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Biochimica et Biophysica Acta 1514 (2001) 100–116

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Phase behavior and glass transition of 1,2-dioleoylphosphatidylethanolamine (DOPE) dehydrated in the presence of sucrose

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Received 15 December 2000; received in revised form 12 June 2001; accepted 15 June 2001

Abstract

The effect of sucrose on the phase behavior of 1,2-dioleoylphosphatidylethanolamine (DOPE) as a function of hydration was studied using differential scanning calorimetry and X-ray diffraction. DOPE/sucrose/water dispersions were dehydrated at osmotic pressures (Π) ranging from 2 to 300 MPa at 30°C and 0°C. The hexagonal II-to-lamellar gel ($H_{II} \rightarrow L_\beta$) thermotropic phase transition was observed during cooling in mixtures dehydrated at $\Pi \leq 35$ MPa. After dehydration at $\Pi \geq 57$ MPa, the $H_{II} \rightarrow L_\beta$ thermotropic phase transition was precluded when sucrose entered the rigid glassy state while the lipid was in the H_{II} phase. Sucrose also hindered the H_{II}-to-lamellar crystalline (L_c), and H_{II}-to-inverted ribbon (P_δ) lyotropic phase transitions, which occurred in pure DOPE. Although the L_c phase was observed in dehydrated 2:1 (mole ratio) DOPE/sucrose mixtures, it did not form in mixtures with higher sucrose contents (1:1 and 1:2 mixtures). The impact of sucrose on formation of the ordered phases (i.e., the L_c, L_β, and P_δ phases) of DOPE was explained as a trapping of DOPE in a metastable H_{II} phase due to increased viscosity of the sucrose matrix. In addition, a glass transition of DOPE in the H_{II} phase was observed, which we believe is the first report of a glass transition in phospholipids.

Keywords: Phosphatidylethanolamine; Sucrose; Dehydration; Glass transition; Phase transition; Phospholipid

1. Introduction

The accumulation of sugars and other compatible solutes is associated with the ability to survive either freezing or desiccation in a diverse array of organisms including nematodes [1], brine shrimp [2], mam-

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphatidylcholine; L_α, lamellar liquid-crystalline phase; L_β, lamellar gel phase; H_{II}, hexagonal II phase; L_c, lamellar crystalline phase; P_δ, inverted ribbon phase; T_m, the $L_\beta \rightarrow L_\alpha$ phase transition temperature; $T^h_{\beta bh}$, the $L_\beta \rightarrow H_{II}$ phase transition temperature determined during heating; $T^c_{\beta bh}$, the $H_{II} \rightarrow L_\beta$ phase transition temperature determined during cooling; T_{bh}, the temperature of the $L_\alpha \rightarrow H_{II}$ phase transition; T_{g_L}, the glass transition temperature of DOPE; T_g, the glass transition temperature of sucrose

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0005-2736/01/$ – see front matter
PII: S0005-2736(01)00372-8

malian cells [3], plant tissues and seeds [4], and even vertebrates [5]. Given that cell membranes are the primary site of freeze-induced [6] and desiccation-induced injury [7–9], studies of the effect of sugars on the stability of biological membranes are required to understand the role of sugars in protecting organisms against freezing and desiccation. Also, there is a growing interest in using liposomes for drug delivery and the effect of sugars on the stabilization of liposomes during dehydration [10].

Most commonly, biological membranes consist of >100 lipid species (phospholipids, sterols, sterol esters, cerebrosides, glycolipids) with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) the predominant phospholipid species. The lyotropic phase behavior of PC species and PC/sugar mixtures has been studied in detail. It has been shown that drying of synthetic PC species and biological membranes causes an increase in the liquid crystalline-to-gel phase transition temperature, T_m, and the lyotropic liquid crystalline-to-gel phase transition that occurs results in the destabilization of liposomes and biological membranes [10,11]. When dried in the presence of sugars, however, the lyotropic phase behavior of phospholipids is modified. The Crowes demonstrated that when DPPC and POPC were dried in the presence of sugars, T_m did not increase but either remained about the same as for fully hydrated lipids or was substantially depressed below the T_m of the fully hydrated species [12,13]. To explain the protective effect of sugars on biological membranes, the Crowes proposed the 'water replacement hypothesis' [14,15]. According to this hypothesis, sugar molecules directly interact with the polar groups of phospholipids which maintains the lateral spacing between polar headgroup in the dry state [16,17]. More recently, however, it has been shown that physical state of the sugar matrix has a major effect on the lipid phase transition of phosphatidylcholine species [18–20]. In particular, Koster et al. observed that depression of the T_m occurred when the glass transition temperature, T_g, of the sugar was greater than the T_m of the lipid [19,20]. In a more detailed study, Zhang and Steponkus [18] demonstrated that depression of the T_m of several different PC species was not simply a consequence of $T_g > T_m$. Rather, depression of the T_m only occurred if the phospholipid was in the L_α phase when the sugar matrix underwent a glass transformation. If the phospholipid was in the L_β phase when the sugar underwent the glass transformation the T_m was elevated during the first warming scan. Therefore, as an alternative to the 'water replacement' hypothesis, Zhang and Steponkus suggested [18] that depression of the T_m is caused by rigidity of the sugar glass rather than a direct lipid–sugar interaction. That is, the lipid phase transition is accompanied by area changes, and the rigid sugar matrix resists the area changes that might create an increase in lateral pressure. This hypothesis has recently been confirmed by Koster et al. [20]. Extensive discussion of the effect of sugars on phase transition of a lipid and lateral pressure effect is given in [21,22].

Phosphatidylethanolamine (PE) species represent another major phospholipid of biological membranes, and PE/sugar systems have been studied in some detail. In particular, it has been shown that when lipids were dispersed in sugar solutions, T_m increased whereas the $L_\alpha \rightarrow H_{II}$ phase transition temperature, T_{bh}, decreased relative to that of lipids dispersed in water (see [23] for a review). At high sugar concentrations, the L_α phase was not detected; instead, a direct $L_\beta \rightarrow H_{II}$ phase transition occurred. Therefore, it was suggested that sugars 'stabilize' the L_β and H_{II} phase at the expense of the L_α phase [24,25]. It should be noted, however, that such a conclusion is in apparent contradiction with the well-established protective effect of sugars on biological membranes.

It should be stressed that most studies of PE/sugar systems have been performed with dried lipids dispersed in solutions containing varied concentrations of sugars. In such cases, only a limited range of water activity (and lipid dehydration) can be achieved. A common way to achieve a greater extent of dehydration is to equilibrate phospholipid dispersions over saturated salt solutions [26]. Such experiments have been performed with PC/sugar mixtures [18–20] and binary lipid mixtures [27–29]. However, PE/sugar mixtures have not been studied at low water contents, and the phase behavior of PE/sugar mixtures at low water activities is poorly understood.

In this study, DOPE was chosen as a model phospholipid because it is the most studied species of PE with unsaturated hydrocarbon chains. Although the phase behavior of DOPE at different hydration levels

has been studied in pure DOPE [30–32], in DOPE/DMSO mixtures [33], and in binary and ternary mixtures with other lipids [27–29], the lyotropic phase behavior of DOPE in the presence of sugars has not been reported in the literature. In the present study, DOPE/sucrose liposomes were dehydrated over saturated salt solutions that provided a wide range of osmotic pressures. The lyotropic phase behavior of the dehydrated DOPE/sucrose mixtures was studied using DSC and X-ray diffraction, with special attention given to the interactions of a glass transition in the sucrose matrix and the phase transitions of DOPE. Another point of interest in the present study was the observation of a glass transition of the DOPE itself, which we believe is the first report of a glass transition in phospholipids.

2. Materials and methods

2.1. Sample preparation

Sucrose was purchased from Fisher Scientific as ACS reagent. DOPE was purchased from Avanti Polar Lipids as a chloroform solution and used without further purification. Chloroform was removed under a stream of N_2 at room temperature followed by holding DOPE under vacuum for at least 15 h. Sucrose solutions (10 wt%) were prepared with double-distilled, deionized water saturated with N_2. The sucrose solutions were added to dry DOPE by weight, and DOPE liposomes were prepared by freeze–thaw cycling (10×) between −196°C (liquid nitrogen) and 1–3°C (in a water bath) with vortexing between cycles. 2:1, 1:1, and 1:2 (mole ratio) DOPE/sucrose suspensions were prepared.

The DOPE/sucrose suspensions were dehydrated by vapor phase equilibration over saturated salt solutions at two temperatures. Specifically, 1:2 and 1:1 DOPE/sucrose mixtures were dehydrated at 30°C, and 1:2, 1:1, and 2:1 DOPE/sucrose mixtures were dehydrated at 0°C. Two dehydration temperatures were chosen because it has been previously shown [30] that the lyotropic behavior of DOPE liposomes was dependent on the dehydration temperature and the phase that the lipid was in during dehydration. To dehydrate the samples for the DSC studies, DSC pans containing liposomes were placed over saturated salt solutions in tightly closed jars; the jars were flushed with argon, and the mixtures were dehydrated for 7–9 days at 30°C and 23 to 28 days at 0°C. After dehydration, the DSC pans were hermetically sealed. One experiment was performed with liposomes composed of DOPE/sucrose, DOPE/trehalose, DOPE/glucose, and DOPE/fructose (1:2 mole ratio) that were dried over phosphorus pentoxide at the room temperature. To dehydrate samples for the X-ray diffraction experiments, Petri dishes with the liposomes were placed over saturated salt solutions in desiccators; the desiccators were evacuated with a vacuum pump and stored at 30°C for 6–10 days. After equilibration, the mixtures were loaded into capillary tubes and then placed over saturated salt solutions for one day before the capillary tubes were flame-sealed. Note that the samples lost a considerable amount of water during loading [30]. To account for such uncontrolled dehydration, the water content of the samples was determined after the X-ray diffraction experiments.

Saturated aqueous solutions of Na_2HPO_4, KNO_3, $BaCl_2$, KCl, NaCl, NaBr, $MgCl_2$, and LiCl were used to provide a range of osmotic pressures [27]. Corresponding osmotic pressures, Π, for those solutions were calculated at 30°C and 0°C as [34]

$$\Pi = -(RT/V_w)\ln(\mathrm{RH}/100) \quad (1)$$

where $R = 8.31$ J mol^{-1} K^{-1} is the universal gas constant, T is the temperature in K, $V_w = 18\times10^{-6}$ m^3 is the partial molar volume of pure water, and RH is the relative humidity of the solution. RH and osmotic pressures for saturated salt solutions used in this study are given in Table 1.

Water content was determined gravimetrically after DSC and X-ray diffraction experiments by drying DOPE/sucrose mixtures in DSC pans with pin holes at 70°C under vacuum until a constant weight was achieved (usually 3.5 h). It has been shown in a separate experiment that sample weight was the same after 3.5 and 24 h of dehydration. The water content, X wt%, was calculated as

$$X = 100(W_i - W_d)/(W_i - W_e) \quad (2)$$

where W_i, W_d, and W_e are the weights of sealed DSC pan+sample before drying, after drying, and the weight of the empty DSC pan+cover, respectively [35].

