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Effect of *n*-alcohols and glycerol on the pretransition of dipalmitoylphosphatidylcholine

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We have systematically investigated the effect of short-chain *n*-alcohols and glycerol on the pretransition of 1,2-dipalmitoylphosphatidylcholine (DPPC) by spectrophotometry. It is found that the *n*-alcohols and glycerol remove the pretransition above a critical concentration for each ligand. In addition, the short-chain *n*-alcohols below the critical concentration decrease the pretransition temperature. The longer the aliphatic chain length of the *n*-alcohol (up to butanol) (a) the greater the decrease in the pretransition temperature, and (b) the lower the concentration necessary to remove the pretransition. However, glycerol differs from the short-chain *n*-alcohols in that it has no significant effect on either the pretransition or the main transition, but it is also capable of removing the pretransition above a critical concentration. It has previously been shown that alcohols have a biphasic effect on the main transition temperature of phosphatidylcholines (Rowe, E.S. (1983) Biochemistry 22, 3299–3305). At high alcohol concentrations, the main transition is not thermodynamically reversible (Rowe, E.S. (1985) Biochim. Biophys. Acta 813, 321–330). Recently, Simon and McIntosh (Biochim. Biophys. Acta (1984) 773, 169–172) have identified that at high ethanol concentration DPPC exists in the interdigitated phase. The critical ligand concentration at which the pretransition disappears coincides with the induction of main transition hysteresis and the biphasic alcohol effect in the main transition. These three effects appear to correlate with the induction of the interdigitated gel state by alcohols and glycerol.

1. Introduction

It is now well established that phosphatidylcholines (PCs) exhibit a low enthalpy transition (the pretransition) in addition to the chain melting main transition [1,2]. A number of studies have been done to determine the effects of small molecules on the pretransition of PCs [3–9]. For example Pringle and Miller [3] from electron spin resonance measurements reported that long-chain (C_{10}–C_{16}) *n*-alcohols increased the pretransition temperature by as much as 5 °C, below 10 mol% concentration of *n*-alcohols. At higher concentrations of *n*-alcohols the pretransition is abolished. Recently O'Leary and coworkers [8] have demonstrated that *cis*- and *trans*- tetradecenol decreased the pretransition temperature of distearoylphosphatidylcholine (DSPC) bilayers. Lee [6] also

Abbreviations: DPPC, 1,2-dipalmitoylphosphatidylcholine; DSPC distearoylphospatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DPHPC, 2-(3-(diphenylhexatrienyl)propanoyl)-3-palmitoyl-L-phosphatidylcholine; PE, phosphatidylethanolamine; Beta-DPPC, 1,3-dipalmitoylphosphatidylcholine; DHPC, 1,2-dihexadecylphosphatidylcholine; PC, phosphatidycholine.

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noted that at 1.5 mM *n*-octanol the pretransition disappeared in dipalmitoylphosphatidylcholine vesicles. Similar observations were made with dodecanol.

We have been characterizing the interaction of short-chain *n*-alcohols (methanol to *n*-butanol) with synthetic phospholipids, by studying the effect of *n*-alcohols on the main phase transition temperature of PCs [10], phosphatidylethanolamines (PEs) [11] and binary PC-PE mixtures [12]. It was demonstrated that the alcohols induce a biphasic melting behaviour in phosphatidylcholines with significant thermal hysteresis at high alcohol concentrations. Recently it has been shown by X-ray diffraction that ethanol at high concentrations induces DPPC to undergo a transition from the bilayer phase to a unusual gel phase in which the lipid hydrocarbon chains from opposing monolayers fully interdigitate [13].

Very few studies have been done on the effects of short-chain *n*-alcohols on the pretransition of PCs. Our earlier studies [10–12] focused on the effect of short-chain alcohols on the main transition but little attention was given to the pretransition. However, studies on the effect of alcohols on the pretransition can provide valuable information regarding the relative interaction of the alcohols with the gel phases of PCs.

