Calorimetric Studies of Freeze-induced Dehydration of Phospholipids

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ABSTRACT Differential scanning calorimetry (DSC) was used to determine the amount of water that freezes in an aqueous suspension of multilamellar dipalmitoylphosphatidylcholine (DPPC) liposomes. The studies were performed with dehydrated suspensions (12–20 wt% water) and suspensions containing an excess of water (30–70 wt% water). For suspensions that contained ≥ 18 wt% water, two ice-formation events were observed during cooling. The first was attributed to heterogeneous nucleation of extraliposomal ice; the second was attributed to homogeneous nucleation of ice within the liposomes. In suspensions with an initial water concentration between 13 and 16 wt%, ice formation occurred only after homogeneous nucleation at temperatures below −40°C. In suspensions containing <13 wt% water, ice formation during cooling was undetectable by DSC, however, an endotherm resulting from ice melting during warming was observed in suspensions containing ≥ 12 wt% water. In suspensions containing <12 wt% water, an endotherm corresponding to the melting of ice was not observed during warming. The amount of ice that formed in the suspensions was determined by using an improved procedure to calculate the partial area of the endotherm resulting from the melting of ice during warming. The results show that a substantial proportion of water associated with the polar headgroup of phosphatidylcholine can be removed by freeze-induced dehydration, but the amount of ice depends on the thermal history of the samples. For example, after cooling to −100°C at rates ≥ 10°C/min, a portion of water in the suspension remains supercooled because of a decrease in the diffusion rate of water with decreasing temperature. A portion of this supercooled water can be frozen during subsequent freeze-induced dehydration of the liposomes under isothermal conditions at subfreezing storage temperature T_s. During isothermal storage at $T_s \geq -40$°C, the amount of unfrozen water decreased with decreasing T_s and increasing time of storage. After 30 min of storage at $T_s = -40$°C and subsequent cooling to −100°C, the amount of water associated with the polar headgroups was < 0.1 g/g of DPPC. At temperatures > −50°C, the amount of unfrozen water associated with the polar headgroups of DPPC decreased with decreasing temperature in a manner predicted from the desorption isotherm of DPPC. However, at lower temperatures, the amount of unfrozen water remained constant, in large part, because the unfrozen water underwent a liquid-to-glass transformation at a temperature between −50° and −140°C.

1. INTRODUCTION

Dehydration of biological and synthetic membranes by air-drying (desiccation) results in membrane destabilization (Crowe and Crowe, 1988). Freeze-induced dehydration also results in membrane destabilization, which is the primary cause of freezing injury (Gordon-Kamm and Steponkus, 1984; Steponkus, 1984; Steponkus and Lynch, 1989; Steponkus and Webb, 1992; Quinn, 1985). However, Crowe et al. (1990) have suggested that freezing and dehydration are "fundamentally different stress vectors." As part of their rationale for this suggestion, they state that "the water hydrogen bonded to the polar head groups (of lipids) is, like that associated with proteins, not freezable" and cite previous differential scanning calorimetry (DSC) studies of Chapman et al. (1967) to support this notion.

Chapman et al. (1967) reported that 0.25 g of H_2O per g of DPPC (20 wt%) remains unfrozen after cooling to −100° at 10°C/min. According to Chapman et al. (1967), the water of hydration that is associated with phosphatidylcholine at hydration levels of approximately 20 wt% or less is unfreezable. However, these results are not consistent with subsequent studies, in which ice formation has been demonstrated in suspensions of DPPC liposomes with an initial water concentration significantly less than 20 wt% (Grabielle-Madelmont and Perron, 1983; Kodama et al., 1982; Ueda et al., 1986). Therefore, the amount of water that remains unfrozen during freezing of phospholipid vesicles is questionable.

We disagree with the suggestion that the water associated with the polar headgroups of phospholipids is "not freezable" and have initiated DSC studies to determine the mass of water that freezes in aqueous suspensions of dipalmitoylphosphatidylcholine (DPPC) liposomes with different thermal histories. The studies include an analysis of possible errors in determining of the amount of water that remains unfrozen, development of a method for the correct determination of the baseline of DSC thermograms, and formulation of a mathematical model for calculation of the amount of water frozen at different subzero temperatures.

2. MATERIALS AND METHODS

The DSC studies were conducted with suspensions of multilamellar DPPC liposomes that were initially either well-hydrated (30–70 wt% water) or dehydrated (12–20 wt% water). Suspensions of fully hydrated multilamellar DPPC liposomes were prepared by mixing dry DPPC (Avanti Polar Lipids, Inc.) with approximately the same mass of degassed, distilled water. The samples were then kept at 55°C for 2 h; at 20-min intervals, the samples were vortexed for ~5 min and sonicated for ~3 min.

Dehydrated samples were prepared by equilibration of fully hydrated lipid suspensions in DSC pans for ≥7 days at 20°C over either saturated salt

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solutions or a 2.5 wt% solution of ethylene glycol (osmotic pressure ≃1 MPa). The saturated solutions that were used included Na_2HPO_4, KNO_3, $BaCl_2$, and KCl, which have osmotic pressures of 2.7, 8.3, 12.8, and 20.4 MPa at 20°C, respectively (Rockland, 1960). Although gravimetric studies of the change in sample weight during equilibration suggested that equilibration was completed during 3–4 days, DSC studies of ice formation in the dehydrated (12–18 wt% water) samples (see section 4.1) have shown that in the samples equilibrated ≤4 days, two exotherms of ice formation were observed during cooling at 10°C/min. The first occurred at temperatures >−40°C after heterogeneous ice nucleation in compartments containing fully hydrated lipids, and the second occurred after homogeneous ice nucleation at temperatures <−40°C. However, after 7 days of equilibration over the saturated salt solutions we observed formation of ice during cooling only after homogeneous ice nucleation at temperatures <−40°C (see section 4.1). These data suggested that a week is required to complete the equilibration of a 10-μl suspension of DPPC liposomes at 20°C over the solutions used in the present study. In addition, parallel studies of the effect of dehydration on the L_α to $L_{\beta'}$ phase transition of DPPC performed by Dr. M. Webb in our lab (personal communication) have shown that a single lipid melting peak is observed after 7 days of equilibration over saturated salt solutions with osmotic pressures up to 150 MPa that corresponds to 0.048 g of water per g of DPPC.

DSC studies were performed in a Perkin-Elmer DSC 7; the samples were first cooled to −100°C and then subjected to varied thermal histories (for example after being cooled to −100°C the samples were warmed to −40°C, at which it was maintained isothermally during 30 min and then cooled again to −100°C). After the DSC studies, the pans were punctured and the samples were dried in a vacuum oven over P_2O_5 at 75°C. The dry weights of the samples were used for calculation of the lipid and water masses in the pans. The mathematical method used for calculation of the mass of water that froze (m_f) is presented in section 3.

3. METHOD FOR CALCULATION OF THE MASS OF FROZEN WATER

3.1. Dependence of DSC output on temperature

Although DSC is frequently used for determining the mass of water that freezes during cooling of aqueous solutions of polymers (Pouchly et al., 1979), proteins (Ali and Bettelheim, 1985; Kakor and Bettelheim, 1991), suspensions of liposomes (Chapman et al., 1967; Grabielle-Madelmont and Perron, 1983; Ter-Minassian-Saraga and Madelmont, 1981, 1982; Ueda et al., 1986) and biological tissues (Ramlov and Hvidt, 1992), the manner in which the baseline, the peak area, and the mass of frozen water were determined is not specified in the majority of these publications. An accurate estimate of the amount of frozen water requires that the baseline be precisely established. This is problematic because of the difference in the specific heat of ice and water there is a shift of the baseline of DSC thermograms before and after ice melting. Therefore, as demonstrated by Pouchly et al. (1979), the baseline on the thermogram is not precisely determined if it is established merely by interpolation between the onset and completion of the ice melting endotherm (see also section 3.2). The theoretical analysis that follows is devoted to the problem of proper determination of the baseline and the reliable calculation of the frozen water mass from DSC data.

At low warming rates, when the change in enthalpy of the sample is negligibly small during the characteristic time of temperature relaxation in the DSC sample holder, the value of the DSC output Y (in J/s) divided by the warming rate τ is equal to the rate of the change in enthalpy (H) with temperature (T). Thus, Y/τ is equal to the thermal capacity of the sample plus the heat absorbed as a result of ice melting during an increase in temperature of 1°C:

$$\frac{Y}{\tau} = \frac{dH}{dT} = C_w m_w + C_l m_l + C_i m_i - \frac{q dm_i}{dT}$$

$$= (C_w - C_i)m_w + C_l m_l + C_i m_w^\circ - \frac{q dm_i}{dT}, \qquad (1)$$

where q is the specific heat of fusion; C_w, C_l, and C_i are the specific heats of water, lipids, and ice, respectively; m_w, m_l, and m_i are the masses of water, lipids, and ice in the sample, respectively; and m_w° is the initial mass of water.

