well with data known for the equivalent bilayer systems (Blume 1979). A recent theoretical study comes to the same conclusion (Georgallas et al. 1984). In this study, for purely technical reasons, we used a film pressure of 25 dynes/cm in order to reduce the transition temperature of DMPA, because at high film pressures and high temperature the monolayers tend to become unstable and film loss can become a problem. The slight reduction in film pressure leaves the general findings unaffected. Figure 4b shows that the change in area, Δf, at the liquid-condensed to liquid-expanded phase transition decreases almost linearly with increasing cholesterol content, in a very similar manner to the transition enthalpy in the corresponding bilayer system. This decrease is mainly due to the condensation effect of cholesterol on the liquid-expanded phase. Trans-gauche isomerization is drastically reduced by cholesterol incorporation and the molecules adopt a more extended conformation. For the corresponding bilayer system this could be clearly shown by ^{2}H-NMR measurements on specifically deuterated phospholipids (Blume and Griffin 1982). The slight condensation effect in the liquid-condensed state is probably caused by change in tilt of the molecules induced by cholesterol incorporation. Dihexadecylphosphatidic acid at pH 7 in the singly charged form is known to have slightly tilted hydrocarbon chains with a tilt angle of 18° at 20 °C (Jähnig et al. 1979). The molecular area at that temperature is 41 Å^2. This is close to what we find for the molecular area in the monolayer at 25 dynes/cm. Removal of the hydrocarbon chain tilt would reduce the molecular area of DMPA to 39 Å^2. This is obviously the effect we observe. In DPPC/cholesterol bilayers this effect is even more pronounced (Ladbrooke et al. 1968; Müller-Landau and Cadenhead 1979). Monolayer experiments on DMPA/cholesterol mixtures have been reported before by Albrecht et al. (1981). A direct comparison of our results with their data is unfortunately not possible as they constructed phase diagrams from isotherms measured at one particular temperature as opposed to our method of measuring isobars. So only some values can be compared and these seem to agree quite well. The construction of a phase dia-

gram from our monolayer data is difficult for the same reasons as for constructing a phase diagram from the DSC-curves for the bilayer system, because the transitions are broad and distinct breaks in the isobars which could be taken as points on phase boundaries are difficult to distinguish. It is evident from the shape of the isobars that the effects of cholesterol on lipid monolayers are very similar to the effects produced in the corresponding bilayer system. Consequently the coupling between the opposing monolayers in the bilayers is apparently only weak (Georgallas et al. 1984).

While the calorimetric and monolayer data on DMPA/cholesterol mixtures confirm earlier results on similar phospholipid/cholesterol mixtures the results of our kinetic experiments give some new insights into the dynamic behaviour of these mixed systems which lead to conclusions concerning the thermodynamic behaviour. The phase transition kinetics of phospholipid bilayers is complex. Overall five relaxation processes have been observed for pure phosphatidylcholine bilayers by relaxation methods such as the laser temperature-jump and the pressure jump technique (Gruenewald et al. 1980; Elamrani and Blume 1983; Gruenewald et al. 1981; Eck and Holzwarth 1984). While the fastest two processes in the 10 ns and 100 ns time range show no or only little change with temperature, the other three slower relaxation processes display marked increases in amplitude and time constant at the transition midpoint and can therefore be ascribed to a cooperative phenomenon. By the pressure jump relaxation technique it was shown that these three processes display a marked dependence on the nature of the lipid head group. Phosphatidic acids show relaxation times ca. two orders of magnitude slower than phosphatidylcholines with the same chain length (Elamrani and Blume 1983). This is a clear indication of the importance of head group interactions for the kinetic behaviour. In the case of phosphatidic acids head group interactions can occur via hydrogen bonds between the phosphate groups such as $O=P-O-H\cdots O=P-O-H\cdots O=P-O-H$ which overcompensate repulsive electrostatic interactions between the negatively charged groups. The results of our pressure jump experiments and also data from our studies of the electrostatically induced phase transition of the same system (Elamrani and Blume 1984) lead us to believe that nucleation, is indeed, the rate determining step in the transition kinetics. That means that the formation of stable liquid-crystalline nuclei in the surrounding gel phase lipid is a slow process. The subsequent growth steps which lead to larger clusters of the opposite phase are faster and are influenced by the cluster size. This mechanism is essentially what has been proposed in the frame work of Fisher's cluster model, first in a different context by Adam (1968) and then by Kanehisa and Tsong (1978). Within this model the slowest relaxation time representing the nucleation step always shows the largest increase in time constant at the transition midpoint. This is what we observe for pure DMPA as well as DMPA/cholesterol bilayers. The slowest relaxation time, τ_1, increases by a factor of 5–20 at T_m whereas τ_2 and τ_3 increase only by a factor of 3–5 (see Figs. 5 and 6). The much slower relaxation times observed for DMPA are therefore not caused by higher cooperativity (the cooperativity in this system is in fact lower than in the corresponding DMPC bilayer) but by slower nucleation because of the stronger attractive head group interactions between the phosphate groups in DMPA.