In the present report we have systematically investigated the effects of several short-chain alcohols on the pretransition of DPPC by spectrophotometry and correlated these effects with their influence on the main transition. In an effort to correlate our melting experiments with alcohols with the extensive X-ray diffraction study of the DPPC-glycerol system [14], we have performed melting experients with DPPC in glycerol solutions.

Materials and Methods

Materials

Synthetic 1,2-dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma. The purity of the lipid was established by a single spot on thin-layer chromatography, and used without further purification. The fluorescent probe 2-(3-(diphenylhexatrienyl)propanoyl)-3-palmitoylphosphatidylcholine (DPHPC) was obtained from Molecular Probes, Eugene, OR. Ethanol ('200 Proof') was from Publicker Industries Co., Linfield, PA. Propanol and butanol were obtained from Aldrich Chemical Co., Milwaukee, WI, and glycerol was from Mallinckrodt Inc., St. Louis, MO.

Spectrophotometric measurements

Hydrated multilamellar dispersions of DPPC were prepared essentially by the method of Bangham et al. [15], to give a final phospholipid concentration of 1.3 mM. In the case of samples containing alcohols, these were added neat in microliter quantities directly to the liposomal aqueous suspension, and incubated for approximately 24 h at 15 °C prior to absorbance measurements.

Absorbance changes in the lipid suspension were used to monitor the phospholipid phase transition. The change in the main phase transition is primarily due to a change in refractive index which accompanies the lipid density change [16]. A contribution to the absorbance change also arises from the change in optical anisotropy of the lipid during the transition. In the case of the pretransition there is a relatively large absorbance change, which has previously been observed [16–18]. The pretransition involves a change in surface topography and tilt of the acyl chain [1,2]. It is likely that these physical changes contribute to the optical anisotropy effect which leads to the change in absorbance [18].

Absorbance measurements were made on a Cary 219 spectrophotometer operating at 400 nm and interfaced to an Apple IIe microcomputer. The temperature was monitored via a built-in thermistor, and the temperature and absorbance data were read directly into the computer. The thermistor was immersed in a jacketted cuvette connected in series with the sample cuvette as previously documented [10]. A uniform heating and cooling rate of 0.75 Cdeg/min was used unless stated otherwise. Data acquisition and manipulation was carried out using BASIC software kindly provided by R.G. Khalifah.

Fluorescence depolarization

Fluorescence depolarization measurements were made using an SLM 8000 T-format spectrofluorimeter. The excitation was at 351 nm using the OSRAM 450 watt (Ozone-free) xenon lamp. There

were three polarizers used in these experiments. The excitation polarizer was set at the vertical polarization mode. The polarizers for the phototubes A and B were set at the vertical and horizontal modes, respectively. The instrument G-factor (Ref. 19, p. 29) was measured using horizontally polarized incident light in order to calculate the fluorescence polarization. The excitation wavelength selection was made using the MC320 monochromator and the emission wavelength was selected using the MC640 monochromator. The bandpass selections were 2 and 16 nm for the excitation and emission monochromators, respectively, in order to minimize photobleaching and maximize sensitivity. The incident and scattered light intensity were minimized using high-pass KV-370 and KV-418 nm filters, for A and B phototubes, respectively. Note that a long wavelength filter was needed for the phototube B, since there is no monochromator to discriminate the fluorescence signal from the scattered light signal. Experiments without the probe showed that the signal from stray scattered light was less than 2% of the fluorescence signal. The data collection was performed in the A/B mode., i.e., as the ratio of the photocounts in the A and B phototubes. This ratio was collected and stored using an Apple II + computer onto to a floppy disk. The temperature was controlled by water circulation from an external programmed bath (Neslab ETP-3 temperature programmer). The temperature data were collected into the computer concurrently (using an Instrulab digital thermometer) with the A/B values, using a thermistor probe immersed in a reference cuvette at the same depth as the beam in the sample cuvette. Both the sample and the reference were stirred by a magnetic stirrer bar throughout the experiment. The heating and cooling experiments were carried out at a heating rate of 1 Cdeg/min. The data were stored on floppy disks and analyzed using a modified version of the BASIC program provided by SLM.

