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# Do Trehalose and Dimethyl Sulfoxide Affect Intermembrane Forces?

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The sugar trehalose is produced in some organisms that survive dehydration and desiccation, and it preserves the integrity of membranes in model systems exposed to dehydration and freezing. Dimethyl sulfoxide, a solute which permeates membranes, is added to cell suspensions in many protocols for cryopreservation. Using a surface forces apparatus, we measured the very large, short-range repulsion between phosphatidylcholine bilayers in water and in solutions of trehalose, sorbitol, and dimethylsulfoxide. To the resolution of the technique, the force–distance curves between bilayers are unchanged by the addition of trehalose or sorbitol in concentrations exceeding 1 kmol · $m^{-3}$. A relatively small increase in adhesion in the presence of trehalose and sorbitol solutions may be explained by their osmotic effects. The partitioning of trehalose between aqueous solutions and lamellar phases of dioleylphosphatidylcholine was measured gravimetrically. The amount of trehalose that preferentially adsorbs near membrane surfaces is at most small. The presence of dimethyl sulfoxide in water (1:2 by volume) makes very little difference to the short-range interaction between deposited bilayers, but it sometimes perturbs them in ways that vary among experiments: free bilayers and/or fusion of the deposited bilayers were each observed in about one-third of the experiments. 

Most cells and tissues do not survive severe dehydration. Nevertheless, some plants and animals recommence approximately normal biological function upon rehydration from water contents of several percent (19 and chapters therein, 28). Cellular dehydration occurs in nature as the result of extracellular freezing or equilibration with an unsaturated atmosphere. The freezing of extracellular solutions concentrates the extracellular solutes and elevates their osmotic pressures, leading to osmotic dehydration of the cells. Although most cells and tissues do not survive freezing, some plants and animals resume biological activity upon thawing (30, 29). Damage to cell membranes is the most studied, and perhaps the most important, form of cellular damage caused by freezing and dehydration. There is therefore interest in the proposition that the membrane is protected in some way. This study aimed to investigate one possible manner of protection.

Different types of membrane damage occur in different cells under different conditions (30, 9, 5, 32, 29). One important type of damage is the loss of membrane semipermeability in the dehydrated state. The membrane strains which have been correlated with loss of semipermeability include liquid–crystal–gel phase transitions, lateral phase separations, and the formation of inverted cylindrical micelles which resemble the inverse hexagonal phase (9, 29).

These deformations in cell membranes are also observed in model systems and can be directly related to the stresses[2] produced in membranes by dehydration. When water contents of cells fall to several percent, typical separations between nonaqueous components such as macromolecules or membranes fall to one or two nanometers. At this range, very large repulsive forces, usu-

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[2] The term "stress" is often used metaphorically. In this article it is used in its usual physical meaning of deforming force per unit area.



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ally called hydration forces, are exerted between membranes (18, 13, 26, 39, 37). These forces, which may reach tens or hundreds of atmospheres, produce highly anisotropic stresses in the membranes (33, 36). In lipid lamellar phases, these stresses produce a contraction in the membrane plane and a thickening of the membrane in the normal direction (20). Demixing of membrane components and formation of the inverted hexagonal phase have also been reported in lipid–water systems at low hydration (6, 11, 4).

Several of the species that survive dehydration and/or freezing produce and accumulate cytoplasmic solutes, especially sugars. These have an obvious osmotic effect: high concentrations of solutes depress the chemical potential of water, and so such solutions need lose proportionally less water to come to equilibrium at any given low chemical potential. Some of the solutes which are produced by drying and freezing tolerant species are reported to be effective at preserving membrane integrity (7, 8). Of these, the sugar trehalose has attracted the most attention (7, 8, 21, 17, 32).

If cryoprotectant and anhydroprotectant solutes have effects beyond their osmotic activity, how do they act? It has been previously suggested that such solutes affect the interfacial properties of membrane lipids. In this picture, these solutes would interact directly with the lipid headgroups and partly replace the water molecules at the membrane interface. Some investigations reported changes in surface tension-area isotherms in lipid monolayers (15, 27). When applied to membranes, such intramembrane changes would imply changes in the stress–strain behavior. Other studies, however, have argued that these effects were due to surface active impurities and that they disappeared when the impurities were removed (3).

