

## Effect of Trehalose and Sucrose on the Hydration and Dipole Potential of Lipid Bilayers

M. del C. Luzardo,\* F. Amalfa,\* A. M. Nuñez,\* S. Díaz,<sup>†</sup> A. C. Biondi de Lopez,<sup>†</sup> and E. A. Disalvo\*

\*Laboratorio de Fisicoquímica de Membranas Lipídicas, Cátedra de Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, and <sup>†</sup>Instituto de Fisicoquímica, Universidad Nacional de Tucumán, Tucumán, Argentina

**ABSTRACT** The water activity in dimyristoylphosphatidylcholine (DMPC) decreases by 60% when the lipid is dehydrated in the presence of trehalose concentrations higher than 0.02 M. In contrast, sucrose in concentrations 10 times higher produced only a 20% decrease in the water activity in the sample. Titrations of a DMPC solution in chloroform yielded 14 water molecules per lipid when pure water was added and seven water molecules per lipid when the titration was done with 0.025 M trehalose. The same concentrations of sucrose produced a turbid solution, which made it impossible to quantify the number of water molecules per lipid. Lipid monolayers spread on an air/water interface showed a decrease from 480 mV in pure water to 425 mV in 0.1 M trehalose. However, the same concentrations of sucrose produced an increase of less than 100 mV. Results obtained with Fourier transform infrared spectroscopy (FTIR) under the same conditions denoted that trehalose binds to the carbonyl groups, while sucrose showed no specific binding. It is concluded that per lipid molecule, 11 of 14 water molecules can be replaced by three trehalose molecules. About four are displaced by changes in the water activity of the bulk solution, and seven by specific interactions with the phospholipids. In this last case, at least two of them are linked to the carbonyls, and this appears to be the cause of the decrease in the dipole potential of the membrane. In contrast, four sucrose molecules displace only three water molecules per lipid, with no effect on the dipole potential or the carbonyl groups.

### INTRODUCTION

The organization of water at a lipid/membrane interface is a matter of interest, because it determines important functional properties of biomembranes, such as the excluded volume, hydration forces, and the dipole potential (Disalvo and de Gier, 1983; Simon and McIntosh, 1989; Rand and Parsegian, 1989). The excluded volume contributes to the barrier of permeability and to the membrane structure (Disalvo and de Gier, 1983). The hydration water also accounts for the repulsion energy between surface membranes, thus counteracting the adhesion, adsorption, and aggregation processes (Simon and McIntosh, 1989; Rand and Parsegian, 1989).

Phase diagrams show that 18 water molecules per lipid is the limit beyond which water in excess is incorporated into the interlamellar space and does not form part of the membrane structure (Marsh, 1990). According to x-ray diffraction studies, a certain number of water molecules, roughly equivalent to a first hydration shell around the phospholipid headgroups, belong to the lipid phase (Simon and McIntosh, 1985). Calorimetry, sedimentation, and equilibrium dialysis experiments have demonstrated that 18–24 water molecules, depending on whether the lipids are stabilized as large or small vesicles, contribute to the bilayer thickness and

hence to the permeability barrier (Jendrasiak and Hasty, 1974; Lis et al., 1982; Wiener et al. 1989, Disalvo and de Gier, 1983). On average, water locates around the polar headgroups with slight penetration of the region of the ester bonds between the glycerol backbone and the fatty acid residues (Wiener et al., 1989).

When anhydrous lipids are hydrated, the first four or five water molecules confer a mobility on the headgroup, glycerol, and carbonyl moieties (Korstanje et al., 1989). This water appears to locate around the phosphate groups, as deduced from infrared spectroscopy measurements in which the displacement of the asymmetrical frequency from 1250 in the dehydrated state to 1230 nm in the hydrated state has been observed (Hübner and Blume, 1998). In an excess of water, it has been postulated that the first layer of water molecules, bound by hydrogen bonds to membrane headgroups, is polarized, and the second layer has no preferential orientation (Marrinck and Berkowitz, 1995; Essman et al., 1995; Nicklas et al., 1991). This polarized water is not affected by the neutralization of the dipoles corresponding to the carbonyl groups or by sucrose, as shown by measurements of dipole potential in monolayers and with FTIR. In contrast, trehalose decreases the dipole potential, which is interpreted to be a consequence of the displacement of polarized water (Diaz et al., 1999).

