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Biochimica et Biophysica Acta 1327 (1997) 41–51

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Solute-induced shift of phase transition temperature in Di-saturated PC liposomes: adoption of ripple phase creates osmotic stress

Walter R. Perkins [a], Xingong Li [a], James L. Slater [a,1], Paul A. Harmon [a,2], Patrick L. Ahl [a], Sharma R. Minchey [a], Sol M. Gruner [b], Andrew S. Janoff [a,*]

[a] *The Liposome Company, Inc., One Research Way, Princeton, NJ 08540, USA*
[b] *Physics Department, Princeton University, Princeton, NJ 08544, USA*

Received 10 December 1996; accepted 7 February 1997

Abstract

We have examined the calorimetric behavior of large liposomes consisting of symmetric saturated chain phosphatidylcholines. Most notably, for systems made in solutions containing solute (e.g., NaCl, glucose, etc.) there was an additional major endotherm just below the main phase transition temperature. The new endotherm was found to represent a population of lipid whose main phase transition was shifted to lower temperature due to an induced osmotic stress across the membrane. Absent for isoosmotic systems, the osmotic stress was created when the liposome internal volume decreased, a consequence of the $L_{\beta'}$ (gel) to $P_{\beta'}$ (rippled) phase transition. That is, rippling of the membrane caused vesicle volume to decrease ($\geq 28\%$) and because the free flow of water outward was restricted by solute, an osmotic gradient was created where none had existed before. The distribution of enthalpy between the new shifted T_m and the expected T_m correlated with the percent of lipid in the outer bilayer and it was concluded that only the outer bilayer sensed the induced stress. Internalized liposome structures were shielded, thus explaining the persistence of the expected T_m in preparations made in solute. The shift in T_m (ΔT_m) was discrete and linearly dependent upon lipid chain length for the PC series di-17:0 ($\Delta T_m \approx 1.4°C$) through di-20:0 ($\Delta T_m \approx 0.6°C$), suggesting a structural change (i.e., lipid packing/orientation) was involved. Although freeze-fracture electron microscopy of stressed and unstressed bilayers revealed no differences in ripple periodicity there were differences in surface features and in vesicle shape. The fact that this phenomenon has gone unnoticed for MLVs is probably due to the fact that these systems are known to exclude solute and thus exist under osmotic compression. © 1997 Elsevier Science B.V.

Keywords: Liposome; Phospholipid bilayer; Solute effect; Differential scanning calorimetry; Interdigitation-fusion vesicle; Calorimetry

1. Introduction

Of the phospholipid bilayer thermal events examined, none have been more scrutinized than the gel-to-liquid crystalline phase transition, (for example Ref. [1]). Although the effects of solutes upon this transition have been examined for both charged and neutral systems [2–4], most studies have focused

* Corresponding author. Fax: +1 (609) 5208250. E-mail: ajanoff@lipo.com
[1] Present address: GeneMedicine, Inc., The Woodlands, TX 77381, USA.
[2] Present address: Merck Manufacturing Division, Merck and Co., Inc., West Point, PA 19486, USA.

PII S0005-2736(97)00042-4

upon lipid dispersed as multilamellar vesicles (MLVs). Because MLVs are known to exclude solute [5,6], it is likely that any quantitative assessment of a solute effect, if noticed at all, would be underestimated.

Previously, we described a new technique which produces large (1–3 μm) liposomes from the fusion of SUVs comprised of saturated chain lipids [7,8]. Termed interdigitation-fusion vesicles (IFVs), these resulting liposomes have high captured volumes and solute appears to be distributed ideally (no osmotic compression). Because of these features, we believed these systems to be ideally suited to study the effect of solute upon what can be considered to be locally planar bilayers.

When IFVs comprised of di-18:0 phosphatidylcholine (PC) were examined by differential scanning calorimetry (DSC), we noted the appearance of an additional major endotherm just below the main T_m for samples made in 150 mM NaCl but not for those made in distilled water [9]. This extra endotherm was also observed for other saturated lipids (di-17:0 through di-20:0 PC) and liposomes made by other formation protocols (i.e., FATMLV and SPLV). The additional endotherm was not observed for di-14:0PC (DMPC) or di-16:0PC (DPPC) liposomes. Recently, Jørgensen [10] reported the appearance of an additional minor endotherm (0.2–0.3 kcal/mol) in MLVs made in 50 mM KCl for the PC series di-17:0 through di-20:0. While Jørgensen and co-workers [11] have since proposed a theoretical model involving a 'lattice melt' to explain the new endotherms, we hypothesize that they result from the same solute effect that we noted previously [9] and have investigated extensively here.