In this study we investigate the possibility that the putative nonosmotic effects of such solutes might be to change the intermembrane force and thus to change the stresses generated in the membranes by such forces. To do this we measure the intermembrane force using the surface forces apparatus (13, 14). Phospholipid bilayers are deposited on molecularly smooth surfaces of mica in water. The forces are measured by the deflection of a calibrated spring, and the separations are measured using optical interferometry. In the surface forces apparatus, both solute and solvent are equilibrated between the bulk solution and the layer between the surfaces. The osmotic pressure of the solution therefore does not contribute to the force, except possibly for the case when the intermembrane separation is comparable with the size of a solute molecule. This makes the surface forces apparatus ideal to investigate putative nonosmotic effects.

To understand the interaction of solutes with membranes, it is also useful to know whether the composition of the solution near the membrane interface is similar to that in bulk or whether the solute partitions in higher or lower concentration in the interfacial region. For this reason, we undertook a series of partitioning experiments to measure solution composition.

We use dioleylphosphatidylcholine (DOPC) as the model membrane system. Phosphatidylcholines are one of the largest classes of lipids in biological membranes. Further, previous investigations of solute–lipid interactions have most often used phosphatidylcholines (e.g., 17, 21), and DOPC bilayers are in fluid state at room temperature. We examined three solutes involved in cryoprotection. Trehalose is the solute whose role in membrane protection has been most investigated, so we chose that sugar for these experiments. We also studied the effects of sorbitol, another cytoplasmic solute, to allow comparison with another sugar. We also investigated the effects of dimethyl sulfoxide ($Me_2SO$) which

is one of the most widely used artificial cryoprotectants and which is also reported to interact with membranes (2).

The short-range intermembrane repulsion was measured in solutions of sugars and $Me_2SO$. The controls were measured in pure water. The strong inter-bilayer repulsion at close approach (hydration-steric force) is at most weakly dependent on a range of dissolved ions (23), although electric double-layer interactions may depend weakly on the pH.

## MATERIALS AND METHODS

*Langmuir–Blodgett depositions and surface forces apparatus.* Forces and separations between surfaces were measured using a variant of the technique described by Israelachvili and Adams (14). The force (precision of about 2%) was obtained as a function of distance (precision ± 0.1–0.2 nm) between two molecularly smooth, curved surfaces. The water used was obtained from an Elga system and degassed. The lipids used were bought from Avanti Polar Lipids and the nominal purity was greater than 99%. Ethanol and chloroform were Merck A.R. grade.

Lipids were dissolved in chloroform, and the solutions were spread on the air–water interface of a Langmuir trough. The solutions were kept under argon in a freezer and were always used within a week of preparation. All glassware was cleaned in sulphochromic acid and rinsed in Elga water.

The depositions were conducted at constant surface pressure and the isotherms were measured in a Langmuir trough (25). All experiments and preparation were conducted at 21 ± 1°C.

Lipid bilayers were deposited on mica surfaces with the Langmuir–Blodgett method. The first monolayer deposited on the mica surface was dimyristoylphosphatidylethanolamine (DMPE) deposited at a surface pressure of 42 $mN \cdot m^{-1}$. This gives a stable, homogeneous hydrophobic surface. The second monolayer was DOPC deposited at a surface pressure of 38.5 $Mn \cdot m^{-1}$. This gives a surface density comparable with that in fluid bilayers. Further details have been given elsewhere (37).

In all surface forces experiments the total volume of solution was 8 ml. Control experiments were performed in (nominally pure) water from the Elga system. Some water was replaced by concentrated sugar and $Me_2SO$ solutions, without unsealing the surface forces apparatus.

This technique of changing the medium from pure water to concentrated solution allows comparison of the forces between the same pair of bilayers and thus allows maximum sensitivity to changes produced by the solutes. Some previous studies have shown that depletion of the bilayers exposed to water may change their interaction (12). It could be argued that the removal of medium removes some lipids in monomer solution[3] in the medium and thus might reduce slightly the lipid density in the bilayers. For this reason we conducted further control experiments in which the concentrated solution was serially diluted. Following the measurements of force–distance curves in the presence of solutes, 4-ml volumes of solution were removed and replaced with pure water up to seven times. This reduced the concentration by a factor of up to 130. The force–distance curves measured following these dilution series were similar to those obtained before the injection of the solutes.