To determine the true baseline, Y was expressed as a sum of B and D:

$$Y = B + D. \qquad (2)$$

B is the part of Y that is independent of ice melting; it equals

$$B = \tau(C_w - C_i)m_{unf} + \tau C_l m_l + \tau C_i m_w^\circ, \qquad (3)$$

where m_{unf} is the mass of the water remaining unfrozen ($m_{unf} = m_w^\circ - m_f$, where m_f is the mass of water that has been frozen). D is equal to the endothermic deviation of the thermogram from the baseline that occurs during melting of the ice:

$$D = \tau(C_w - C_i)m + \tau q dm/dT = \tau d(qm)/dT, \qquad (4)$$

where m is the mass of ice that melts. $D = \tau d(qm)/dT$ because $dq/dT = d(H_w - H_i)/dT = C_w - C_i$, *where* H_w and H_i are the specific enthalpies of water in the sample and ice. The deviation is zero at low temperatures before ice begins to melt at T_1 and approaches $\tau(C_w - C_i)m_f$ at the end of ice melting ($T \to T_2$) when $dm/dT \to 0$. Consequently, at T_2, the baseline on the thermograms should pass through the point O, for which the Y coordinate equals:

$$Y_O = [dH/dT - (C_w - C_i)m_f]\tau. \qquad (5)$$

For this reason, we assumed that the baseline may be linearly interpolated (see Figs. 1b, 8b, and 10) from temperatures $<T_1$ to temperature T_2. This assumption was used for calculation of heat absorbed during ice melting from the DSC output data. After the baseline is established, the deviation D can be calculated from the DSC output data. Subsequently, the mass of ice that melted can be calculated using differential Eq. 4 or the integral form of Eq. 4:

$$m(T) = [1/\tau q(T)] \int_{T_1}^{T} D\, dT. \qquad (6)$$

Eq. 6 represents Kirchhoff's law for frozen solutions. In Eq. 6 the specific heat of fusion is equal to the sum of the specific heat $q_o(T)$ of the ice → water transformation and the heat δq_h of mixing (hydration) of 1 g of pure water with the lipid suspension that contains m_l/m grams of lipid and m_{unf}/m grams of water at a given subzero temperature T. The dependence of q_o on temperature is shown in Franks (1982); the heat of hydration for a DPPC bilayer is unknown. To calculate m_{unf} from DSC data, we assumed that $\delta q_h \ll q_o$ and $q(T)$ can be taken equal to $q_o(T)$.

Note that an erroneous estimation of the baseline strongly affects the calculated values of m_f and m_{unf}. For example, if the baseline of the thermogram obtained for the melting of pure ice (see Figs. 1 *b* and 7 *b*) was determined by interpolation between point S^* (the apparent onset of an ice-melting endotherm) and point F (the end of the endotherm), one would erroneously estimate the heat of fusion as 320 J/g of water, which is the value reported by Chapman et al. (1967), rather than the true value (333.88 J/g). Moreover, if the baseline is established by simple interpolation and used to determine the mass of water frozen in fully hydrated liposome suspensions, one erroneously reaches the conclusion that the unfrozen water content of DPPC liposomes is constant at about 20 wt%.

3.2. Calculation of m_f when a sample contains only pure water

When determining the amount of heat absorbed during the ice → water phase transformation during warming, it is important to note that the melting of ice begins at temperatures lower than 0°C in a polycrystalline sample (Kvlividze et al., 1974; Nenow and Trayanov, 1989). The freezing point of water between ice crystals is decreased for the same physical reason as is the freezing point of pure water between lipid bilayers, i.e., the occurrence of hydration forces in thin films

of water (Cevc and Marsh, 1987; Derjaguin, 1980; Lis et al., 1982; Marsh, 1989; Rand and Parsegian, 1989). As a result, the chemical potential and freezing point of water in a film between ice crystals decreases with decreasing thickness of the film. Because at a constant hydrostatic pressure the thickness of the film of pure water between ice crystal surfaces is a function only of temperature, the total water mass that remains unfrozen between ice crystals should be proportional to the total surface area of the ice crystals.

A typical thermogram obtained during warming (5°C/min) of a sample of distilled water that was cooled to -80°C at 10°C/min and the calculated baseline are shown in Fig. 1, *a* and *b*. It can be seen from Fig. 1 *b* that a detectable deviation of the thermogram from the baseline, which corresponds to the onset of ice melting, begins at $T_1 \simeq -50$°C. For this reason, we suggest that, in a sample that contains only water, the amount of unfrozen water between ice crystals is negligibly small at temperatures <-50°C and not detectable by DSC, but at temperatures >-50°C the ice starts to melt, increasing the mass of the water located between ice crystals.

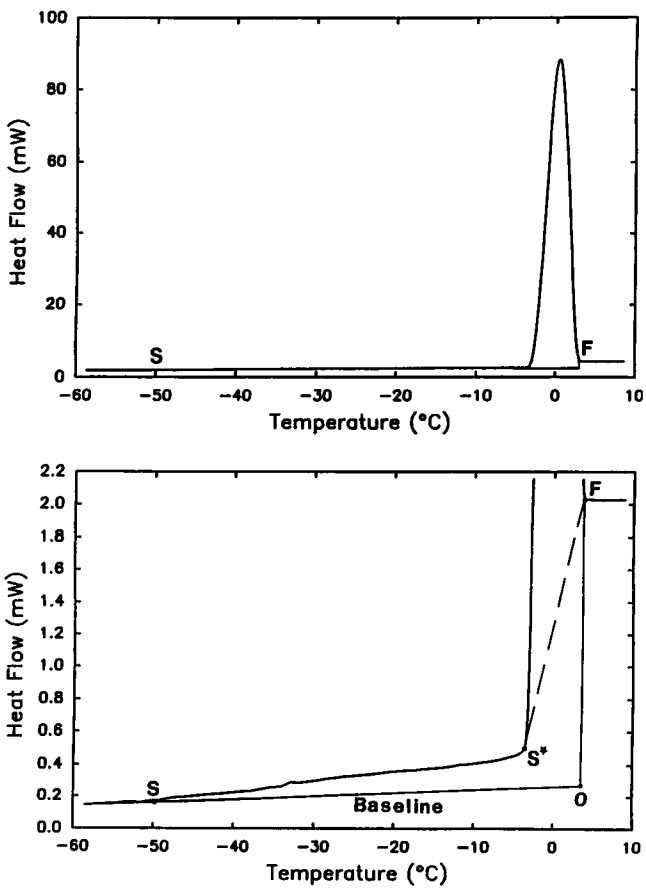

FIGURE 1 (*a*) The dependence of DSC output on temperature during warming at 5°C/min. The sample contains 9.87 g of pure water. (*b*) The dependence of DSC output on temperature from *a* presented in a smaller scale. Points *S* and *S** correspond to the actual (at temperature T_1 that equals approximately -50°C) and apparent points of the beginning of ice melting, respectively. Point *F* corresponds to the end of the endothermal peak at temperature T_2. The point *O* corresponds to the linear continuation of the baseline up to temperature T_2.

In other words, if the sample contains only pure ice, then at temperatures <-50°C

$$m_f = m_w^\circ, \tag{7}$$

$$B = \tau m_w^\circ C_i. \tag{8}$$

and the *Y* coordinate of the *O* point and position of the baseline are determined by Eqs. 7 and 8 together with Eq. 5. To confirm that this is correct, the mass of ice that melts during warming (m_f) was calculated from DSC data by estimating the baseline using Eq. 8 and using the value for heat of the fusion $q^\circ = 333.88$ J/g; the calculated mass of ice that melted was ±1% of the mass of water in the DSC pan that was determined gravimetrically. This variability of ±1% was probably due to a small instability in the DSC that affects the area calibration. Indium was used as a standard for area calibration of the DSC; the area of the melting peak per unit mass of indium varied ±1%. This variability may result from a decrease in the level of liquid nitrogen in the DSC and small variations in helium flow.

The temperature dependence of the portion of pure water remaining unfrozen in the gaps between ice crystals is shown in Fig. 2. This dependence was calculated from the partial areas of the endotherm at 5°C intervals during warming (5°C/min) of samples cooled to -100°C at 10°C/min. The water remaining unfrozen on the surface of ice crystals should be taken into consideration as a portion of water that may remain unfrozen at subzero temperatures.

3.3. Calculation of m_f in aqueous lipid suspensions

In section 3.2, it was shown that $B = \tau m_w^\circ C_i$, because the mass of water that freezes in a sample of pure water during cooling to $T < -50$°C equals the mass of water in the sample ($m_f = m_w^\circ$). However, the portion of water m_f/m_w° that freezes in a liposome suspension is initially unknown. For this reason, m_f and the *Y* coordinate of the point *O* (Y_O) cannot be found independently as was possible for a sample of pure

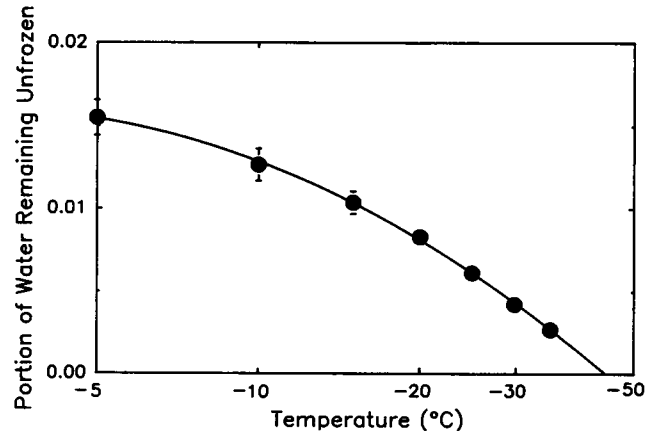

FIGURE 2 Effect of temperature on the portion of water remaining unfrozen in the gaps between ice crystals.

water. In turn, the calculated value of m_f depends principally on the proper determination of Y_O and the baseline. The values of m_f and Y_O can be calculated using an iterative procedure. In the first step of this procedure, the coordinate of point O (Y_O^1 is determined so that

$$Y_O^1 \simeq \left[\frac{dH}{dT} - \int_{T_1}^{T_2} DdT(C_w - C_i)/q_o\right]\tau. \quad (9)$$

As a first approximation, the baseline is a straight line between a point with coordinates (T_2, Y_O^1) and point S on the thermogram at T_1, which is the onset of ice melting. There is no formal way to determine T_1 because the amount of heat absorbed at the onset of melting is very small. In the majority of the measurements, ice melting (detectable as a resolvable deviation from the baseline) starts somewhere between −50 and −40°C.