The samples for fluorescence were prepared by mixing appropriate volumes of chloroform solutions of DPPC and DPHPC and the solvent was blown off using nitrogen gas for 1 h to form a dry thin film. The suspension of the mixed PC was prepared in 0.1 M NaCl 0.001 M Na_2EDTA (pH 7.18) at a DPPC concentration of 1.3 mM. The ratio of the probe DPHPC to DPPC was 1 : 500. The sample was excited at 351 nm and emission was monitored at 430 nm. The concentration of DPPC in the sample for melting experiments was 0.13 mM. Appropriate volumes (less than 40 μl) of alcohol were added and the sample was incubated at room temperature for 15 min before starting the heat scan. The cooling scans were performed immediately following the heating scan.

Results

Fig. 1 shows the measurement of the pretransition and main transition of DPPC liposomes. Trace (a) illustrates a representative heating curve of DPPC in the absence of alcohol, observed by monitoring the change in absorbance at 400 nm as a function of temperature. The melting curve exhibits two abrupt changes in absorbance centered at 33.8° C and 41° C which represent the pretransition and main transition respectively. The temperature for these transitions are in agreement with those reported in the literature using a variety of methods [16,20–23].

The cooling curve shown in trace (b) depicts the change associated with the main transition of the DPPC bilayers at 41° C, but the absence of an absorbance change at or near the pretransition temperature is apparent. If this sample is stored at a temperature of 15° C overnight, the initial absorbance is restored, and the heating curve shown in trace (a) is reproduced. This demonstrates the slow reversible kinetics of the pretransition, which has been reported by others [22,24–26].

The effect of alcohols on the pretransition of DPPC was measured for methanol, ethanol, pro-

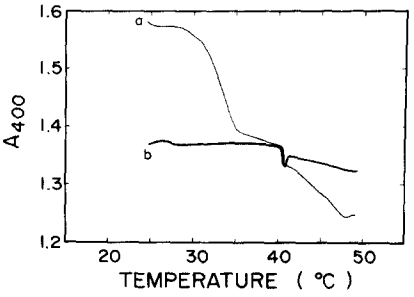

Fig. 1. Absorbance at 400 nm of DPPC as a function of temperature. Thin line, heating scan; thick line, cooling scan.

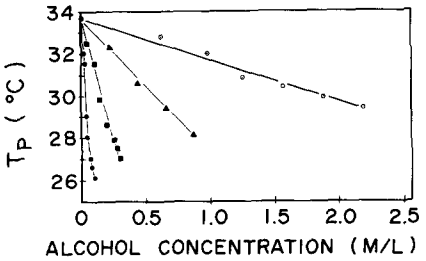

Fig. 2. Shift in the pretransition midpoint temperature of DPPC as a function of alcohol chain length and concentration for ⊙, methanol; ▲, ethanol; ■, *n*-propanol and ●, *n*-butanol.

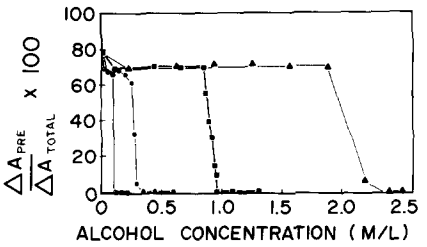

Fig. 4. The alcohol concentration dependence of the percentage of the pretransition signal of DPPC in the presence of ▲, methanol; ■, ethanol; ●, *n*-propanol and ⊙, *n*-butanol.

panol and butanol. Because of the kinetic effects mentioned above, only heating scans were studied, and the samples had been equilibrated below 15 °C at least overnight before measurements were made. Fig. 2 shows plots of the apparent pretransition midpoint temperature as a function of aqueous alcohol concentration for methanol, ethanol, propanol, and butanol. The plot depicts a linear decrease in temperature up to a critical alcohol concentration for each alcohol. It is also observed in Fig. 2 that the slope of each plot is dependent upon the alcohol chain length. The longer the chain length of the alcohol, the more negative the slope. The finding that the pretransition temperature is decreased by the alcohols indicates that the alcohols interact preferentially with the $P_{\beta'}$ phase relative to the $L_{\beta'}$ phase. The increase in slope with increasing alcohol chain length suggests that there is some hydrophobic character to the interaction of alcohol with the $P_{\beta'}$ phase.