In this study, we present details of the solute effect and demonstrate a correlation between lipid chain length and the temperature separation between the two main transitions. We have also correlated the ratio of enthalpies between the shifted T_m and expected T_m for DSPC with (1) lamellarity (a measure of the amount of lipid in the outermost bilayer), (2) the concentration of solute, and (3) the concentration gradient across the membrane. From these and other data, we propose that for closed bilayer systems, the T_m can be shifted to lower temperature due to an induced osmotic stress, a consequence of liposome shrinkage when the bilayer adopts the $P_{\beta'}$ ripple phase. For multilamellar liposomes, the shifted T_m represents that population of lipid in the outermost bilayer. The endotherm at the expected T_m is derived from the inner bilayer structures that are shielded from the osmotic stress by the outermost bilayer. The size of the temperature shift (ΔT_m) is relatively independent of the osmotic differential. To determine whether this discrete shift of T_m is due to a specific structural change, we examined, using freeze-fracture electron microscopy, the morphology of stressed and unstressed membranes. While no differences in ripple periodicity were noted, vesicle shape and ripple patterns were noticeably different for stressed and unstressed membranes.

2. Materials and methods

2.1. Chemicals

All phosphatidylcholines (PCs), dipalmitoyl-PC (di-16:0), diheptadecanoyl-PC (di-17:0), distearoyl-PC (di-18:0), dinonadecanoyl-PC (di-19:0), and diarachidoyl-PC (di-20:0), were purchased from Avanti Polar lipids (Alabaster, AL). Octaethylene glycol monododecyl ether (C_{12}-E_8) was obtained from Fluka (Buchs, Switzerland). NBD-PE (di-16:0) was purchased from Molecular Probes (Eugene, OR).

2.2. Preparation of liposomes

MLVs were made by hydrating lipid powder in the appropriate solution at a temperature of at least 10°C above its phase transition temperature for 1 h with intermittent vortexing. Typically, solutions were buffered (pH 7.4) with either 10 mM Tris or 2 or 10 mM Hepes. Frozen and thawed MLVs (FATMLVs) were made from these MLVs by freezing and thawing the sample 10 times between liquid nitrogen and a water bath heated to 10°C above the phase transition temperature of the lipid. For interdigitation-fusion vesicles, precursor small unilamellar vesicles (SUVs) were made from the MLVs via probe sonication and used within 2 h. Ethanol (absolute) was added to ~2 ml of SUVs at 20 mg/ml lipid (room temperature) such that the final ethanol concentration was between 2 and 4 molar. A gelatinous intermedi-

ary comprised of fused lipid bilayer sheets formed which, after 1 h, was heated to 10°C above the phase transition temperature of the lipid. Upon heating the sheets spontaneously formed large predominantly unilamellar liposomes [7]. Lipid concentrations were determined by phosphate analysis [12]. Osmolarity of solutions was determined using a model 5500 Vapor Pressure Osmometer from Wescor, Inc. (Logan, UT, USA).

2.3. Liposome-captured volume

Captured volumes were measured by the previously described ViVo method [6,8] using the electron spin resonance (ESR) probe 4-trimethylammonium-TEMPO (CAT1). External probe concentrations were determined by assay of the supernatant above the liposome pellet following centrifugation ($10\,000 \times g$ for 20–30 min) [8].

2.4. Lamellarity by NBD-PE fluorescence quenching

To vary lamellarity, we made MLVs, FATMLVs, and IFVs as indicated above except all samples included 0.5 mol% NBD-DPPE which was incorporated prior to solvent removal. Samples were made in either 300 mM glucose plus 10 mM Hepes or 150 mM NaCl plus 10 mM Hepes. For IFVs, the final concentration of ethanol in the IF process was varied to produce liposomes of various lamellarity; ethanol was prediluted and mixed 1:1 with SUVs to avoid locally high concentrations upon mixing. To determine the percent of lipid in the outer monolayer we used the quenching (dithionite) assay previously described [13]. To obtain access to the vesicle interior for 100% quenching, C_{12}-E_8 was added at 10% (w/v) to each sample followed by heating to 60°C for 10 min.

2.5. Differential scanning calorimetry

Calorimetry was performed using a MC2 Ultrasensitive Scanning Calorimeter (MicroCal, Northhampton, MA). Scans were performed at a rate of 20°C/h. Samples were equilibrated at the starting temperature for 50 min prior to each scan and typically the second or third scan reported. Sample concentration was typically 1–3 mg/ml lipid.

2.6. Electron microscopy

For freeze-fracture electron microscopy, 1–3 μl of sample was placed between a pair of Balzers copper double replicating holders and frozen from the desired temperature in liquid propane. For freezing from 45°C, samples in copper holders were held in a chamber equilibrated with saturated water vapor and maintained at 45°C. Temperature was measured by a thermocouple probe located adjacent to the sample. After 10 min at 45°C, samples were removed and plunged into liquid propane. The sample was fractured (at -100°C and 10^{-6}–10^{-7} mbar) and shadowed with platinum ($\angle 45°$) and carbon in a Balzers BAF 400 freeze-fracture device. Replicas were cleaned overnight in 5% hypochlorite (commercial bleach), washed in distilled water, mounted on 300 mesh grids and viewed with a Philips 300 TEM. A waffle type grating from Ladd Research Industries (Burlington, VT) was used to determine magnification.