After the baseline is established in the first approximation, the mass m_f^1 of frozen water in the first approximation is calculated from Eq. 6. Next, using m_f^1, the Y coordinate of point O in the second approximation is calculated as follows:

$$Y_O^2 = [dH/dT - (C_w - C_i)m_f^1]\tau. \quad (10)$$

Consequently, in the second approximation of m_f we have:

$$m_f^2 = m_f^1 + (Y_O^2 - Y_O^1)(T_2 - T_1)/2q_o. \quad (11)$$

The value m_f^2 is used to begin the third iteration step. Iteration is complete when the relative change in m_f after the next iteration becomes <0.1%. In practice, two iterations are sufficient to achieve this precision.

As noted above, the expected error in the calculation of the frozen water mass is ~1% of the total mass. This small error in a sample containing a low concentration of lipid may result in a high error in the calculated mass of water per g of lipids remaining unfrozen, which equals:

$$\frac{1\% \cdot m_f}{m_l} = 1\% \cdot \frac{m_f}{m_w^o}\frac{m_w^o}{m_l} \simeq \frac{1\% \cdot m_w^o}{m_l}. \quad (12)$$

Therefore, if $m_w^o/m_l = 10$, the expected error in the estimation of the amount of water that remains unfrozen per g of DPPC at low temperatures is approximately 0.1 g/g; if $m_w/m_l < 1$, the expected error will be <0.01 g/g. In order to decrease this error, we used concentrated suspensions of multilamellar liposomes.

4. RESULTS AND DISCUSSION

4.1. Ice formation in suspensions of DPPC vesicles

The kinetics of ice formation in suspensions of DPPC vesicles during cooling was strongly dependent on the initial water content. In suspensions that contained ≥0.219 g water per g of lipid (18 wt% water), two exothermic events of ice formation were observed during cooling to −100°C at 10°C/min. For example, in a sample containing 0.235 g water per g of lipid (19 wt% water), the onset of the first exothermic event (Fig. 3, *plot A*) was at −20°C, with the completion at approximately −35°C. The onset of the second exothermic event (Fig. 3, *plot A*) was at −42°C, with the peak at −45°C. However, in suspensions that were dehydrated over supersaturated salt solutions (mass of water per mass of DPPC $m_w/m_l < 0.2$), the first exothermic peak was almost undetectable and ice formed primarily at temperatures <−40°C. For example, Fig. 3, *plot B*, shows a typical thermogram obtained for a sample containing 15 wt% of water during cooling at 10°C/min, in which the first exothermic event was not detectable, and the onset of the exotherm of ice formation was at −43°C.

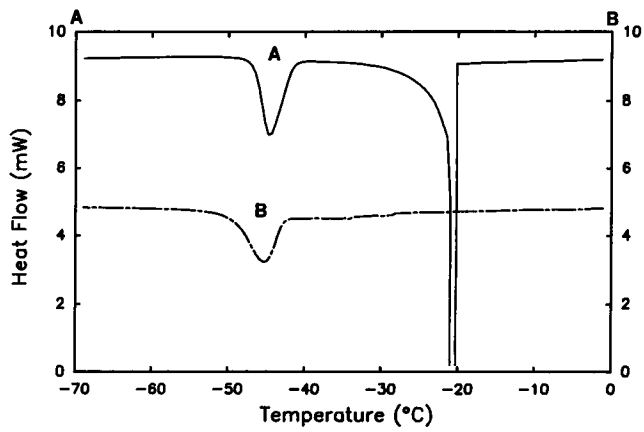

FIGURE 3 DSC thermograms of aqueous suspensions of multilamellar DPPC liposomes during cooling at 10°C/min. Thermograms A and B correspond to different initial water concentrations: A = 19 wt%; B = 15 wt%

In aqueous solutions, the temperatures of heterogeneous and homogeneous ice nucleation decrease with increasing concentration of solute or initial osmotic pressure of the solutions (Franks, 1982). For this reason, we studied the dependencies of the temperature T_I corresponding to the onset of the first exothermic event and the temperature T_{II} corresponding to the peak of the second exothermic event on the mass of water per g of DPPC (Fig. 4). The temperatures

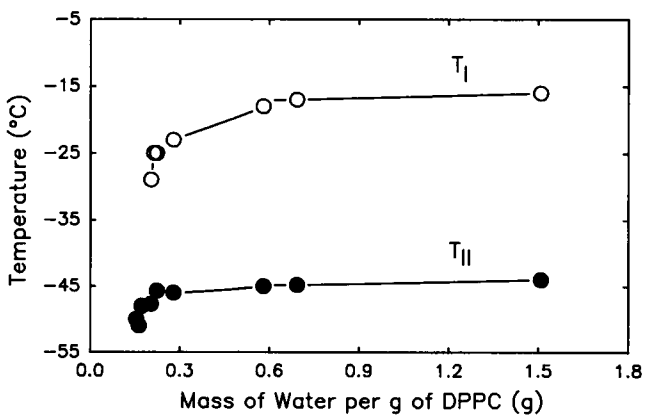

FIGURE 4 Dependencies of T_I (temperature of the onset of ice formation after heterogeneous nucleation) and T_{II} (temperature of the peak of the ice formation exotherm after homogeneous nucleation) on initial water content per gram of lipids measured at a cooling rate of 10°C/min.

T_I and T_{II} occur within the temperature ranges at which heterogeneous and homogeneous nucleation normally occur in supercooled aqueous solutions (Franks, 1982). In addition, the temperatures T_I and T_{II} decrease with decreasing water mass per g of lipid (Fig. 4), which is qualitatively consistent with decreases in the temperatures of heterogeneous (T_{het}) and homogeneous (T_{hom}) nucleation of ice with decreasing chemical potential of water in aqueous solutions. Therefore, we believe that the first and the second events of ice formation correspond to heterogeneous and homogeneous nucleation of ice, respectively. This interpretation agrees with observation of Talsma et al. (1992) who also reported the appearance of a second event of ice formation at −40°C during cooling of fully hydrated suspensions of multilamellar DPPC liposomes. Wu et al. (1991) also observed a second exotherm at about −35°C in suspensions of multilamellar DPPC liposomes in D_2O and suggested that the second exothermic event was attributable to the "freezing of the lipid polar headgroups." This suggestion contradicts experimental data obtained by Grabielle-Madelmont and Perron (Grabielle-Madelmont and Perron, 1983), and Talsma et al. (1992), as well as the results of the present study. Grabielle-Madelmont and Perron (1983) specifically noted that the second exotherm observed during cooling cannot be associated with lipids because subsequent heating does not give rise to an endotherm in the temperature range where the second exothermic event occurs. This is in agreement with the thermogram presented in Wu et al. (1991). We found that if the sample was cooled to −50°C and then warmed to −10°C, at which ice is still present in the sample, the second endothermic event does not occur during subsequent cooling. This observation provides direct evidence that the second exothermic event is attributable to formation of ice in the sample rather than to "freezing of the lipid polar headgroups." In the studies of Wu et al. (1991) the second nucleation event occurred at −35°C, which is the homogeneous nucleation temperature of D_2O ice (Angell, 1982)—a fact that is not considered in their publication.

The appearance of the two nucleation events can be explained as follows. A suspension of lipid vesicles consists of many continuous water subsystems, separated from each other by lipid bilayers. During cooling, ice crystals are expected to occur first by a heterogeneous nucleation mechanism in some portion of those subsystems. For example, in suspensions containing an excess of water, the extraliposomal space is a large, continuous subsystem, and ice crystals will first nucleate in the extraliposomal space, where the distance between bilayers is greater than that inside the multilamellar liposomes. After nucleation of ice in the extraliposomal volume, the crystals will quickly grow between liposomes transforming the extraliposomal water into ice. In a dehydrated suspension, the liposomes will be tightly packed so that it will be difficult to distinguish intra- and extraliposomal spaces on the basis of distance between the bilayers. Nevertheless, under these conditions, water in a subsystem that contains centers of heterogeneous ice nucleation may also freeze as a result of ice growth in the subsystem. However, the water in the subsystems that contain no centers of heterogeneous ice nucleation will remain supercooled until the supercooled water diffuses through bilayers to the regions that contain ice crystals or until the ice formation occurs between the bilayers as a result of homogeneous nucleation. The rate of freeze-induced dehydration of the subsystems containing supercooled water is limited by the permeability of the bilayers to water. Therefore, the slower the cooling rate, the greater the amount of water that may be removed from the subsystems by freeze-induced dehydration and the less the amount of water that will freeze after homogeneous nucleation.