At a particular alcohol concentration, different for each alcohol, the pretransition abruptly disappears. Fig. 3 shows a comparison of the melting transition curves of DPPC liposomes in the pres-

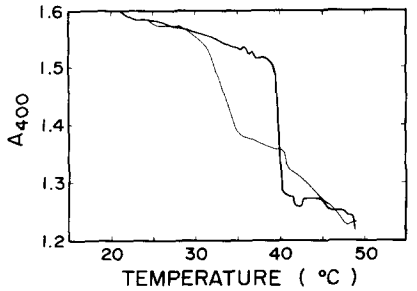

Fig. 3. Absorbance at 400 nm of DPPC as a function of temperature for heating scans in the absence (thin line) and presence (thick line) of 1.30 mol/l ethanol.

ence and absence of 1.30 mol/l ethanol, demonstrating that the pretransition has disappeared at this alcohol concentration. It is interesting to note that the absorbance change resulting from the main transition has increased significantly; no explanation for this change is readily apparent. The corresponding cooling curve for the high ethanol concentration sample (data not shown) depicts significant hysteresis in the main transition similar to that shown in Fig. 5 for propanol.

The disappearence of the pretransition as a function of alcohol concentration for four alcohols is shown in Fig. 4. Here is plotted the effect of alcohol concentration on the relative magnitude of the absorbance change resulting from the pretransition as a fraction of the total change for both the pre- and main transitions. For ethanol the suppression of the pretransition in DPPC liposomes is observed to take place over a very narrow ethanol concentration range (0.85–0.98 mol/l for ethanol). At lower concentrations (less than 0.85 mol/l), the magnitude of the pretransition remains constant, being independent of alcohol concentration. In order to determine whether the change in relative magnitude of the pretransition was an artifact of the heating rate, absorbance measurements were conducted using a range of scan rates. The results showed that the transition temperature and magnitude of the absorbance changes were independent of scan rate when measurements were made at rates less than 1.5 Cdeg/min. Similar results were obtained for all four alcohols as shown in Fig. 4.

Fig. 5 shows the main transition midpoint temperature (T_m) of DPPC as a function of propanol concentration, measured by DPHPC fluorescence depolarization and absorbance. The transition

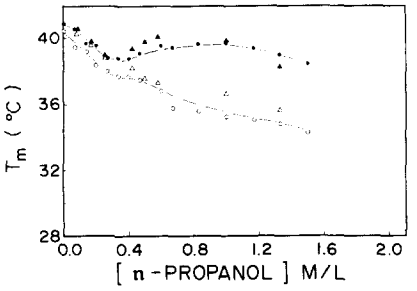

Fig. 5. Plot of the main transition temperature, T_m of DPPC for different total concentrations of *n*-propanol, from DPHPC fluorescence polarization measurements (●, ○), and from absorbance measurements (▲, △). The closed and open symbols are for T_m valus obtained from heating and cooling scans respectively.

midpoints from both heating and cooling scans are shown. The effect of propanol on the apparent melting temperature of DPPC as measured during heating scans is biphasic in nature. At relatively low alcohol concentrations (less than 0.3 mol/l) the effect of propanol is to linearly reduce the transition temperature. With a further addition of propanol the transition temperature increases, levels out and the apparent saturation occurs at approximately 0.6 mol/l. Similar results were previously obtained with ethanol and methanol using absorbance measurements [10,11]. The concentration at which the inflection in T_m and the hysteresis occur depends on the alcohol chain length. As seen here also, thermal hysteresis becomes apparent, beginning at the propanol concentration where the break in T_m occurs. The threshold concentration for the inflection of T_m and the onset of hysteresis for four alcohols are summarized in Table I. Based on our previous work [10,11] and that of Simon and McIntosh [13] these two phenomena occur concurrently with the induction of the interdigitated gel phase.