*Contamination of sugars by surfactants.* Arnett *et al.* (3) report that some sugars, as supplied by chemical companies, are contaminated with surfactants. Their report cast some doubt on the reported effects of sugars on the properties of phospholipid monolayers. We therefore tested each of

[3] The solubility of double chain lipids in water is very low and so this effect is understandably small.

the sugars used for surfactant impurities and, where necessary, we treated the sugars to remove the impurities.

To test for surface active contaminants, a solution of sugar was poured into a large Langmuir trough and the surface tension was measured as a function of time. A Teflon barrier was then swept across the surface to reduce the area on the side of the tension balance by a factor of 10. The surface tension was then measured as a function of time. Surface tensions were measured by the Wilhelmy method (22). Absolute accuracy was $\pm$ 3 mN $\cdot$ m$^{-1}$. The sensitivity is rather better, however, and measurements were repeatable to about $\pm$ 0.5 mN $\cdot$ m$^{-1}$. Thus, it was possible to measure small differences between samples.

Surface active impurities were found in sucrose and trehalose but not in sorbitol. They were removed using activated charcoal. Ten grams of activated charcoal was added to the solution which was then vigorously agitated by passing through it a stream of nitrogen gas for 10 min. The solution was then filtered through a sintered borosicilate glass filter to remove the charcoal. The solutions that were used in the surface forces apparatus were further filtered with a 200-nm Millipore filter.

For the treated and filtered solution of trehalose, the measured surface tension was 72 mN $\cdot$ m$^{-1}$. The change measured before and after compression and aspiration were both 0.1 $\pm$ 0.5 mN $\cdot$ M$^{-1}$. From these measurements we conclude that the treatment with activated carbon is sufficient to remove measurable quantities of surfactant contaminants.

*Partitioning of trehalose.* We prepared samples containing DOPC and 2 *M* trehalose solution (quantities about 150 and 700 mg, respectively). Samples were prepared in two different ways: in the first method sugar was added to the excess water above a lamellar phase containing no solutes, while in the second a lamellar phase with a concentrated sugar solution was diluted with water. In control experiments, DOPC was mixed with water. In all cases a thorough mixing was achieved by centrifugation, vortex mixing, and sonication. Both methods gave similar results, indicating that there was equilibration between the interlamellar solution and the excess solution.

The mixtures were then centrifuged until complete phase separation. The trehalose content of the aqueous solution was determined gravimetrically and an assay of nitrogen and phosphorus conducted to ensure that there was no lipid in the dry matter.

## RESULTS AND DISCUSSION

### *Surface Forces Measurements*

The forces between two DOPC bilayers immersed in 1.5 M trehalose and control experiments with pure water are shown in Fig. 1. Results of experiments with 2 *M* sorbitol and $Me_2SO$–water in volume ratio 1:2 are not shown as figures. The distance on the abscissa in the figure refers to the distance between the hydrophobic surfaces at the midplanes of the deposited bilayers. To convert this to intermembrane separation, one must subtract the thickness of one bilayer (further details in 23).

A summary of results for the series of experiments is given in Table 1. Within the limits of sensitivity of this technique neither the decay length of the short-range repulsion nor its value at any separation is changed by the presence of trehalose, sorbitol, or $Me_2SO$. The adhesion is somewhat increased by all three compounds (Table 1).

The similarity of force–distance curves with and without solutes, especially at small separation, is not what one would naively expect if there were a strong interaction between solutes and the bilayer surface. If, for instance, solutes were adsorbed on the surface, one might expect that an

