Colloids and Surfaces A, **129**, 425-434 (1997)

Freezing-induced hydration forces between phosphatidylcholine bilayers - the effect of osmotic pressure

Yong Hyeon Yoon*, James Pope+ and Joe Wolfe*[1]
*School of Physics, University of New South Wales, Sydney 2052 AUSTRALIA
+ School of Physics, Queensland University of Technology, Brisbane 4001 AUSTRALIA

Abstract

Quantitative nuclear magnetic resonance was used to measure the freezing behaviour of lamellar phases of phosphatidylcholine in water and in solutions of sorbitol. Both solute and solvent were deuterated in different series of experiments to allow the calculation of the partitioning of solute and solvent molecules between the lamellar phase and unfrozen bulk solution. Sorbitol, as well as water, was found to redistribute between these phases as a function of temperature. The results show a strong, repulsive, interlamellar force which decreases approximately exponentially with hydration. Compared to measurements on lipid/water systems and solute/water systems, the hydration of the lamellar phase containing solutes is slightly less than the sum of the hydrations of lipid and solute at any given chemical potential of water. For a lamellar phase with a given quantity of lipid, interlamellar sorbitol and water, reduction of chemical potential of water is greater than that due to lipid acting alone plus that due to solute acting alone.

Introduction.

This study is inspired by the desire to understand the mechanism of freezing induced dehydration damage in cells. This is one of several types of freezing damage in which colloidal effects have been proposed to explain the ultrastructural damage–in this case the stresses which are produced by hydration forces in systems with low water contents [1,2]. Cellular membranes are often modelled by lipid bilayers. In this paper we measure and analyse the hydration behaviour of bilayer membranes at freezing temperatures and in the presence of high concentrations of osmotically active solute.

When tissues or suspensions of cells are frozen relatively slowly, freezing almost always occurs first in the extracellular solution. This concentrates the extracellular solutes in the small remaining unfrozen volume and causes severe osmotic dehydration of the cell. The dehydration is often sufficiently severe that the non-aqueous components of the cell, including membranes, are forced into separations of order 1 nm. At this range, the strong, repulsive hydration interaction produces large anisotropic stresses in membranes, and these often result in strains such as lateral compression, part fusion of membranes, lateral phase separations and formation of non-bilayer phases [3-7]. Such deformations have been associated with freezing-induced damage in cells (reviewed by [2]; see also [8,9]). The place of dehydration-induced membrane strains in freezing damage is represented in Figure 1.

We have reported a technique for measuring hydration forces between model membranes at freezing temperatures in pure water [14]. The situation is considerably complicated by the presence of osmotically active solutes, and in this paper we report experiments which determine the hydration forces between phosphatidylcholine bilayers in the presence of sorbitol. Phosphatidylcholine is a major component of biological membranes, and is often used in model studies. Sorbitol is a sugar accumulated by some freezing tolerant species. The effect of some cryobiologically important solvents on the forces between dioleylphosphatidylcholine bilayers has been studied by [15] using the surface forces apparatus. In that technique, osmotic effects due to

[1] Address for correspondence. J.Wolfe@unsw.edu.au

the solute are minimised because of the free exchange of solutes and solvent between the interlamellar layer and a very large solution reservoir. For sorbitol, that study found no measurable specific effect on the intermembrane forces at concentrations exceeding 1 kmol.m^{-3}, and little effect on bilayer structure. Sorbitol is therefore a suitable solute for investigating osmotic effects on interlamellar forces.