Table 1
Relative humidities (RH) and osmotic pressures for saturated salt solutions at 0°C and 30°C, and hydration of amorphous sucrose and DOPE at 30°C

Saturated aqueous solution of	30°C				0°C	
	RH	Osmotic pressure (MPa)	Sucrose hydration (wt% water)	DOPE hydration in the H_{II} phase at 30°C (wt% water) [30]	RH	Osmotic pressure (MPa)
Na_2HPO_4	97	4.3	51.7 ± 0.4	13.3 ± 0.3	98	2.5
KNO_3	92.3	11.2	–	10.8 ± 0.2	96.3	4.8
$BaCl_2$	89	16.3	37.7 ± 0.1	9.4 ± 0.6	94	7.8
KCl	83.6	25.1	28.2 ± 0.8	7.1 ± 0.4	88.6	15.3
NaCl	75.1	40.1	20.7 ± 1.0	5.4 ± 0.1	75.5	35.4
NaBr	56.0	81.1	10.8	3.1 ± 0.1	63.5	57.3
$MgCl_2$	32.4	157.7	4.2 ± 0.3	0 (extrapolated)	33.7	137.2
LiCl	11.3	305.2	–	0 (extrapolated)	11.2	276.1

RH values are from [55] with an exception of Na_2HPO_4 and $BaCl_2$. At 30°C, RH values for Na_2HPO_4 and $BaCl_2$ at 30°C are from [54]. At 0°C, RH values for Na_2HPO_4 and $BaCl_2$ were extrapolated from 5°C to 0°C using data from [56].

2.2. DSC and X-ray diffraction experiments

DSC experiments were performed with a Perkin–Elmer DSC-7 instrument. An empty aluminum pan was used as a reference. The instrument was calibrated using the melting points of water and indium at a heating rate of 10°C/min, and the calibration was checked at a higher heating rate of 40°C/min. The water and indium melting temperatures obtained at 40°C/min were 0.7 to 1.5°C higher than those obtained at 10°C/min. Such a difference is not significant for the purposes of this study, and temperature correction for heating rate was not used. DOPE/sucrose mixtures were studied at heating and cooling rates of 10°C/min and 40°C/min. The more rapid scanning rate did not cause changes in the general appearance of the DSC curves of DOPE/sucrose mixtures. Minor differences in the measured temperatures were observed at the two scanning rates. For example, the difference in the L_β-to-H_{II} phase transition temperatures measured at heating rates of 40°C/min and 10°C/min was (2.5 ± 1.0)°C, and the difference in the T_g was (2.3 ± 2.5)°C. As a rule, samples were cooled to ≤ −120°C followed by immediate heating to ≥ 30°C; cooling-heating cycles were repeated several times. Also, samples that contained higher water contents (non-dehydrated dispersions and after dehydration at 2.5–16 MPa) were cooled to −10°C to −25°C to avoid ice formation. DSC pans usually contained 1–3 mg of DOPE/sucrose mixture (dry weight). Samples dehydrated at 0°C were transferred into the pre-cooled DSC instrument at 0°C. The lipid L_β-to-H_{II} and H_{II}-to-L_β phase transition temperatures reported in this paper were determined as the peak temperature in the DSC warming curves ($T^h_{\beta bh}$) and the onset temperature on the DSC cooling curves ($T^c_{\beta bh}$), respectively. The glass transition temperatures were reported as the onset temperatures.

X-ray diffraction powder patterns were obtained at the Cornell High Energy Synchrotron Source on the D1 station as previously described [30].

3. Results

3.1. DSC and X-ray diffraction results

3.1.1. The effect of osmotic pressure on thermotropic phase behavior of the DOPE/sucrose mixtures

Representative DSC heating curves of DOPE/sucrose (1:1) mixtures dehydrated at 0°C are shown in Fig. 1. DSC heating curves for pure DOPE liposomes are shown for comparison. DSC curves of 1:2 and 2:1 DOPE/sucrose mixtures dehydrated at 0°C, and the 1:1 and 1:2 mixtures dehydrated at 30°C had a similar appearance. The exceptions were the 1:1 and 1:2 mixtures dehydrated at 41 MPa at 30°C and the 2:1 mixture dehydrated at 57 MPa at 0°C as described later in the paper.

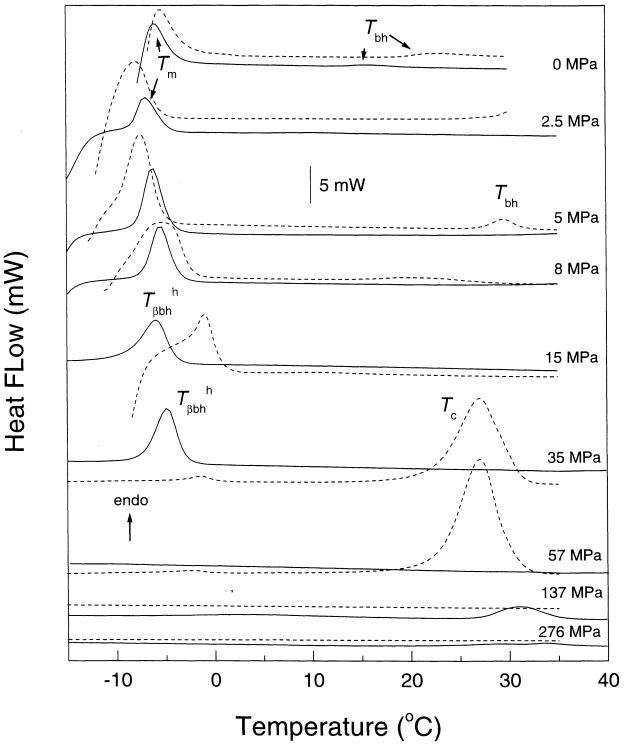

Fig. 1. DSC heating curves of DOPE/sucrose mixtures (1:1 mole ratio, solid lines) and DOPE (dashed lines) dehydrated at 0°C over saturated salt solutions that ranged in osmotic pressure from 2.5 to 276 MPa. Ice formation was avoided. Scanning rates: 40°C/min. T_{bh}, the $L_\alpha \rightarrow H_{II}$ phase transition; $T^h_{\beta bh}$, the $L_\beta \rightarrow H_{II}$ phase transition; T_c, the $L_c \rightarrow H_{II}$ phase transition.

A weak endothermic event, T_{bh}, due to the $L_a \rightarrow H_{II}$ phase transition was observed in the fully hydrated, 1:1 DOPE/sucrose mixtures and in samples dehydrated at 2.5 MPa; however, it was not observed in samples dehydrated at ≥ 5 MPa. Hence, the 'main' thermal event was assigned to the $L_\beta \rightarrow L_\alpha$ phase transition (T_m) in fully hydrated samples and in the DOPE/sucrose mixtures dehydrated at 2.5 MPa, and to the $L_\beta \rightarrow H_{II}$ phase transition ($T^h_{\beta bh}$) in mixtures dehydrated at 5–35 MPa. Note that a direct $L_\beta \rightarrow H_{II}$ phase transition has been reported in PE/sugar mixtures formed from dried PE species that were hydrated with highly concentrated sugar solutions [24,25]. The assignment of the thermal event was confirmed in the X-ray diffraction experiments. Fig. 2 shows X-ray diffraction patterns of the 1:2 DOPE/sucrose mixture dehydrated at 16 MPa at 30°C as an example. Three small-angle diffraction rings indexing $1:\sqrt{3}:2$ and a broad wide-angle diffraction ring at 0.45 nm indicative of the H_{II} phase were observed at 30°C (Fig. 2A). When the sample was cooled to −40°C, two small-angle diffraction rings with ratio 1:4 were observed (Fig. 2B; the second ring was very weak and is not shown in the figure). In the wide-angle region, a relatively sharp ring with a d spacing of 0.43 to 0.44 nm which is indicative the lamellar gel phase was detected (Fig. 2B). When the sample was heated from −40°C to 0°C, the sharp wide-angle diffraction ring disappeared leaving a broad diffraction at 0.45 nm. Three small-angle diffraction rings with a $1:\sqrt{3}:2$ ratio re-appeared indicating that the lamellar-to-hexagonal phase transition occurred.

After dehydration at osmotic pressures ≥ 57 MPa, the appearance of the DSC heating curves of the DOPE/sucrose mixtures changed dramatically, i.e., the 'main' $T^h_{\beta bh}$ peak resulting from the L_β-to-H_{II} phase transition was no longer observed (Fig. 1). In pure DOPE, the T_c thermal event, which is associated with the lamellar crystalline phase, L_c, was observed after dehydration at 35 and 57 MPa at 0°C [30]. No significant thermal events were observed in pure DOPE samples dehydrated at 137 and 276 MPa when DOPE entered the inverted ribbon phase, P_δ [30]. The effects of sucrose on formation of the P_δ phase, the $L_\beta \rightarrow H_{II}$ thermotropic phase transition, and formation of the L_c phase are described below in Sections 3.1.2, 3.1.3 and 3.1.4, respectively.

3.1.2. *The effect of sucrose on formation of the P_δ phase*

DOPE forms the inverted ribbon phase at extreme dehydration [30,32]. The inverted ribbon phase, P_δ, consists of ribbon-like strips of bilayer indefinitely long in one direction of finite width and thickness [36,37]. Previously [30], we observed that the 'disappearance' of the thermal event associated with the $L_\beta \rightarrow H_{II}$ phase transition in DOPE liposomes dehydrated at ≥ 137 MPa was due to formation of the P_δ phase. However, the absence of the $T_{\beta bh}$ thermal event in the DOPE/sucrose mixtures dehydrated at ≥ 57 MPa was the result of a different mechanism. DSC heating curves for dehydrated 1:2 DOPE/sucrose mixtures were compared with the DSC curves for DOPE in the P_δ phase in Fig. 3, which shows that the thermal event at −35°C, which we consider as a 'signature' of the P_δ phase of DOPE [30], was not observed in the 1:2 DOPE/sucrose mixtures de-