Trehalose and sucrose have been reported to be efficient cryoprotectants, which is ascribed to the replacement of water at the membrane structure (Crowe et al., 1984b). Trehalose is effective in replacing water when the bilayers were dried under drastic conditions (Crowe et al., 1984a,b; Tsvetkova et al., 1988; Alonso-Romanowski et al., 1989). However, it is not clear how many water molecules can be displaced by trehalose and sucrose from the lipid bilayer in

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Address reprint requests to Dr. E. A. Disalvo, Laboratorio de Fisicoquímica de Membranas Lipídicas, Cátedra de Química General e Inorgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 2°P, 1113 Buenos Aires, Argentina. Tel.: 54-11-4964-8249; Fax: 54-11-4964-8274; E-mail: eadisal@satlink.com.

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an excess of water. In addition, as the structure and dynamics of the membrane are influenced by the surrounding medium and, vice versa, the membrane (the lipids) exerts a strong influence on the liquid vicinity (Nicklas et al., 1991), it would be important to know how many of those water molecules affect the magnitude of the dipole potential, which of them can be replaced by sugars in an excess of water, and which are the sites or chemical groups at which the exchange with water is taking place. Knowledge of these details would give insight into the mechanisms of the protective action of the different saccharides for membranes under stress and into the number of water molecules at the hydration layer around the chemical groups of the phospholipids.

The purpose of this work is to determine the number of water molecules that must be displaced by trehalose and sucrose to affect the dipole potential. With that aim, the water activity in dimyristoylphosphatidylcholine (DMPC) samples after dehydration in the presence of different trehalose and sucrose ratios was determined. These results were compared with the hydration number of the lipids obtained in the formation of reversed micelles of the same lipid in chloroform. In parallel, the dipole potential was determined in monolayers spread on the interface of solutions having different concentrations of these sugars. The specific interaction of these sugars with the lipids was quantified by measurements of FTIR spectroscopy. From the comparison of these results, the amount of polarized and nonpolarized water and the different strengths with which they are bound to the phospholipid can be inferred.

## MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Birmingham, AL) and used as received after its purity was checked by thin-layer chromatography.

Trehalose and sucrose were from Fluka or Merck and purified by recrystallization in ethanol. Final purity was checked by thin-layer chromatography. Deuterated water was from Sigma Chemical Co. KCl for monolayer experiments was roasted at 700°C before use. All other chemicals were of analytical grade, and the solution was prepared in Milli-Q water.

## Water activity in lipid dispersions

Samples for water activity measurements in mixtures with different sugar/lipid ratios were prepared by weighing an exact amount of a lipid film (10 mg) evaporated from a chloroform solution quantified by phosphorous and an exact weighed amount of the sugar. The mixtures were hydrated in an exact weight of water (usually 1 ml) and dispersed above the transition temperature.

The samples were dried at 45°C to constant weight in a stove, and the value of water activity was determined with a Novasina water activity measurement device. Then the sample was dehydrated at 70°C under vacuum in a SpeedVac centrifuge, and the final water activity was determined in the same way. The percentage of decrease in water activity was calculated from the relative difference of those values for each sugar/lipid ratio.

The water activity value ( $a_w$  value) designates the equilibrium of water determined by the partial water pressure on the surface of a substance. The  $a_w$  value depends on the composition, the temperature, and the water content of the substance. The determination of the  $a_w$  value is made via a sorption isotherm based on the measurement of the water content. However, this method provides much less accurate results than a direct determination of  $a_w$  measurements.

The correct determination of  $a_w$  requires thermal and humidity equilibrium

The  $a_w$  value was determined in an electrolytic Novasina sensor, which is free of hysteresis and measures the values within a tolerance of 0.005  $a_w$ . The Novasina instrument is calibrated with a salt of known water activity at controlled temperature (25°C). The sample is placed in a temperature-controlled sample holder, and measurements are taken when the equilibrium is reached.