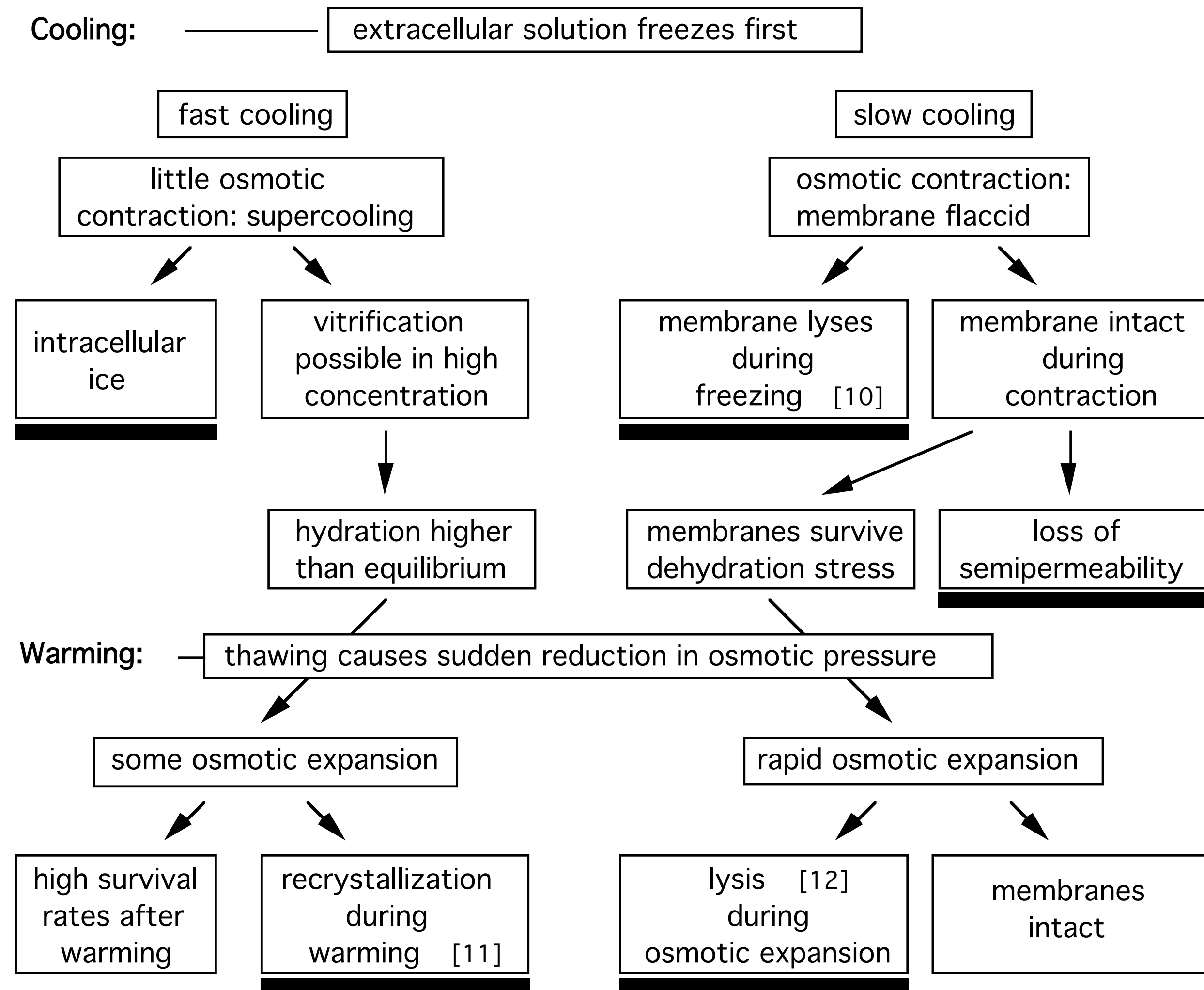

Figure 1 shows some of the mortal dangers (indicated by dark bars) awaiting cells subjected to freezing and thawing. Cooling rates are divided into fast or slow by the characteristic time for hydraulic equilibration across the plasma membrane: $\tau = r/3\Pi_0 L_P$ where r is a cellular dimension, Π_0 the osmotic pressure of the suspending medium and L_P is the hydraulic conductivity of the membrane [13]. For slower cooling rates, such as occur in nature, the water can leave the cell faster than heat does, so the cell remains in hydraulic equilibrium with its surroundings, which usually means substantial osmotic contraction for even modest sub-freezing temperatures. For faster cooling rates, such as are used for artificial cryopreservation, there is insufficient time for water to equilibrate by leaving the cell, so supercooling occurs rather than extensive osmotic contraction. The cellular damage that inspires this study occurs to cells which survive slow freezing to reach very low hydration levels, but which then suffer a range of ultrastructural strains and which lose semipermeability.

The method previously described by us for obtaining hydration force curves at freezing temperatures uses quantitative deuterium nuclear magnetic resonance (NMR) spectroscopy to determine the hydration of the bilayers, and hydraulic equilibrium to determine the hydration force [14]. Some of the experiments reported here use D_2O whose deuterium NMR signal can readily be resolved into ice and unfrozen components because the vastly different molecular rates of motion in the two phases produce signals of greatly differing linewidth. Other experiments use H_2O and deuterated sorbitol so that the distribution of solute can be resolved into the fractions in the interlamellar solution and the bulk solution. The lamellæ were formed from phosphatidylcholines (PC): either egg yolk phosphatidylcholine (EYL) or dioleylphosphatidylcholine (DOPC). We also

measured the freezing behaviour of sorbitol/D_2O solutions to determine the osmotic behaviour of the solute in this temperature range.

