

## $\alpha,\alpha$ -Trehalose—Water Solutions. 1. Hydration Phenomena and Anomalies in the Acoustic Properties

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The topic dealt with in this paper concerns an experimental study, performed by means of ultrasonic techniques, on aqueous solutions of  $\alpha,\alpha$ -trehalose, an effective bioprotector against dehydration. The ultrasonic velocity and density data show that, in these aqueous solutions, the mixing process is not ideal. The behavior of the excess compressibility as a function of concentration can be interpreted by supposing that  $\alpha,\alpha$ -trehalose molecules form intramolecular hydrogen bonds by folding over at high concentration. When water molecules are added, they spread out again, thereby releasing hydrogen bond sites for water interactions and increasing the water–sugar interaction strength. Furthermore, the temperature dependence of the hydration number has been evaluated, and its value at room temperature is very close to that given in the literature.

### General Considerations

Clear evidence is found in literature<sup>1–5</sup> that trehalose ( $\alpha$ -D-glucopyranosyl- $\alpha$ -D-glucopyranoside), a nonreducing disaccharide of glucose, is synthesized by various molecular and cellular systems, e.g. plant seeds,<sup>6</sup> spores,<sup>7</sup> cysts of certain crustaceans.<sup>8</sup> In particular some species of soil-dwelling organisms<sup>9</sup> can pass, during dry times, into a state of suspended animation involving a possible chemical action of trehalose. These species can be dehydrated nondestructively and reversibly because of their ability to synthesize this disaccharide. They thus succeed in surviving for long periods (up to 120 years) in drought conditions and then recover full viability upon rehydration. Other sugars, such as maltose, sucrose, and glucose, have shown similar properties to protect against dehydration, but trehalose is significantly more effective. Green and Angell<sup>1,2</sup> have related this effectiveness to the fact that trehalose possesses the highest glass-transition temperature in the presence of water. It is a widely held view that the high viscosity and the attendant suppression of crystallization of the solid phase play an important role in maintaining for decades the structural and functional integrity of organisms, which reach an anhydrobiotic state by passing into the vitreous state. Grigera et al.<sup>3</sup> have recently performed a molecular dynamics (MD) simulation of aqueous solutions of  $\alpha,\alpha$ -trehalose at room temperature by using the SPC/E model.<sup>6</sup> They found that both the structural and dynamical properties of water are only slightly perturbed by the presence of the disaccharide, which tends to bond up to 10 water molecules, creating a hydrophilic surface with typical hexagonally coordinated units.<sup>10</sup> Therefore, they suggest that no trehalose-induced destructuring effect occurs in the mixture and that the protective action on biological structures can be attributed to the fact that trehalose may replace a certain number of water molecules that are hydrogen-bonded to the structure in a way similar to water itself. Furthermore, Grigera et al. show that the trehalose molecule is able to have also internal hydrogen bonds (between the two hexapyranose rings) and that, regarding the hydrogen bond network, the particular structural environment in which 10 water molecules fit with each  $\alpha$ -

trehalose is energetically favored. The ability of the trehalose molecule to have a *closed* and an *open* structure depending on the existence of intramolecular H bonds can explain, as will be shown below, the behavior of measured structural parameters as a function of concentration and temperature. Crowe et al.,<sup>4</sup> in their extensive work on this subject, have suggested that the removal of water molecules from the headgroup region of cell bilayer membranes increases the headgroup packing, thus forcing the dry bilayer from the liquid crystalline to the gel phase. Upon rehydration, dry membranes, which are in the gel phase at room temperature, undergo a phase transition from the gel back to the liquid crystalline phase, during which leakage may occur because of packing defect regions. Trehalose protects membrane integrity during drying and rehydration, since it lowers the transition temperature ( $T_{\text{mel}}$ ) of the dry membranes by replacing the water between the lipid headgroups (water replacement hypothesis). In such a way, in the opinions of these authors, it prevents the phase transition and the accompanying leakage upon rehydration.