To characterize the rate of freeze-induced dehydration of the liposomes, we performed the following experiments. A fully hydrated suspension of DPPC multilamellar liposomes was first cooled at 10°C/min to −25°C to obtain ice crystals by heterogeneous nucleation. The sample was then warmed to storage temperatures of either −10, −15, or −25°C and held isothermally for varying times. Under these conditions, a portion of the supercooled water located inside the liposomes diffused through the bilayers to the extraliposomal ice; the remaining intraliposomal water froze (the second event of ice formation) during subsequent cooling at 10°C/min at temperatures <−40°C after homogeneous nucleation of ice crystals inside the liposomes. In Fig. 5 the area of the exothermic peak per g of lipid is plotted as a function of time at different storage temperatures (T_s). From Fig. 5 it is seen that the amount of ice formed during the second event of ice formation decreased with increasing time of storage at T_s.

The area of the exotherms (per g of lipids) corresponding to the second ice formation event decreased with decreasing initial concentration of water (Fig. 6) so that ice formation in samples containing ≤13 wt% water was undetectable by DSC. Although DSC was not sufficiently sensitive to detect

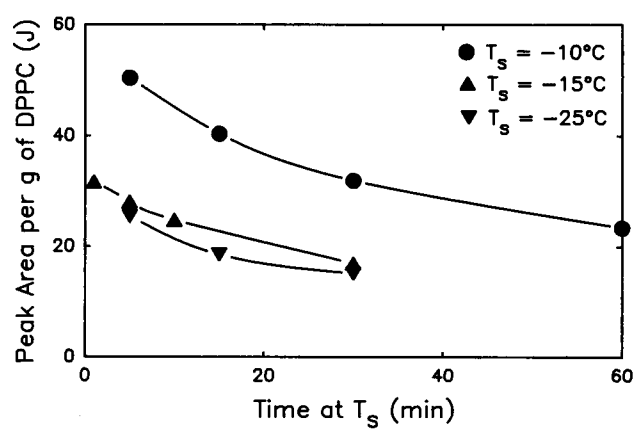

FIGURE 5 Dependence of the specific area (per g of lipids) under the second exotherm of ice formation on storage time at T_s. Samples containing a fully hydrated suspension of DPPC multilamellar liposomes were first cooled at 10°C/min to −25°C < T_{het} and then warmed to T_s = −10, −15, and −25°C at which the samples were isothermally held for varied times. Subsequently, the samples were cooled at a rate of 10°C/min to determine the area under the second exotherm of ice formation at temperatures <−40°C.

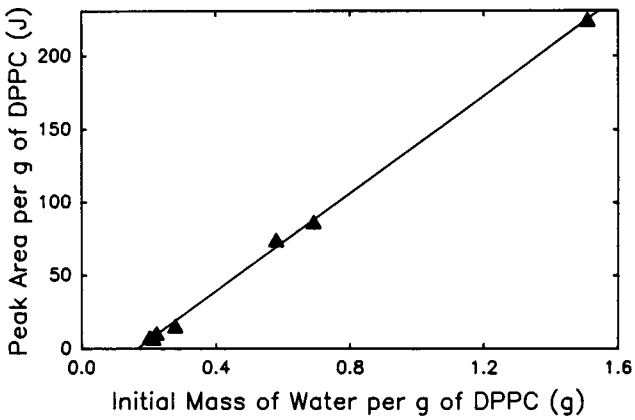

FIGURE 6 Dependencies of heat released per g of lipids under the second exotherm of ice formation on initial water content (per g of lipids) in a suspension of multilamellar DPPC liposomes. Peak areas were measured at a cooling rate of 10°C/min.

ice formation in the DPPC suspensions containing ≤13 wt% of water ($m_w/m_l \leq 0.147 \pm 0.012$), ice nucleation must have occurred in these suspensions because an endothermic event during warming, which resulted from the melting of ice, was detected. However, for samples in which $m_w/m_l = 0.135 \pm 0.002$ (≃12 wt% water) after dehydration over saturated solutions of KCl, there was no detectable ice melting endotherm during warming. This suggests that rates of ice nucleation and growth are very small in samples containing ≤12 wt% water. However, the failure to detect ice nucleation events in samples containing ≤12 wt% water does not mean that the water is unfreezable. A part of this water may still be removed from the polar head groups of the lipids by freeze-induced dehydration, if ice crystals were present.

As discussed above, the supercooled water in subsystems inside liposomes that do not contain centers of heterogeneous ice nucleation may freeze after homogeneous ice nucleation inside the subsystems or may be removed by freeze-induced dehydration. The rate of freeze-induced dehydration is limited by the rate of the diffusion of water molecules from the subsystems containing supercooled water through lipid bilayers to the surface of ice crystals located in the extraliposomal space. Therefore, at a given cooling rate, some portion of freezable water may remain supercooled inside the liposomes. If the concentration of this water is ≥12 wt%, it may freeze after homogeneous ice nucleation in the subsystems. However, because we did not observe ice formation in the samples containing ≤12 wt% water, water in the dehydrated subsystems containing ≤12 wt% water may remain supercooled during cooling. This supercooled water may contribute to the erroneous estimates of the amount of unfreezable water.

4.2. Ice melting during warming of frozen suspensions of DPPC vesicles

In DPPC samples containing an excess of water, one endothermic peak was observed during warming (Fig. 7 *a*). It is

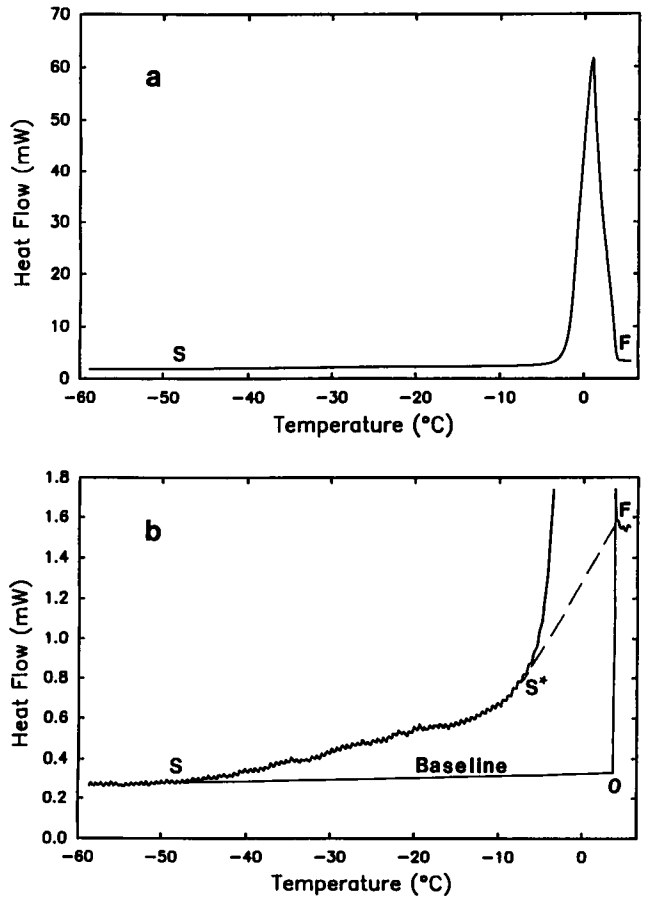

FIGURE 7 (*a*) The dependence of DSC output on temperature during warming at 3°C/min. The sample contains a fully hydrated suspension of DPPC multilamellar liposomes. The sample was first cooled to −100°C at 100°C/min, then warmed at 100°C/min to the storage temperature −30°C at which it was stored for 30 min. Subsequently the sample was cooled to −100°C at 100°C/min and then studied during warming. (*b*) The dependence of DSC output on temperature from *a* presented in a smaller scale. Points *S* and *S** correspond to the actual and apparent beginning of ice melting respectively. Point *F* corresponds to the end of the endothermal peak at temperature T_2. The point *O* corresponds to the linear continuation of baseline up to temperature T_2.

seen from Fig. 7 *b* that the onset of ice melting (as determined by the beginning of the deviation of the endotherm from the baseline) occurred between −50 and −40°C, which is in agreement with the results obtained for samples of pure water (Fig. 1 *b*). During subsequent warming from −50°C, the endothermic heat flow increased continuously with increasing temperature while ice was melting. Upon completion of the ice → water transformation, the heat flow rapidly decreased to the value $D = \tau(C_w - C_i)m_f$ (Figs. 7, *a* and *b*). However, in DPPC samples containing 14 to 18 wt% water, two endothermic peaks were observed (see *thermogram A* in Fig. 8) during warming. The first broad peak started at about −50°C to −40°C and ended at temperatures ≤−3°C; the main part of the second peak was between −3° and 0°C. With increasing initial water concentration, the area of the second peak increased greatly and the boundary between the first and the second peaks became less pronounced (see Fig. 9).