3. Results and discussion

Recently we reported a new method of making liposomes of high captured volume comprised of di-saturated PCs [7]. These liposomes are made from precursor SUVs via an ethanol-induced interdigitation-fusion process [14], hence the term interdigitation-fusion vesicles or IFVs. By varying process parameters, liposomes that are predominantly unilamellar can be formed. Unlike MLVs [5,6], these systems do not exclude solute and, because of their low lamellarity and relatively large size, exhibit large captured volumes [7,8].

In the course of characterizing these DSPC IFVs by DSC, we noted the appearance of an additional major endotherm at a temperature just below the expected T_m (Fig. 1). This endotherm persisted on repeated scanning and, in fact, was observed in a DSC scan of a DSPC IFV sample that had been heated to 70°C for 72 h. To confirm this phenomenon, we examined the temperature dependence

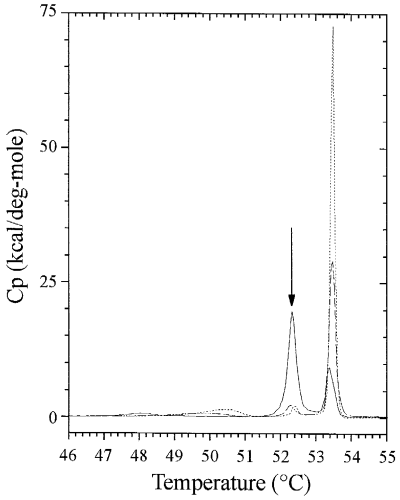

Fig. 1. DSC heating scans of DSPC IFVs (solid line), FATMLVs (dashed line), and MLVs (dotted line). The arrow indicates the position of the new endotherm at approximately 52.3°C; the 'expected' main endotherm is at approximately 53.4°C. Samples were made in 150 mM NaCl plus 10 mM Tris as described in Section 2. For the IFVs, 4 M ethanol was used during formation. For all three samples, the combined integrated areas under the two peaks (total enthalpy from 51.5°C to 54°C) were 10.6 kcal/mol. The separations between T_m values (ΔT_m) were 1.04°C (IFVs), 1.07°C (MLVs), and 1.18°C (FATMLVs). Pre-transitions were noted between 47°C and 51°C.

of the polarization anisotropy of DPH fluorescence, incorporated into the DSPC IFV membrane at 0.5 mol%, and found there to be two major transitions corresponding to the transitions observed by DSC (data not shown). Likewise, we found that the phenomenon was unrelated to lipid purity or residual ethanol.

Although smaller in magnitude, we found the extra endotherm to also be present in the DSC scans of both MLVs and FATMLVs made in buffer solution (see Fig. 1). The magnitude of the new transition was lowest for MLVs and highest for IFVs. The obvious major difference between these systems is their lamellarity. Also because MLVs are known to exclude solute [5,6], we suspected that solute distribution played a role in producing the extra endotherm.

Shown in Fig. 2 are the DSC scans of IFVs and MLVs made in 10 mM buffer solution with and without 150 mM NaCl. Both MLVs and IFVs made without salt exhibited essentially only the 'expected' endotherm which corresponded to that of the higher temperature transition for the cases when salt was present. It is not clear if there was indeed a new endotherm for MLVs in the absence of saline because signal and noise amplitudes were similar; additionally, these liposomes were not solute-free but contained 10 mM buffer. When salt was present, IFVs exhibited two major endotherms while for MLVs, the new endotherm was minor by comparison to the 'expected' endotherm. Interestingly, the sum of the enthalpies of the two IFV endotherms was equal to that of the single gel-to-liquid crystalline phase transition of the system made without salt (10.6 kcal/mol). This suggests that the new transition represents a subpopulation of lipid undergoing chain melting at a lower temperature. Close inspection of the pre-transition of IFVs made in salt (top panel) revealed that this transition was also shifted to lower temperature. In fact, there appeared to be a shoulder (indicated by the arrow in top panel inset) at approxi-

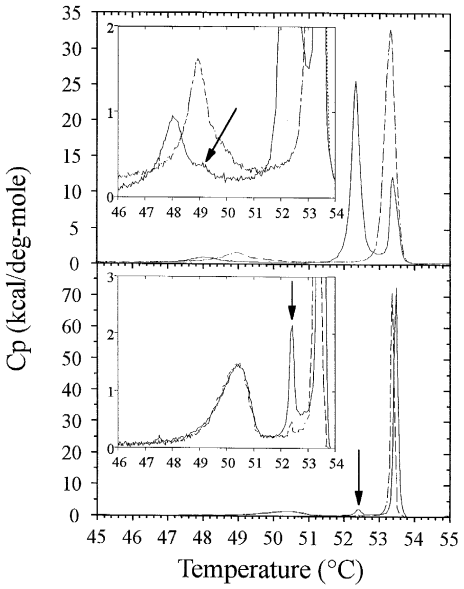

Fig. 2. DSC heating scans of DSPC IFVs (top panel) and MLVs (bottom panel) for samples made in either 150 mM NaCl plus 10 mM Tris (solid lines) or in 10 mM Tris alone (dashed lines). The additional endotherm in MLVs is noted by an arrow (bottom panel). The panel insets allow a closer examination of the pre-transitions. Note that the pre-transitions for IFVs with and without salt are separated by approximately 1°C while those for MLVs overlap. Both IFV pre-transitions are lower in temperature than those for the MLV systems. The arrow of the top panel inset indicates a shoulder to the shifted pre-transition of IFVs in salt. IFV formation (i.e., captured volume) was not affected by salt.

mately 49°C corresponding to an unshifted pre-transition. From a qualitative examination of several samples where the ratio of the enthalpies of the main transition endotherms varied, it appeared that the ratio of enthalpies for the pre-transitions was proportionate to that for the main T_m. Quantitation of these transitions was made difficult by their broadness and was, therefore, not attempted. The persistence of the pre-transition in these DSPC systems suggests that this phenomenon is unrelated to the splitting of the T_m caused by calcium which not only obliterated the pre-transition in DSPC systems but also affected DPPC [15].