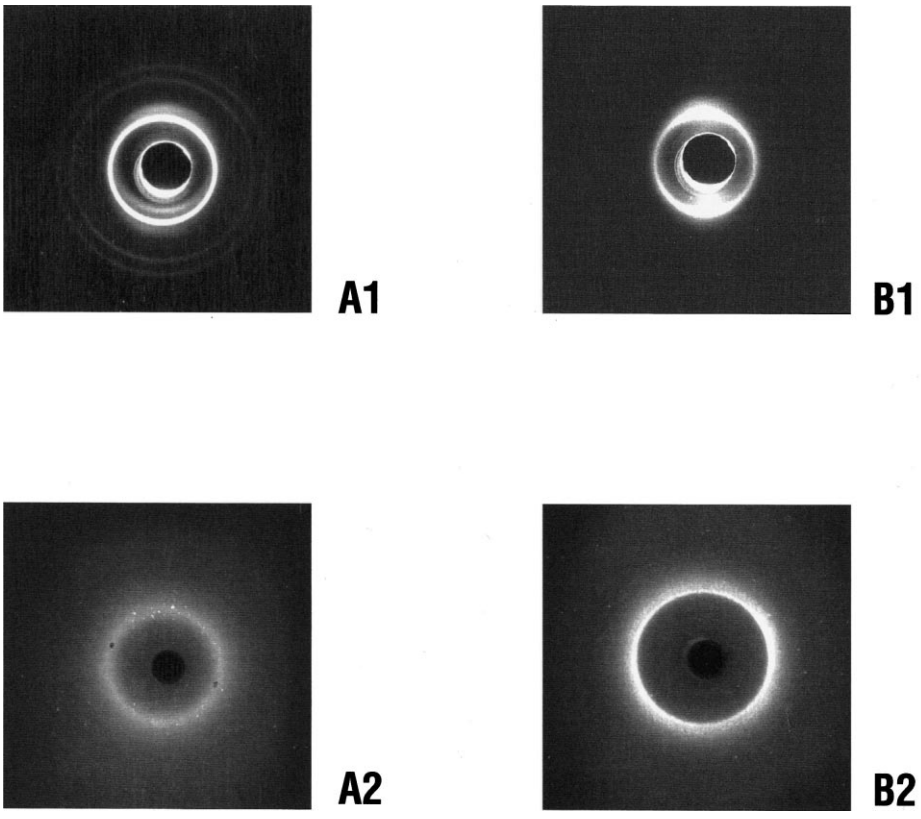

Fig. 2. X-ray diffraction patterns of a 1:2 DOPE/sucrose mixture dehydrated at 16 MPa at 30°C (water content 10.4 wt%). A1: 30°C, small angle, $d=5.62$ nm; A2: 30°C, wide angle, $d=0.45$ nm; B1: −41°C, small angle, $d=6.08$ nm; B2: −40°C, wide angle, $d=0.44$ nm.

hydrated at osmotic pressures of 137 and 276 MPa. DSC curves for the 1:2 and 2:1 DOPE/sucrose mixtures had a similar appearance. Hence, the DSC data suggest that the P_δ phase did not form in the DOPE/sucrose mixtures. Moreover, X-ray diffraction patterns of the DOPE in the P_δ phase and 1:2 DOPE/sucrose mixtures dehydrated at high osmotic pressures (≥ 137 MPa) are different (Fig. 4). The P_δ phase in DOPE had partially ordered hydrocarbon chains as evidenced from observation of a relatively sharp wide-angle diffraction ring, which was not detected in the DOPE/sucrose mixtures.

Hence, both DSC and X-ray diffraction results suggest that the P_δ phase was not formed in the DOPE/sucrose mixtures at extreme dehydration – which is different from the behavior of pure DOPE liposomes. The absence of a sharp wide-angle diffraction ring indicates that this is a phase with disordered hydrocarbon chains such as the lamellar liquid crystalline, cubic, or inverted hexagonal phase. Lack of higher-order small-angle diffractions did not allow us to identify the phase based on the X-ray diffraction data alone. We suggest that DOPE in the DOPE/sucrose mixtures that were dehydrated at osmotic pressures ≥ 57 MPa remained in the H_{II} phase, and that the H_{II} phase in these mixtures did not undergo a thermotropic phase transition to the L_β phase in the temperature range used in the DSC experiments (i.e., −140°C to 50°C).

3.1.3. *The effect of the glass transition of sucrose on the $L_\beta \rightarrow H_{II}$ thermotropic phase transition*

Dehydration had a relatively weak effect on the $L_\beta \rightarrow H_{II}$ phase transition temperature, $T_{\beta bh}$, of DOPE (Fig. 1) whereas the T_g of sucrose significantly increased with dehydration and approached the $T^c_{\beta bh}$ after dehydration at 41 MPa (Fig. 5). The increase in the T_g of sucrose close to the $T^c_{\beta bh}$ coincided with changes in the thermotropic behavior of DOPE as shown in Fig. 5. During cooling of the 1:2 DOPE/sucrose mixture dehydrated at 25 MPa, an exothermic peak was observed at approx. −35°C

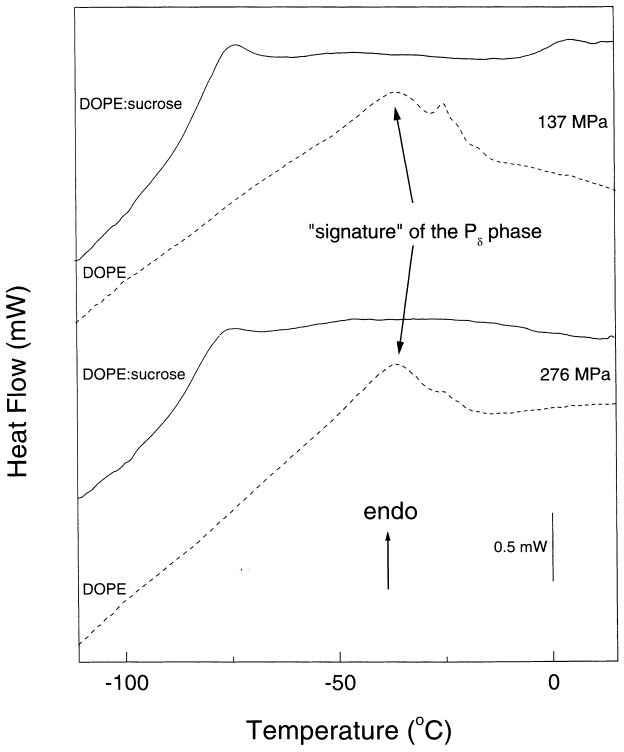

Fig. 3. DSC heating curves of DOPE/sucrose mixtures (1:2 mole ratio, solid lines) and pure DOPE (dashed lines) dehydrated at 137 MPa and 276 MPa at 0°C. Heating rates: 40°C/min. DSC curves of DOPE in the P_d phase typically had a broad endotherm centered at approx. −35°C, which we considered to be the 'signature' of the P_d phase.

and assigned to the $H_{II} \rightarrow L_\beta$ phase transition (Fig. 5, upper curve). In this mixture, the T_g of sucrose was much lower than the temperature of the $H_{II} \rightarrow L_\beta$ phase transition (−70°C vs. −35°C). In the mixture that was dehydrated at 41 MPa, the H_{II} phase was not converted to the L_β phase during cooling as evidenced by the absence of a strong exothermic peak on the DSC cooling curve (compare cooling curves for the mixtures dehydrated at osmotic pressures 25 and 41 MPa in Fig. 5). A strong exotherm (T_{exo}) was detected during heating, after sucrose transformed from a glassy to liquid state. We suggest that this exotherm corresponds to the H_{II}-to-L_β phase transition. At further dehydration at ≥81 MPa the T_g of sucrose reached −30°C, and the H_{II}-to-L_β thermotropic phase transition did not occur during either cooling or heating as evidenced by an absence of any significant exothermic event. In these mixtures, only the T_g of sucrose, and a low temperature endotherm T_{gL} (discussed below in Section 3.1.5) were detected. A lack of thermal events that can be associated with phase transitions of DOPE in mixtures dehydrated at ≥57 MPa suggests that DOPE remained in the H_{II} phase in the temperature interval of the DSC experiments. Note that pure DOPE dehydrated at 41 and 81 MPa at 30°C did show the $H_{II} \rightarrow L_\beta$ thermotropic transitions [30].

Indirect support for the phase assignment was obtained from a low-temperature thermal event, T_{gL}, which was observed in the mixtures dehydrated at ≥41 MPa. These samples remained in the H_{II} phase after cooling; hence, the T_{gL} thermal event is associated with the H_{II} phase. Thermal cycling experiments were performed to support the suggestion that the T_{gL} thermotropic event is associated with the H_{II} phase (Fig. 6). In the thermal cycling experiment, when heating was interrupted and the sample was cooled down immediately after the $H_{II} \rightarrow L_\beta$ phase transformation, the T_{gL} was not observed during the second heating, i.e., when the sample converted to the L_β phase. Hence, T_{gL} is an attribute of the H_{II} phase rather than the L_β phase, and observation of the T_{gL} in DOPE/sucrose mixtures was considered as a 'signature' of the H_{II} phase. In addition, we suggest that the lack of higher-order, small-angle reflections observed in these mixtures also favors the H_{II} phase assignment because the disappearance of higher-order reflections with dehydration is typical for the H_{II} phase [38]. We conclude that sucrose prevented the $H_{II} \rightarrow L_\beta$ thermotropic phase transition in the DOPE/sucrose mixtures dehydrated at ≥57 MPa. Most likely, this 'stabilization' of the H_{II} phase has a kinetic basis, as considered in Section 4.

It should also be mentioned that, in the 1:2 mixtures dehydrated at ≥81 MPa, two thermal events were detected on the DSC heating curves instead of one T_g (Fig. 5, bottom curve). Observation of two T_g events may be explained by the appearance of two compartments of sucrose molecules and will be discussed in more detail in a separate publication (in preparation).

3.1.4. The effect of sucrose on formation of the L_c phase

Another important difference in the phase behavior of DOPE and DOPE/sucrose mixtures is associ-

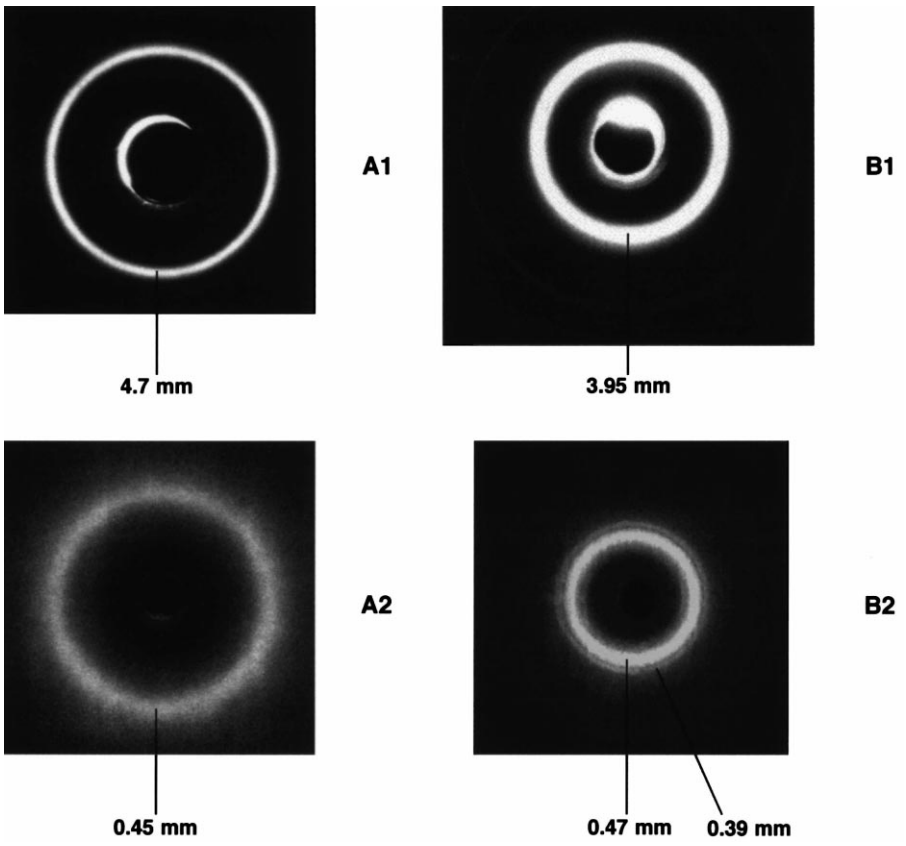

Fig. 4. X-ray diffraction patterns of the DOPE/sucrose mixtures (1:2 mole ratio) (A_1, A_2) and pure DOPE (B_1, B_2) after dehydration at osmotic pressures ≥140 MPa. The patterns were obtained at room temperature. Subscripts 1 and 2 refer to the small-angle and wide-angle diffraction patterns, respectively. Pure DOPE was in the P_δ phase. *d* spacings are shown.