From the relative decrease in  $a_w$  for each sugar/lipid ratio, the amount of water can be estimated, taking as a reference value 18 water molecules per lipid for phosphatidylcholine-in-water phase diagrams (Marsh, 1990). These values were compared with those obtained by titrating a solution of phospholipids in chloroform.

## Water/lipid ratio in reversed micelles

A lipid solution of 18 mM DMPC in chloroform, evaluated by phosphorous analysis, was titrated with water and aqueous solutions of different concentrations of trehalose or sucrose at 25°C. After each addition a brief sonication was imposed to achieve a transparent solution. The final point was taken as that at which the addition of an excess of water promoted the appearance of turbidity in the sample. To visualize the final point, small amounts of methylene blue was included in the aqueous solutions, and the changes followed spectrophotometrically. The ratio of water to lipid was calculated from the volume (in microliters) of aqueous solution added for each sugar concentration.

## Dipole potential in monolayers

The dipole potential was determined in monolayers of DMPC spread on an air-water interface, following the procedure described before (MacDonald and Simon, 1987; Luzardo et al., 1998). Aliquots of an evaluated chloroform solution of lipids were added to the air-water interface, exhaustively cleaned by suction. The titration of the surface of solutions of different sugar concentrations with a chloroformic solution gave a variation of the surface potential as a function of the lipids until a constant potential was reached above 20 nmol of lipids. These values were constant at constant temperature and characteristic for each concentration of sugar in the subphase.

Temperature was measured with a calibrated thermocouple immersed in the subphase and maintained within  $\pm 0.5^\circ\text{C}$ .

The values of potential across the interface ( $V$ ), either with or without lipids, were determined by using the following expression:

$$V = V_{\text{Ag/AgCl}} - V_{\text{grd}} = V_{\text{solution}} - V_{\text{grd}},$$

where  $V_{\text{Ag/AgCl}}$  is the potential of the reference electrode and  $V_{\text{grd}}$  is the potential of the shield covering the ionizing electrode. For each trehalose or sucrose concentration a different value of  $V$  without lipids was obtained. The addition of lipids to the interface produced in all cases an additional decrease. The values of the dipole potential were calculated as the difference between the potential across the interface without lipid at each sugar concentration and that obtained when the interface of that solution was saturated by the lipid monolayer under the conditions described above.

The standard deviations of dipole potentials of different measurements are included in Fig. 3.

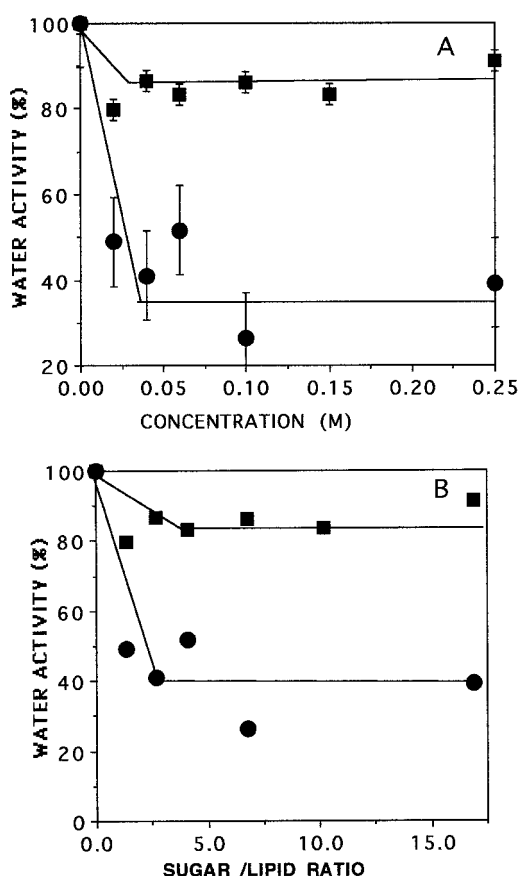


FIGURE 1 Water activity of DMPC after dehydration in the presence of trehalose and sucrose. (A) Water activity as a function of sugar concentration. (B) Water activity as a function of the sugar/lipid ratio. ●, Trehalose; ■, sucrose. Lines are used only to indicate the trend. The 100% activity corresponds to the hydration water of the lipids obtained by drying lipids, after dispersion in pure water, under vacuum at 70°C.