Other di-saturated PC systems. Because we observed no additional endotherms in the calorimetric scans of either DMPC or DPPC IFVs made in salt, we questioned whether the salt-induced shift of T_m was specific to DSPC. This turned out not to be the case. Shown in Fig. 3 are the DSC scans of IFVs made with salt for the series di-17:0 PC through di-20:0 PC. For all four lipids, the combined enthalpies of the two main endotherms for samples in salt solution were equivalent to the enthalpies of the single endotherm for samples made without salt (not shown). In all cases the single endotherm for similarly made samples without salt present was at a temperature corresponding to the higher temperature transition for samples made with salt present. Interestingly, the separation between the two peaks (ΔT_m) was chain length-dependent (see Fig. 3 bottom). While not understanding the cause, we were not too surprised since lipid phase transition temperatures (e.g., pre and main), as well as the separation between them, exhibit chain length dependence [1]. These data convinced us that the discrete shift of T_m was not specific to DSPC and was indeed an effect of salt. We also believed that the effect of salt upon the bilayer had to be rudimentary in nature in order to explain the chain length dependence. To learn more detail about the salt effect, we next examined the relationship between salt concentration and the ratio of the two main endotherms.

Effect of salt concentration. All of the samples we examined thus far were made in buffer alone (10 mM Tris or Hepes) or buffer plus 150 mM NaCl. To define the effect that salt concentration had on the magnitude of the new endotherm, as well as the separation between endotherms, we made DSPC IFVs

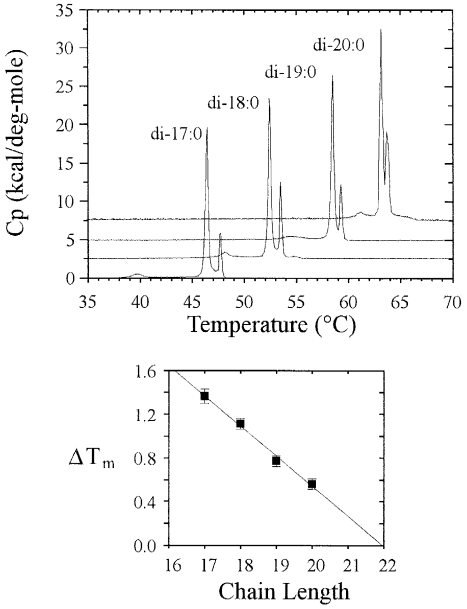

Fig. 3. DSC heating scans of IFVs comprised of various di-saturated chain PCs made in 150 mM NaCl plus 20 mM Tris (top panel). All IFVs were formed using 3 M ethanol which was removed by an extensive centrifugation-washing procedure (see Section 2). Shown in the bottom panel are the separations (ΔT_m) between peak positions of the two major endotherms as a function of chain length. All data were for IFVs in 150 mM NaCl with 10 or 20 mM Tris. The mean values of ΔT_m for the samples made in saline were 1.33°C for di-17:0 PC, 1.14°C for di-18:0 PC (DSPC), 0.72°C for di-19:0 PC, and 0.54°C for di-20:0 PC(DAPC). The solid line is a fit of the data using linear regression. Error bars represent S.D. ($n \geq 3$).

in solutions containing 5 to 300 mM NaCl (see Fig. 4). While the ratio of the enthalpies demonstrated a dependence on salt concentration, the temperature at which the new endotherm was positioned remained relatively unchanged with only a minor increase ($<$ 0.2°C) between 5 and 300 mM NaCl (see Fig. 4, inset). The captured volumes of all samples were measured and found to be similar (mean of 13.4 ± 2.8 $\mu l/\mu mol$ lipid), indicating that samples were morphologically similar.