ated with formation of the lamellar crystalline phase, L_c. When DOPE liposomes were dehydrated at osmotic pressures of 35 and 57 MPa at 0°C, the L_c phase formed [30]. DOPE samples in the L_c phase had several sharp, wide-angle X-ray diffraction rings and characteristic DSC heating curves with a strong endothermic peak, T_c, at ~25°C, which was not present in the second heating scan [30].

DSC heating curves for 1:2, 1:1, and 2:1 DOPE/sucrose mixtures as well as pure DOPE after dehydration at 35 and 57 MPa at 0°C are shown in Fig. 7. After dehydration at 35 MPa (Fig. 7A), none of the three DOPE/sucrose mixtures formed the L_c phase (i.e., the T_c peak was not observed); however, after dehydration at 57 MPa (Fig. 7B), the L_c phase appeared in the 2:1 mixture (i.e., the T_c peak was detected) but not in the 1:1 and 1:2 mixtures. Sucrose inhibited formation of the L_c phase in sucrose-rich mixtures (1:1 and 1:2 DOPE/sucrose, Fig. 7). At a lower sucrose content (2:1 DOPE/sucrose ratio), sucrose inhibited formation of the L_c phase at 35 MPa but not at 57 MPa. Also note that the T_{g_L} thermal event was not observed in the 2:1 DOPE/sucrose mixture in the L_c phase whereas it appeared on the second scan after DOPE was converted to the H_{II} phase (Fig. 7B, inset). When 2:1 DOPE/sucrose mixtures were dehydrated at 140 MPa, the peak associated with the L_c phase of DOPE was not present (Fig. 8), and the DSC curves were similar to those of the 1:1 and 1:2 mixtures with the T_{g_L} at −90°C indicative of the H_{II} phase. Hence, the L_c phase was formed at the intermediate osmotic pressure (57 MPa), but the H_{II} phase existed at both lower (35 MPa) and higher (140 MPa) osmotic pressures. We suggest that, in the 2:1 DOPE/sucrose mixture dehydrated at >57 MPa, the L_c phase is the thermodynamically stable phase, and the H_{II} phase which was observed after dehydration at 140 MPa is a metastable phase in relation to the L_c phase. Indeed, the L_c phase of DOPE is characterized by

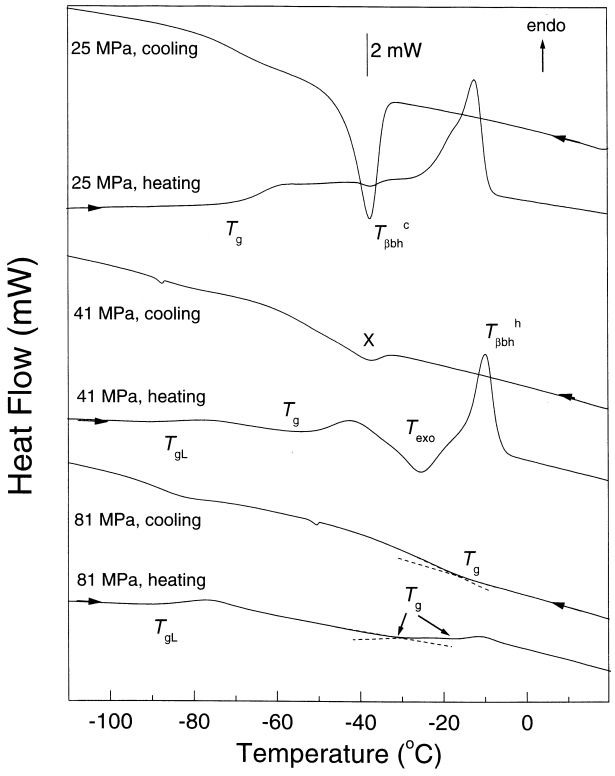

Fig. 5. DSC cooling and heating curves of the DOPE/sucrose mixtures (1:2 mole ratio) after dehydration at osmotic pressures of 25, 41 and 81 MPa at 30°C. Arrows show the direction of the temperature change: ← cooling, → warming. Scanning rates: 40°C/min. T_{g_L}, the glass transition temperature of DOPE; $T_{\beta bh}^h$, the $L_\beta \rightarrow H_{II}$ phase transition; $T_{\beta bh}^c$, the $H_{II} \rightarrow L_\beta$ phase transition; T_g, the glass transition temperature of sucrose; T_{exo}, the $H_{II} \rightarrow L_\beta$ phase transition completed during warming; X, onset of the $H_{II} \rightarrow L_\beta$ phase transition during cooling.

slow kinetics of formation [30], and it is possible that the L_c phase in the DOPE/sucrose mixtures was precluded because of kinetic effects. An alternative explanation for the observation of the L_c phase at the intermediate osmotic pressure is that a reentrant $H_{II} \rightarrow L_c \rightarrow H_{II}$ phase transition occurred; for example a reentrant $L_\alpha \rightarrow H_{II} \rightarrow L_\alpha$ lyotropic phase transition was reported for DOPE [39]. Available data did not allow us to distinguish between these two possibilities to explain the effect of sucrose on formation of the L_c phase.

3.1.5. Glass transition of DOPE

A low-temperature thermotropic event, T_{gL}, was observed in the mixtures dehydrated at ≥ 41 MPa (Fig. 5). The appearance of this thermal event resembles a glass transition. One way to determine if the T_{gL} thermal event corresponds to a glass-to-liquid transition is to conduct annealing experiments. In glass-forming materials, annealing below T_g results in an enthalpy recovery peak on a DSC heating curve, with the peak intensity increasing with annealing time [40]. The kinetics of the enthalpy recovery obeys a stretched-exponential law (Eq. 3).

$$\Delta H_t = \Delta H_\infty \ (1-\exp(-t/\tau)^\beta) \qquad (3)$$

where ΔH_t and ΔH_∞ are the measured and maximal enthalpy recoveries, respectively, t is the annealing time, τ is the mean relaxation time, and β is a relaxation time distribution parameter; $\beta = 1$ corresponds to a single relaxation time whereas low β values correspond to a broad distribution of τ.

Two samples of the 1:2 DOPE/sucrose mixtures that were dehydrated at 81 at 30°C ($T_{gL} = -89.1 \pm 1.1$°C) and 140 MPa at 0°C ($T_{gL} = -88.5 \pm 1.5$°C) were annealed at three temperatures

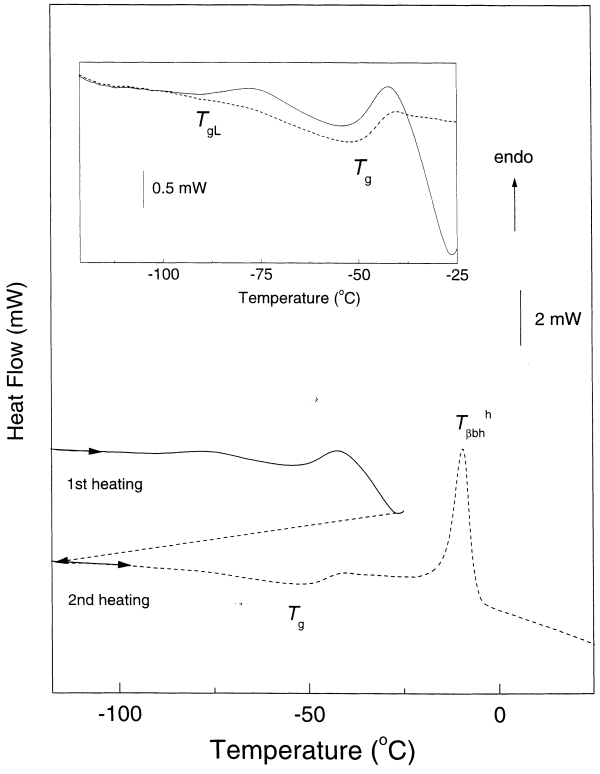

Fig. 6. DSC heating curves of a 1:2 DOPE/sucrose mixture dehydrated at 41 MPa at 30°C obtained in a thermal cycling experiment. Inset: magnified low-temperature portions of the first (solid line) and second (broken line) DSC heating curves.

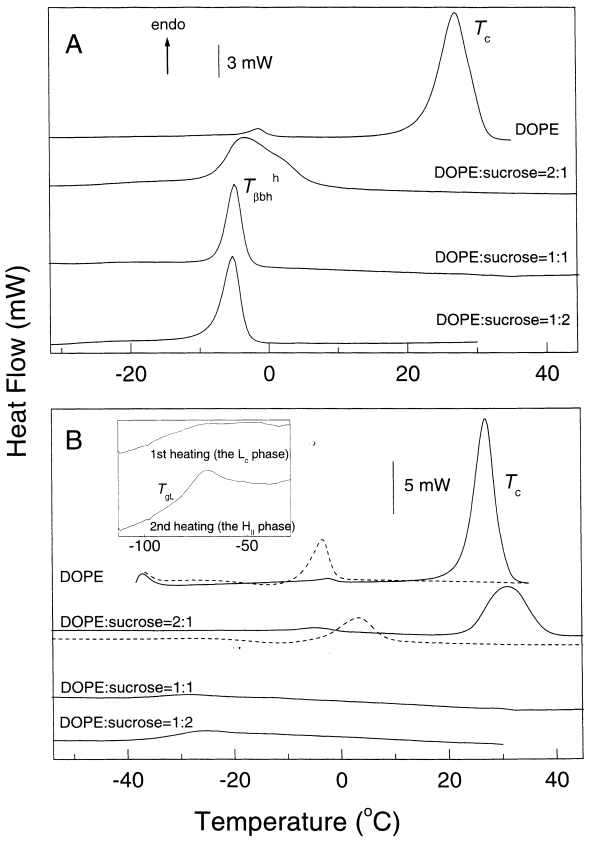

Fig. 7. DSC heating curves of pure DOPE and 2:1, 1:1, and 1:2 (mole ratio) DOPE/sucrose mixtures dehydrated at 0°C at 35 MPa (A) and 57 MPa (B). Heating rate: 40°C/min. Solid lines, first heating; dashed lines, second heating. Inset: low-temperature portion of the DSC heating scans of the 2:1 DOPE/sucrose mixture dehydrated at 57 MPa at 0°C showing the glass transition of DOPE.