Freezing a mixture of lipids and water, with or without solutes, produces virtually pure ice at zero hydrostatic pressure whose chemical potential is readily calculated from the temperature [18]. In hydraulic equilibrium, this determines the chemical potential of water in the unfrozen water in the sample. The chemical potential of interlamellar water can be decomposed in different ways. We treat the lamellæ as macroscopic boundaries which exert a force on each other[2]. At small separations, which are the region of interest both in this paper and in cryobiology, this force is large and repulsive, and is usually attributed to the hydration force [16,17]. In the absence of solutes, the pure water phase between the lamellæ has a hydrostatic pressure P_{lam} which is equal in magnitude to the interlamellar force per unit area, F, but opposite in sign. Thus, for lipid/water samples at freezing temperatures, the temperature determines μ_{ice} and thus F. The intensity of the NMR signal yields the total amount of unfrozen water, from which the interlamellar separation can be deduced, and this yields a hydration force which falls exponentially with separation and whose characteristic length is about two hundred pm [14].

Consider now the system containing PC, water and sorbitol. Sorbitol is readily soluble in water but is not expected to dissolve in the hydrocarbon region of the lamellæ. It can therefore partition into the interlamellar water and into a bulk aqueous solution phase (see Fig 2). Under appropriate conditions, it might also crystalise, although the last possibility was not observed under any of the conditions in this study. Here we report the details of the coexistence of ice, sorbitol solution and a PC/sorbitol/water lamellar phase.

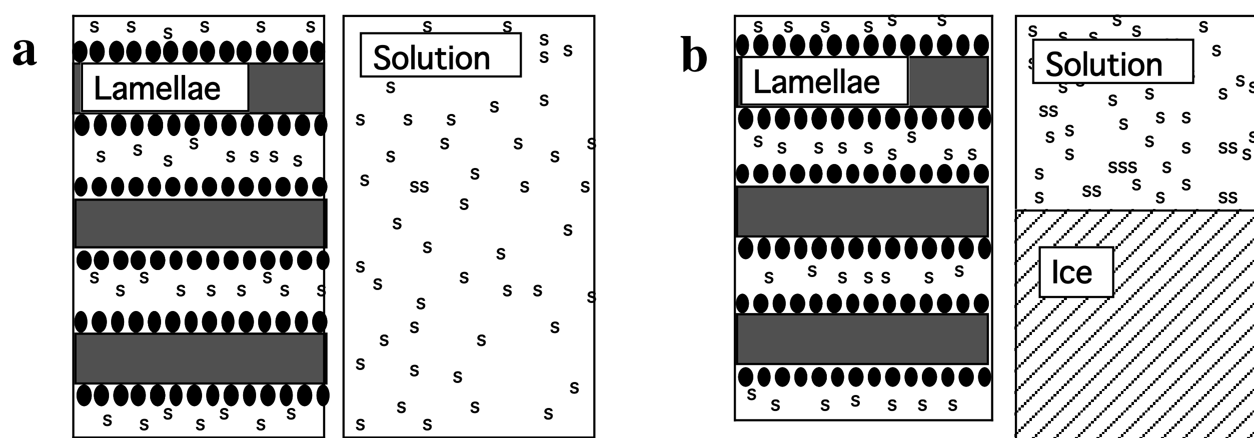

Figure 2. This figure shows the two observed phase coexistence regimes of the lipids, water and solutes studied here. We neglect the finite but small concentration of lipid monomers in water, and we assume that the ice is a pure phase. (a) shows the situation observed above freezing: a bulk aqueous solution of the solute coexists with a lipid lamellar phase whose aqueous layers contain solutes. (b) shows the situation at a freezing temperature. Ice coexists with a concentrated bulk solution whose concentration at equilibrium is determined by the temperature (freezing point depression). The chemical potential of the water in this solution is lower than that in (a), so some water leaves the lamellar phase. This exodus of water is however opposed by the repulsive forces between the lamellæ. The concentration of solutes in the lamellar phase of (b) is greater than that in (a). The value of the concentration depends on, among other things, whether or not the solutes permeate the bilayers. If they do, the increasing concentration in the bulk phase causes solute to partition into the lamellar phase. For such a permeating solute, the bulk solution may disappear entirely if the ratio of solute:lipid in the whole sample is sufficiently small. For a permeating solute, the initial water content of the sample is irrelevant to the composition of the bulk solution and lamellar phases at freezing temperature: more water simply means more ice.