We next made DSPC IFVs in either 300 mM glucose, sucrose, or mannitol. Importantly, two major peaks were observed in the DSC profiles as had been observed for samples made in NaCl; the separation between the two transitions was 1.3–1.4°C as compared to 1.1–1.22°C for samples made in NaCl. When an IFV sample made without salt (single en-

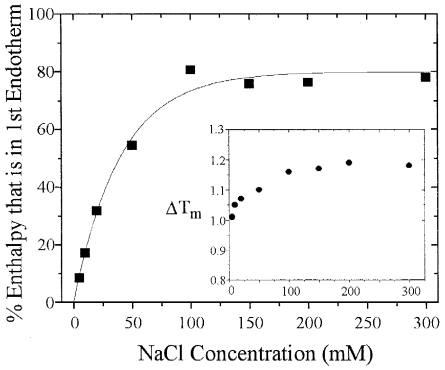

Fig. 4. Percent of combined enthalpy in the 1st (new) endotherm as a function of NaCl concentration. The line is a manual fit of the data where $f(x) = 80 \cdot (1 - \exp(-x/40))$. Liposomes (DSPC) were made by the IF process using 4 M ethanol. The inset depicts the separation between the peak positions of the two endotherms (ΔT_m) as a function of salt concentration.

dotherm) was mixed with a NaCl solution (final concentration 150 mM NaCl) or a glucose solution (300 mM glucose) the DSC scans of the mixtures exhibited only a minor 'new' endotherm and a broadened higher temperature T_m. However, when the samples were heated for an extended period of time (60 min) above the T_m prior to DSC the shifted new endotherm was quite prominent for the sample made in glucose but only slightly larger for the sample mixed with NaCl. Most likely, the higher membrane permeability of glucose allowed more of it to cross into the internal aqueous space [16,17]. This suggested to us that solute must be present on the inside of the vesicle in order to obtain the shifted endotherm.

The role of solute in producing the shifted T_m. The ability of glucose, sucrose, and mannitol to elicit the new endotherm indicated that the solute effect was not due to a specific salt interaction. Although lipid phase behavior has been shown to be affected by osmotically driven dehydration, in these cases quite high concentrations of solute are needed and produce an increase in T_m [18,19], inconsistent with the observation we report here. Moreover, while sugars are known to interact with the bilayer surface, and can in fact stabilize bilayer structure during dehydration by replacing interfacial water [20], this does not explain the effect of NaCl. The requirement for solute to be present in the interior aqueous space to produce the shifted endotherm led us to believe that osmotic stress was involved. In fact, a downward shift in T_m has been observed for osmotically stressed (2 M sucrose in/0.1 M out) DPPC vesicles [21]. Additionally, Evans and co-workers [22,23] have shown that application of tension upon DMPC bilayers via mechanical stretching lowers the main phase transition temperature. All of the liposomes we examined thus far had been made under conditions where the vesicle aqueous interior was putatively isoosmotic with the exterior aqueous phase. However, as the bilayer rearranges from the $L_{\beta'}$ phase to the $P_{\beta'}$ phase, vesicle volume decreases [22], a consequence of surface area reduction as the membrane surface becomes corrugated [24]. We hypothesized that the volume change associated with adoption of the ripple phase could create an interior hyperosmotic condition if solute were present and unable to cross the bilayer. The outward flow of water would then be restricted and tension would form in the membrane as it is stressed [22]. While the volume reduction for DSPC vesicles upon rearranging from $L_{\beta'}$ to the $P_{\beta'}$ ripple phase has not been determined, a volume reduction of 20–30% has been reported for DPPC vesicles undergoing this transition [22]. A volume reduction (loss of water with retention of solute) of this magnitude would yield an osmotic differential as high as 125 mOsM for a sample made in 150 mM NaCl. That is, by assuming no solute migration across the bilayer, the change in vesicle volume can be directly related to the change in solute concentration as:

$$\frac{V_P}{V_L} = \frac{[S]_L}{[S]_P} \approx \frac{OsM_L}{OsM_P} \quad (1)$$

where V_L and V_P are the internal vesicle volumes for $L_{\beta'}$ and $P_{\beta'}$ phases, respectively, and $[S]_L$ and $[S]_P$ are the corresponding concentrations of solute, which are proportional to interior osmotic strength (OsM). Note that the thermal expansion of water itself from 20 to 60°C is minor ($\sim 1\%$) when compared to these expected volume changes. By assuming a permeability coefficient of 10^{-5} cm/s for water through gel state DSPC [16], we estimate that a vesicle volume change of 25% could occur within 4 min for a unilamellar vesicle of 1 μm diameter (see Ref. [16] for flux equation). Since the pre-T_m and main phase transitions are separated by ~ 4°C, our scan rate of

20°C/h would allow more than enough time for these volume changes to occur. Jørgensen [10] saw no scan rate dependence between 4 and 60°C/h for DSPC.

To test this induced osmotic stress hypothesis, we added glycerol (500 mM) to a DSPC IFV sample made with 300 mM glucose present. Because of its high membrane permeability [25], we expected that glycerol would diffuse during the DSC heating scan and offset any osmotic gradient, thus removing the shifted peak from the scan. Without added glycerol, the shifted endotherm was prominent and accounted for approximately 80% of the total enthalpy for the two endotherms. With added glycerol (the sample was first heated and cooled to allow glycerol equilibration), the shifted endotherm was greatly reduced. In fact, after two heating scans, the ratio of the two peaks had changed dramatically and the expected endotherm now accounted for 80% of the total enthalpy (data not shown). That this reversal was caused by glycerol permeation and not abrogation of the ripple phase was confirmed by the continued presence of the pre-transition with glycerol present.