(−95°C, −100°C and −105°C) for different time periods. An enthalpy recovery peak was observed in annealed samples (Fig. 9A). For quantitative measurements of the enthalpy recovery, the DSC curves for annealed samples were subtracted from the DSC heating curves obtained on the second scans. The representative curves are shown in Fig. 9B demonstrating that the enthalpy recovery event increased with annealing time. In addition, peak temperature increased with annealing time which is typical for glass-forming materials [41]. The areas under the peaks were determined with the aid of MicrocalOrigin software and were plotted against annealing time as shown in Fig. 10. The experimental data were fitted to a stretched-exponential function (Eq. 3) using MicrocalOrigin software with ΔH_∞, τ, and β as fitting parameters. The fitting results are given in Fig. 10 as solid lines. β was determined to be from 0.3 to 0.5 which corresponds to a wide distribution of relaxation times for DOPE molecules. Note that such β values are typical for other glass forming materials; for example, a β value of 0.36 was reported for the glass transition of a liquid crystalline polymer [42]. Mean relaxation times were approximately 70 min at −95°C and 750 min at −100°C. The observation of the time-dependent enthalpy recovery, and the fact that the enthalpy recovery is described by the stretched exponential equation provide strong support for the assignment of the T_{gL} thermal event to the glass transition.

To determine if the glass transition at −90°C occurs only in DOPE/sucrose mixtures, mixtures of DOPE with either trehalose, fructose, or glucose were studied. The representative DSC heating curves of the mixtures dried over P_2O_5 at room temperature (22°C) are shown in Fig. 11. The T_{gL} step was ob-

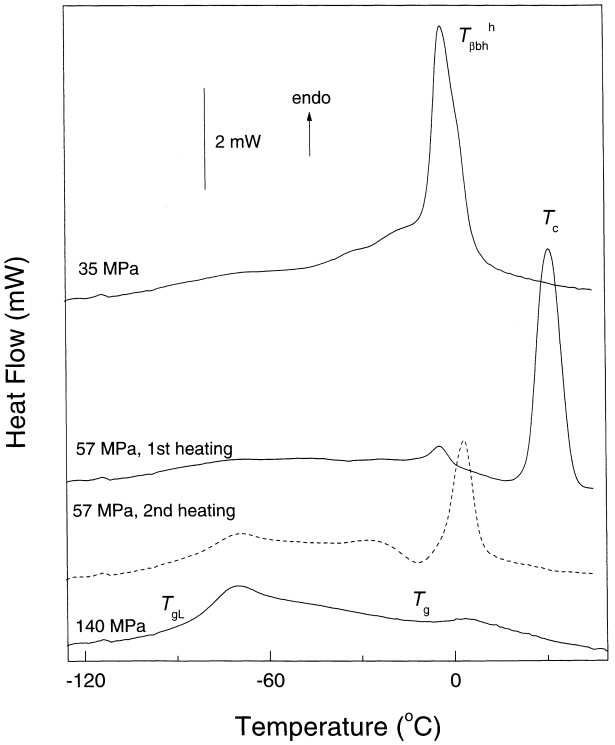

Fig. 8. DSC heating curves of the 2:1 DOPE/sucrose mixtures dehydrated at 0°C. Heating rate: 40°C/min. See Figs. 1 and 5 for the assignment of thermal events. Broken curve demonstrates an apparent irreversibility of the thermal behavior of the L_c phase.

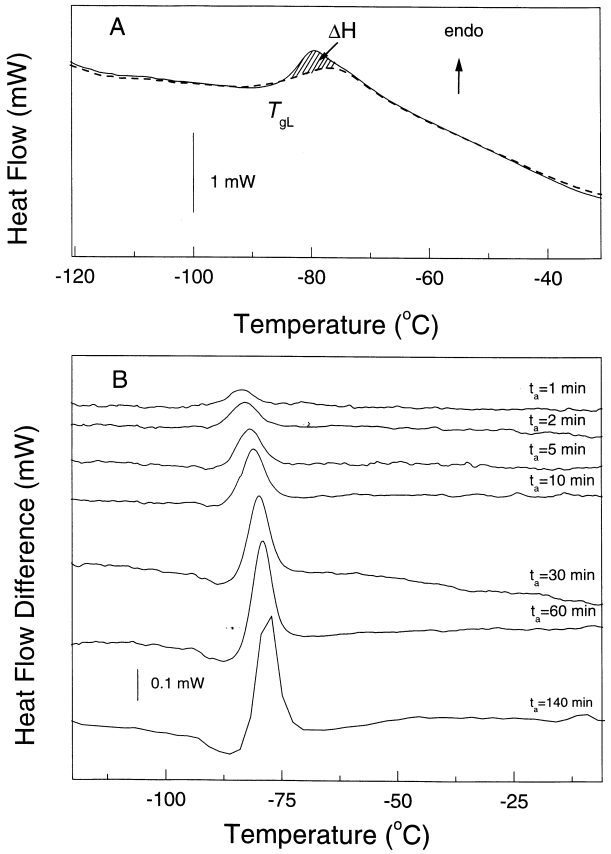

Fig. 9. DSC heating curves of the 1:2 DOPE/sucrose mixture dehydrated at 81 MPa at 30°C and annealed at −95°C. Water content 8.3 wt%. (A) Annealing time 30 min; solid line, first scan (after annealing); broken line, second scan (with eliminated thermal history); ΔH and shaded area, enthalpy recovery. (B) Differential DSC curves obtained by subtracting the second DSC heating curve (with the eliminated thermal history) from the first DSC heating curve (with the enthalpy recovery endotherm). The sample was annealed at −95°C for various times as indicated.

served at the same temperature of −90°C in all four mixtures and was independent of the sugar type that was present in the DOPE/sugar mixture. This is strong evidence that the thermal event at −90°C is associated with the DOPE phase rather than the sugar phase.

3.2. Empirical phase diagram

In this section, we present a summary of thermotropic and lyotropic phase transitions of DOPE/sucrose mixtures as a function of the water content and osmotic pressure. Fig. 12 presents a summary of the lyotropic phase transitions of DOPE and DOPE/sucrose mixtures at 0°C and 30°C. At 30°C, the H_{II}-to-P_δ lyotropic phase transition occurred in pure DOPE dehydrated at osmotic pressures between 81 and 158 MPa whereas the H_{II} phase existed in the DOPE/sucrose mixtures over the entire range of osmotic pressures. At 0°C, the L_α-to-L_c-to-P_δ lyotropic phase transitions occurred in DOPE whereas in the DOPE/sucrose mixtures the P_δ phase was not observed at all, and the L_c phase was detected only at a lower sucrose content and in a very narrow range of osmotic pressures.

Changes in the sequence of thermotropic phase transitions of DOPE as a result of dehydration are summarized using the composition triangle of a DOPE/sucrose/water system (Fig. 13). For mixtures with a relatively high water content (>50 wt%, field

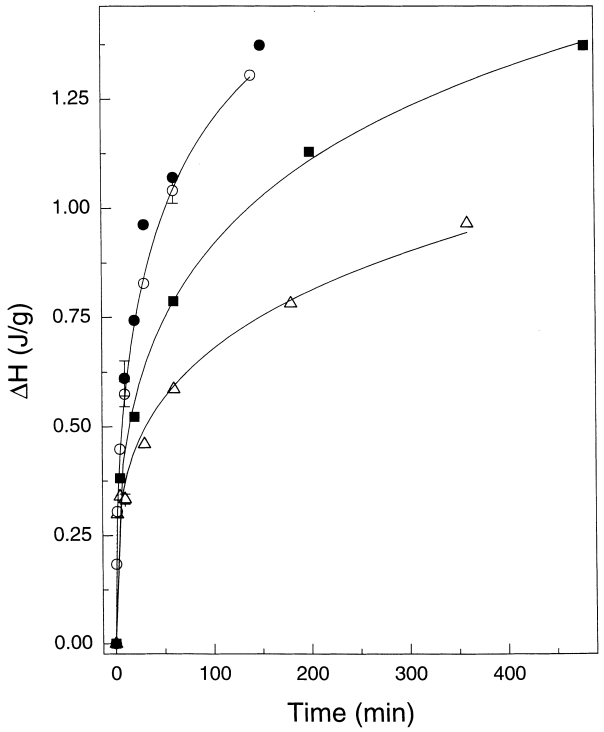

Fig. 10. Enthalpy recovery vs. annealing time for two 1:2 DOPE/sucrose mixtures dehydrated at 81 MPa at 30°C (8.3 wt% water, $T_{gL} = -89.1 \pm 1.1$°C, open symbols) and at 137 MPa at 0°C (4.3 wt% water, $T_{gL} = -88.5 \pm 1.5$°C, closed symbols). ○,●, annealing at −95°C; ■, annealing at −100°C; Δ, annealing at −105°C. Curves represent fitting of the experimental data to stretched exponential Eq. 3. Similar T_g values and enthalpy recovery patterns for two samples with different water content imply that the hydration of DOPE phase in these two samples was similar.

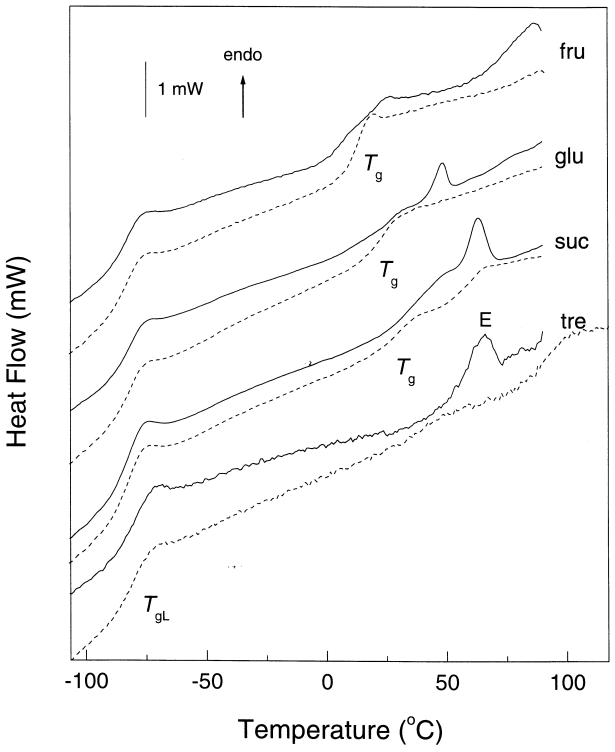

Fig. 11. DSC heating curves of 1:2 DOPE/sugar mixtures dehydrated over phosphorus pentoxide at room temperature. Sugars were as follows: fructose (fru), glucose (glu), sucrose (suc), and trehalose (tre). Solid lines, first heating; dashed lines, second heating. T_{g_L} corresponds to the glass transition of DOPE, T_g corresponds to the glass transition of the sugar. Endothermic peaks (E) on the first heating curves of DOPE/glucose, DOPE/sucrose, and DOPE/trehalose mixtures were assigned to the enthalpy recovery. Two T_g events were observed for DOPE mixtures with disaccharides (sucrose and trehalose), which are thought to be due to the existence of two sugar compartments.