## FTIR

FTIR measurements were made with DMPC from Avanti Polar Lipids, dispersed in D<sub>2</sub>O or in D<sub>2</sub>O solutions of trehalose and sucrose, at temperatures below and above the phase transition, in a Perkin-Elmer 1600 spectrometer, using a cell with AgCl windows and thermostatted with a temperature control to within  $\pm 0.1^\circ\text{C}$ . The resolution of the equipment under the conditions employed in this work was  $2\text{ cm}^{-1}$ . For each condition a total of 1024 scans were carried out. The spectra were analyzed using the mathematical software Grams. The deconvolution algorithm was applied to define the contours of overlapped bands. The bands in the sugar/lipid mixtures were assigned to the carbonyl by comparison with lipids dispersed in D<sub>2</sub>O without sugars. The displacement of the corresponding frequencies was calculated from the spectra for the two carbonyl group populations. The mean values were obtained from spectra in these conditions from a total of four different batches of samples. The standard deviation of the frequency shift calculated from pool of data was about  $\pm 1.5$  in all conditions assayed.

## RESULTS

The water activity remaining in DMPC samples dehydrated in the presence of trehalose or sucrose depends on the

concentration and the type of sugar (Fig. 1 A). For convenience, the data of Fig. 1 A are plotted as a function of the sugar/lipid ratio (Fig. 1 B). At a mole trehalose/lipid ratio of  $\sim 3$ , the percentage of water activity decreases to 40% and remains constant for higher ratios. In contrast, for a similar sucrose/lipid ratio the water activity remains near 90% (Fig. 1 B). This means that the number of water molecules in equilibrium with the vapor phase depends on the type of sugar in contact with the lipid.

The titration with water of a chloroformic solution of anhydrous DMPC gives a limit of 14 water molecules per lipid at 25°C. When this titration was carried out by increasing the trehalose concentration in the aqueous solution, seven water molecules per lipid was obtained for four moles of trehalose per lipid (Fig. 2). In contrast, the titration with a sucrose solution formed a precipitate, and no homogeneous solution could be obtained to quantify the water/lipid ratio (data not shown).

The resulting dipole potential of DMPC monolayers spread at 20°C on the surface of solutions with increasing concentration of trehalose decreased with trehalose concentration (Fig. 3). In contrast, sucrose produces a slight increase in the dipole potential in the same range of concentration.

FTIR assays of lipids dispersed in aqueous solutions of the sugars (Fig. 4) show that the frequencies of vibration of the carbonyls are clearly displaced to lower values by trehalose. However, sucrose produces a slight effect on one carbonyl population and a significant shift to higher frequencies of the vibration modes for the other.

## DISCUSSION

Considering that the procedures of dehydration followed in this work allow us to eliminate the water molecules that are not firmly bound to the bilayer structure, the water activity remaining in the sample after we dry a sample hydrated in water would correspond to the 18 water molecules of the

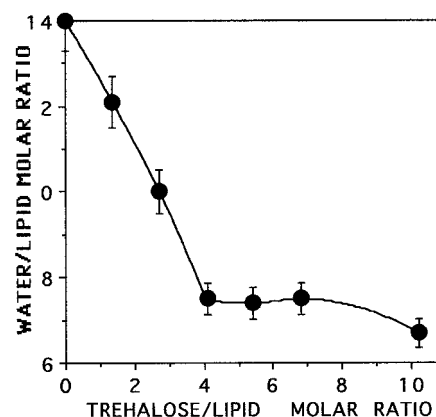


FIGURE 2 Number of water molecules per DMPC as a function of the concentration of trehalose.