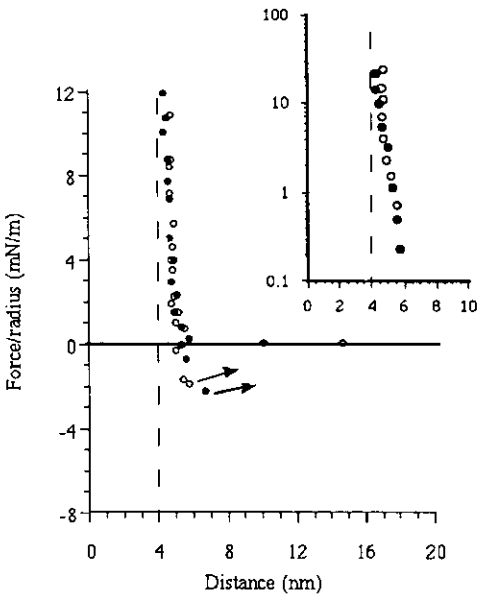


FIG. 1. Force *vs* distance curves for DOPC in pure water (open symbols) and 1.5 *M* trehalose solution. The distance refers to that between the hydrophobic surfaces at the mid-planes of the deposited bilayers. To convert this to intermembrane separation, one must subtract the thickness of two DOPC monolayers. This thickness cannot be measured to the same precision as the distance measurements in this experiment, so the absolute separation is known less precisely than is the relative separation. The uncertainty in bilayer thickness does not concern the comparative experiments done here, because all comparisons were between control and experiment on the same membranes. An estimate of the thickness of a DOPC bilayer deposited under the conditions used here can be obtained in the manner described in ref.23. It is 4.0 ± 0.4 nm. On the figure, the vertical dashed line at 4.0 nm may be regarded as the *approximate* position of the zero for intermembrane separation, but statements about absolute membrane separation must be made with caution because of the relatively large uncertainty in the value of membrane thickness. The forces are expressed divided by the radius of the cylindrical surfaces upon which the bilayers are deposited. These curves are equivalent to energy of interaction as a function of distance for two plane surfaces.

increased repulsive force would be encountered at very close approach. The increase in adhesion caused by the solutes sorbitol and trehalose can be explained by their osmotic effect. Both solute molecules are larger than water, and so it is plausible that, when the surfaces are brought together, they are excluded from the region of closest approach. This region would then have a lower solute concentration and a lower osmotic pressure. We show under the Appendix that the required region of solute exclusion has a thickness of only molecular dimensions, and thus that this effect is large enough to explain the observed change in adhesion. For $Me_2SO$ the origin of the increase in adhesion is not clear. This molecule permeates membranes and thus it is not easy to analyze its osmotic effect on adhesion.

$Me_2SO$ exhibits further behavior that was not observed with trehalose nor sorbitol and which varied among experiments. In about one-third of the $Me_2SO$ experiments, freely suspended bilayers were formed after a first force measurement (multiple bilayer formation in the surface forces apparatus is described by 1). In another one-third of the cases the two bilayers deposited on the mica fused into one bilayer (fusion in the surface forces apparatus is discussed by Horn (13) and Wolfe *et al.* (37)). In the remaining one-third of the experiments, the force–distance curves were consistent with neither fusion nor the presence of free lamellae (as in Fig. 1).

Such a behavior suggests that the elastic properties of the bilayers may be affected by the presence of $Me_2SO$. This hypothesis can be checked because any decrease in the bending modulus enhances the repulsive undulation forces between bilayers (1) and consequently increases the repeat distance of the lamellar phase structure. To investigate this possibility, the repeat distances were measured by small-angle X-ray scattering performed on a lamellar phase of DOPC in excess water (DOPC and water were 1:3 by mass) and on a lamellar phase of DOPC in excess $Me_2SO$ solution. (The DOPC and solution were 1:3 by mass, and solution was water and $Me_2SO$ 3:1 by

TABLE 1
Change in the Force Measurements Due to the Cryoprotectant

| | Slope (nm) | Adhesion ($mN \cdot m^{-1}$) | Distance at 1 $mN \cdot m^{-1}$ (nm) |
|---|---|---|---|
| DOPC: (pure water) | 0.33 | 1.2 | 5.6 |
| | Increase in slope (nm) | Increase in adhesion ($mN \cdot m^{-1}$) | Increase in distance at 1 $mN \cdot m^{-1}$ (nm) |
| Trehalose | $0.01 \pm 0.4$ | $0.8 \pm 0.8$ | $-0.1 \pm 0.4$ |
| Sorbitol | $-0.01 \pm 0.01$ | $1.2 \pm 0.3$ | $0.1 \pm 0.0$ |
| $Me_2SO$ | $0.00 \pm 0.06$ | $1.5 \pm 1.0$ | $0.3 \pm 1.0$ |

*Note.* Three parameters are used: (a) the slope of a semi-log plot of the force between surfaces vs the separation. The slope was measured in the force range 2.5 to 25 $nM \cdot m^{-1}$, (b) the adhesion obtained during the separation process, and (c) the distance observed when the value of $F/R$ is 1 $nM \cdot m^{-1}$ (the distance is the thickness of two DOPC monolayers + water/solution layer). For each cryoprotectant, the difference between values from the experiment with the cryoprotectant and from the control experiment (DOPC bilayers in pure water) is given.

mass). For the two lamellar phases, the repeat distances were the same within 5%.