1), three DOPE phases, i.e., L_β, L_α, and H_{II}, were observed depending on the temperature. These samples demonstrated thermotropic phase behavior that was typical for pure DOPE liposomes, i.e., the thermotropic $L_\beta \rightarrow L_\alpha \rightarrow H_{II}$ phase transition sequence was observed during heating. Upon moderate dehydration (water content ~20–40 wt%, field 2), the L_α phase was not detected, and a direct thermotropic $L_\beta \rightarrow H_{II}$ phase transition was observed in these samples. Further dehydration (field 3) resulted in disappearance of the L_β phase as sucrose entered the glassy state. The H_{II} phase alone existed over a broad temperature range (−120°C to 50°C), and thermotropic DOPE phase transitions were not detected. At low sucrose contents (field 4), the L_c phase formed which underwent a thermotropic $L_c \rightarrow H_{II}$ phase transition during heating. The P_δ phase formed in pure DOPE only at the extreme dehydration (at osmotic pressures ≥137 MPa), and was not observed in DOPE/sucrose mixtures.

The $H_{II} \leftrightarrow L_\beta$ phase transition temperatures of DOPE and the liquid ↔ glass transformation temperatures of sucrose for the 1:2, 1:1, and 2:1 DOPE/sucrose mixtures are shown in Fig. 14 as functions of osmotic pressure. The $L_\beta \rightarrow H_{II}$ phase transition temperature (i.e., the $T^h_{\beta bh}$ which was determined during warming) did not change much with dehydration whereas the $H_{II} \rightarrow L_\beta$ phase transition temperature ($T^c_{\beta bh}$ measured during cooling) decreased with osmotic pressure. T_g of sucrose increased with dehydration. The T_g of sucrose determined during cooling was higher than the T_g determine during heating. It should be also mentioned that the $T_{\beta bh}$ in mixtures dehydrated at 30°C was lower than the $T_{\beta bh}$ for the mixtures dehydrated at 0°C whereas both T_g values (Fig. 14) and water content (Fig. 16, see below) for mixtures dehydrated at 30°C and 0°C were similar. We did not attempt to investigate the difference in the $T_{\beta bh}$ between mixtures dehydrated at 0°C and 30°C any further.

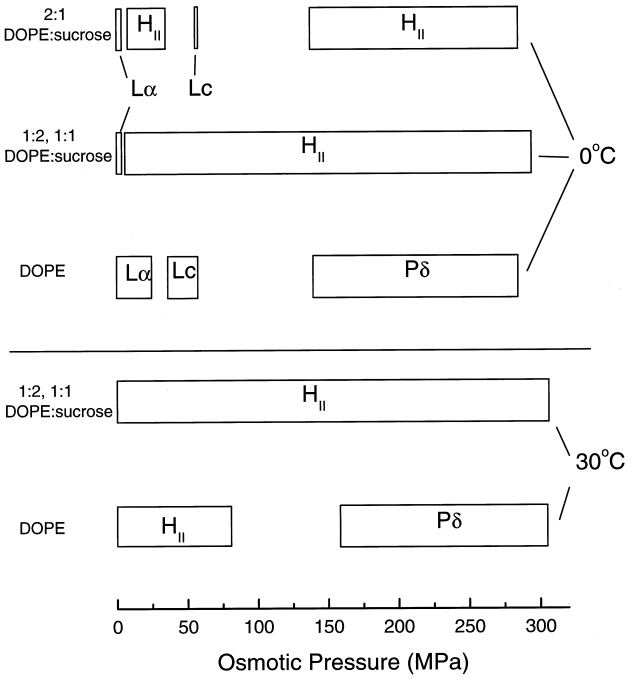

Fig. 12. Summary of the lyotropic phase transitions of DOPE dehydrated in the presence and absence of sucrose.

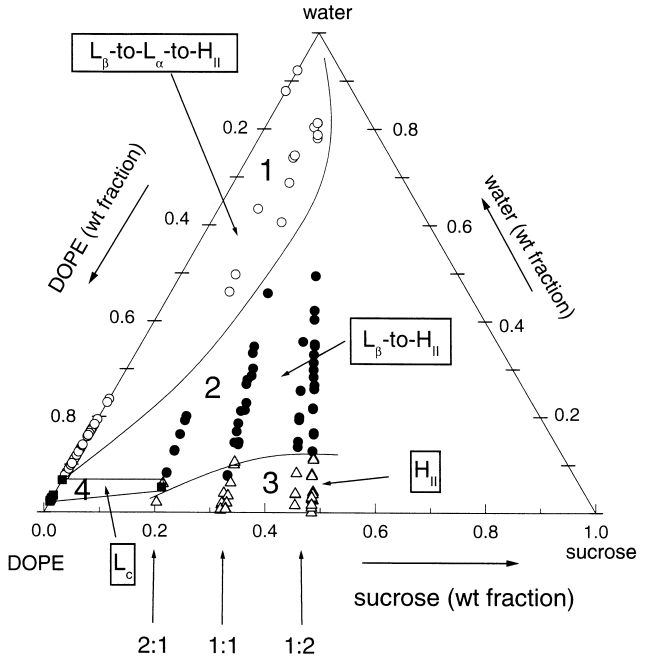

Fig. 13. Composition triangle of the water/DOPE/sucrose system. Symbols represent the composition of the mixtures that were studied. Field 1 (○): $L_\beta \rightarrow L_\alpha \rightarrow H_{II}$ thermotropic phase transition observed during heating. Field 2 (●): $L_\beta \rightarrow H_{II}$ thermotropic phase transition observed during heating. Field 3 (△): only the H_{II} phase exists in the temperature range −100°C to 50°C. Field 4 (■): the $L_c \rightarrow H_{II}$ thermotropic phase transition observed during heating. DOPE/sucrose mole ratio in the studied mixtures is shown. The 2:1 DOPE/sucrose mixtures were dehydrated at 0°C whereas the 1:1 and 1:2 mixtures were dehydrated at 0°C and 30°C. The appearance of DSC curves of the 1:1 and 1:2 mixtures dehydrated at 0°C and 30°C was similar.

Water contents of the DOPE/sucrose mixtures are shown as a function of osmotic pressure in Fig. 15 and Fig. 16. The water content increased with increasing sucrose content (Fig. 15). Fig. 16 compares the water content of the 1:2 (Fig. 16A) and 1:1 (Fig. 16B) DOPE/sucrose mixtures dehydrated at 30°C and 0°C. In addition, a theoretical hydration of the 1:1 and 1:2 mixtures assuming that sucrose and the H_{II} phase of DOPE in the DOPE/sucrose mixtures dehydrate independently of each other is shown as a dotted line. The hydration values of sucrose and the H_{II} phase of DOPE that were used for the calculations are given in Table 1. For both mixtures dehydrated at osmotic pressures >8 MPa, experiments at 0°C and 30°C gave similar results between each other and the theoretical calculation. After dehydration at a lower osmotic pressure (5 MPa), the water content of both the 1:2 and 1:1 mixtures that were dehydrated at 0°C was higher than both the calculated value and the experimental value determined for the samples dehydrated at 30°C. It is possible that hydration equilibrium was not achieved at osmotic pressures of 5 MPa and 0°C due to insufficient equilibration time. For all other dehydration conditions,

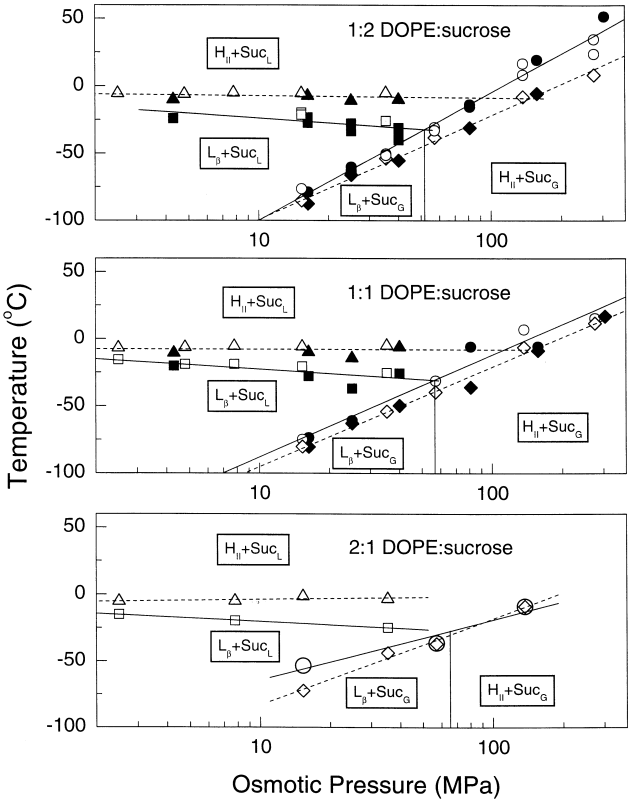

Fig. 14. The $L_\beta \rightarrow H_{II}$ phase transition temperature of DOPE determined during heating ($T^h_{\beta bh}$), the $H_{II} \rightarrow L_\beta$ phase transition temperature of DOPE determined during cooling ($T^c_{\beta bh}$), and T_g of sucrose in DOPE/sucrose mixtures as a function of osmotic pressure. □,■, $T^c_{\beta bh}$ measured during cooling; ○,●, T_g measured during cooling; △,▲, $T^h_{\beta bh}$ measured during warming; ◇,◆, T_g measured during warming. Open symbols are for the mixtures dehydrated at 0°C, and closed symbols are for the mixtures dehydrated at 30°C. Scanning rate: 40°C/min. For 1:2 DOPE/sucrose mixtures, only the first T_g of sucrose is plotted. Suc_G and Suc_L are for sucrose in the glassy and liquid state, respectively. The solid vertical line represents the boundary between the L_β+Suc_G and H_{II}+Suc_G phase fields. Other lines are linear regressions of the experimental data. Solid lines, linear fit of $T^c_{\beta bh}$ of DOPE and T_g of sucrose measured during cooling; dashed lines, linear fit of $T^h_{\beta bh}$ of DOPE and T_g of sucrose measured during warming. Error bars are not shown because most are smaller than the symbol size.