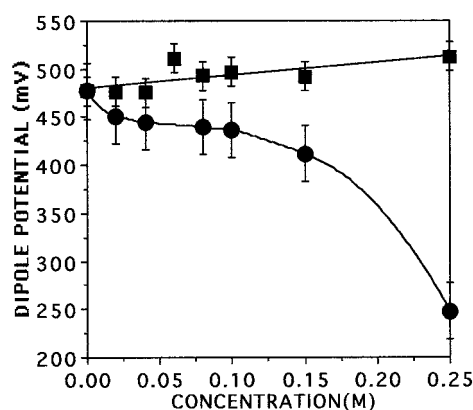


FIGURE 3 Effect of trehalose (●) and sucrose (■) on the dipole potential of DMPC monolayers spread in an air/aqueous solution interface at 20°C.

hydration sphere (Disalvo and de Gier, 1983; Jendrasiak, and Hasty, 1974; Wiener et al., 1989). Taking this value as a reference, the decrease of 60% reported in Fig. 1 represents ~11 water molecules displaced by about four trehalose molecules. The lower number of water molecules remaining in contact with the lipid at the highest trehalose concentrations is seven. For the same trehalose/lipid ratio, the hydration number obtained by titrating a chloroformic solution of anhydrous lipids is also about seven.

It is interesting to note that the hydration number obtained when anhydrous lipids dissolved in chloroform are titrated with water is 14, that is, four molecules lower than the 18 water molecules reported for bilayers in excess water (Marsh, 1990). This suggests that four water molecules are not firmly bound to the lipids and therefore are not incorporated into the reverse micelle. Water inside the reverse micelles would be more tightly bound than those four molecules, but they may be replaced by trehalose, however. The data from monolayers and FTIR show that trehalose interacts with the carbonyls of lipids in an excess of water. Thus this interaction would displace water in the tight hydration

sphere. Molecular dynamic simulations have shown that trehalose can replace water molecules in the water lattice without perturbations (Donnamaria et al., 1994). This seems to be a consequence of the flexibility of the trehalose molecule. This would explain the fact that reverse micelles can be formed in the presence of trehalose, because phospholipids would create similar energetic stabilization, either in trehalose solution or in water. A rough calculation from the data of Fig. 2 can be made, considering that each trehalose molecule forms eight hydrogen bonds. Thus each trehalose molecule is equivalent, in terms of hydrogen bonding, to four water molecules. The four trehalose molecules replacing water would be equivalent to 16 water molecules. A total of 23 water molecules results, when that number is added to the remaining 7 water molecules not displaced by trehalose. This hydration is comparable to the amount of water associated with phosphatidylcholine stabilized in sonicated vesicles (Disalvo and de Gier, 1983). This suggests that the hydration state in reverse micelles is similar to that found in sonicated vesicles.

As described in the Results, it was impossible to form reversed micelles when the water solution contained sucrose. However, an estimation of water molecules displaced by this sugar can be made from the relative changes in water activity. It is observed that the relative decrease of water activity is only 20%, representing three or four water molecules, i.e., the same difference obtained with trehalose between the water activity method and the titration in chloroform. According to the monolayer and FTIR data, sucrose does not interact with the phospholipid groups, and because it does not form inverted micelles, it is inferred that this sugar cannot replace water molecules in the tightly bound hydration sphere as trehalose does. Thus the action of sucrose on bilayers in an excess of water is only colligative; that is, it can extrude water from the bilayer by an osmotic effect. It has been suggested that the hypertonic stress increases the transition and pretransition temperatures of DMPC, as a consequence of a calculated decrease in water activity in the bulk solution as the sugar concentration is increased (Cevc, 1991). In addition, refractive index changes observed in vesicles under osmotic stress have been ascribed to alterations in the extent of hydration and/or lipid packing of the phospholipid (White et al., 1996).

Sucrose displaces only four water molecules. The 60% decrease reported in Fig. 1, equivalent to 11 water molecules, indicates that trehalose seems to extrude those four water molecules and seven additional ones (Fig. 2). The decrease in the dipole potential of DMPC monolayers and the FTIR measurements suggest that the effect of trehalose on the hydration of the phospholipids takes place in an excess of water, by a direct interaction with the phospholipids. The decrease in dipole potential of ~75 mV at 0.1 M trehalose indicates that the dipole density in the bilayer is decreased. This could be ascribed to an expansion of the area per lipid or by a decrease in polarized water. The