We expected that increasing the external salt concentration to match the increased internal salt concentration should also obviate osmotic stress and thus prevent the shifted T_m. All of the data shown (Figs. 1–4) indicates that the shift in T_m is discrete in nature even when small osmotic differences are induced (Fig. 4). This being the case, we expected that bilayers experiencing even the slightest osmotic differential should melt at the lower temperature transition. We expected therefore to be able to estimate the volume reduction associated with the DSPC $L_{\beta'}$ to $P_{\beta'}$ transition by titrating the concentration of salt needed to prevent the appearance of the shifted T_m. As shown in Fig. 5, the lower temperature transition for a DSPC IFV sample made in 289 mOsM saline and diluted into solutions of increasing salt concentration was systematically decreased. For the cases where the external osmotic strength was 571 mOsM (Fig. 5a), the shifted endotherm was completely absent in the first heating scan. The external osmotic strength marking the disappearance was somewhere between 399 and 571 mOsM. From these osmotic strengths and assuming an initial internal osmotic strength of 289 mOsM (OsM_L) and that the disappearance of the new endotherm coincided with a zero

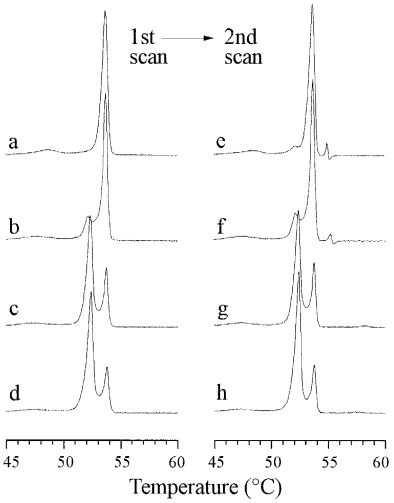

Fig. 5. DSC heating scans (temperature versus Cp) for DSPC IFVs made in 150 mM NaCl and 10 mM Hepes at pH 7.4 and diluted into solutions of varying salt concentration. The final osmotic strengths of the NaCl solutions into which the liposomes were diluted were (a,e) 571 mOsM, (b,f) 399 mOsM, (c,g) 324 mOsM, and (d,h) 289 mOsM (isoosmotic with interior). Exterior osmotic strength was measured directly (osmometer) from supernatants following centrifugation. Tracings on the left (a through d) are of the first heating scans and those on the right (e through h) are the second heating scans.

osmotic differential (external osmotic strength = OsM_p), we estimated from Eq. (1) that the volume reduction associated with adoption of the ripple phase was between 28% and 49%, the magnitude being consistent with the volume change reported for DPPC [22].

Several attempts were made with DPPC to evoke a shifted endotherm by making the interior hyperosmotic. However, while a minor shifted endotherm of lower temperature was noted, it was neither discretely shifted (varied) nor stable as it was absent in the second heating scans (data not shown). Using mechanical calorimetry, Evans and co-workers [22,23] have quite successfully demonstrated the dependence of the main phase transition temperature upon tension for large DMPC bilayers. It would therefore seem that an estimation of membrane tension, knowing the osmotic difference, should be possible for the disaturated systems examined here [26]. However, because the shift in T_m (ΔT_m) for lipids > 16 carbons in length was discrete (i.e., little or no dependence on

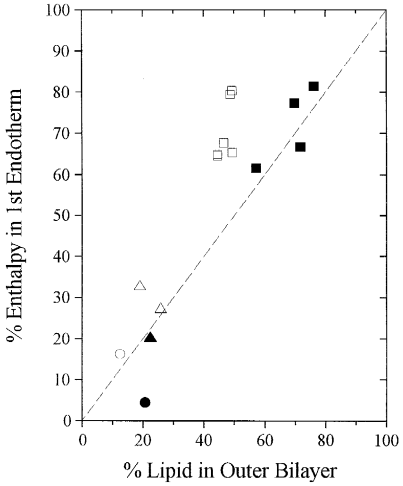

Fig. 6. Percent of total enthalpy that occurs in new lower temperature endotherm as a function of the lipid distribution. Liposome samples were made in 10 mM Hepes buffer (pH 7.4) with either 150 mM NaCl (solid symbols) ($n = 6$) or 300 mM Glucose (open symbols) ($n = 9$); MLVs (circles), FATMLVs (triangles), and IFVs (squares). Percent lipid in the outer bilayer was obtained from the lamellarity measurement (2 times the % lipid in the outer monolayer). The dashed line is the expected relationship if the new endotherm arises only from lipid in the outer bilayer and the 'old' endotherm arises from internal bilayers; % of the total enthalpy in new endotherm = % lipid in outer bilayer.

the osmotic differential), such an analysis would be problematic. The lack of discreteness for the shift in DPPC's endotherm and its instability suggested that DPPC bilayers are inherently different from those of longer chain lipids. Needless to say, a clearer understanding of the physical basis that causes the shift to be so discrete is required before any modeling can proceed.