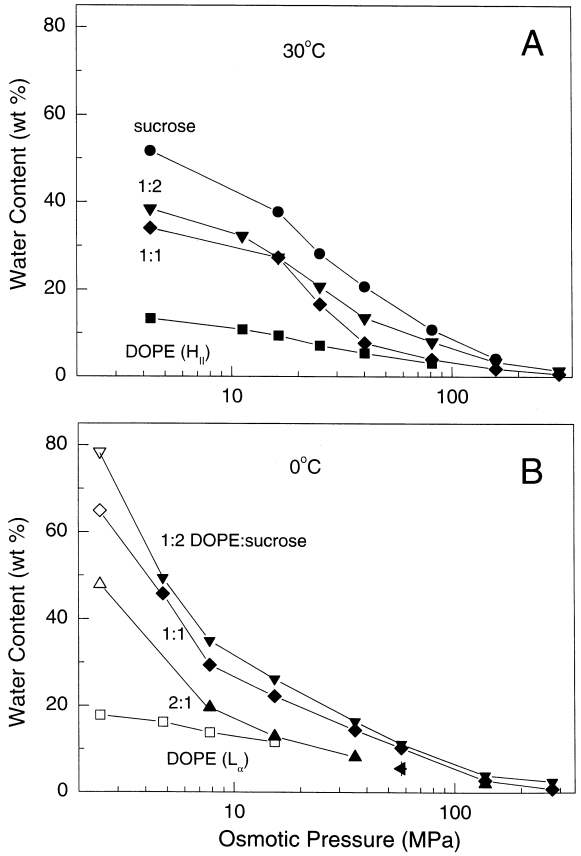

Fig. 15. Desorption isotherms of DOPE, sucrose, and DOPE/sucrose mixtures at (A) 30°C and (B) 0°C. ●, sucrose; ▼,▽, DOPE/sucrose (1:2 mole ratio) (DOPE in the H_{II} and the L_α phase, respectively); ♦,◇, DOPE/sucrose (1:1 mole ratio) (DOPE in the H_{II} and the L_α phase, respectively); ▲,◀,△, DOPE/sucrose (2:1 mole ratio) (DOPE in the H_{II}, the L_c, and the L_α phase, respectively); ■,□, DOPE in the H_{II} and the L_α phase, respectively. Lines are given as a visual aid. Error bars are not shown; in the majority of cases, they are smaller than symbol size.

the close correspondence between the water content after dehydration at 30°C and 0°C and the calculated values indicates that hydration equilibrium was achieved in the experiments.

4. Discussion

4.1. Effect of sucrose on formation of the ordered and partially ordered phases of DOPE

It has been shown for PC/sugar mixtures composed of various PC species (DPPC, DMPC, DOPC) and various sugars (trehalose, sucrose, glucose, fructose) that the $L_\beta \rightarrow L_\alpha$ phase transition temperature, T_m, is depressed below the T_m of the fully hydrated lipid if the PC is in the L_α phase when the sugar phase forms a glass as a result of dehydration [18]. In the present study, we observed that a glass transformation of sucrose also affected the H_{II}-to-L_β thermotropic phase transition of PE species. Depending on how close the T_g of sucrose was to the $T^c_{\beta bh}$ of DOPE, the H_{II}-to-L_β thermotropic phase transition in the DOPE/sucrose mixtures either (i) occurred during cooling or (ii) started during cooling and was completed during warming or (iii) did not occur at all. Indeed, in the mixtures dehydrated at osmotic pressures ≤ 35 MPa, when T_g was much lower than $T^c_{\beta bh}$ ($T_g/T^c_{\beta bh} \leq 0.91$ as determined from Fig. 14), the $H_{II} \rightarrow L_\beta$ phase transition occurred during cooling.

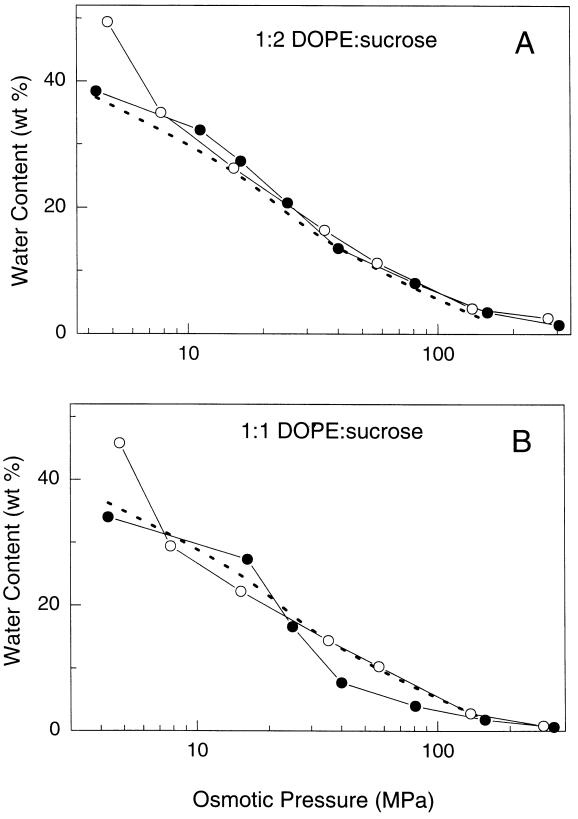

Fig. 16. Comparison of the experimental water desorption data and calculated water content for 1:2 (A) and 1:1 (B) DOPE/sucrose mixtures. Open symbols, experimental data obtained at 0°C; filled symbols, data obtained at 30°C. Dashed line, calculated values assuming that DOPE and sucrose hydrated independently of each other. Error bars are not shown; in the majority of cases, they are smaller than the symbol size.

As T_g approached the $T_{\beta bh}$ with dehydration ($T_g/T^c_{\beta bh} = 0.96$ at 41 MPa), the $H_{II} \rightarrow L_\beta$ phase transition was not completed during cooling, i.e., it started during cooling and was completed during warming (see results above for 1:2 mixtures dehydrated at 41 MPa, Figs. 5 and 6). With further dehydration at osmotic pressures ≥ 57 MPa, when $T^c_{\beta bh} \approx T_g$, the $H_{II} \rightarrow L_\beta$ phase transition did not occur during either cooling or warming. We estimated the viscosity of the sucrose matrix at the $T^c_{\beta bh}$ at different hydrations using the relationships between viscosity and T_g/T given in [43], and correlated the viscosity of the sucrose matrix with changes in the $H_{II} \rightarrow L_\beta$ phase behavior as follows. The $H_{II} \rightarrow L_\beta$ phase transition occurred during cooling when the viscosity was $< 10^4$ Pa s ($T_g/T = 0.91$); however it was not completed during cooling when the viscosity at the T_m was 10^7–10^8 Pa s ($T_g/T = 0.96$), and did not occur at all when viscosity was $> 10^{10}$ Pa s ($T_g/T = 0.99$). Therefore, we submit that viscosity of sucrose plays a major role in preventing formation of the L_β phase of DOPE, i.e., the $H_{II} \rightarrow L_\beta$ thermotropic phase transition in dehydrated mixtures of DOPE/sucrose. Recently, Wolfe and Bryant also suggested that solution viscosity may hinder dynamic phase transitions in phospholipid/sugar mixtures [21].

We suggest that the highly viscous sucrose solution created a rigid immobile matrix around the H_{II} phase, such that the H_{II}-to-L_β phase transition (which requires rearrangement of the phospholipid molecules from a nonbilayer to a bilayer structure) was greatly impeded. This 'viscosity' mechanism was first proposed by Zhang and Steponkus [18] to explain depression of the T_m in PC/sugar mixtures. However, there is a difference between the effect of sucrose on the phase behavior of PC and PE species. In PC species, the T_m was depressed but formation of the L_β phase was not precluded by transformation of sucrose into a glassy state and the $L_\alpha \rightarrow L_\beta$ phase transition ultimately occurred. We suggest that prevention of the H_{II}-to-L_β phase transition by sucrose is a kinetic rather than a thermodynamic phenomenon, in large part because of the observation that in the DOPE/sucrose mixtures dehydrated at 41 MPa, the transition started during cooling and was not completed until the subsequent warming scan.

Furthermore, we observed that sucrose affected formation of two other ordered phases, namely the L_c phase and the P_δ phase. Formation of the L_c phase of DOPE occurred after equilibration at 35 and 57 MPa at 0°C for 2 or more days [30]. The L_c phase, however, was not observed in either 1:2 or 1:1 DOPE/sucrose mixtures in the present study. At the lower sucrose content, i.e., the 2:1 DOPE/sucrose mixture, the L_c phase formed after dehydration at 57 MPa but not at 35 MPa (Fig. 7). The $H_{II} \rightarrow P_\delta$ lyotropic phase transition occurred in pure DOPE that was dehydrated at high osmotic pressures (> 140 MPa) [30,32] but was not observed in the DOPE/sucrose mixtures in this study. The viscosity mechanism, which plays a major role in the effect of sucrose on the $H_{II} \rightarrow L_\beta$ phase transition, may be involved in the $H_{II} \rightarrow L_c$ and $H_{II} \rightarrow P_\delta$ phase transitions as well; however, additional studies are required to consider other possible mechanisms (see [21] for a review of effects of solutes on phase transitions of phospholipids).

The analysis of the water content vs. osmotic pressure data suggest (Fig. 16) that DOPE and sucrose hydrate independently of each other. The independent hydration of the lipid and sucrose phases was reported also for dioleoylphosphatidylcholine/sucrose mixtures [44]. Nevertheless, sucrose has a dramatic impact on the phase behavior of DOPE in the H_{II} phase. Thus, we suggest that although sucrose and DOPE hydrate independently of each other, sucrose influences the phase behavior of DOPE in an indirect manner, i.e., by the viscosity mechanism.

4.2. Glass transition of DOPE

We submit that the T_{g_L} thermal event observed at −90°C in the DOPE/sucrose mixtures dehydrated at ≥ 57 MPa is the glass transition of DOPE. The assignment of the T_{g_L} to the glass transition was confirmed in annealing experiments when enthalpy recovery was observed in samples annealed at temperatures below T_{g_L} (Figs. 9 and 10). The glass transition of DOPE was observed when DOPE was in a liquid crystalline state (most likely the H_{II} phase), and was not observed when DOPE was in either the L_β or the L_c phase (Figs. 6 and 7, insets). This may be the reason why the T_{gL} was observed only in the presence of sucrose because sucrose prevented formation of the ordered L_β and the L_c phases. The glassy state is of major importance in

different fields of applied science, i.e., food science, pharmaceutical science, cryobiology and anhydrobiology (see [45–49] for examples). However, to the best of our knowledge, there are no reports of a glass transition in phospholipids, even though membrane vitrification was predicted theoretically [50], and glass transitions in other classes of liquid crystalline materials have been studied extensively (see [51] for example). The properties of a glass transition occurring in typical amorphous materials and more ordered materials, such as liquid crystals, are qualitatively similar. In particular, the glass transition is accompanied by a stepwise change in the specific heat capacity, and physical aging behavior is similar in both amorphous and liquid crystalline materials. On the other hand, there is a difference in the type of motion that is responsible for the liquid/glass transition: in regular amorphous materials, the liquid/glass transition is associated with the isotropic translational motion of molecules whereas the glass transition in some of the liquid crystalline materials is due to highly anisotropic rotational motion [52,53]. It is possible that the glass transition in DOPE is associated with a rotational motion of DOPE molecules along the hydrocarbon chain axis rather than with a translational diffusion of the molecules as expected in regular glass-forming materials.