Although the role of trehalose as a *bioprotector* is well established and widely reported in the literature, mostly from a biological point of view, the molecular processes involved remain unclear and few data exist on the microscopic mechanisms governed by hydrogen bond interactions.

This paper reports first results from a program of research devoted to a quantitative analysis of the physical properties of water–trehalose systems. Before studying the time (using dynamic neutron and light scattering techniques) and spatial (using small-angle neutron-scattering and small-angle X-ray-scattering techniques) properties of the relevant correlation functions, we wanted to clarify the role of hydrogen bonds in determining the unusual molecular properties of this system through the analysis of ultrasonic velocity and density measurements.

Ultrasonic techniques<sup>11,12</sup> can be a rich source of information on structural changes that take place in liquid mixtures such as trehalose–water solutions. In the framework of physical acoustics theory such techniques are also able to furnish useful information concerning the trehalose hydration number,  $n_{\text{H}}$ , and its temperature dependence. It will be shown that the concentration dependence of the adiabatic compressibility as a function

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**TABLE 1: Ultrasonic Velocity  $v_s$  and Density  $\rho$  Data as a Function of Temperature and Weight Fraction  $\phi$** 

$\phi$	$T = 30\text{ }^{\circ}\text{C}$		$T = 40\text{ }^{\circ}\text{C}$		$T = 50\text{ }^{\circ}\text{C}$		$T = 60\text{ }^{\circ}\text{C}$	
	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$
0.188	1.079 91	1571.3	1.074 22	1588.1	1.071 02	1599.0	1.064 05	1605.1
0.322	1.141 99	1652.2	1.139 84	1664.0	1.131 56	1670.3	1.128 51	1673.0
0.387	1.174 02	1692.2	1.170 08	1699.3	1.165 63	1702.1	1.160 12	1702.2
0.487	1.229 03	1744.5	1.222 23	1750.4	1.219 98	1752.3	1.214 35	1752.8
$\phi$	$T = 70\text{ }^{\circ}\text{C}$		$T = 80\text{ }^{\circ}\text{C}$		$T = 85\text{ }^{\circ}\text{C}$			
	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$	$\rho\text{ (g cm}^{-3}\text{)}$	$v_s\text{ (m/s)}$		
0.188	1.059 82	1606.1	1.053 12	1603.7	1.052 31	1601.1		
0.322	1.121 61	1672.4	1.119 08	1665.3	1.115 71	1660.1		
0.387	1.156 07	1701.9	1.149 68	1696.2	1.148 99	1696.2		
0.487	1.209 03	1750.4	1.204 53	1744.2	1.201 86	1740.8		
0.559					1.233 80	1759.4		
0.655					1.292 14	1817.3		
0.703					1.333 32	1861.5		
0.760					1.378 57	1901.1		
0.791					1.405 32	1927.2		

of temperature allows us to deduce hydration numbers  $n_H$  that turn out to be very close to those extracted from the molecular simulation study mentioned previously.<sup>3</sup>