---

[2] Once could instead define the pressure of the entire lamellar phase to be zero and introduce an energy of hydration of the lamellæ. This energy would decrease with increasing hydration and go to zero at mole ratios of order 20:1. Physically, the two systems of accounting can be simply reconciled: the pressure in the hydration force picture equals the derivative of the energy of hydration with respect to partial molar volume of water in the hydration energy picture. For the benefit of readers who may be surprised by the idea of large negative hydrostatic pressures in the interlamellar water, we point out that cavitation is unlikely even at very large suctions: the water is bounded by highly hydrophilic surfaces and its thickness is less than the critical radius for cavitation.

Materials and methods

Materials: Egg yolk phosphatidylcholine (EYL) was bought from Sigma and dioleoylphosphatidylcholine (DOPC, MW=786.12) from Avanti Polar-lipids, Inc. D_2O with nominal purity 100% was bought from Sigma. Sorbitol was purchased from ICN Biochemicals. Sorbitol-1,1,6,6-d4 with minimum 98 atom % deuterium was bought from Matheson. All were used without further purification.

Quantitative NMR: A Bruker MSL 200 spectrometer operating at 30.720 MHz was used for the ^2H NMR measurements. A cooling system employing boil-off gas from a liquid nitrogen dewar provided temperature control with a precision of 0.1 K when employing a specially designed (home built) NMR probe insert. The method was previously described by [14], and further details are given by [18]. The spectral width was adjusted usually in the range 2 kHz - 40 kHz, depending on the type of samples used. The typical $\pi/2$ pulse length was ~8 μs. Data file size was chosen to be 4 k to 8 k. The number of acquisition was typically in the range 64 to 256. The recycle time between consecutive acquisitions was usually 1 to 3 seconds.

The temperature sensitivity of the radio frequency NMR coil and associated electronics was calibrated by measuring the total signal in samples which do not freeze over the range of the experiment. Perdeuterated methanol was used for one calibration. D_2O was also used over a limited range of freezing temperatures by performing cooling experiments and recording the total signal as a function of temperature over the range of supercooling. The temperature controller was calibrated by measuring the melting temperature of D_2O, which was set to 276.97 K [19].

Exchange between hydrogen and deuterium: The hydroxyl groups of sorbitol can exchange protons with D_2O. This would produce DHO and H_2O in D_2O, resulting in the possibility that the composition of the ice and water phases might be different and so the unfrozen fraction could not accurately be determined. To minimise this effect, the exchangeable protons were replaced with deuterons. Sorbitol was dissolved in excess D_2O. The ratio of the OD groups of the D_2O to the exchangeable OH groups of sugar was 10:1. The solution was then dried in an oven until the crystalline form of the sugar was obtained. This procedure was repeated. After two repetitions, we expect 99% of the exchangeable OH groups in sorbitol to be replaced by OD. All the sorbitol used in D_2O solutions was "hydroxyl group deuterated" in this way. The deuterons in the OD groups of the solutes and those of the D_2O contribute to the NMR signal and the contribution from the solutes can be subtracted because the number of solutes is known. Sorbitol-1,1,6,6-d4 was used as a deuterated solute in H_2O solution. In this case the deuterium atoms are not in the hydroxyl groups and therefore are not exchangeable with H_2O.

Two methods were used to prepare EYL samples. The lipid was purchased dissolved in chloroform and methanol. In the first method, about 2 ml of solution (containing about 200 mg of EYL) was dried in a stream of dry nitrogen to remove most of the solvent, then placed in a desiccator with P_2O_5. The pressure in the desiccator was then reduced by vacuum pump for 12 hours, at the end of which time the EYL formed a fine powder. The desiccator was opened in a nitrogen atmosphere and about 50 mg of lipid was then transferred to an NMR tube. An appropriate amount of aqueous solution was added to the sample which was then weighed. The sample was temporarily sealed with a plastic cap and removed from the nitrogen atmosphere. In the second method, the lipid solution was transferred directly to a pre-weighed NMR tube which was placed in a vacuum desiccator with P_2O_5 as above. After pumping for 12 hours to reduce the EYL to a fine powder, aqueous solution was added in an amount determined by weighing, in the laboratory atmosphere, before temporary sealing. The exposure to the atmosphere was less than one minute, and we expect that the adsorption of water from the air by the sample in the NMR tube was insignificant. The hydration behaviours of samples produced by the two methods were indistinguishable.