Within this model some of the observations on the kinetics of cholesterol/DMPA bilayers can now be described quite reasonably. Incorporation of cholesterol has a spacer effect on the phospholipid head groups and perturbs the hydrocarbon chain packing as well. Due to this effect we would expect a decrease in relaxation times as the formation of nucleation sites should be facilitated because of the disorder already induced in the neighbourhood of cholesterol molecules. Also the number of nucleation sites should increase because of the perturbation by cholesterol. What we observe for the slowest process is initially a decrease in time constant but an increase in the amplitude A_1 (see Figs. 7 and 8). This increase in amplitude for the slowest process thus reflects, in our opinion, the increased number of nucleation sites and the decrease in τ_1 the faster kinetics of nucleation. Processes 2 and 3 also display a decrease in time constants but to a much larger extent (see Fig. 8 for τ_3). If one or both of these processes reflect the growth of the nuclei to larger clusters the strong decrease of the cooperative unit size of the system as reflected by the calorimetric results (see Fig. 2b) can serve as a simple explanation. Lower cooperativity means smaller cluster size and hence faster kinetics. The cooperative unit size at 5 mol% cholesterol has decreased by a factor of 2 and τ_3 by a factor of 2.5–3. The nucleation step should be less affected by the lower cooperativity as a nucleation site presumably consists, a priori, only of a few molecules, a number certainly smaller than the size of the whole cluster. It should be mentioned that the relative amplitude of the fast process, which in pure DMPA bilayers accounts for ca. 50% of the total amplitude decreases to only 20% at 5 mol% cholesterol while the amplitude A_1 of the slowest process increases to 40% and this when the total amplitude A_t is still almost the same as in pure DMPA. It could be that this fast process can be ascribed to non-cooperative transitions of individual

molecules to a liquid-crystalline state, however without the formation of stable nucleation sites (Eck and Holzwarth 1984). If this interpretation is correct then the effect of cholesterol would be to change the ratio of stable to unstable nucleation sites. Within the microscopic interaction model suggested by Mouritsen et al. (Mouritsen et al. 1983; Mouritsen 1983; Mouritsen and Zuckermann 1985) this fast process could correspond to the formation of isolated "intermediate type" lipid and the slower process to the formation of small "liquid lipid" clusters stabilized at the boundary by "intermediate type" lipid.

