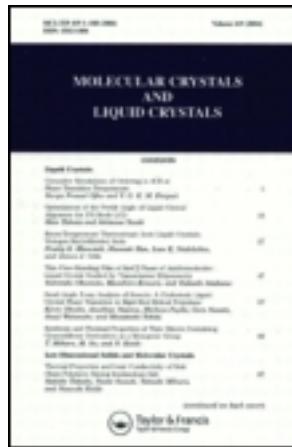


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## Hydration Properties of Disaccharide Aqueous Solutions

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**Abstract** In order to ascertain if disaccharides, besides maintaining the membrane bilayer in a liquid crystalline phase, also obstruct the formation of ice which would damage the membrane structure, we have analysed the interaction mechanisms of trehalose, maltose, and sucrose with water, by performing density and ultrasonic velocity measurements at different concentration values. Secondly, to analyse the effects of trehalose, maltose and sucrose, on the hydrogen bond network of water, we have performed Raman scattering measurements on aqueous solutions of the three disaccharides as a function of concentration. In particular we have focused the attention on the intramolecular OH stretching contribution to evidence differences that could be sufficient to account for the different effectiveness as bioprotectors. What emerges from these studies is that the trehalose-water system, characterized, in respect to the other disaccharides, by the highest value of the hydration number, reveals an extensive destructuring effect on the adjacent water molecules.

**Keywords** aqueous solution; disaccharide; ultrasonic measurements; density measurements, Raman scattering

## INTRODUCTION

Early studies on *Artemia* cysts found that the ability to survive desiccation was coincident with the accumulation of trehalose, a non-reducing disaccharide of glucose[1,2]. Many other anhydrobiotic organisms, including fungal spores, yeast cells, certain soil-dwelling animals, may persist without water for decades and, in some cases centuries, resuming rapidly their metabolism when rehydrated[3-6]. A common feature in these organisms is the synthesis induced by heat shock of a large amounts of trehalose, a non reducing disaccharide of glucose ( $\alpha$ -D-glucopyranosil  $\alpha$ -D-glucopyranoside)[7-11]; its analogue in higher plants appears to be sucrose, which may in some cases make up as much as 50% of the dry weight[12].

Thanks to the extraordinary properties of the disaccharides, during last years numerous indications of a growing technology for stabilizing biomaterials of various composition during air-drying or freeze-drying have been reported. Disaccharides such as sucrose and trehalose have been used widely as excipients during freeze-drying of a variety of materials including products in the pharmaceutical industry[13]. In particular the thermo protective and thermostabilization effect of trehalose on enzymes[14], liposomes[15] as well as on isolated biological membranes or proteins[12,14-16] has been analysed opening new horizons for industrial and medical applications. It has also been predicted that trehalose can be used as an ingredient for dried and processed food, as well as a non-toxic cryoprotectant of vaccines and organs for surgical transplants[13].

The mechanisms by which trehalose mediates stress tolerance has not been completely determined. Green and Angell in their impressive works[7,8] have concluded that the bioprotection from destruction by freezing is related to the high glass transition temperature of trehalose, which implies an higher water content at the transition.

The water replacement hypothesis, first proposed by Crowe[12,17] asserting the bonding of the hydrogen with the polar headgroups of the lipids that constitute biomembranes, accounts for how the nonreducing disaccharides trehalose and sucrose preserve the integrity of biological structures. As the systems are dried or frozen, these interactions replace those of the water of hydration at the membrane-fluid interface. In such a way, in the opinions of these authors, it prevents the phase transition and the accompanying leakage upon rehydration. Findings of heat of solution calorimetry[18] confirm that trehalose is a more effective hydrogen bond donor rather than maltose and sucrose. With respect to lipid membranes, trehalose can

depress the phase transition temperature of membranes so that they remain in the liquid-crystal state even when dry. It is hypothesized that this prevents membrane leakage during rehydration, thereby preserving cellular viability. Many studies have shown that in different membrane systems *in vitro*, trehalose maintains the membrane bilayer in a liquid-crystalline phase when water is removed. In the same way, it has been shown that sucrose can act in water replacement to maintain membrane phospholipids in the liquid-crystalline phase and to prevent structural changes in soluble proteins[19].

Evidently the bioprotective effectiveness of trehalose reflects a complex array of interactions at the structural, physiological and molecular levels. Many of the mechanisms remain cryptic, but it is clear that they involve interactions that derive from the unique properties of the water molecules.