DOPC was purchased as a powder and samples were prepared in the second manner described for EYL. Because the exact amounts of lipid and solution are known only after weighing, it is not possible to produce samples with exactly the same composition. In all cases the sample composition is well known, however, and the variations in composition among samples did not hinder the analysis of results.

The temporarily sealed tubes were centrifuged at ~1000 g. The bottom of the tube, containing the sample, was then frozen in liquid nitrogen. The other end was quickly flame sealed to produce a

size appropriate (about 20 mm long) for NMR measurement while keeping frozen the end containing the sample. After sealing, the sample was mixed by further centrifugation for several hours with intermittent reversal of the sample orientation.

For solution samples without lipid, about 50 to 100 μl of solution was added to a pre-weighed NMR tube and re-weighed. The sample was then frozen and flame sealed as described above.

A range of treatments were employed to achieve good mixing: samples were also sonicated for 24 hours at room temperature, and subjected to ten or more cycles of freezing and thawing and aged for several days at room temperature.

Freezing runs: The samples were first cooled to 253 K to initiate crystallisation of water and allowed to equilibrate at least for 30 min. Measurements were usually carried out during warming, with occasional returns to lower temperatures to ensure that there was no thermal hysteresis apart from supercooling. The sample equilibration at each successive temperature was monitored. In most cases, 20 minutes of equilibration per 1 K increase in temperature was sufficient to ensure that the signal amplitude did not change appreciably with time. At some temperatures the signal was monitored for several hours following this equilibration, and no further changes were observed. The process is described in more detail by [14] and [18].

Results and discussion

Figure 3a is a deuterium NMR spectrum of a sample of DOPC/sorbitol/D_2O at T = 263 K. It shows two distinct components. The narrow central peak is attributed to water molecules moving isotropically in domains of bulk solution. The broad component is attributed to water in the lamellar phase which exhibits anisotropic reorientation on the NMR time scale, giving rise to the observed powder spectrum. The resolved splitting arises from incomplete motional averaging of the (spin I = 1) quadrupolar coupling of the deuterons in the water molecules of the lamellar phase. The spectral component due to ice is so wide (~ 150 kHz) that it forms the baseline in this plot. The solution component can be well fitted by a Lorentzian line shape, and its integral calculated. Once this component is subtracted, the remaining powder spectrum can be integrated. These two integrals are proportional to the amount of water in the two phases.

It is also possible to look at the distribution of the solute directly using NMR. Figure 3b is a deuterium NMR spectrum of a sample of EYL/sorbitol$_{d4}$/H_2O. The signal to noise ratio is not as good as in 3a, because there are fewer deuterons in the sample. (Although the solute has four deuterons per molecule and the solvent two, there are many more solvent than solute molecules.) A simulation of this spectrum is also plotted, but it is not clearly seen as it overlaps the data very closely. The simulation is the sum of two components which are also shown individually in Figure 3b: a Lorentzian with a line width of 70 Hz and a Gaussian with a line width of 240 Hz. The width of the Lorentzian as a function of temperature agrees well with that measured for sorbitol$_{d4}$/H_2O solutions (data not shown, see [18]). The Lorentzian is attributed to sorbitol in the solution phase, and the Gaussian to sorbitol in the lamellar phase. The width of the spectral component of a putative crystalline phase would again be much wider than the scale shown here. The amounts of solute in the solution and in the lamellar phase can be calculated from the integral of the components of the simulated spectrum in this case. However there is a possible error of up to several percent in such data, because there is some latitude in the range of parameters (linewidths and relative intensities of the two components) for which the simulations fit the experimental spectra. From such data, the amount of solute in the two phases can be determined as a function of temperature, and this is shown in Figure 3c. The sum of the two components equals the total amount of sorbitol in the sample. Note however that the relative proportions of the two components vary with temperature: the sorbitol content of the lamellar phase falls and that of the solution rises with rising temperature. As the temperature rises and ice thaws, the extra-lamellar solution becomes more dilute, and so the sorbitol diffuses from the lamellar phase into the solution phase.