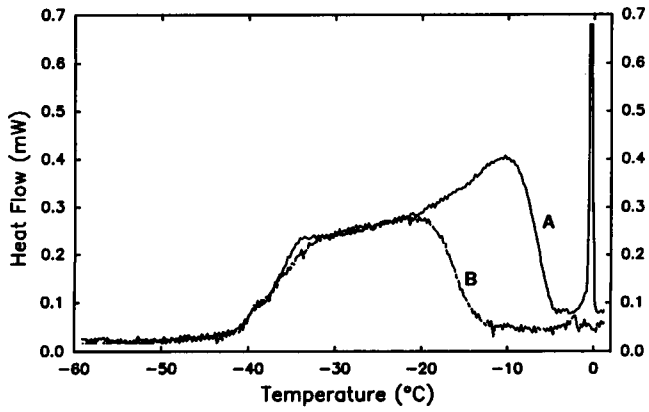

FIGURE 8 The dependence of DSC output on temperature (thermogram) during warming at 5°C/min. Curve A is for a suspension of multilamellar DPPC liposomes that were dehydrated over a saturated solution of Na_2HPO_4 (osmotic pressure = 2.7 MPa). Curve B is for a suspension of multilamellar DPPC liposomes dehydrated over a saturated solution of $BaCl_2$ (osmotic pressure = 12.8 MPa). The sample was first cooled to −100°C at 100°C/min, then warmed at 100°C/min to a storage temperature of −40°C at which it was stored for 30 min. Subsequently, the sample was cooled to −100°C at 100°C/min and then studied during warming

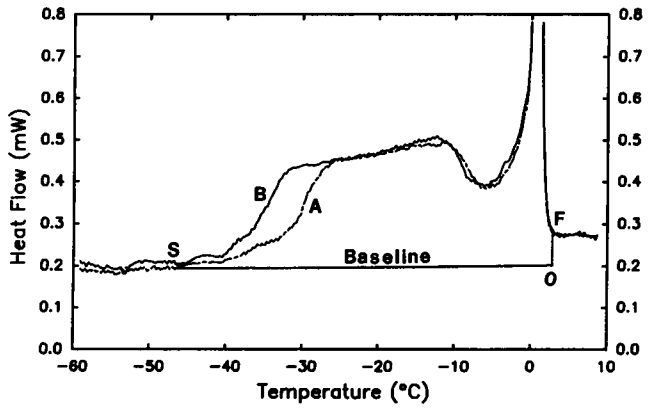

FIGURE 9 The dependencies of DSC output on temperature obtained for a suspension of multilamellar DPPC liposomes dehydrated over a 2.5 wt% EG solution (osmotic pressure = 1 MPa). The sample was first cooled to −100°C at 100°C/min, then warmed at 100°C/min to the storage temperature −30°C (*A*) and −40°C (*B*) at which it was stored for 30 min. Subsequently, the sample was cooled back to −100°C at 100°C/min and then studied during warming at 5°C/min.

The appearance of the two peaks has been observed in several previous DSC studies, with several different explanations given for the origin of the second peak (Franks et al., 1977; Grabielle-Madelmont and Perron, 1983; Körber et al., 1982; Ueda et al., 1986). Although Grabielle-Madelmont and Perron (1983) and Ueda et al. (1986) have suggested that the second peak occurs because in addition to the "intermediately bound" water some amount of "free" water is present in the sample, Franks et al. (1977) proposed that the appearance of the second ice melting peak may be an instrumentation effect. They observed a second ice melting peak during warming of frozen solutions of polyvinylpyrrolidone and hydroxyethyl starch and suggested that a temperature gradient in the sample pan results in distillation of water from the sample onto the lid of the sample pan. This interpretation is consistent with subsequent studies in which the second peak did not appear in the thermogram if the samples were covered by oil (Körber et al., 1982).

We agree with interpretation proposed by Franks et al. (1977) that the appearance of the second ice melting peak is an instrument effect. We found that if suspensions of DPPC liposomes were covered with Cargille immersion oil, the second endotherm did not appear. Without this precaution, a portion of water evaporated from the sample and condensed on the lid of the pan, which had a lower temperature than the sample because of the temperature gradient in the DSC sample holder. At temperatures >0°C this process of water evaporation stops when the chemical potential of water in the solution becomes equal to the chemical potential of water condensed on the lid. In other words, water from the sample evaporates and the osmotic pressure in the sample increases until the product of the osmotic pressure and specific volume of water in the sample is smaller than the product of the specific entropy of water and the difference in temperature of the solution in the pan and the lid. At subzero temperatures, the chemical potential of water in a partially frozen sample is less than or equal to chemical potential of water in ice, which is a function only of temperature. For this reason, the chemical potential of water frozen on the lid, which has a lower temperature, is lower than the chemical potential of water in the sample. Therefore evaporation of water from the frozen sample will continue at subzero temperatures. The rate of this evaporation is very small because membrane permeability to water decreases with decreasing temperature. Nevertheless, during slow freezing or storage of the sample at subzero temperatures, which is required to complete freeze-induced dehydration, some amount of water will evaporate from the sample and form pure ice on the lid.

We found that the area and amplitude of the second endothermic peak per g of lipids decreased with decreasing initial concentration of water in the samples and became undetectable for samples containing 0.147 ± 0.012 g of water per g of lipid after equilibration over a saturated solution of $BaCl_2$, which has an osmotic pressure of ≈12.8 MPa (see *thermogram B* in Fig. 8). At positive temperatures, this is consistent with the decrease in the difference in chemical potentials of water in the sample and water on the lid with increasing osmotic pressure of the sample, and is inconsistent with the process of water evaporation from a frozen sample, because the thermodynamic driving force for water evaporation from the frozen sample is independent of the initial (before freezing) value of the chemical potential of water (osmotic pressure) in the sample. Therefore, we suggest that distillation of water from the samples onto the lid of the sample pan occurs primarily when the samples are kept inside the DSC prior to cooling. Consequently, for reliable measurement of T_m in samples with an osmotic pressure <12.8 MPa, the samples should be covered by oil. However, addition of the oil precludes reliable measurements of the lipid and water masses in the sample. For this reason, in the

experiments directed at measuring the mass of the water remaining unfrozen, samples were not covered with oil.

Note that the process of water evaporation from the sample and condensation of this water on the lid is occurring inside the sealed pan. Therefore, the amount of water that remains unfrozen in the sample is equal to the difference between the initial amount of water in the sample and the total amount of ice that formed inside the pan, i.e., the sum of the masses of pure water frozen on the lid and the water frozen in the sample. Thus, it is necessary to measure the area of both ice melting endotherms and not just that of the sample (see Fig. 9).

4.3 The effect of step freezing

As shown in section 4.1, during cooling of DPPC suspensions containing ≥18 wt% water, ice first forms in a portion of the structural subsystems (presumably the extraliposomal compartments) that contains centers of heterogeneous nucleation. Although the rate of freeze-induced dehydration is limited by the rate of diffusion of water molecules, a significant portion of freezable water may be removed from the subsystems that do not freeze before homogeneous nucleation in the intraliposomal subsystems if the sample is cooled at a sufficiently slow cooling rate. As discussed in section 4.1, if the concentration of water in the unfrozen subsystems decreases to values ≤12 wt%, the probability of homogeneous ice nucleation becomes very small. For this reason, some water may remain supercooled even after slow cooling to −100°C. In order to decrease the amount of supercooled water, samples first were cooled rapidly (at 100°C/min) to −100°C to effect homogeneous nucleation of ice in the subsystems that did not contain centers of heterogeneous ice nucleation. However, because we did not know if all freezable water in the sample froze after rapid cooling to −100°C and how the thermal history affected the amount of ice that formed in the sample, an additional isothermal period was included after cooling to −100°C. The protocol of temperature change consisted of three steps:

Step 1

Cooling at 100°C/min to −100°C to obtain ice crystals in both the extra- and intraliposomal compartments.

Step 2

Warming at 100°C/min to storage temperature T_s = −20, −30, or −40°C at which the sample was maintained isothermally during a storage time t_s that varied from 0 to 30 min to complete freeze-induced membrane dehydration.

Step 3

Cooling at 100°C/min to −100°C to establish the baseline at temperatures significantly below which ice melting first occurs.

Storage at T_s significantly affected the subzero behavior of the sample during warming. First, the magnitude of the deviation of the thermogram from the baseline, which represents the onset of melting, increased with increased storage time (Fig. 10). Second, after 30 min of storage at T_s, the temperature at which the first detectable melting of ice began decreased with decreasing storage temperature; this was observed in a suspension containing 18 wt% water (Fig. 9) and a suspension that contained 50 wt% water (Fig. 11). These results indicate that the amount of water that freezes in the DPPC suspensions increases with increased storage time and decreased storage temperature; the amount of water remaining unfrozen as a function of the storage time and storage temperature is presented in Figs. 12 and 13, respectively.

Fig. 12 shows the effect of storage time at −40°C on the amount of water remaining unfrozen in a suspension of DPPC liposomes that initially contained 0.22 g water per g of lipid (18 wt% water). After 30 min of storage at −40°C and subsequent cooling to −100°C, the amount of water remaining unfrozen was approximately 0.015 g/g of lipids less than the amount remaining unfrozen after zero time of storage. The data presented in Fig. 12 also suggest that, after first cooling to −100°C, freeze-induced dehydration of lipids at $T_s \geq -40$°C is completed after 30 min. Fig. 13 shows the dependence of the amount of water remaining unfrozen in a sample that contained 18 wt% water on storage temperature after 30 min of storage and subsequent cooling to −100°C; the amount of water that remained unfrozen in the sample decreased with decreasing storage temperature. This decrease in the amount of unfrozen water was a result of diffusion of supercooled water of hydration from lipids to ice (freeze-induced dehydration).