Effect of vesicle morphology on ratio of endotherms. The magnitude of the higher temperature 'expected' T_m was greatest in MLVs and lowest in IFVs suggesting a morphological link (Fig. 1). Both IFVs and MLVs possess internalized liposome structures which might not experience the osmotic gradient felt by the outermost bilayer. Shown in Fig. 6 are data relating the percent of total lipid found in the outer bilayer (from an NBD-PE (0.5 mol%) fluorescence quenching assay, see Section 2) and the percent of the total enthalpy found in the first (shifted) transition. Samples were made by various formation protocols (MLV, FATMLV, and IFV) and in 10 mM buffer plus either 150 mM NaCl or 300 mM glucose. The dashed line of Fig. 6 represents the theoretical relationship between the distribution of enthalpy and the percentage of lipid in the outermost bilayer, assuming that it is only the outermost bilayer that experiences osmotic stress and exhibits the shifted T_m. For samples made in NaCl, the agreement with theoretical prediction was quite good except for the MLV sample. This was not surprising and was consistent with the fact that MLV systems are known to exclude solute [5,6] which would offset the osmotic induced stress caused by vesicle shrinkage. The value for MLVs made in glucose fell near the predicted value probably because during vesicle formation glucose was not excluded, a likely consequence of its greater membrane permeability as compared to NaCl [16,17]. For the other samples made in glucose, data fell above the theoretical curve suggesting that not just lipid in the outermost bilayer experienced osmotic stress. If for some liposomes, glucose leaked across the outermost bilayer at the L_β to $P_{\beta'}$ transition, the internal liposomes would then experience an osmotic differential. Consequently, enthalpy would be shifted into the lower temperature endotherm. Thus, vesicles with fewer bilayers per liposome would be more affected than those that are multilayered.

Discreteness of shift in T_m. As indicated from all the data, the shift of T_m to lower temperature was discrete. Because IFV samples comprised of DMPC and DPPC and made iso-osmotically did not exhibit the prominent shifted endotherms, we suspected that both the discreteness and chain length dependence originated from the ability (or lack thereof for DMPC and DPPC) of the membranes to undergo some specific structural change (e.g., ripple periodicity). Matuoka et al. [27] have observed a metastable secondary ripple structure of longer periodicity only for PC samples where the number of carbons in the chains was ≥ 15. Although this work was conducted for samples in distilled water, it did raise the possibility that the chain length-dependent shift in T_m observed here arose from some definable change in membrane structure.

To assess vesicle morphology, we employed freeze-fracture electron microscopy. Because we wanted to capture structural details of liposomes in the $P_{\beta'}$ phase, we chose di-17:0 PC IFVs for techni-

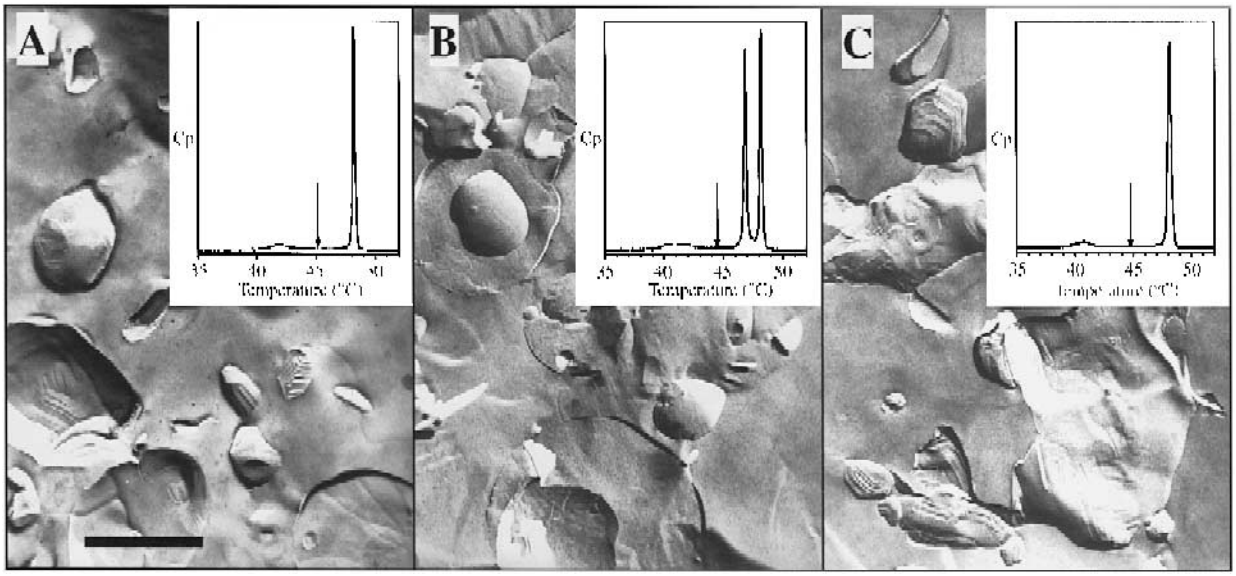

Fig. 7. Freeze-fracture electron micrographs of di-17:0 PC IFVs made in (A) distilled water, (B) 150 mM NaCl (10 mM HEPES, pH 7.4), or (C) made in 150 mM NaCl and diluted into 300 mM NaCl (10 mM Hepes, pH 7.4). All samples were frozen without cryoprotectant (see Section 2) at the temperatures indicated by the arrows in the insets; the corresponding DSC scans are located in the figure insets. Ripple structure ($\lambda \approx 141$Å, see text) was noted in all samples. IFVs made in water and frozen from room temperature ($L_{\beta'}$) were spherical in shape (not shown). Bar represents 1 μm.