In Fig. 5 the T_m values are shown for both the absorbance measurements made using the Cary spectrophotometer and fluorescence depolarization of DPHPC. A comparison of the results obtained using the two techniques shows that even though there are small differences in the values of T_m, the overall results are consistent with each other. It has been reported [27] that the value of T_m obtained with DPHPC-labelled DPPC was slightly smaller than the value obtained without the probe by calorimetry, even if the probe concentration was less than 1 mol%. This is in agreement with our observation reported here.

In order to relate the changes in the main transition and pretransition behavior with alcohol to the induction of interdigitation, we have also studied the effect of glycerol on DPPC, because this system has been well studied by X-ray diffraction and scanning calorimetry techniques [14]. It was shown in that study that glycerol at a mol fraction of more than 0.45 induces the interdigitated gel phase in DPPC. Figs. 6–8 illustrate our results obtained with DPPC in glycerol. Fig. 6 shows the effect of glycerol concentration (plotted in terms of mol fraction of glycerol, X_g) on the main transition measured from the temperature dependence of the absorbance at 400 nm. The T_m values observed from the heating scans range from 41.3 to 42.4° C. These T_m values are in agreement

TABLE I
Aqueous ligand concentration (mol/l)

Ligand	Disappearance of pretransition	Break in T_m	Onset of pronounced hysteresis
Methanol	2.00	2.00–2.40 [a]	2.00
Ethanol	0.98	0.87–1.10 [a]	0.97
n-Propanol	0.32	0.27–0.40	0.40
n-Butanol	0.11	0.10–0.13	0.10
Glycerol	8.60 ($X_g = 0.45$)	–	8.60 ($X_g = 0.45$)

[a] See Ref. 11.

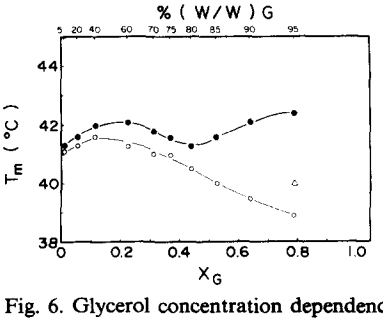

Fig. 6. Glycerol concentration dependence of the main transition temperature. Glycerol concentration is represented as mol fraction (X_g) and as weight percent units. ●, heating scan; ○, cooling scan. △ illustrates another transition seen in 95% (w/w) glycerol solution in the cooling scans.

with the values reported earlier using calorimetry [14]. As seen here, the T_m values obtained from cooling scans show significant thermal hysteresis for $X_g > 0.45$. Unfortunately, the corresponding calorimetry cooling scan measurements have not yet been reported for DPPC at different concentrations of glycerol. These observations of thermal hysteresis with glycerol are similar to the results obtained with *n*-propanol (see Fig. 5). However, it may be noted that glycerol does not decrease the T_m values (during the heating scans) unlike the case with alcohols, and in fact increases them slightly. At the highest concentration of glycerol studied here, $X_g = 0.79$, we observed two transitions in the cooling scans. Similar multiple transitions have been reported for DPPC suspended in pure glycerol (i.e., $X_g = 1.0$) [28].

Fig. 7 shows the effect of glycerol concentration on the pretransition temperature (T_p). Unlike the case with *n*-alcohols (see Fig. 4) the T_p does not decrease with increasing concentration of glycerol but varies from 35.5 to 37.5 °C. For $X_g >$ 0.45 there is no pretransition. This value for X_g correlates well with the minimum concentration of glycerol required to induce the interdigitated phase [14].

Fig. 8 shows the effect of glycerol concentration on the fractional contribution to the absorbance changes due to the pretransition phenomenon. As in the case with *n*-alcohols this fractional change reduces to zero, at this concentration of glycerol ($X_g = 0.45$). However, in Fig. 8 the fractional contribution in the case of glycerol is not a constant at lower concentrations of glycerol but in fact decreases gradually between X_g of 0 and 0.45. A possible factor is that the addition of the large amounts of glycerol used in these experiments is enough to significantly reduce

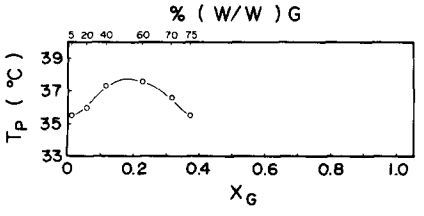

Fig. 7. Glycerol concentration dependence of the pretransition temperature.