The decomposition of spectra can be done more precisely for spectra like 3a than for spectra like 3b because in 3a the difference in spectral shape and width of the two components is more pronounced, and because the larger number of contributing deuterons gives a better signal to noise ratio. Fortunately, the composition of the different phases can be determined entirely from D_2O spectra. Deuterium spectra of sorbitol/D_2O solutions over the range 261 to 286 K (data not shown, see [18]) give the amount of unfrozen water as a function of temperature, and thus the composition

of a sorbitol solution at any temperature - i.e. the freezing behaviour. From spectra like 3a we calculate the amount of water in the solution phase of the lipid/solute/water sample, and from the solution freezing behaviour we calculate the amount of solute in the solution phase of that sample. The rest of the (unfrozen) solute is therefore in the lamellar phase. This indirect method of determining the solute partitioning gives data that agree with those gained from spectra like 3b, but are more precise.

The determination of the water contents of the two different phases is made directly from D_2O spectra. In Figure 4 the hydration of three different phases as a function of temperature is shown. One set of data is for DOPC/D_2O without solutes. The other data set is for a sample of DOPC/sorbitol/D_2O in total mole ratio 1/0.52/20.1. The solid and dashed lines in that figure refer to the hydration components of the DOPC/sorbitol/D_2O determined as described above. The solid line is the hydration of the lamellar phase, and the dashed line that of the solution phase. The hydration of the lamellar phase containing solutes is larger than that of the solute-free phase by several waters per lipid, the amount increasing with temperature.

How can the hydration of such a phase be analysed? The chemical potential of water is

$$\mu = \mu^o + kT \, ln \, a_w + Pv_w \qquad (1)$$

where a_w is the activity of water and v_w its partial molecular volume. In our accounting the repulsive force F per unit area between the lamellæ supplies the hydrostatic pressure term because $P = - F$. Solutes obviously affect the chemical potential via the entropy term $kT \, ln \, a_w$, and in principle they may also affect F. Let us define the osmotic contribution of the solutes thus:

$$\Pi_s \equiv \frac{kT}{v_w} \, ln \, a_w$$

$$\text{whence } \Psi \equiv \frac{\mu - \mu^o}{v_w} = \Pi_s - F \qquad (2)$$

where Ψ thus defined is called the water potential [20].

The vertical axis in Figure 5 plots $-\Psi$ as a function of hydration for lamellar phases with and without solutes. For the DOPC/D_2O sample, $\Psi = P = -F$, where F is the repulsive force per unit area between the lamellæ. Its approximately exponential dependence on hydration is similar to that reported by other authors [21] at room temperature, but a little smaller.

At any given chemical potential, Figure 5 shows that the hydration of the lamellar phase was higher in the presence of solutes. The two other lines on Figure 5 represent two very simplistic ways of treating these data for the lamellar phase with solutes present. Both use the independently measured hydration of the solute in a sorbitol/D_2O solution. The dotted line was obtained by subtracting from the lamellar hydration the hydration of the solute in a simple solution at that temperature. The fact that this line falls below that for the pure lamellar phase in the absence of solute shows that the hydration of the lamellar phase with solute present is less than the sum of that of the lipids plus that of the solute, but not by a great deal. To obtain the solid line, the osmotic pressure of a solution having the composition of the interlamellar layer is added to Ψ of the lamellar phase with solute present. This line falls above that for the lamellar phase without solute, showing that the freezing point depression in the lamellar phase with solute present is more than the sum of those produced by the lipids and the solute separately. So the hydrations of the lipid molecules and of the solute molecules are not simply additive, and neither are their colligative effects. One could argue that osmotic pressure of the interlamellar layers should be larger than that measured for a solution having the same composition. The interlamellar layers are not very much larger than molecular dimensions and one might expect that the solute molecules are excluded from a volume close to the lamellæ. Such an excluded volume effect would lower the solid line in the figure and bring it closer to the data for the lamellar phase without solutes.

Could there be specific effects on the bilayer hydration due to the presence of sorbitol, or non-specific effects other than sorbitol's osmotic effect? Working in a system that minimised osmotic effects, Pincet and co-workers [15] did not measure specific effects, but they worked at lower concentrations (1 $kmol.m^{-3}$) than those reported here. It is difficult to answer this question

unequivocally from the data in this study because it is difficult to estimate the osmotic effect of the interlamellar solutes, as discussed above. Nevertheless, we can cite some possibilities. First, the presence of sorbitol in water in high concentrations (mole ratios of order 1:30) might be expected to affect water structure and therefore the hydration force. Second, it is possible that sorbitol interacts with the bilayers and/or affects the way that the lipid headgroups interact with water. Of the many conceivable such interactions, an exclusion of the solutes from a region near the lipid headgroups would be one of the simplest and would be qualitatively consistent with the results reported here and with those of [15].