4.4. Amount of water that is in thermodynamic equilibrium with ice at a subzero temperature

In further studies to decrease the amount of freezable water that remained unfrozen we used the three-step protocol of

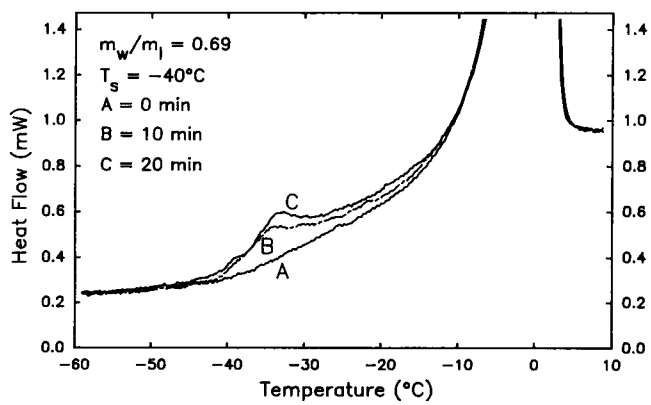

FIGURE 10 The effect of the storage time at −40°C. A fully hydrated suspension of multilamellar DPPC liposomes was first cooled to −100°C at 100°C/min, then warmed at 100°C/min to the storage temperature −40°C at which it was stored for 0 min (*A*); 10 min (*B*); and 20 min (*C*). The sample was then cooled to −100°C at 100°C/min and then studied by DSC during warming at 10°C/min.

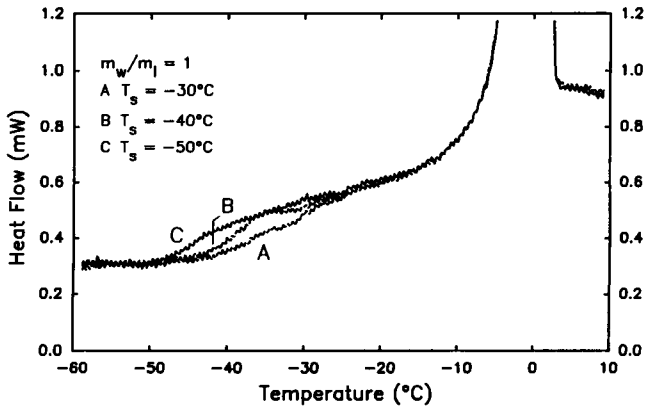

FIGURE 11 Effect of the storage temperature on amount of frozen water. A fully hydrated suspension of multilamellar DPPC liposomes was first cooled to −100°C at 100°C/min, then warmed at 100°C/min to a storage temperature of −30°C (*A*), −40°C (*B*), and −50°C (*C*) at which it was stored for 30 min. Subsequently, the sample was cooled to −100°C at 100°C/min and then studied by DSC during warming at 10°C/min.

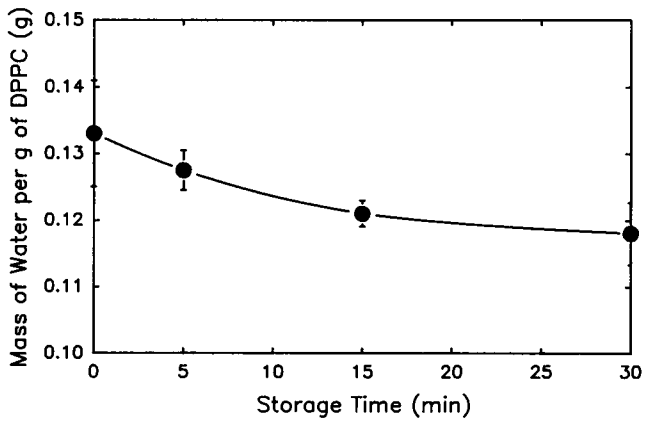

FIGURE 12 Effect of storage time at −40°C on the amount of water remaining unfrozen per g of lipids in suspensions dehydrated over a 2.5 wt% solution of ethylene glycol (osmotic pressure = 1 MPa).

temperature change described in section 4.3. The samples were first cooled to −100°C at 100°C/min and then stored for 30 min at T_s = −30 or −40°C and then cooled again to −100°C at 100°C/min. If we assume that 30 min at T_s is sufficient to complete freeze-induced dehydration (Fig. 12) and approach thermodynamic equilibrium in the sample, then during subsequent warming the water in the sample will be in thermodynamic equilibrium with ice at least at $T > T_s$. The mass of ice that melted at different subzero temperatures was calculated from the partial areas of the endotherm using the integral Eq. 6. Subsequently, the mass of water per gram of lipid that remained unfrozen was calculated.

The dependence of the amount of unfrozen water on temperature was measured for suspensions containing excess water (water concentration 30–70 wt% water) and 0.193 ± 0.13 g water per g of lipid (16.2 wt% water). After freezing to −100°C, the samples were stored for 30 min at −30°C and then cooled again to −100°C; measurements were performed during warming at 3°C/min. After this cooling protocol,

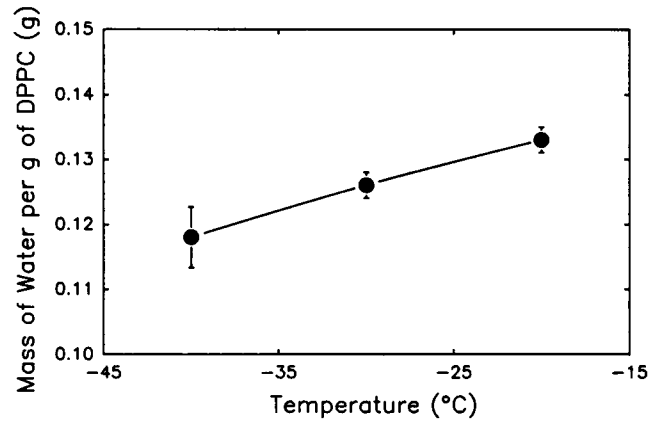

FIGURE 13 Effect of storage temperature on the amount of water remaining unfrozen per g of lipids in suspensions dehydrated over a 2.5 wt% solution of ethylene glycol (osmotic pressure = 1 MPa). Storage time was 30 min.

0.13 ± 0.01 g and 0.11 ± 0.02 g of water per g of lipid remained unfrozen for suspensions with an initial water concentration of 30–70 wt% and 16.2 ± 1.2 wt%, respectively.

The dependencies of the unfrozen water mass per g of DPPC on temperature and the corresponding osmotic pressure π in frozen suspensions are shown in Fig. 14. At equilibrium, the osmotic pressure in a partially frozen sample is dependent only on temperature and, as it was shown by Toner and Cravalho (Toner et al., 1990), can be approximated by Hoffman's equation:

$$\pi = q^{\circ}(T - T_o)T/wT_o^2, \tag{13}$$

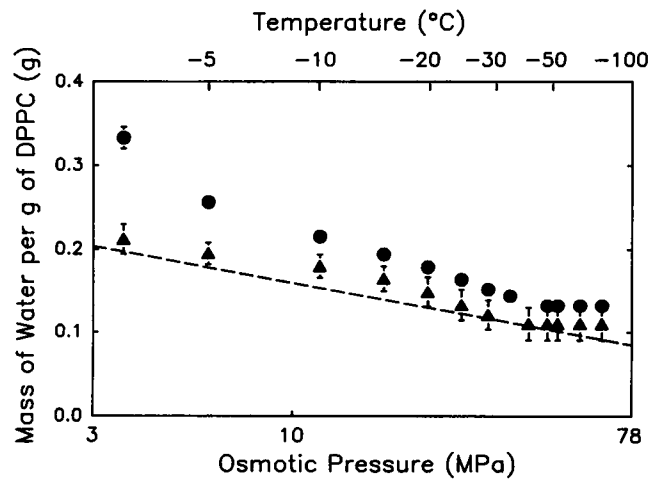

FIGURE 14 Dependence of unfrozen water mass per g of lipids in fully hydrated suspensions of multilamellar DPPC liposomes (●) and suspensions that contain 0.193 ± 0.013 g of water per g of DPPC (△), which were effected by dehydration over a saturated solution of sodium phosphate, on temperature and osmotic pressure in frozen suspensions. The samples were cooled to −100°C at 100°C/min, then warmed at 100°C/min to the storage temperature −30°C at which the samples were stored for 30 min. Subsequently, the samples were cooled to −100°C at 100°C/min and then studied during warming at 3°C/min. The long-dashed line corresponds to that presented in Fig. 17.

with an accuracy of 2%, where T_o = 273K, w is the specific volume of water. Eq. 13 was used to calculate the osmotic pressure of the partially frozen suspensions and subsequent theoretical prediction of the amount of water remaining unfrozen at subzero temperatures using the desorption isotherm presented in Fig. 17 (see Appendix 1). For suspensions that contained 0.193 ± 0.13 g water per g of lipid (16.2 wt% water), the experimental values of the unfrozen water mass per g of lipid agreed with that predicted from the desorption isotherm (*dashed line* in Fig. 14) down to approximately −50°C. To determine experimentally the amount of water that remains unfrozen in thermodynamic equilibrium with ice at temperatures ≤−50°C, one should use the step freezing protocol with T_s <−50°C. We did not perform these experiments. However, we can suggest that the amount of unfrozen water shown for temperatures <−50°C (Fig. 14) are probably overestimated. The experimental basis for this suggestion is, first, the dependence of the amount of water that freezes during cooling on the thermal history and, second, the decrease in the temperature T_1 at which the thermogram deviates from the baseline with decreasing T_s (see Figs. 9 and 11). Thus, the osmotic pressure of water between DPPC bilayers is not strongly dependent on temperature between at least +20° and −50°C, and the desorption isotherm data can be used for the prediction of the amount of unfrozen water in aqueous suspensions of DPPC liposomes at freezing temperatures. However, for fully hydrated suspensions, the calculated amount of water remaining unfrozen was substantially higher than that predicted by the desorption isotherm. Two factors may contribute to the increased amount of water remaining unfrozen in suspensions containing excess water.