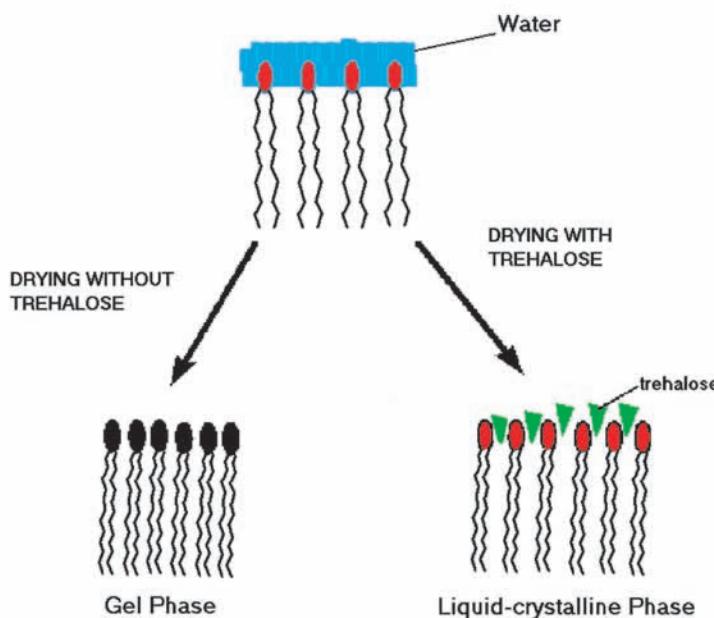


Figure 1 Effect of water replacement of trehalose on membrane lipids.

In order to ascertain if disaccharides, besides maintaining the membrane bilayer in a liquid crystalline phase, also obstruct the formation of ice which would damage the membrane structure, we have analysed the interaction mechanisms of trehalose, maltose, and sucrose with water, by performing density and ultrasonic velocity measurements at different concentration values. Secondly, to analyse the effects of trehalose, maltose and sucrose, on the hydrogen bond network of water, we have performed Raman scattering measurements on aqueous solutions of the three disaccharides as a function of concentration. In particular we have focused our attention on the intramolecular OH stretching contribution to evidence differences that could be sufficient to account for the different effectiveness as bioprotectors. What emerges from these studies is that the trehalose-water system, characterized, in respect to the other disaccharides, by the highest value of the hydration number, reveals an extensive destructuring effect on the adjacent water molecules. These results make plausible the hypothesis that the crystallization process in presence of disaccharides is obstructed by a reduction of the amount of freezable water. The relative effectiveness for preserving biological membranes can be connected with the *magnitude* of the destructuring effect on the tetrahedral hydrogen bonded network; in this frame trehalose reveals to be the most effective.

#### EXPERIMENTAL SET-UP

The investigated samples were purchased from Aldrich-Chemie. The solutions were prepared by weight using double distilled deionized water. Care was taken in order to obtain stable, clear and dust-free sample; ample time was allowed for equilibration. In addition, the solutions were filtered with 0.45  $\mu\text{m}$  Amicon filters and stored in the dark to minimize biological and photo-chemical degradation.

Density measurements were performed using the standard picnometer technique. Ultrasonic velocity measurements were performed by the pulse echo technique using a home-made acoustic interferometer working at the frequency of 3 MHz. We have checked, by performing measurements at some values of concentration and for frequencies from 3 MHz to 20 MHz, that the sound velocity is almost frequency-independent in this range indicating that we are not in the presence of possible relaxation processes. The electronic equipment consisted of a standard Matec Inc. apparatus and the measurements,

performed using the echoes overlapping method, were reproducible within 0.05%.

The samples for the Raman light scattering measurements were sealed in optical quartz cells of inner diameter 5 mm and then mounted in an optical thermostat especially built to avoid any unwanted stray-light contributions. The measurements were performed with a temperature stability better than 0.1°C. The samples of high purity, as well as the optical purity of the sample holder, ensured to collect data with good signal to noise ratio and with high reproducibility.  $I_{VV}$  and  $I_{VH}$  Raman spectra were obtained by a high resolution fully computerized Spex-Ramalog 5 triple monochromator in a 90° scattering geometry. Vertically polarized radiation of an INNOVA 70 Series Ar-Kr gas mixed laser operating in the 4579÷6764 Å was used as an excitation source. To reduce fluorescence we chose the 6471 Å laser line. The laser power was maintained at approximately 1 W. The detection apparatus consisted of a photon counting system whose outputs were processed on line by a computer. The scattered photons were automatically normalized for the incoming beam intensity in order to ensure good data reproducibility. Isotropic scattering intensities were calculated from the parallel and perpendicular components of the scattered light by:  $I_{iso} = I_{VV} - 4/3 I_{VH}$

## RESULTS AND DISCUSSION

To get information on the solute-solvent interaction strength, ultrasonic velocity measurements were performed on trehalose, maltose and sucrose aqueous solutions as a function of concentration. In fact, through the evaluation of the adiabatic compressibility coefficient, we can get information on the disaccharide hydration number,  $n_H$ .