Conclusions

i) The NMR freezing technique described here using deuterium labelling of the solute and solvent alternately allows one to separate hydration components of lamellar and bulk solution phases, and therefore to measure the partitioning of solute and solvent into those phases as a function of temperature.

ii) At a given freezing temperature, the hydration of a lamellar phase of DOPC including sorbitol is a little less than the sum of the hydrations of both individually.

iii) The observed freezing point depressions or reductions in chemical potential of water due to DOPC and sorbitol are not simply additive. For a lamellar phase with a given quantity of lipid, interlamellar sorbitol and water, the reduction of chemical potential of water is greater than that due to lipid acting alone plus that due to sorbitol acting alone.

Acknowledgement. We thank Peter Rand for helpful advice on sample preparation.

References

[1] Wolfe, J. (1987) "Lateral stresses in membranes at low water potential". Aust. J. Plant Physiol., 14, 311-318.

[2] Bryant, G. and Wolfe, J. (1992) "Interfacial forces in cryobiology and anhydrobiology" Cryoletters 13, 23-36.

[3] LeNeveu D.M., R.P. Rand and V.A. Parsegian, (1976). Measurement of forces between lecithin bilayers. Nature , 259, 601-603.

[4] Horn R.J, (1984). Direct measurements of the forces between two lipid bilayers and observation of their fusion. B.B.A., 778, 224-228.

[5] Helm C, Israelachvili JN and McGuiggan P (1989) "Molecular mechanisms and forces involved in the adhesion and fusion of amphiphilic bilayers" Science 246, 919-922.

[6] Bryant G., Pope, J.M. and Wolfe, J. (1992) "Low hydration phase properties of phospholipid mixtures: evidence for dehydration-induced fluid-fluid separations." Eur Biophys J., 21, 223-232.

[7] Webb, M.S., Hui, S.W. and Steponkus, P.L. "Dehydration-induced lamellar-to-heaxagonal-II phase transitions in DOPE/DOPC mixtures", Biochim. Biophys. Acta, 1145, 93-104, 1993.

[8] Gordon-Kamm W.J. and P.L. Steponkus, (1984). Lamellar-to-hexagonal II phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. Proc. Natl. Acad. Sci. U.S.A., 81, 6373-7.

[9] Uemura, M., Joseph, R.A. and Steponkus, P.L. (1995) "Cold acclimation of *Arabidopsis thaliana*. Effect of plasma membrane composition and freeze-induced lesions", Plant Physiol., 109, 15-30.

[10] Steponkus, P.L., Stout, D.G., Wolfe, J. and Lovelace, R.V.E. (1985) "Possible role of transient electric fields in freezing-induced membrane destabilisation". J. Memb. Biol. 85, 191-198.

[11] Rall, W.F., Reid, D.S. and Polge, C. (1984) "Analysis of slow-warming injury of mouse embryos by cryomicroscopical and physiochemical methods" Cryobiol. 21, 106-121.

[12] Wolfe, J., Dowgert, M.F. and Steponkus, P.L. (1986) "Mechanical study of the deformation and rupture of the plasma membranes of protoplasts during osmotic expansions". J. Memb. Biol., 93, 63-74.

[13] Wolfe, J. and Bryant, G. (1992) "Physical principles of membrane damage due to dehydration and freezing". *In* "Mechanics of swelling: from clays to living cells and tissues", T.K. Karalis, ed., NATO ASI Series H vol 64, pp 205-224.

[14] Yan, Z., Pope, J. and Wolfe, J. (1993) "Nuclear magnetic resonance spectroscopy of frozen phosphatidylcholine-D_2O suspensions: a new technique for measuring hydration forces". J. Chem. Soc. Faraday Trans., 89, 2583-2588.

[15] Pincet, F., Perez, E. and Wolfe, J. (1994) "Do trehalose and dimethylsulphoxide affect inter-membrane forces?" Cryobiology 31, 531-539.

[16] Lis, L.J., M. McAlister, N. Fuller, R.P. Rand & V.A. Parsegian (1982). Interactions between neutral phospholipid bilayer membranes. Biophys J., 37, 657-666.

[17] Israelachvili, J.N. (1977) Measurement of forces between surfaces immersed in electrolyte solutions. Faraday Disc. Chem. Soc. 65, 22-24

[18] Yoon, Y. (1996) "A Nuclear Magnetic Resonance Study of the Freezing Behaviour of Lipid-Water-Solute Mixtures and Some Simple Biological Systems" Doctoral dissertation, University of New South Wales, Sydney.