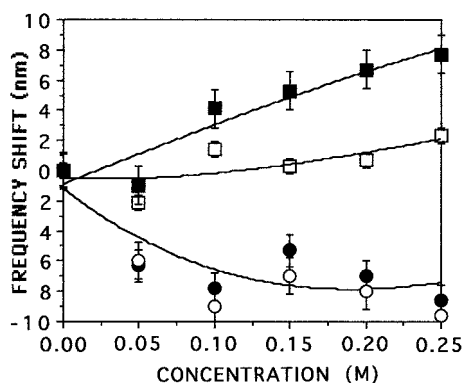


FIGURE 4 Effect of trehalose (○, ●) and sucrose (□, ■) on the characteristic frequency of vibration of  $sn_1$  (○, □) and  $sn_2$  (●, ■) carbonyls.



replacement of ester-linked dipalmitoylphosphatidylcholine (DPPC) by ether-linked dihexadecylphosphatidylcholine (DHPC) produced a decrease of  $\sim 118$  mV, indicating that carbonyls contribute to the dipole potential (Gawrisch et al., 1992). However, by different considerations, they claim that the major contribution to the dipole potential is the first layer of water at the lipid interface.

Recent studies concluded that the bands giving maxima at 1745 and 1728  $\text{cm}^{-1}$  are due to distinct hydrated subpopulations of the carbonyls. The higher absorption band represents non-hydrogen-bonded carbonyls, and the lower absorption band represents hydrogen-bonded C=O groups (Jackson and Mantsch, 1993; Arrondo and Goñi, 1998). However, in the gel and liquid crystalline phases of DMPC, the sn-2 C=O group is more hydrated than the sn-1 C=O (Hübner and Blume, 1998).

The shift of the frequency of the carbonyls to lower values indicates that trehalose binding to both carbonyl populations saturates at 0.1 M trehalose, a concentration at which seven water molecules are displaced. The dipole potential is decreased in 75 mV in this range of concentration. Taken together with the results in reverse micelles and water activity, part of the water in the tightly bound water is polarized at the carbonyl groups.

In the present set-up, dipole potential was measured in monolayers saturated with lipids in the surface of solutions of different sugar concentrations. Under these conditions, a decrease of 75 mV is observed at 0.1 M trehalose. A further decrease to 250 mV is observed at higher concentrations. Studies of the behavior of the film as a function of trehalose concentration, in which the surface pressure is controlled, are in progress.

We suggest that some water molecules may be polarized at the sn-2 carbonyls. However, it must be considered that trehalose may intercalate in the plane of the membrane binding to the sn-1 C=O. There is a report which showed that phospholipid monolayers on an aqueous phase are expanded laterally when trehalose is added to the subphase (Johnston et al., 1984). It has been argued that monolayer effects were mostly caused by surface-active impurities (Arnett et al., 1986). However, this point was thoroughly examined, using several brands of sugars that were purified in ethanol (Alonso-Romanowski et al., 1989). Under these conditions, it was found that the partitioning of a fluorophore was greatly enhanced by trehalose, consistent with a bilayer expansion, but was not affected by sucrose (Alonso-Romanowski et al., 1989).

According to the present results, trehalose appears to be able to intercalate between the phospholipid headgroups, as suggested by the interaction with the carbonyls. Previous results have shown that trehalose can act as a spacer between the lipids, affecting the water permeability of the bilayer and increasing the area per molecule (Viera et al., 1992). In light of those results and the present ones, in which an interesting stoichiometry for trehalose and sucrose

is derived, it seems unlikely that those specific effects, so well correlated by three different methodologies, could be ascribed to impurities.

However, trehalose has been shown to increase the packing density of polar headgroups of phospholipid in unilamellar vesicles (Nishiwaki et al., 1990). This is consistent with the increase in the transition temperature observed in bilayers in an excess of water (Crowe and Crowe, 1991), but not with the monolayer experiments. The stronger hydrogen bonds created by the sugar with the lipids in a lateral array appear to support the interpretation that the trehalose increase in area is due to an increase in lateral interactions of trehalose with the lipids, thus increasing the transition temperature slightly.