cal ease since the temperature range for $P_{\beta'}$ is lower than that for the other systems and because this system exhibited the largest separation between the pre- and main phase transitions (see Fig. 3 bottom). Shown in Fig. 7 are electron micrographs of samples frozen from approximately 45°C that were made in water (Fig. 7A) or 150 mM NaCl solution and diluted into either the same buffer solution (Fig. 7B) or a solution where the final external salt concentration was 300 mM NaCl (Fig. 7C). While the sample diluted into 150 mM NaCl exhibited two major endotherms, the sample diluted into 300 mM NaCl and that made in distilled water both exhibited only the expected T_m in their calorimetric scans. For the samples exhibiting only the expected T_m (i.e., that were made in distilled water and those made with 150 mM NaCl and then diluted into 300 mM NaCl), liposome structures were non-spherical with ripple patterns that changed direction frequently with tortuous paths. For the sample exhibiting the shifted T_m (Fig. 7B), all outermost bilayer structures, and some internalized liposome structures, were spherical in shape and ripples were primarily unidirectional with few deviations. These features seemed to be consistent with the notion that those membranes were stretched. All structures exhibiting ripple structure with short range turns and tortuous paths were identified as being internalized liposomes encompassed by a membrane with the stretched appearance described above. Regardless of whether the membrane was an outermost bilayer or part of an internalized liposome, there were no notable differences in ripple periods (141 ± 8 Å) for all of the samples; this periodicity is consistent with a previous reported value [28]).

We believe that the discrete nature of the shift of T_m must involve some lipid packing parameter which, when defined, will also explain the chain length dependence noted in Fig. 3B. While no change in ripple periodicity was noted, stretching of the membrane by osmotic stress may induce a change in ripple amplitude [24], chain tilt, glycerol backbone orientation, or some other specific structural change that might account for the discrete shift in chain melting temperature. Additionally, an altered structural component in the ripple phase might also explain why the $L_{\beta'}$ to $P_{\beta'}$ transition (recall Fig. 2) is lowered rather than increased as one might expect. Although many details remain to be answered, the

link between osmotic stress and the shift in T_m for the PC series di-17:0 through di-20:0 appears to be clear.

4. Conclusions

We have presented the details of what appears to be a rather general solute effect upon disaturated long chain PC liposome systems. This effect, as noted by a discrete downward shift in T_m (and pre-T_m), was demonstrated for IFVs made in solutions containing either NaCl, glucose, sucrose, or mannitol. Liposomes made without these solutes did not exhibit the shifted endotherm. We conclude that the shift in T_m is due to an induced osmotic stress across the membrane, a consequence of vesicle volume reduction upon adoption of the $P_{\beta'}$ phase (see Fig. 8). Jørgensen [10] mentioned that he had also observed minor endotherms in samples prepared in distilled water (data was not shown). Although we have only observed the shifted endotherm for samples made with solute, there could be conditions other than osmotic stretching that favor the formation of the domains exhibiting the shifted T_m. For example, Hinz and Sturtevant [29] noted a minor endotherm in a dispersion of DSPC in water when the sample was rapidly cooled from above the T_m prior to the DSC heating scan. The endotherm was absent in scans when the sample had been cooled slowly. In conclusion, we do not believe the new endotherm reported here represents a new lipid transition per se but rather is the main T_m shifted to lower temperature for that subpopulation of lipid subjected to osmotic stress.

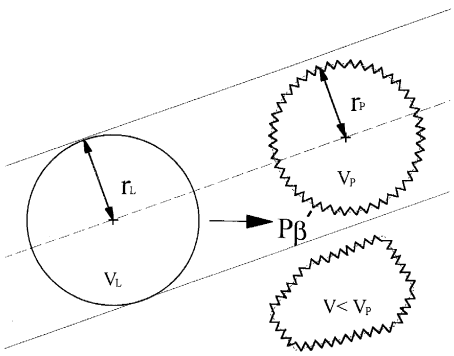

Fig. 8. Schematic representation of the change in vesicle geometry upon adoption of the ripple phase. Where spherical shape is maintained, the effective radius (r_P) is less than r_L with a concomitant reduction in total volume ($V_P < V_L$). If solute does not cross the bilayer then an osmotic pressure differential results (see Eq. (1)). Note that a fractional change in surface area yields a proportional change in volume, which is independent of the absolute radius; that is, $\Delta A/A = K \cdot \Delta V/V$, where $K = (1 - x^3)/(1 - x^2)$ and x is the fractional change in radius (r_P/r_L). Where spherical shape is lost the decrease in volume would be greater.

Acknowledgements

We thank Dr. Robert Reed for gas chromatographic analyses of samples for residual ethanol. This work was funded by The Liposome Company, Inc.

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