At ca. 10 mol% cholesterol content discontinuities in the thermodynamic as well as the kinetic behaviour of the system can be observed. In the calorimetric experiments this is manifested in the dependence of the cooperative unit size or the maximum values of the calorimetric peaks on cholesterol content (see Fig. 2b). In other phospholipid/cholesterol systems it has been observed before that at low cholesterol concentration the DSC thermograms can be decomposed into a sharp and an underlying broad peak and that the sharp component disappears at higher (20 to 25 mol%) cholesterol content (Mabrey et al. 1978; Estep et al. 1978). The abrupt changes occurring at this particular cholesterol concentration have been interpreted in terms of phase separation in the solid state or alternatively by a percolation model (Mabrey et al. 1978; Rubinstein et al. 1979; Lentz et al. 1980; Snyder and Freire 1980). NMR-measurements of ^{13}C and ^{2}H-labelled phospholipids in mixtures with cholesterol revealed no evidence for phase separation in the gel state nor in the temperature range of the phase transition region of phospholipid/cholesterol mixtures. Instead small clusters of lipids having different properties and exchanging by lateral diffusion seem to exist (Wittebort et al. 1982; Blume and Griffin 1982). In contrast, mixtures of two different phospholipids display phase separation in the transition region as two distinctly different NMR-signals characteristic of gel and liquid-crystalline lipid are observed (Blume et al. 1982). These findings are supported by recent x-ray and electron diffraction results on DMPC/cholesterol mixtures. These diffraction experiments gave no indication for the existence of larger domains with different composition. Instead the properties of DMPC/cholesterol bilayers changed continuously, which again can be contrasted with results for mixtures of two different phospholipids where phase separation with large domains is observed (Hui 1981; Hui and He 1983). A different explanation for the abrupt changes of various properties occurring at particular cholesterol concentrations has been suggested by Jähnig (1981a, b). Jähnig has developed some concepts for lipid/protein interactions within the frame work of the Landau theory which are also applicable for lipid/cholesterol interactions. It is now generally agreed that the phase transition of phospholipids is of first order though close to a critical point (Jähnig 1981a, b; Mouritsen et al. 1983; Mouritsen and Zuckermann 1985; Mitaku et al. 1983). That implies that in pure lipid bilayers a somewhat broadened phase transition is observed with a coexistence of fluid and ordered domains within the transition region. Experimentally this can indeed be verified by ^{2}H-NMR spectroscopy where two distinctly different NMR signals are seen inside the transition region of a phospholipid (Blume et al. 1982). Because this transition is relatively close to a critical point incorporation of foreign molecules which perturb the packing of the lipid molecules can change the nature of the phase transition to second-order. This occurs at a certain critical concentration of the perturbing molecule. A plot of the response function of, for instance, the relaxation time vs the concentration should then pass through a maximum at the critical concentration (Jähnig 1981a, b). We believe that this suggestion offers an explanation for most of our experimental findings. Within this model the intermediate maximum in the relaxation times at ca. 10 mol% cholesterol is an indication for a change of the nature of the phase transition from first- to second-order. The discontinuity in the bilayer behaviour is not only apparent from the plot of τ_1 vs cholesterol content in Fig. 8 but also from a comparison of Figs. 5 and 6. It can clearly be seen that τ_1 as a function of temperature increases at T_m much more for the sample with 10 mol% cholesterol (a factor of ca. 20) than for pure DMPA where τ_1 changes only by factor of 10 at T_m. The other relaxation times τ_2 and τ_3 show a similar behaviour, though the effect is more pronounced for the slowest process. Above the critical concentration the phase transition becomes seond-order, i.e. a continuous cooperative transition without domain structure is observed. Jähnig (1981b) in his paper gave a simple equation derived from geometrical arguments for the calculation of the critical concentration. For an incorporated protein of 15 Å radius the critical lipid/protein ratio is 54, corresponding to a concentration of ca. 2 mol%. Inserting a value for the radius of the cholesterol molecule of ca. 3.5 Å one obtains a critical ratio of ca. 27 corresponding to 3.5 mol%. Jähnig corrected by a factor of 2 because the rigid part of cholesterol is only half as long as an acyl chain. Still the critical concentration comes out too low as the experimentally observed values are much higher. One has to keep in mind however, that the equation is based on a oversimplification of the whole system. In particular non-ideal mixing is not taken into account. This

effect would increase the critical concentration to values depending on the extent of non-ideality of the mixture. It is known that the nature of the fatty acyl chains and the structure of the lipid head group control the thermodynamic behaviour. For lipids with longer chains the critical concentration would probably be higher and indeed with DPPC discontinuities in the behaviour of the system are observed at ca. 20 to 30 mol% (Mabrey et al. 1978; Estep et al. 1978). For distearoyl- and diarachidoylphosphatidylcholine/cholesterol mixtures the critical concentration seems to be even higher as judged by DSC measurements (Blume, unpublished observation).

A consequence of the change of the nature of the phase transition from first- to second-order would also be that the transition enthalpies determined from the DSC scans at high cholesterol content should not be interpreted as latent heats of melting because for a second order transition the latent heat no longer exists because the transition is continuous. The broad peaks in the calorimetric scans reflect a continuous change of the heat capacity of the system.