First, the higher values of unfrozen water obtained for fully hydrated suspensions includes the water that remains unfrozen at ice crystal surfaces. The amount of this water is proportional to the product of the surface area of ice crystals and the thickness of the water film on the ice surface, which is a function only of temperature. The surface area of ice is unknown, but we can expect that it increases with increasing initial water concentration in the sample. This may explain why the data obtained for dehydrated suspensions are closer to those predicted from the desorption isotherm. Because the mass of unfrozen water is proportional to the sum of the surface area of lipid bilayers and the surface area of ice crystals, the slope of the dependence of m_w/m_l on log π (Fig. 14) for fully hydrated suspensions may be significantly smaller than that for dehydrated suspensions. Note, that water remaining unfrozen between ice crystals in a sample of pure water (Fig. 2) cannot be compared with the amount of water remaining unfrozen on the surface of ice crystals in frozen suspensions of liposomes because the surface area of ice in the suspension of liposomes should be greater (the size of crystals smaller) than that in a sample containing only water.

Second, the higher amounts of unfrozen water calculated for fully hydrated suspensions could be the result of an error in calculation of m_f, which comes from the assumption that the specific heat $q_o(T)$ of the ice → water transformation is much greater than the specific heat of mixing (hydration) δq_h of pure water with the lipid suspension that contains m_l/m grams of lipid and m_{unf}/m grams of water ($\delta q_h \ll q_o$) (see section 3.1). If $\delta q_h > 0$, the values of m_{unf} that were calculated assuming $\delta q_h = 0$ are overestimated. Because the enthalpy of DPPC hydration decreases (δq_h increases) with increasing initial water concentration (Cevc and Marsh, 1987), the value of this overestimation may also increase with increasing initial water concentration.

In order to estimate the effect of the heat of hydration (δq_h) of DPPC, we determined the amount of water that remains unfrozen in suspensions with varied levels of initial water content (0.135–0.219 g water per g of lipid). The suspensions were first cooled to −100°C at 100°C/min, then warmed to −40°C at 100°C/min and stored for 30 min, and then cooled again to −100°C. The DSC measurements were conducted during warming at 5°C/min.

The dependence of the mass of unfrozen water per g of DPPC on subzero temperature and osmotic pressure $\pi = (\mu_o - \mu_i)/w$ in frozen suspensions at the same temperature is shown in Fig. 15 for initial water contents of 0.145 ± 0.012 (▲), 0.171 ± 0.007 (▼), and 0.209 ± 0.012 g (●) per g of lipid. It is seen that the mass of water remaining unfrozen decreased as the initial water content in the samples decreased. The amount of water remaining unfrozen after storage at −40°C for 30 min and subsequent cooling to −100°C as a function of the initial water content is plotted in Fig. 16. The amount of water remaining unfrozen in the suspensions containing an initial water content >0.2 g/g of lipid is ≈0.12 g/g of lipid. However, at an initial water content <0.2 g/g of DPPC, the mass of unfrozen water strongly decreases with decreasing initial water content and approaches 0.1 ± 0.003 g of water per g of lipid for samples with an initial water

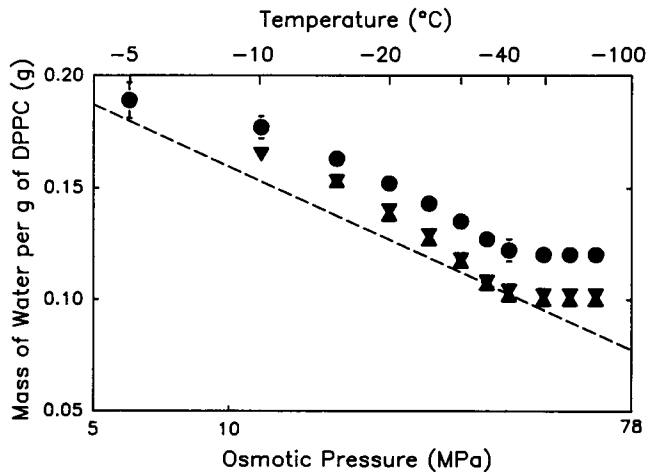

FIGURE 15 Dependence of the unfrozen water mass per g of lipids in dehydrated suspensions of multilamellar DPPC liposomes with initial water contents 0.145 ± 0.012 (▲), 0.171 ± 0.007 (▼), and 0.209 ± 0.012 g (●) per g of lipid on temperature and osmotic pressure in frozen suspensions. The long-dashed line corresponds to that presented in Fig. 17. The samples were cooled to −100°C at 100°C/min, then warmed at 100°C/min to a storage temperature −40°C at which the samples were stored for 30 min. Subsequently, the samples were cooled to −100°C at 100°C/min and then studied during warming at 5°C/min.

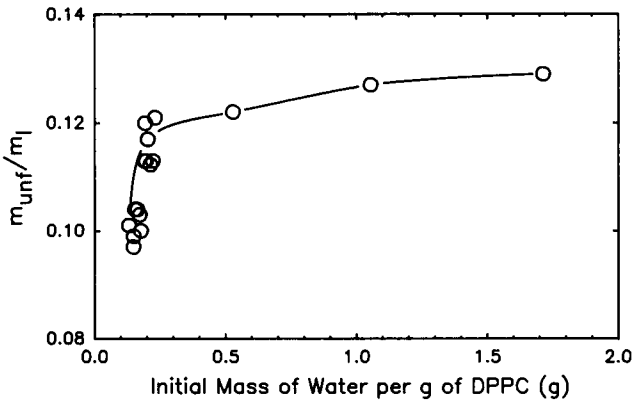

FIGURE 16 Dependence of the amount of the water remaining unfrozen per g of a suspension of multilamellar DPPC liposomes on the initial amount of water per g of lipids. The samples, which contained varied initial amounts of water, were cooled to −100°C at 100°C/min, then warmed at 100°C/min to −40°C at which the samples were stored for 30 min. Subsequently, the samples were cooled to −100°C at 100°C/min and then studied by DSC during warming. The ice melting endotherms were determined at a warming rate of 5°C/min.

content of 0.145 ± 0.012 g/g of lipids. Because the amount of ice formed in dehydrated samples is small, it seems that this decrease of 0.02 g of water per g of DPPC lends support to the possibility that the values of the amount of water remaining unfrozen are overestimated because of the assumption that $\delta q_h \ll q_o$. The overestimation occurs because the heat released during mixing of water with lipids after ice melting is not included in the calculations. If this is so, we can calculate that the amount of heat released during mixing of 0.1 g of water with 1.0 g of a dehydrated suspension of DPPC liposomes is ≥6.7 J. However, to obtain the final proof of this, the heat of hydration should be measured directly.

5. CONCLUSIONS

Previous reports have suggested that the water of hydration associated with the polar headgroups of phospholipids (e.g., 0.25 g/g of DPPC) is unfreezable (Crowe et al., 1990). This suggestion is based on DSC measurements of the amount of water that freezes in aqueous suspensions of multilamellar DPPC liposomes (Chapman et al., 1967). However, accurate measurement of the amount of water that freezes in aqueous solutions and/or suspensions of lipid vesicles by DSC is not a trivial problem for two reasons. First, because of the substantial difference between the specific heats of ice and water, reliable calculation of the heat absorbed during melting of ice requires the development of methods for the correct estimation of the baseline on DSC thermograms. The failure to detect any freezable water of hydration may be attributable to the erroneous estimation of the baseline. For example, we found that if the baseline was determined by interpolation between the point of the apparent onset of an ice melting endotherm and the point corresponding to the completion of the endotherm, one would estimate that the unfrozen water content of DPPC liposomes is constant at about 0.25 g/g and independent of the thermal history of the sample. Second, because the rate of freeze-induced dehydration depends on the temperature and spatial distribution of ice crystals within the sample, the mass of ice that forms in the sample depends on the thermal history. If ice crystals form only between multilamellar liposomes (extraliposomal ice formation), the rate of freeze-induced dehydration is limited by the rate of the diffusion of intraliposomal water through the bilayers. If ice crystals nucleate in both the extra- and intraliposomal compartments, the time required for the dehydration strongly decreases, because water inside the liposome does not have to diffuse through the bilayers to be frozen. Consequently, different amounts of water will remain supercooled after different cooling protocols. This supercooled water may contribute to the erroneous determination of the amount of water that is in thermodynamic equilibrium with ice in a partially frozen sample. Neither of these concerns have been discussed in most of the publications (Ali and Bettelheim, 1985; Chapman et al., 1967; Grabielle-Madelmont and Perron, 1983; Kakor and Bettelheim, 1991, Ter-Minassian-Saraga and Madelmont, 1981, 1982; Ueda et al., 1986) devoted to DSC measurements of the amount of freezable water in aqueous solutions and/or biological suspensions.