*Partitioning*

These measurements were performed to determine whether the putative trehalose–phospholipid interaction caused the sugar to partition preferentially into or near the solution–membrane interface. These gravimetric measurements have limited sensitivity, but they provide a useful control for the surface forces experiments because they impose limits on the extent to which the solution composition may differ between the bulk aqueous solution and the thin solution layer between approaching bilayers.

The weight of the dried material allowed us to calculate the extent to which sugar was adsorbed into the interlamellar solution. This is expressed as the average number $r$ of trehalose molecules per lipid molecule by which the concentration of trehalose in the lamellar water exceeds the concentration of trehalose in the excess solution. The value for $r$ is $0.06 \pm 0.22$.

This is consistent with the results of the force measurements. We would have expected a substantial adsorption of trehalose at the lipid–water interface to increase the intermembrane repulsion at very close approach, but this was not observed. The negative results of the partitioning experiments show that there is unlikely to be a substantial difference between the composition of the bulk solution in the surface forces apparatus and the solution layer between the approaching membranes.

*Conclusions: Possible Implications for Cryo- and Anhydrobiology*

All experiments gave largely negative results. Within the accuracy of the measurements, there is no specific effect on the inter-bilayer force of trehalose and sorbitol at concentrations of 1.5 and 2.0 *M*. No substantial adsorption at the membrane water interface was observed for trehalose or sorbitol.

What do these results imply for the effect of solutes on membranes in cells? It is important to note that, in these experiments, the applied force is the independent variable. The only likely osmotic effect of the solutes on intermembrane interaction is that discussed above: the exclusion of solutes at very small separation. Otherwise, the technique used here is independent of the osmotic pressure of the interlamellar medium. This makes the technique ideal for investigating specific interactions and, in this case, shows that specific effects of trehalose or sorbitol on the forces between

DOPC bilayers, if they exist, are small. The reason why some solutes are more effective than others remains unknown.

Several possible differences among solutes may be relevant. One is the molecular volume: at very high concentrations, when the solute volume is a substantial fraction of that of the solution, large solutes would maintain a greater membrane separation and lower inter- and intramembrane stresses (5, 36). At very high concentrations, nonideal osmotic behavior may also distinguish among different solutes, and we are currently studying this possibility using a forces technique that allows us to work at freezing temperatures (39). Finally, not all solutes may achieve the same aqueous concentration during dehydration. The different effects of different solutes may depend on their tendency to crystalize (10, 16).

## APPENDIX

### *Osmotic Effect on Adhesion*

The separation $h$ between the surfaces is a function of $r$, the radial distance from the axis of symmetry upon which $h$ has its minimum value $h_0$. To estimate the osmotic force, we use the following simple model. Suppose that the solutes are entirely excluded from the area with separation less than some value, $h$, and that for larger values of separation the water activity has its bulk value. The reduction of pressure in the region from which solutes are excluded will equal the osmotic pressure $\Pi$ of the bulk solution. This osmotic contribution to the adhesion gives an attractive force $F = \pi r^2 \Pi$ which, for this geometry, yields

$$F/R = 2\pi(h - h_0)\Pi.$$

The sugars in these experiments increased adhesion by approximately $\Delta F/R \cong 1$ mN $\cdot$ m$^{-1}$. The osmotic pressures were of the order of 4 MPa. If the osmotic effect alone is responsible for the increased adhesion, then $(h - h_0)$ is about 0.04 nm. The value of $h_0$, the minimum of separation between the membranes, cannot be given with precision because it depends on the definition of the surface of the membrane and on the thickness of the membranes, which is not measured independently in these experiments. Nevertheless, the required value of $(h - h_0)$ is smaller than molecular sizes, and so solute exclusion and osmotic effects may account for the measured change in adhesion (Fig. A).

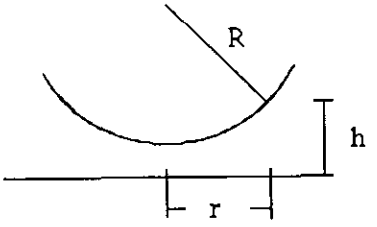


FIG. A. The geometry of the surface forces apparatus is locally equivalent to a sphere of radius $R$ near a plane.

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