Summarizing the results of our kinetic experiments we can say that in addition to the well-known effects of cholesterol upon structure, dynamics and calorimetric behaviour of lipid bilayers the incorporation of cholesterol also leads to drastic changes in the kinetic behaviour. A general decrease of relaxation times with increasing cholesterol content was observed. This was also shown for a related system, i.e. DMPC/cholesterol (Eck and Holzwarth 1984). The changes of the relative amplitudes of the different relaxation times enabled us to give a reasonable explanation for the mechanism of the transition. The large increase of the amplitude of the slowest process seems to be due to an increased number of nucleation sites produced by cholesterol incorporation. This means that the slowest process is indeed the nucleation step. The other two observed relaxation times decrease much faster with cholesterol concentration and can probably be ascribed to the growth steps. As the cooperativity is decreased the clusters become smaller. We could also show that at a certain concentration the relaxation times pass through an intermediate maximum and we interpret this, in agreement with suggestions made by Jähnig (1981 a), as being caused by a change of the nature of the phase transition from first- to second-order.

At this point we want to add some speculations concerning the possible biological relevance of this phenomenon. Many biological membranes contain cholesterol and in the past this was mainly interpreted with respect to an increased stability and decreased permeability being important in these systems. In our view the finding that the phase transition becomes continuous at low cholesterol content is of particular relevance for a biological membrane. A drop in temperature can now no longer lead to phase separation into gel and liquid-crystalline lipid domains because the membrane stays homogeneous. Consequently the permeability of the membrane which otherwise would be enhanced, as larger molecules permeate preferentially through boundaries between the different domains, stays low. In addition protein aggregation with concomitant functional inhibition caused by phase separation into gel and liquid crystalline lipid domains in mixtures of phospholipids without cholesterol is prevented.

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References

Adam G (1968) Ionenstrom nach einem depolarisierenden Sprung im Membranpotential. Z Naturforschg 236: 181–197

Albrecht O, Gruler H, Sackmann E (1978) Polymorphism of phospholipid monolayers. J Phys (Paris) 39: 301–313

Albrecht O, Gruler H, Sackmann E (1981) Pressure-composition phase diagrams of cholesterol/lecithin, cholesterol/phosphatidic acid, and lecithin/phosphatidic acid mixed monolayers. A Langmuir film balance study. J. Colloid Interface Sci 79: 319–338

Blume A (1979) A comparative study of the phase transition of phospholipid bilayers and monolayers. Biochim Biophys Acta 557: 32–44

Blume A (1980) Thermotropic behavior of phosphatidylethanolamine-cholesterol and phosphatidylethanolamine-phosphatidylcholine-cholesterol mixtures. Biochemistry 19: 4908–4913

Blume A (1983) Apparent molar heat capacities of phospholipids in aqueous dispersion. Effects of chain length and head group structure. Biochemistry 22: 5436–5442

Blume A, Griffin RG (1982) Carbon-13 and deuterium nuclear magnetic resonance study of the interaction of cholesterol with phosphatidylethanolamine. Biochemistry 21: 6230–6242

Blume A, Wittebort RJ, Das Gupta SK, Griffin RG (1982) Phase equilibria, molecular conformation and dynamics in phosphatidylcholine/phosphatidylethanolamine bilayers. Biochemistry 21: 6243–6253

Calhoun, WI, Shipley GG (1979) Sphingomyelin-lecithin bilayers and their interaction with cholesterol. Biochemistry 18: 1717–1722

Chapman D, Wallach DFH (1968) Recent physical studies of phospholipids and natural membranes. In: Chapman D (ed) Biological membranes. Academic Press, London New York, pp 125–202

Demel RA, de Kruijff B (1976) The function of sterols in membranes. Biochim Biophys Acta 457: 109–132

Eck V, Holzwarth JF (1984) Fast dynamic phenomena in vesicles of phospholipids during the phase transition. In: Mittal KL, Lindman B (eds) Surfactants in solution. Plenum Press, New York, pp 2059–2079

Elamrani K, Blume A (1983) Phase transition kinetics of phosphatidic acid bilayers. A pressure jump relaxation study. Biochemistry 22:3305–3311

Elamrani K, Blume A (1984) Phase transition kinetics of phosphatidic acid bilayers. A stopped-flow-study of the electrostatically induced transition. Biochim Biophys Acta 769: 578–584

Estep TN, Mountcastle DB, Biltonen RL, Thompson TE (1978) Studies on the anomalous thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine-cholesterol mixtures. Biochemistry 17:1984–1987

Estep TN, Mountcastle DB, Barenholz Y, Biltonen RL, Thompson TE (1979) Thermal behavior of synthetic sphingomyelin-cholesterol dispersions. Biochemistry 18: 2112–2117