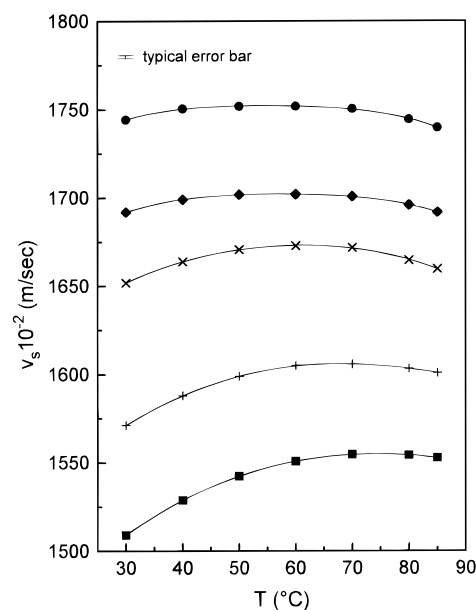
### Experimental Section

The solutions were prepared by weight, using  $\alpha,\alpha$ -trehalose dihydrate, purchased from Aldrich-Chemie, and doubly distilled deionized water. Care was taken in order to obtain stable, clear, and dust-free samples; ample time was allowed for equilibration, and the solutions were stored in the dark to minimize biological and photochemical degradation. Ultrasonic velocity measurements were performed by the pulse echo technique using a homemade acoustic interferometer working at a frequency of 3 MHz. We have checked, by performing measurements at some values of concentration and for frequencies from 3 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 corresponding density measurements, necessary to evaluate the adiabatic compressibility of the solutions by the formula  $\beta = (\rho v_s^2)^{-1}$ , were performed using a standard picnometer technique. The temperature range investigated was 30–85 °C with a temperature control better than 0.1 °C. In Table 1 we report the measured values of the solution sound velocity  $v_s$  and density  $\rho$  at different water content and as a function of temperature.

### Results and Discussion

Figure 1 shows the temperature variation of acoustic velocity for pure water and for weight fraction values of  $\phi = 0.188$ , 0.322, 0.387, and 0.487, with  $\phi = n_t M_t / (n_t M_t + n_w M_w)$ ,  $n_t$ ,  $n_w$ ,  $M_t$ , and  $M_w$  being the mole number and the molecular weights of trehalose and water, respectively. The most noteworthy features are quite evident: the data show an increase of velocity with concentration, a clear indication of trehalose–water interactions<sup>11–14</sup> that give rise to the formation of more incompressible units, and, as a function of temperature, a maximum that shifts toward lower temperatures as the trehalose concentration increases.

Regarding the temperature dependence of the ultrasonic velocity, one can see from Figure 1 that when the trehalose content is raised, this anomalous behavior is progressively attenuated, leading to a shift of the velocity maximum temperature toward lower values. This phenomenon has already been observed in many electrolytic solutions,<sup>13</sup> and it can be related

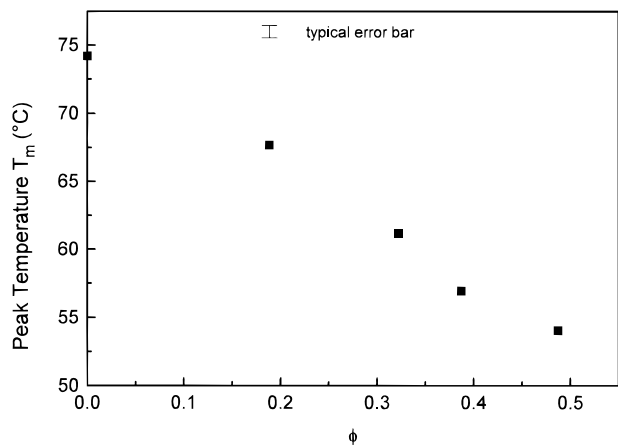


**Figure 1.** Behavior of ultrasonic velocity  $v_s$  as a function of temperature at weight fraction values of  $\phi = 0$  (■),  $\phi = 0.188$  (+),  $\phi = 0.322$  (×),  $\phi = 0.387$  (◆), and  $\phi = 0.487$  (●). Continuous lines represent the fit results.