[19] Weast, R.C. (1990) "CRC Handbook of Chemistry and Physics" CRC Press, Boca Raton, Fl.

[20] Slatyer, R.O. (1967) "Plant Water Relationships" Academic, London.

[21] Ulrich, A.S., Sami, M. and Watts, A. (1994) "Hydration of DOPC bilayers by differential scanning calorimetry". Biochim. Biophys. Acta, 1191, 225-230.

Figure captions

Figure 1
This figure shows some of the mortal dangers (indicated by dark bars) awaiting cells subjected to freezing and thawing. Cooling rates are divided into fast or slow by the characteristic time for hydraulic equilibration across the plasma membrane: $\tau = r/3\Pi_o L_P$ where r is a cellular dimension, Π_o the osmotic pressure of the suspending medium and L_P is the hydraulic conductivity of the membrane [13]. For slower cooling rates, such as occur in nature, the water can leave the cell faster than heat does, so the cell remains in hydraulic equilibrium with its surroundings, which usually means substantial osmotic contraction for even modest sub-freezing temperatures. For faster cooling rates, such as are used for artificial cryopreservation, there is insufficient time for water to equilibrate by leaving the cell, so supercooling occurs rather than extensive osmotic contraction. The cellular damage that inspires this study occurs to cells which survive slow freezing to reach very low hydration levels, but which then suffer a range of ultrastructural strains and which lose semipermeability.

Figure 2.
This figure shows the two phase coexistence regimes of the lipids, water and solutes studied here. We neglect the finite but small concentration of lipid monomers in water, and we assume that the ice is a pure phase. (a) shows the situation observed above freezing: a bulk aqueous solution of the solute coexists with a lipid lamellar phase whose aqueous layers contain solutes. (b) shows the situation at a freezing temperature. Ice coexists with a concentrated bulk solution whose concentration at equilibrium is determined by the temperature (freezing point depression). The chemical potential of the water in this solution is lower than that in (a), so some water leaves the lamellar phase. This exodus of water is however opposed by the repulsive forces between the lamellæ. The concentration of solutes in the lamellar phase of (b) is greater than that in (a). The value of the concentration depends on, among other things, whether or not the solutes permeate the bilayers. If they do, the increasing concentration in the bulk phase causes solute to partition into the lamellar phase. For such a permeating solute, the bulk solution may disappear entirely if the ratio of solute:lipid in the whole sample is sufficiently small. For a permeating solute, the initial water content of the sample is irrelevant to the composition of the bulk solution and lamellar phases at freezing temperature: more water simply means more ice.

Figure 3
(a) is a deuterium NMR spectrum of a sample of DOPC/sorbitol/D_2O at T = 263 K. (b) is a deuterium NMR spectrum of a sample of EYL/sorbitol$_{d4}$/H_2O at 264 K. (c) shows the number of sorbitol$_{d4}$ in the lamellar (■) and the bulk solution (●) phases. The total number of sorbitol per EYL in this sample is 0.76.

Figure 4
The total hydration for samples of DOPC/D_2O (⚲) and DOPC/D_2O/sorbitol (○). The total sample hydration is expressed as a mole ratio of water per lipid but note that, in the presence of solutes, not all of the water is interlamellar. The hydration of the sample with sorbitol is divided into the hydration of the lamellar phase (solid line) and the water in the unfrozen extra-lamellar solution (broken line). There are 0.52 sorbitol molecules per lipid molecule in this sample. The freezing point depression is greater in the presence of solutes than in the absence of solutes, so there are more data for DOPC/D_2O.

Figure 5
(Minus one times) the water potential ($\Psi \equiv (\mu - \mu^o)/v_w$) is plotted on a logarithmic axis as a function of hydration of DOPC lamellar phases. In the absence of solutes (□), an approximately exponential force law is obvious. The dashed line is an exponential fit to the data of Ulrich et al (1994) for the hydration force in DOPC lamellar phases at room temperature. The water potential in the presence of solutes is also shown (■) as a function of hydration. In the presence of solutes, the chemical potential of water must be more negative to achieve the same hydration. The dotted line is the result of subtraction from the hydration of the lamellar phase the hydration of the solutes acting alone. The solid line is the result of adding to the Ψ of the lamellar phase including sorbitol the osmotic pressure (Π_s) of a bulk solution with the same composition as the inter-lamellar layer.