We assume, following a molecular model [20], that the volume of the solution  $V$  can be partitioned into two contributions: the hydration volume,  $V_H$ , where significant interactions between the disaccharide and water occur, and the bulk water volume,  $V_w$ . Following this model the volume of the solution can be written as  $V = n_d V_H + (n_w - n_d n_H) V_w$ , where  $n_d$  and  $n_w$  are the mole numbers of disaccharide and of water, respectively. Under the hypothesis of negligible compressibility for hydrated units, we obtain the solute-solvent interaction strength,  $n_w/n_d(1 - \beta/\beta_w)$ , being  $\beta$  and  $\beta_w$  the adiabatic compressibility of the solution and of water,

respectively. From the extrapolation at infinite dilution we can evaluate the hydration number by the formula:

$$n_H = \lim_{n_d \rightarrow 0} \frac{n_w}{n_d} \left( 1 - \frac{\beta}{\beta_w} \right) \quad (1)$$

In Figure 2, as an example, the behaviour of the solute-solvent interaction strength as a function of the weight fraction,  $\phi$ , is reported for trehalose, maltose and sucrose at  $T=25^\circ\text{C}$ . The continuous lines represent the result of a polynomial fitting procedure. It clearly emerges that, in respect to the other disaccharides, the trehalose-water system is characterized, in all the investigated concentration range, by both the highest value of the interaction strength, and of the hydration number.

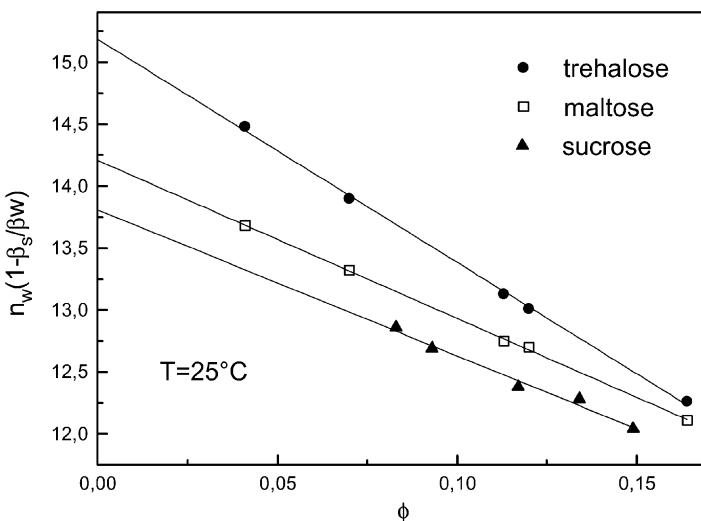


FIGURE 2 Behaviour of the solute-solvent interaction strength of the three disaccharides as a function of concentration at  $T=25^\circ\text{C}$ . The continuous lines represent the result of a polynomial fitting procedure.

In order to get further information on the effects of disaccharides on the hydrogen bonded network of water, we have analysed the intramolecular O-H stretching spectral region.

The existence of an isosbestic point in the isotropic spectrum of pure water suggested to some authors[21] the decomposition of each spectrum into an “open” and a “closed” contribution (see Figure 3).

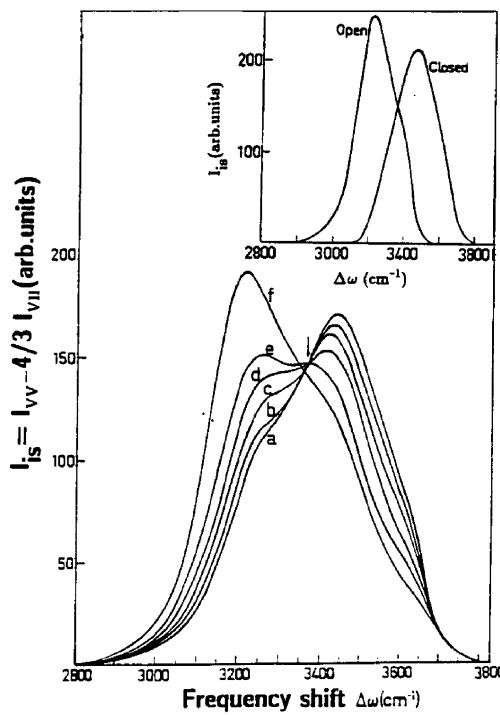


FIGURE 3 Isotropic Raman spectra of the OH stretching bands of liquid water at various temperatures. a: T=95°C, b: T=80°C, c: T=60°C, d: T=40°C, e: T=20°C, f: T=24°C. (P. Migliardo et al. *Physical Chemistry of Aqueous Systems: Meeting the Needs of Industry*, edito da H. J. White, J. V. Sengers, D. B. Neumann, J. C. Bellows, Begell House New York, 317 (1995))