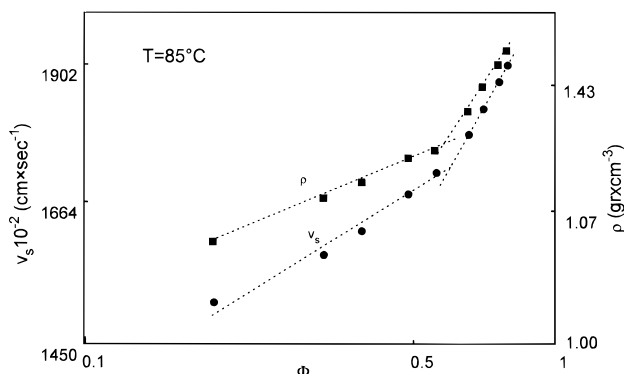
to the building up of a more compact trehalose-imposed structure.<sup>3</sup> It is presumed, and now confirmed by very recent Raman results concerning the OH stretching region,<sup>15</sup> that with increasing trehalose content, the bulk water concentration gradually decreases until a limit is reached where all the water molecules are involved in hydration. The sound velocity vs temperature data, for each concentration, were fitted with a power law<sup>16</sup> according to

$$v_s = \sum_{n=0}^4 a_n T^n$$

Because the positions of the maxima are not well defined, it is unclear whether a transition between two distinct molecular configurations or a more graduate transition takes place. However, from the fitting procedure we have extracted the behavior of the maximum of temperature  $T_m$  as a function of concentration. The evolution of this latter quantity is shown in Figure 2, while in Figure 3 the values of ultrasonic velocity  $v_s$  and density  $\rho$  as a function of trehalose weight fraction  $\phi$  are reported on a log–log scale. These latter data refer to a temperature of 85 °C for which all the systems investigated are in the liquid phase. The behavior of the data shown seems to



**Figure 2.** Behavior of peak temperature  $T_m$  as a function of weight fraction  $\phi$ .



**Figure 3.** Log-log plots of ultrasonic velocity  $v_s$  and of density  $\rho$  as a function of weight fraction  $\phi$ . Dashed lines represent a guide for the eye.

suggest that the system gradually evolves from a structure characteristic of water, in which the dissolved trehalose acts as a *perturbing* element, to a structure in which solute interactions dominate.

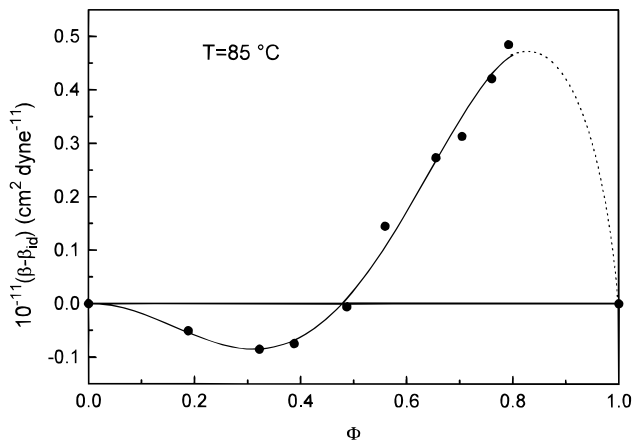
If we try to calculate the sound velocity of the solutions starting from that of the pure components, several models can be used. A simple but significant procedure<sup>17,18</sup> consists of considering the solution properties as resulting from an ideal mixing process. Therefore, the ideal volume of the system can be written as

$$V_{id} = \frac{n_t V_t + n_w V_w}{n_t + n_w} \quad (1)$$

where  $V_t$  and  $V_w$  are the molar volumes of trehalose and water, respectively. Taking the derivative with respect to pressure and using the definition of the adiabatic compressibility,  $\beta = -(1/V)(\partial V/\partial P)_s$ , we find

$$\beta_{id} = \frac{\rho_w n_t M_t \beta_t + \rho_t n_w M_w \beta_w}{\rho_w n_t M_t + \rho_t n_w M_w} \quad (2)$$

where  $\beta_t$ ,  $\rho_t$  and  $\beta_w$ ,  $\rho_w$  are the adiabatic compressibility and the density of trehalose and of water, respectively. The calculated  $\beta_{id}$  values can be compared with the experimental ones obtained from  $v_s$  and  $\rho$ . We have evaluated the departure from the ideal, namely, the difference  $(\beta - \beta_{id})$ , at each temperature and concentration. Figure 4 shows, as an example, the resulting isotherm at  $T = 85^\circ\text{C}$  as a function of weight fraction  $\phi$ . The values relative to pure trehalose have been extrapolated from the values at higher water content. As can be seen, the experimental compressibility does not follow the



**Figure 4.** Behavior of excess compressibility as a function of weight fraction  $\phi$ . The continuous and the dashed lines represent a guide for the eye.