Although the T_g of DOPE observed in this study is very low (approx. −90°C), the T_g may be different in other phospholipid species or classes. Indeed, in a subsequent study of DOPE and several other PE and PC species as well as lipid extracts from plant membranes, we observed the phospholipid glass transition at much higher temperatures. For example, T_g of DMPC with 3 wt% water was detected at approx. 20°C (in preparation). The observation of a glass transition in synthetic phospholipids and lipid extracts of plant membranes suggests that the glass transition may be a general phenomenon in biological membranes. The transformation of a biological membrane to a glassy state during cooling or desiccation may be of significant practical importance. The storage of biological membranes in a glassy state (i.e., below T_g) would be expected to increase the chemical and physical stability because of a dramatic decrease in molecular mobility. For example, both lipid/protein and lipid/lipid demixing would be impeded in the glassy state. Alternatively, it is possible that a glass transformation in a biological membrane at ultra-low temperatures may have detrimental effects. It is well known that when either the cooling or warming rate is relatively rapid in the temperature range around T_g, fracturing of the glass often occurs. Such fracturing of the membrane (i.e., the plasma membrane, which plays a central role during freeze–thaw cycle [6]), may be an additional factor to consider in the freeze-induced destabilization of cellular membranes – especially in cryopreservation protocols that involve quenching of the samples in liquid nitrogen.

5. Conclusions

It has been shown that the dehydration-induced formation of the ordered (L_c) and partially ordered (P_δ) phases of DOPE as well as the thermotropic $H_{II} \rightarrow L_\beta$ phase transition at moderate and low hydrations are prevented in DOPE/sucrose mixtures. A 'viscosity hypothesis' is invoked to explain the role of sucrose in precluding formation of the L_β, and possibly the L_c and P_d phases. Specifically, we suggest that at low hydrations, the extremely viscous sucrose matrix kinetically traps DOPE in the H_{II} phase precluding the thermotropic $H_{II} \rightarrow L_\beta$ phase transition and the lyotropic $H_{II} \rightarrow L_c$ and $H_{II} \rightarrow P_d$ phase transitions. It should be stressed, however, that additional studies with other solutes having different T_gs and viscosity profiles need to be performed to confirm the suggested mechanism.

Finally, DOPE underwent a glass transition at −90°C when it was in a liquid crystalline phase – most likely the H_{II} phase. In our more recent studies, we observed a glass transition in other synthetic phospholipids as well as in lipid extracts of plant membranes. The results suggest that biological membranes may enter the glassy state at low temperatures, which might have important practical implications.

Acknowledgements

This work was supported by a grant (DE-FG02-84ER13214) from the United States Department of Energy. We wish to thank Dr. Ernest Fontes for his

expert assistance with the X-ray diffraction experiments, and Dr. Joe Wolfe for his critical reading of the manuscript and constructive criticism.

References

[1] K.A.C. Madin, J.H. Crowe, J. Exp. Zool. 193 (1975) 335–342.
[2] J.S. Clegg, Comp. Biochem. Physiol. 14 (1965) 135–143.
[3] N. Guo, I. Puhlev, D.R. Brown, J. Mansbridge, F. Levine, Nat. Biotechnol. 18 (2000) 168–171.
[4] K.L. Koster, A.C. Leopold, Plant Physiol. 88 (1988) 829–833.
[5] K.B. Storey, Comp. Biochem. Physiol. A: Physiol. 117A (1997) 319–326.
[6] P.L. Steponkus, Annu. Rev. Plant Physiol. 35 (1984) 543–584.
[7] M. Crevecouer, R. Deltour, R. Bronchart, Planta 132 (1976) 31–41.
[8] T. Senaratna, B.D. McKersie, Plant Physiol. 72 (1983) 620–624.
[9] J.H. Crowe, L.M. Crowe, Cryobiology 19 (1982) 317–328.
[10] J.H. Crowe, L.M. Crowe, in: G. Gregoriadis (Ed.), Liposome Technology, 2nd Edition, Vol. 1, CRC Press, Boca Raton, FL, 1993, pp. 229–252.
[11] J.H. Crowe, F.A. Hoekstra, L.M. Crowe, Proc. Natl. Acad. Sci. USA 86 (1989) 520–523.
[12] L.M. Crowe, C. Womersley, J.H. Crowe, D. Reid, L. Appel, A. Rudolph, Biochim. Biophys. Acta 861 (1986) 131–140.
[13] L.M. Crowe, J.H. Crowe, Biochim. Biophys. Acta 946 (1988) 193–201.
[14] J.H. Crowe, L.M. Crowe, J.F. Carpenter, A.S. Rudolph, C.A. Wistrom, B.J. Spargo, T.J. Anchordoguy, Biochim. Biophys. Acta 947 (1988) 367–384.
[15] J.H. Crowe, L.M. Crowe, R. Mouradian, Cryobiology 20 (1983) 346–356.
[16] J.W. Crowe, F.A. Hoekstra, K.H.N. Nguyen, L.M. Crowe, Biochim. Biophys. Acta 1280 (1996) 187–196.
[17] J.H. Crowe, B. Spargo, L.M. Crowe, Proc. Natl. Acad. Sci. USA 84 (1987) 1537–1540.
[18] J. Zhang, P.L. Steponkus, Cryobiology 33 (1996) 624–625.
[19] K.L. Koster, M.S. Webb, G. Bryant, D.V. Lynch, Biochim. Biophys. Acta 1193 (1994) 143–150.
[20] K.L. Koster, Y.P. Lei, M. Anderson, S. Martin, G. Bryant, Biophys. J. 78 (2000) 1932–1946.
[21] J. Wolfe, G. Bryant, Cryobiology 39 (1999) 103–129.
[22] J. Wolfe, Aust. J. Plant Physiol. 14 (1987) 311–318.
[23] R. Koynova, J. Brankov, B. Tenchov, Eur. Biophys. J. 25 (1997) 261–274.
[24] R.D. Koynova, B.G. Tenchov, P.J. Quinn, Biochim. Biophys. Acta 980 (1989) 377–380.
[25] C.A. Wistrom, R.P. Rand, L.M. Crowe, B.J. Spargo, J.H. Crowe, Biochim. Biophys. Acta 984 (1989) 238–242.
[26] V.A. Parsegian, R.P. Rand, N.L. Fuller, D.C.D.C. Rau, Methods Enzymol. 127 (1986) 400.
[27] M.S. Webb, S.W. Hui, P.L. Steponkus, Biochim. Biophys. Acta 1145 (1993) 93–104.
[28] M.S. Webb, T.C. Irving, P.L. Steponkus, Biochim. Biophys. Acta 1239 (1995) 226–238.
[29] M.S. Webb, T.C. Irving, P.L. Steponkus, Biochim. Biophys. Acta 1326 (1997) 225–235.
[30] E.Y. Shalaev, P.L. Steponkus, Biochim. Biophys. Acta 1419 (1999) 229–247.
[31] R.P. Rand, N.L. Fuller, Biophys. J. 66 (1994) 2127–2138.
[32] W. Pohle, C. Selle, Chem. Phys. Lipids 82 (1996) 191–198.
[33] Z.-W. Yu, W.P. Williams, P.J. Quinn, Arch. Biochim. Biophys. 332 (1996) 187–195.
[34] S. Glasstone, Textbook of Physical Chemistry, Van Nostrad, Princeton, NJ, 1946, pp. 669–670.
[35] E.Y. Shalaev, P.L. Steponkus, Thermochim. Acta 345 (2000) 141–143.
[36] V. Luzzati, in: D. Chapman (Ed.), Biological Membranes, Vol. 1, Academic Press, London, 1968, pp. 71–123.
[37] J.M. Seddon, G. Cevc, in: G. Cevc (Ed.), Phospholipids Handbook, Dekker, New York, 1993, pp. 403–454.
[38] J.M. Seddon, Biochim. Biophys. Acta 1031 (1990) 1–69.
[39] K. Gawrisch, V.A. Parsegian, D.A. Hajduk, M.W. Tate, S.M. Gruner, N.L. Fuller, R.P. Rand, Biochemistry 31 (1992) 2856–2864.
[40] I.M. Hodge, J. Non-Cryst. Solids 169 (1994) 211–266.
[41] S. Montserrat, J. Polym. Sci. B: Polym. Phys. 32 (1994) 509–522.
[42] J.F. Mano, N.T. Correia, J.J. Moura-Ramos, S.A. Andrews, G. Williams, Liquid Crystals 20 (1996) 201–217.
[43] C.A. Angell, R.D. Bressel, J.L. Green, H. Kanno, M. Oguni, E.J. Sare, J. Food Eng. 22 (1994) 115–142.
[44] Y.H. Yoon, J.M. Pope, J. Wolfe, Biophys. J. 74 (1998) 1949–1965.
[45] L. Slade, H. Levin, Crit. Rev. Food Sci. Nutr. 30 (1991) 115–160.
[46] B.C. Hancock, G. Zografi, J. Pharm. Sci. 86 (1997) 1–12.
[47] F. Franks, Pure Appl. Chem. 69 (1997) 915–920.
[48] M.J. Burke, in: A.C. Leopold (Ed.), Membranes, Metabolism, and Dry Organisms, Cornell University Press, Ithaca, NY, 1986, pp. 358–363.
[49] S.L. Shamblin, X. Tang, L. Chang, B.C. Hancock, M.J. Pikal, J. Phys. Chem. B 103 (1999) 4113–4121.
[50] M.V. Voinova, Thermochim. Acta 280/281 (1996) 465–477.
[51] M. Sorai, Thermochim. Acta 88 (1985) 1–16.
[52] M. Moller, J.H. Wendorff, M. Werth, H.W. Spiess, J. Non-Cryst. Solids 170 (1994) 295–299.
[53] V.E. Dreval', G.A. Lushcheikin, V.G. Kulichikhin, Vysokomol. Soedin. Ser. A 39 (1997) 1958–1965.
[54] L.B. Rokland, Anal. Chem. 32 (1960) 1375–1376.
[55] L. Greenspan, J. Res. Natl. Bur. Stand. A: Phys. Chem. 81A (1977) 89–96.
[56] L.B. Rokland, Anal. Chem. 81A (1977) 89–96.