The first one has been attributed to the O–H vibration in tetrabonded H<sub>2</sub>O molecules that have an “intact bond” and originate low density patches in the system, while the second one would correspond to the O–H vibration of H<sub>2</sub>O molecules that have a not fully developed hydrogen bond (distorted bond). The open Gaussian component is centered near 3210 cm<sup>−1</sup>, while the closed component is centered near 3420 cm<sup>−1</sup>[22]. With declining temperature, one finds that the “open” contribution increases indicating an enhanced hydrogen bonding which gives rise to patches of four bonded water molecules.

In the case of disaccharide aqueous solutions, also because of the relatively low pure sugar contribution, such an approach has been demonstrated still valid [23]. A further key observation resulting from an inspection of the Raman spectra of disaccharides aqueous solutions is that no additional Gaussian components at higher frequencies were required to fit the OH-stretching contour, that is, no components, whatever, near 3500 and 3620 cm<sup>−1</sup> were required at high concentration. The higher frequency components arise when hydrogen bonds are broken and their absence in spectra indicates that free O–H groups are absent. The numerical curve-fit coefficients resulting from the decomposition procedure for three different concentrations are reported in Tab.1. As we can see the most noteworthy feature is that, by decreasing the water content, one observes a marked decrease of the percentage area of the “open” contribution which tends to a plateau value for  $\phi \geq 0.166$ . These findings are consistent with the picture of a disaccharides destructuring effect on the H<sub>2</sub>O tetrabonded network of molecules that originates low density conformations similar to that of supercooled water. In addition, for the same concentration the integrated area values decrease. This indicate that the addition of sugars tends to destroy the configurational arrangement of water which would give rise, by cooling, to ice.

In conclusion, the peculiar trend of the intramolecular mode suggests that among the three disaccharides, trehalose promotes the *most substantial* changes in the intramolecular spectral band characteristic of pure water. As a result, the amount of freezable water is reduced and the crystallization process is hindered.

TREHALOSE					
	Peak	PkCtr	Wid	Area	%Area
$\phi = 0.166$	open	3210	171	3.58e+04	20.01
	closed	3417	271	9.915e+04	79.99
$\phi = 0.09$	open	3210	171	3.58e+04	27.7
	closed	3417	271	9.915e+04	72.3
$\phi = 0.038$	open	3210	171	3.58e+04	42.94
	closed	3417	271	9.915e+04	57.06
MALTPOSE					
	Peak	PkCtr	Wid	Area	%Area
$\phi = 0.166$	open	3191	181	3150	21
	closed	3386	261	1.184e+04	79
$\phi = 0.09$	open	3191	169	4.107e+04	31.50
	closed	3386	261	8.932e+04	68.50
$\phi = 0.038$	open	3191	218	1.534e+05	44.72
	closed	3386	292	1.897e+04	55.28
SUCROSE					
	Peak	PkCtr	Wid	Area	%Area
$\phi = 0.166$	open	3201	201	3444	22.51
	closed	3390	363	1.186e+04	77.49
$\phi = 0.09$	open	3201	176	4.632e+04	34.37
	closed	3390	265	8.843e+04	65.63
$\phi = 0.038$	open	3201	209	1.568e+05	45.62
	closed	3390	280	1.869e+05	54.38

TABLE 1 Numerical curve fit coefficients relative to the isotropic spectra of the three disaccharides at three different weight fraction values.

### CONCLUDING REMARKS

In the present work we have analysed the hydration mechanisms of disaccharides with water. The results obtained by analysing the intramolecular OH stretching spectral contribution, are consistent with the picture of a disaccharides destructuring effect on the H<sub>2</sub>O tetrabonded network of molecules that originates low density conformations similar to that of supercooled water. In addition the

peculiar trend of the intramolecular mode suggests that a more marked destructuring effect on the tetrahedral hydrogen bonded network, occurs in the presence of trehalose rather than in the presence of sucrose or maltose.

What emerges from these data is that the disaccharides function on water is to provide a structure which enable the OH group on the sugar residues to take up spatial and orientation positions that are not compatible with those of ice. In this frame, the trehalose-water system, characterized, in respect to the other disaccharides, by the highest value of the hydration number, reveals a more marked destructuring effect on the adjacent water molecules.

In order to "simulate" the interactions among the membrane hydrophilic groups and the disaccharides, new measurements in a confined geometry are actually in progress.

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