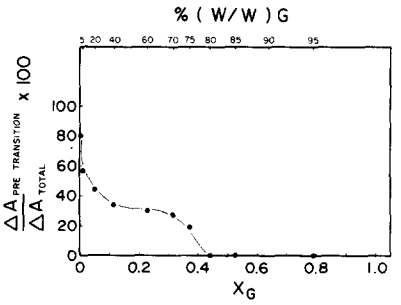

Fig. 8. The glycerol concentration dependence of the percentage of the pretransition signal of DPPC.

the relative refractive index difference between the solvent and the membrane bilayer. Since the measured absorbance is proportional to the refractive index of the solvent the addition of glycerol leads to a reduction in the initial absorbance for the sample [18]. It is not clear whether this effect can explain the changes in the apparent relative contribution of the pretransition to the total transition or not. However, the possibility of a gradual induction of a new phase is ruled out by the X-ray diffraction data [14] for the system.

The results obtained in this investigation are summarized in Table I. The concentration of alcohol at which the pretransition disappears, as compared with the inflection in T_m and concentration at which the onset of hysteresis occurs, are summarized for each of the alcohols investigated, and for glycerol. As seen here, there is good correlation among these values for each alcohol, suggesting that all three changes are characteristic of the induction of the transition of DPPC from the gel phase to the interdigitated gel phase.

Discussion

The main observations reported in this paper are: (1) The short-chain *n*-alcohols (methanol to *n*-butanol) decrease the pretransition temperature of 1,2-DPPC, (2) the longer the aliphatic chain length of the *n*-alcohol (up to butanol) the greater the decrease in T_p at comparable alcohol concentrations, (3) above a critical concentration of the alcohols (unique for each alcohol) the pretransition disappears, (4) glycerol differs from the *n*-alcohols in that it does not decrease T_p but it

does induce hysteresis in the main transition and removes the pretransition at a critical concentration, (5) the disappearance of the pretransition appears to correlate with the induction of the interdigitated phase in DPPC.

The pretransition that is observed in most of the saturated PCs has been interpreted as the transition from the planar bilayer phase ($L_{\beta'}$) to the $P_{\beta'}$ phase in which ripple formation is observed on the surface of the bilayer [1,29,30]. It is generally believed that the acyl chains are tilted with respect to the bilayer surface in the $L_{\beta'}$ phase of the PCs in order to maximize the stabilizing van der Waals interaction between the chains, and at the same time minimize the steric crowding at the headgroup. However, what is not clear are the molecular details of the forces that drive the transition from $L_{\beta'}$, to $P_{\beta'}$, even though limited attempts have been made to explain it in theoretical terms [31–33].

Our results on the effect of short-chain alcohols on the pretransition can be contrasted with those in the literature with longer-chain *n*-alcohols [6]. Earlier studies with longer-chain *n*-alcohols (C_{10}–C_{16}) showed that these alcohols increased the pretransition temperature by as much as 5 Cdeg [3,34], whereas all the *n*-alcohols we have studied decreased the pretransition temperature. In addition, it is important to note that the effect of *n*-alcohols on the pretransition depends not only on the chain length of the *n*-alcohol but also that of the PCs. For example, recently O'Leary et al. [8] demonstrated that both *cis*- and *trans*-tetradecanol increased the T_p of the DMPC bilayers but had the opposite effect with DSPC bilayers. Moreover, with DPPC bilayers, the effect was complex with the *trans*-tetradecanol increasing the T_p while the *cis* isomer decreased it. These authors attributed the different results to the importance of the chain matchings between the lipid and the alcohols.