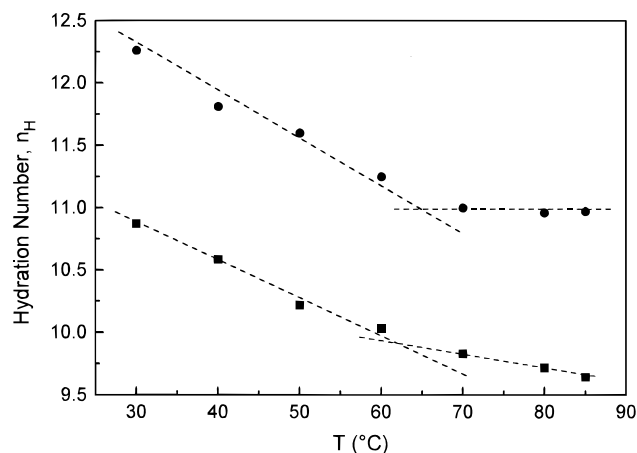
ideal behavior, indicating that the mixing process is not ideal. The most striking feature of the  $(\beta - \beta_{id})$  behavior is the marked positive excess compressibility at high trehalose content. From a general viewpoint the sign and magnitude of such deviations depend on the strength of the interaction between unlike molecules.<sup>18</sup> Systems of not too different molecular size usually mix with an increase in volume and positive excess compressibility if strong interactions between unlike molecules are absent. In addition, positive excess compressibilities tend to become less positive and then increasingly negative as the strength of the interaction between unlike molecules increases. This occurrence has been recently verified in poly(ethylene glycol) aqueous solutions.<sup>17,19–21</sup> The change from positive to increasingly negative excess values, which in our case occurs when  $\phi$  diminishes, can be qualitatively interpreted in terms of a closer approach of unlike molecules, leading to a reduction in compressibility and volume. In our case the existence of a percentage of intramolecular H bonds between the two trehalose rings, especially at high sugar content because of the saturation of some hydrogen bond sites, makes the trehalose–water system less interacting at high concentration. However, when the number of water molecules increases, the trehalose structure opens up, releasing its sites for the interaction with water molecules, and this causes the decrease of the positive excess compressibility to slightly negative values. Therefore, we suggest that  $\alpha, \alpha$ -trehalose, which consists of two hexapyranose rings connected via a 1–1 glycosidic linkage, initially folded on itself, by the addition of water molecules,  $\alpha, \alpha$ -trehalose opens its two rings. Obviously, such a hypothesis must be carefully checked, and small-angle neutron-scattering (SANS) and nuclear magnetic resonance (NMR) experiments have been planned.

Furthermore, from the obtained data we can get information on the trehalose hydration number,  $n_H$ . We assume, following a molecular model,<sup>11,12</sup> that the volume of the solution  $V$  can be decomposed into two parts: (1) the hydration volume where significant interactions between trehalose and water occur and (2) regions where bulk water exists. According to this model, the volume of the solution  $V$  can be written as

$$V = n_t V_H + (n_w - n_t n_H) V_w \quad (3)$$

where  $n_H$  and  $V_H$  are the hydration number and the molar volume of the hydrated sugar. Taking the derivative with respect to pressure at constant entropy, we find

$$V\beta = -n_t \frac{\partial V_H}{\partial P} + (n_w - n_t n_H) V_w \beta_w \quad (4)$$



**Figure 5.** Temperature dependence of trehalose hydration number  $n_H$  at  $\phi = 0.188$  (●) and  $\phi = 0.322$  (■). Dashed lines represent a guide for the eye.