Here we propose an iterative procedure for correct estimation of the baseline in the DSC thermograms (see section 3.3), which is required for an accurate calculation of the amount of ice that melts during warming of frozen specimens. Using the iterative procedure and the three-step protocol of temperature change (see section 4.3), which includes storing of the sample for varied periods of time to control the extent of freeze-induced dehydration, we found that the amount of water remaining unfrozen in a suspension of multilamellar DPPC liposomes decreased with increasing time of storage (Fig. 12) and decreasing storage temperature T_s. For this reason, to measure the amount of water that remains unfrozen at a subzero temperature T in a suspension of multilamellar DPPC liposomes in thermodynamic equilibrium with ice, the three-step protocol should include storage at $T_s \leq T$ for the time required to complete freeze-induced dehydration at T_s. For example, we have established that 30 min of storage is sufficient to complete freeze-induced dehydration at $T_s \geq -40$°C.

Using the values of the partial areas under the endotherm of ice melting, we calculated the amounts of water per g of DPPC remaining unfrozen at subfreezing temperatures. For dehydrated suspensions (12–20 wt% water) at $T > -50$°C the dependence of the amount of unfrozen water per g of DPPC on osmotic pressure in the frozen suspensions was in agreement with the dependence of the amount of water per g of DPPC on the osmotic pressure (<50 MPa) at 20°C. This means that the desorption isotherms for DPPC at subzero temperatures are similar to that at +20°C. For suspensions with an excess of water (30–70 wt% water) the amount of water remaining unfrozen was significantly higher than that in dehydrated suspensions. This was because the amount of water remaining unfrozen is the sum of the water associated

with the polar headgroups of phosphatidylcholine and that which is associated with the surface of ice crystals. Therefore, the amount of unfrozen water per g of DPPC obtained for dehydrated suspensions is closer to the amount of unfrozen water associated with the polar headgroups than that obtained for suspensions containing an excess of water.

From the desorption isotherm for DPPC at 20°C (see Appendix 1) we can estimate that the amounts of water remaining unfrozen at −20°C, −50°, and −140°C are estimated to be 0.13, 0.10, and 0.08 g/g of DPPC, respectively. However, for dehydrated suspensions (12–20 wt% water) at $T <$ −50°C the amount of water remaining unfrozen was a constant of 0.1 ± 0.003 g water per g of DPPC. This is probably because time required to complete lipid freeze-induced dehydration at temperatures <−50°C becomes very high and an insignificant amount of water freezes between −50°C and the temperature at which the unfrozen portion of the sample forms a glass during cooling.

In aqueous solutions (Franks, 1982) the temperature of liquid-to-glass transformation T_g increases with decreasing water concentration, therefore one may also expect that T_g in lipid suspensions also increases with decreasing amount of water per g of lipid. For this reason, T_g should be higher than that for pure water, which vitrifies at −140°C (Johari et al., 1990). However, T_g cannot be higher than T_m. Therefore, the extent of lipid dehydration during freezing is limited. The maximum dehydration that can be achieved in a frozen suspension of DPPC liposomes is the dehydration for which $T_g = T_m = T'_g$. Although the values of T'_g and the corresponding amount of unfrozen water per g of lipid (W'_g) for a suspension of multilamellar DPPC liposomes remain to be determined, our measurements allow for estimation of the values T'_g and W'_g as follows. First, we can say that T'_g is lower than the temperature at which some freezable water is still detectable. For this reason, T'_g is lower than the temperature (approximately −50°C) at which the beginning of ice melting during warming is detectable by DSC. Therefore, we can estimate that $-140°C < T'_g \leq -50°C$. Using this inequality, from the desorption isotherm one can estimate that $0.08 < W'_g \leq 0.10$ g/g of DPPC, which is the limit of the amount of water that can be removed by freeze-induced dehydration. Nevertheless, this limit is much smaller than the 0.25 g of water per g of DPPC that was reported by Chapman et al. (1967). Therefore, we do not find experimental support for the suggestion of Crowe et al. (1990) that the water associated with the polar head groups of phospholipids is unfreezable.

APPENDIX 1: DESORPTION ISOTHERM

At positive constant temperature, the osmotic pressure of water between lipid bilayers is a continuous function of the interbilayer distance h (Lis et al., 1982; Marsh, 1989; Rand and Parsegian, 1989):

$$\pi = \pi_o \exp(-h/h_o), \tag{14}$$

where π_o is the amplitude and h_o is a decay length. From Eq. 14, the equivalent to the desorption isotherm dependence between π and mass of water per g of lipids (m_w/m_l) can be given as follows:

$$m_w/m_l = -(a_l \mu_w h_o / 2\mu_l w) \cdot (\ln \pi - \ln \pi_o), \tag{15}$$

where the surface area of DPPC bilayer per lipid molecule $a_l = 0.5$ nm^2 (Rand and Parsegian, 1989), and $\mu_l = 733$ g/mol and $\mu_w = 18$ g/mol, which are the molecular masses of DPPC and water, respectively.

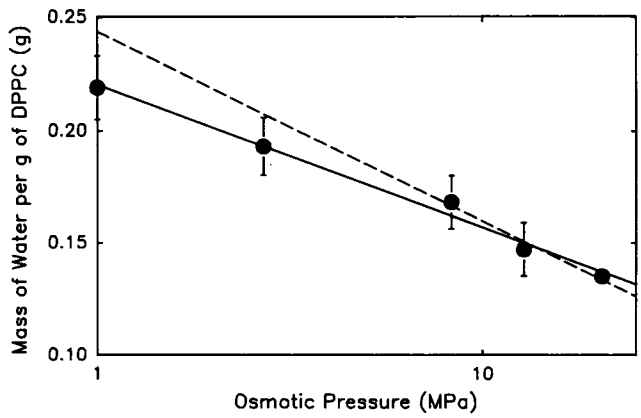

FIGURE 17 Desorption isotherm for DPPC/water mixture measured at 20°C. A linear regression of all data is shown by a solid line; a linear regression of the part of data for osmotic pressures ≥ 8.3 MPa is shown by a dashed line.

It is seen from Eq. 15 that m_w/m_l varies linearly with the logarithm of osmotic pressure. The desorption isotherm (the dependence of m_w/m_l on log π in Fig. 17) was obtained by gravimetric measurement of the amount of water in DPPC suspensions equilibrated at 20°C over a 2.5 wt% solution of ethylene glycol (osmotic pressure ≃ 1 MPa) and over saturated salt solutions of Na_2HPO_4 (2.7 MPa), $KN0_3$ (8.3 MPa), $BaCl_2$ (12.8 MPa), and KCl (20.4 MPa). A linear regression (*solid line* in Fig. 17) fits the data presented in Fig. 17 with a coefficient of determination $R = 0.985$. The values of parameters h_o and π_o, which were calculated from the coefficients of this linear regression, are $1.4 \cdot 10^{-8}$ cm and 2754 MPa, respectively. These values differ from those ($h_o \simeq 2.4 \cdot 10^{-8}$ cm and $\pi_o \simeq 500$ MPa) previously reported for DPPC (see reviews, Marsh (1989) and Rand and Parsegian (1989)). There could be two reasons responsible for this difference. First, in the previous studies (see reviews, Marsh (1989) and Rand and Parsegian (1989)) desorption isotherms were measured at temperatures >20°C. According to the phase diagram of DPPC-water mixtures (Cevc and Marsh, 1987) at 20°C and water concentrations <20 wt%, the crystalline phase (L_c) is the equilibrium state of DPPC. However, at temperatures >20°C the desorption isotherm may have different values for h_o and π_o because DPPC may be in a phase ($L_{\beta'}$, $P_{\beta'}$, or L_α) (Cevc and Marsh, 1987) that is different from the crystalline phase (L_c).

Second, at high water contents (low osmotic pressure), the dependence of π on h can differ from that at low water contents. Marsh (1989) used this suggestion to explain the abnormally high value of π_o (3980 MPa) and the small value of h_o ($1.4 \cdot 10^{-8}$ cm) that were obtained for egg phosphatidylcholine/cholesterol (1:1 mol/mol) in Lis et al. (1982). In addition, it was shown (Rand and Parsegian, 1989) that at 25°C the dependence of π on h for DPPC was significantly different from that corresponding to Eq. 14 at $h >1.5$ nm ($m_w/m_l \simeq 0.3$). It is possible that at 20°C this deviation starts at lower water contents. For this reason, to predict the water content at high (>20.4 MPa) osmotic pressures we used the values of h_o and π_o calculated from a linear regression of the data presented in Fig. 17 for osmotic pressures ≥ 8.3 MPa (*dashed line* in Fig. 17). The values of parameters h_o and π_o calculated from the coefficients of this linear regression are $1.9 \cdot 10^{-8}$ cm and 780 MPa, respectively. These values of h_o and π_o are closer to the values previously published in (Marsh, 1989; Rand and Parsegian, 1989).

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