Georgallas A, Hunter DL, Lookman T, Zuckermann MJ, Pink DA (1984) Interaction between two sheets of a bilayer membrane and its internal lateral pressure. Eur Biophys J 11:79–86

Gruenewald B, Blume A, Watanabe F (1980) Kinetic investigations on the phase transition of phospholipid bilayers. Biochim Biophys Acta 597:41–52

Gruenewald B, Frisch W, Holzwarth JF (1981) The kinetics of the formation of rotational isomers in the hydrophobic tail region of phospholipid bilayers. Biochim Biophys Acta 641:311–319

Hui SW (1981) Geometry of phase-separated domains in phospholipid bilayers by diffraction contrast electron microscopy. Biophys J 34:383–395

Hui SW, He NB (1983) Molecular organization in cholesterol-lecithin bilayers by X-ray and electron diffraction measurements. Biochemistry 22:1159–1164

Jähnig F (1981a) Critical effects from lipid-protein interactions in membranes I. Theoretical description. Biophys J 36:329–343

Jähnig F (1981b) Critical effects from lipid-protein interactions II. Interpretation of experimental results. Biophys J 36:344–357

Jähnig F, Harlos K, Vogel H, Eibl H (1979) Electrostatic interactions at charged lipid membranes. Electrostatically induced tilt. Biochemistry 18:1459–1468

Kanehisa MI, Tsong TY (1978) Cluster model of lipid phase transitions with application to passive permeation of molecules and structure relaxations in lipid bilayers. J Am Chem Soc 100:424–432

Knoche W, Wiese G (1976) Pressure jump relaxation with optical detection. Rev Sci Instrum 47:220–221

Ladbrooke BD, Williams RM, Chapman D (1968) Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. Biochim Biophys Acta 150:333–343

Lentz BR, Barrow DA, Hoechli M (1980) Cholesterol-phosphatidylcholine interactions in multilamellar vesicles. Biochemistry 19:1943–1954

Mabrey S, Mateo PL, Sturtevant JM (1978) High-sensitivity scanning calorimetry study of mixtures of cholesterol with dimyristoyl- and dipalmitoylphosphatidylcholines. Biochemistry 17:2464–2468

Marcelja S (1974) Chain ordering in liquid crystals II. Structure of bilayer membranes. Biochim Biophys Acta 367: 265–276

McIntosh TJ (1980) Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphatidylcholine bilayers. A molecular packing model. Biophys J 29:237–246

Mitaku S, Jippo T, Kataoka R (1983) Thermodynamic properties of the lipid bilayer transition. Pseudocritical phenomena. Biophys J 42:137–144

Mouritsen OG (1983) Studies on the lack of cooperativity in the melting of lipid bilayers. Biochim Biophys Acta 731: 217–221

Mouritsen OG, Zuckermann MJ (1985) Softening of lipid bilayers. Eur Biophys J 12:75–86

Mouritsen OG, Boothroyd A, Harris R, Jan N, Lookman T, MacDonald L, Pink DA, Zuckermann MJ (1983) Computer simulation of the main gel – fluid phase transition of lipid bilayers. J Chem Phys 79:2027–2041

Müller-Landau F, Cadenhead DA (1979) Molecular packing in steroid-lecithin monolayers. Part II. Mixed films of cholesterol with dipalmitoylphosphatidylcholine and tetradecanoic acid. Chem Phys Lipids 25:315–328

Nagle JF (1976) Theory of lipid monolayer and bilayer phase transitions: Effect of headgroup interactions. J Membr Biol 27:233–250

Pink DA, Chapman D (1979) Protein-lipid interactions in bilayer membranes: A lattice model. Proc Natl Acad Sci USA 76:1542–1546

Rubinstein JLR, Smith BA, McConnell HM (1979) Lateral diffusion in binary mixtures of cholesterol and phosphatidylcholines. Proc Natl Acad Sci USA 76:15–18

Snyder B, Freire E (1980) Compositional domain structure in phosphatidylcholine-cholesterol and sphingomyelin-cholesterol bilayers. Proc. Natl Acad Sci USA 77:4055–4059

Wittebort RJ, Blume A, Huang TH, Das Gupta SK, Griffin RG (1982) Carbon-13 nuclear magnetic resonance investigations of phase transitions and phase equilibria in pure and mixed phospholipid bilayers. Biochemistry 21: 3487–3502