Because sucrose does not produce a decrease in the frequency of the carbonyl groups and does not affect the dipole potential, the four water molecules displaced by this sugar are not polarized. It must be noted that sucrose has an effect on the carbonyl frequencies that is qualitatively different from the effect of trehalose and, in addition, is different for the two populations. It can be inferred that the sn<sub>1</sub> group is not accessible to sucrose, which is a reasonable expectation from the location of this group in the bilayer interface. Thus no intercalation of sucrose and area expansion should be expected. The other carbonyl population displaces to higher frequencies, i.e., opposite that occurring with increasing hydration. Therefore, it may be suggested that the osmotic action of sucrose would promote a dehydration of part of the carbonyl population, probably that exposed to water at the sn<sub>2</sub> position. The slight increase in dipole potential suggests that the sucrose effect may increase, to some extent, the packing of the interfacial region, thus increasing the number of dipoles per unit area.

It has previously been speculated that the protective action of trehalose is due to modification of the water structure and dynamics (Kawai et al., 1992), to stabilization of the biological structure by direct interaction (Donnamaria et al., 1994; Crowe and Crowe, 1988), or to a reduction of the interface between lipid and water (Takahashi et al., 1997). From the anomalous hydration properties of trehalose it has been suggested that this sugar can effectively lower the mobility of water molecules bonded to the disaccharide and, therefore, maximize the stability by strengthening the apolar interaction by the stabilization of the surrounding water structure (Kawai et al., 1992). However, results from molecular dynamics support the view that the ability of trehalose to protect against water stress is due to stabilization of the biological structure (Donnamaria et al., 1994). In light of the present results, the stabilization can be ascribed to a direct interaction of the sugar with the carbonyl groups replacing 11 water molecules in a 3–4:1 trehalose/lipid mole ratio. The model suggests that one trehalose molecule fits into the membrane interface, replacing about three water molecules.

At the highest concentration used in this work (0.25 M), trehalose cannot replace the remaining seven water molecules, following the present procedures of dehydration (see Figs. 1 B and 2). Consistently, at those concentrations, seven water molecules can be sequestered by the lipids in chloroform. Thus these water molecules are in a very tightly bound sphere. These last seven water molecules can be displaced in drastic procedures, and they appear to be bound to phosphate groups (Nagase et al., 1998; Crowe and Crowe, 1988). The decrease in the transition temperature of DPPC upon the addition of sugar appears when the water content is less than 8 mol/mol of lipid (Nagase et al., 1997), which is congruent with the data in Figs. 1 and 2. According to Raman and NMR results, the conformation of the  $\text{PO}_4^-$  group is determined by the first four or five water molecules. (Bush et al., 1980; Korstanje et al., 1989). In addition, it has been shown that when the water content in the samples was as low as 0.2 moles of water per mole of lipid, trehalose was able to shift to lower values the antisymmetrical stretching frequency of the  $\text{P}=\text{O}$  bond, suggesting that this sugar can form hydrogen bonds in the absence of water (Crowe and Crowe, 1988). In conditions of extreme dehydration sucrose can also interact by hydrogen bonding with the phosphates. This could be why both sugars are comparable in terms of efficiency in freeze-drying or drying procedures (Strauss et al., 1986). However, based on measurements of carboxyfluorescein retention, Crowe et al. (1985) found that trehalose is much more effective than sucrose in preserving sonicated vesicles, but that the two sugars are similar for extruded vesicles. They suggest that the apparent difference in the abilities of these two sugars to preserve dry liposomes may be related to fundamental differences in their mode of interaction with the bilayer. This point is now clear from the present results.

In conclusion, the present results allow us to describe the hydration structure of a bilayer interphase as being composed of three or four water molecules that are displaced by sucrose or trehalose in a colligative way and are not polarized; seven water molecules tightly bound to carbonyls that are displaced only by trehalose and are polarized, mostly at the carbonyls; and seven water molecules very tightly bound to phosphates that can be replaced by sucrose or trehalose if dehydration is extremely drastic.

The sum of these three types of water is congruent with the total of 18 reported as the nonsolvent water, contributing to the barrier permeability and hydrating the bilayer phase. It follows from these results that the mechanisms of action of trehalose and sucrose are entirely different, probably because of the stereochemical distribution of the OH groups in equatorial and axial positions. However, in drastic conditions, under which water is displaced completely, the two sugars may have similar protective actions. The present results allow us to infer that the dipole potential of a lipid membrane might be modulated by the selective displacement of water molecules by sugars having a specific spatial

distribution of OH, to interact with the carbonyls at the lipid interface.

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