There could be a number of factors that can result in the shift in the pretransition temperature. The fact that the short-chain *n*-alcohols shift the T_p to lower values suggests that the alcohols interact preferentially with the $P_{\beta'}$ phase over the $L_{\beta'}$ phase. The fact that the effectiveness of the alcohols in decreasing T_p increases with increasing alcohol chain length indicates that the $P_{\beta'}$ phase has more hydrophobic character than the $L_{\beta'}$ phase. This could be because the phospholipid molecules in the staggered (ripple) configuration expose part of the acyl chain with which the *n*-alcohols can interact.

The observation that short-chain *n*-alcohols decreased the T_p also raises the possibility that these alcohols decrease the energy difference between the $L_{\beta'}$ phase and the $P_{\beta'}$ phase by altering the packing or tilt in the $L_{\beta'}$ phase. The interaction of *n*-alcohols with the gel phase could result in changing (decreasing) the acyl chain tilt of the $L_{\beta'}$ phase from 30°. Indeed, according to a theoretical analysis of Hawton and Keeler [33], a decrease in the tilt angle could result in an increase in the difference between T_m and T_p, which is in agreement with our observation reported here. The suggestion of removal of 'ordered' water by *n*-alcohols [35] from the surface of the bilayer is consistent with our observation that the ripple phase is more hydrophobic than the $L_{\beta'}$ phase. This again emphasises the importance of surface hydration on the chain tilt during the pretransition process [1,2]. The fact that glycerol has no significant effect on the pretransition and the main transition temperatures below a mol fraction of 0.45 is most likely because it is hydrophilic and it does not partition to any significant extent into the hydrophobic region of the membrane as compared to the *n*-alcohols.

A number of amphipathic molecules, including glycerol, methanol and ethanol has been shown to induce the formation of the interdigitated phase [13,14,36]. In this gel phase the acyl chains of DPPC are fully interdigitated with a bilayer thickness of 30 Å. As shown by X-ray diffraction in the case of glycerol the interdigitated phase occurs at $X_g = 0.45$, the same concentration at which the pretransition disappears as reported here. In the case of ethanol it was shown by X-ray diffraction that the interdigitated phase appears between 0.8 and 1.2 mol/l of ethanol [13]. The results reported here demonstrate that the disappearance of the pretransition occurs at the same concentration (0.98 mol/l ethanol) as the onset of marked hysteresis in the main transition and the break in the concentration dependence of T_m. Similar observations were made with other short-chain alcohols. The appearance of the T_m break and hysteresis

have previously been attributed to the induction of the interdigitated phase [11]. In addition to the amphipathic molecules mentioned above, the thiocyanate ion has been shown to induce the formation of the the interdigitated phase in DPPC [37].

The disappearance of the pretransition at a critical concentration of alcohol or glycerol may be explained by the observation that the critical concentration appears to correspond to the threshold concentration required to induce the interdigitated phase. The narrow concentration range over which the pretransition disappears (Fig. 4) suggests that the transition from the $L_{\beta'}$ to the interdigitated phase is highly cooperative.

The concurrence of the pretransition disappearance and the induction of the interdigitated phase indicates that the interdigitated phase does not undergo a pretransition. The reason why the interdigitated phase of DPPC does not have a pretransition is not clear, but it may be related to the lack of acyl chain tilt and a reduction in the steric interactions among the headgroups – two factors believed to be important for the existence of the pretransition. However, it should be noted that there are two cases in which pretransition has been observed in membranes that do not have tilted chains and the acyl chains are fully interdigitated [38,39].

In summary, we have observed that the short-chain *n*-alcohols decrease the pretransition temperature, and at comparable concentrations, the longer the aliphatic chain of the *n*-alcohol, the greater the decrease in the pretransition temperature. We have also observed that above a critical concentration the pretransition disappears. This critical concentration has been identified as the minimum concentration required to induce the formation of the interdigitated phase. In agreement with our earlier reports we have found that this critical concentration is also the concentration at which: (a) the onset of significant thermal hysteresis in the main transition temperature takes place and (b) the break in the alcohol concentration dependence occurs. These results qualitatively suggest that the three observations mentioned above may be used as a method to detect the probable existence of the interdigitated phase in the PCs.

Acknowledgments

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