where  $V = (n_w M_w + n_t M_t)/\rho$ . On the hypothesis of *negligible compressibility for hydrated units*,  $\partial V_H/\partial P = 0$ , we then obtain for the hydration number the formula

$$n_H = \frac{n_w V_w \beta_w - V \beta}{n_t V_w \beta_w} \quad (5)$$

The values of  $n_H$  have been evaluated for the two most diluted aqueous solutions investigated, namely, for 82 and 40 water molecules per trehalose molecule. Their temperature dependence is reported in Figure 5. As can be seen, the values of the hydration number, for the most diluted solution, go from  $\sim 12.2$  at 30 °C to  $\sim 11$  at 70 °C, while for the more concentrated solution they go from  $\sim 10.9$  at 30 °C to  $\sim 9.8$  at 70 °C. At higher temperatures the hydration numbers stay almost constant.

It is well-known that the main drawback in calculating hydration numbers from ultrasonic measurements is the assumption that the compressibility of the trehalose together with its closely associated water molecules is negligible.<sup>11</sup> Inherent in this model is the further assumption that the compressibility of water molecules near, but not inside, the primary hydration shells is the same as that of pure water. Therefore, in general, the hydration number derived in this way can be different from those derived by means of *local* probes such as neutron scattering or extended X-ray absorption fine structure (EXAFS) also because of differences in the models used with the various approaches and critical assumptions underlying these models. Taking into account these weak points, one should be cautious when interpreting the results. Furthermore, apart from simple interactions between water and trehalose, other interactions could come into play, especially at high concentration, and as a consequence, the compressibility behavior could be related to the structural arrangement of the whole system rather than to specific entities. However, despite the simplicity of the model, in our case the values obtained for  $n_H$  at room temperature are in excellent agreement with those calculated by Grigera et al.<sup>3</sup> by means of the above-mentioned MD simulation performed on trehalose aqueous solutions ( $n_H = 10$ ). The slight decrease of hydration number with decreasing water content can be attributed to a competitive mechanism among the trehalose molecules, which at the highest dilution are able to tie a greater number of water molecules into the primary and secondary hydration shells of the sugar. Therefore, at 30 °C about 12 water molecules have enough time to orientate out of the bulk water and to join trehalose in its diffusional motion. Furthermore, the amount of water for each trehalose molecule decreases with increasing temperature and approaches an almost constant value at the highest temperatures. This trend can be ascribed

to enhanced thermal motions at higher temperatures that lead to lower residence times of water molecules in the nearby hydration of the sugar. Therefore, the average number of water molecules moving together with trehalose will be lower, decreasing the hydration number. It should be pointed out also that with increasing temperature, owing to the rupture of a certain fraction of hydrogen bonds in water, the number of water molecules available for bonding with trehalose also increases. The hydration number is determined by the net result of these two opposing effects. At temperatures higher than about 70 °C, these effects compensate and the hydration number tends to a constant value.

## Concluding Remarks

This work reports new results of an experimental study of  $\alpha,\alpha$ -trehalose–water mixtures as a function of temperature and concentration by means of ultrasonic velocity and density measurements. The data indicate that the mixing process is not ideal and that structural changes take place in these solutions. The deviation of measured compressibility from the ideal state furnishes information on the trehalose–water interaction strength and suggests that when the amount of water increases, the two sugar hexapyranose rings open up. Furthermore, the trehalose hydration number and its temperature dependence have been determined. The values obtained indicate that with increasing temperature the number of water molecules in the sugar hydration shells decreases until a constant value is reached at the highest temperatures.

To elucidate the physical mechanisms underlying the configurational questions examined in the present study, further investigations, using optical and neutron-scattering techniques, are needed. We have already initiated work of this kind, and we hope that it will provide more insight into the intriguing properties of hydrogen bonding in the sugar–water system.

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