

Trehalose and Other Sugar Solutions at Low Temperature: Modulated Differential Scanning Calorimetry (MDSC)

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Aqueous solutions of trehalose, sucrose, fructose, and glucose at concentrations from 0.2 to 70 wt % (mole fractions from 0.01% to 18.9%) have been investigated using modulated differential scanning calorimetry (MDSC). The apparent (nonequilibrium) heat of melting (H_m^{ne}), heat of freezing (H_f^{ne}), and depression of the melting and freezing temperatures have been measured as a function of sugar concentration. Under the fixed experimental protocol and at any fixed concentration, the trehalose solution shows the lowest H_m^{ne} and H_f^{ne} among the four sugars. This may be due to an increased interaction between this sugar and water molecules. Trehalose also has the highest glass transition temperature among the four sugars. These two facts may be linked with the role of trehalose in cryopreservation. We observed that, for some solutions, H_m^{ne} increases with the lowering of cooling temperature and the total time the sample remains at temperatures below the equilibrium freezing point. This increase is likely to be associated with structural changes occurring in the ice.

1. Introduction

The primary goals of this research are to gain a better understanding of the freezing and melting processes in sugar solutions and to reveal the mechanism of cryopreservation of sugars. To accomplish this, we explored the behavior of sugar solutions at low temperatures (from just below 0 to $-100\text{ }^{\circ}\text{C}$) using the technique of modulated differential scanning calorimetry (MDSC). Solutions of trehalose and sucrose, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$, MW 342.30, and fructose and glucose, $\text{C}_6\text{H}_{12}\text{O}_6$, MW 180.16, in pure deionized water have been investigated. We measure the amount of heat released or absorbed during freezing and melting, as well as the freezing/melting point depression by the sugars.

Since the discovery that some organisms survive dehydration using sugars such as trehalose and, furthermore, that sugars such as trehalose and sucrose have the ability to preserve liposomes and membranes during freeze-drying^{1–4} studies of the physical chemistry of such solutions at supercooled temperatures have attracted increasing attention. The ultimate aim of these studies is to discover the molecular mechanism of this kind of cryopreservation. It has been reported that the efficiency for cryopreservation varies from one sugar to another, with trehalose being the most efficient among the sugars investigated to date.⁵ For example, by testing the preservation of structural and functional integrity of membranes at low water activities, Crowe et al.⁵ found that the effectiveness of preservation of sugars from the highest to the lowest is in the following order: trehalose, lactose, maltose, cellobiose, sucrose, glucose, fructose, sorbitol, raffinose, myo-inositol, and glycerol. To explain the origin of this order, a number of techniques such as DSC,^{6,7} NMR,^{8–10} Raman spectroscopy,¹¹ electron spin resonance,¹² dynamic mechanical thermal analysis,¹³ ultrasonic techniques,¹⁴ and computer simulations^{15,16} have been employed to explore the intrinsic properties and structure of sugar solutions. The DSC

measurements of whole organisms have also been reported.¹⁷ In addition, investigations into various aspects of aqueous solutions of sugars are also motivated by the potential application of sugar as a cryoprotectant for freeze-dried foods.^{18–21}

Green and Angell²² have examined the glass transition of solutions of trehalose, maltose, sucrose, and glucose and found that trehalose solution has the highest glass transition temperature among these four sugars. Further, they reported that the glass transition temperatures of these four sugar solutions have an order from the highest to the lowest similar to the cryopreservation effectiveness order reported by Crowe et al.⁵ They concluded that there is a relationship between the high effectiveness in the cryopreservation of trehalose and its high glass transition temperature. It is natural to assume that there is some intrinsic molecular property of trehalose which explains both phenomena, but at present this property is still unknown. In this paper, we report MDSC results of solutions of trehalose, sucrose, fructose, and glucose as a function of concentration. The experimental data we obtained, such as heat of freezing and melting, as well as the freezing/melting point depression, will contribute to the future usage of sugars and the full explanation of their cryopreservation. In addition, the exploration of the behavior of sugar solutions at low temperatures is also relevant to the better understanding of supercooled aqueous solutions and ice nucleation phenomena and is important to fields such as meteorology, agriculture, and other industries.^{23–25}

MDSC was chosen for this study because it yields not only the same information about the heat associated with thermal events as conventional DSC, but it also has the capability to discriminate between irreversible phenomena and reversible phenomena such as crystallization. It is most probable that the quantities measured in this work, such as heat of melting and the heat of freezing, are nonequilibrium values. For this reason, we use the notations H_m^{ne} and H_f^{ne} to represent the measured

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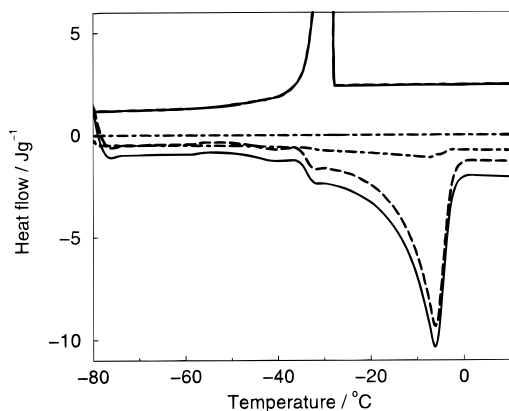


Figure 1. A typical MDSC run for 50 wt % trehalose solution with cooling/heating rate of $3\text{ }^{\circ}\text{C min}^{-1}$: conventional heat flow (solid line), nonreversible heat flow (dashed line), and reversible heat flow (dash-dot line).

heat of melting and heat of freezing, to discriminate them from the equilibrium values.

2. Experimental Method

Each sugar solution was prepared by weighing the relevant sugar and deionized distilled water in the desired percentage using an accurate electronic balance (accuracy $\pm 0.01\text{ mg}$). All sugars were reagent grade from Sigma-Aldrich Pty. Ltd. and used without further purification. For trehalose (α,α -trehalose) the weight percentages are 0.5%, 1%, 5%, 10%, 20%, 35%, 50%, and for sucrose, glucose (β -D-glucose), and fructose, an additional concentration of 70% was prepared. Each mixture of water and sugar was gently stirred with a glass rod over a hot water bath (approximately $40\text{ }^{\circ}\text{C}$) until a transparent solution was obtained. This solution was then kept in a dark place for 12–24 h before use to ensure it was in equilibrium. During the storage of the solution for equilibration, we observed that trehalose recrystallized from the solution with concentration of 50 wt % after 12 h storage. For this reason, the trehalose solution with concentration of 50 wt % was prepared and used immediately in order to avoid recrystallization.

The MDSC experiments were carried out on a DSC 2920 apparatus (TA Instruments) equipped with modulation capability and a liquid nitrogen cooling accessory. The DSC cells were purged with dry nitrogen to prevent air condensation. To make samples for the MDSC, each solution of total mass 10–20 mg was sealed hermetically into an aluminum pan. An identical empty aluminum pan was used as a reference. Calibration for both temperature and heat was performed using pure In metal and deionized distilled water.

In this paper, except where noted, all of the heating/cooling rates used are from 0.5 to $3.0\text{ }^{\circ}\text{C min}^{-1}$ with a modulation of $\pm 0.5\text{ }^{\circ}\text{C}$ over 30 s. Since the DSC 2920 apparatus can record the thermal events occurring during both cooling and heating, our experiments use the same rate for cooling and heating. The MDSC1-1 program was employed to analyze MDSC data and to determine the values of H_m^{ne} , H_f^{ne} , freezing temperature, melting temperature, and the glass transition temperature.

A typical MDSC run is illustrated in Figures 1 and 2. Figure 1 shows the curves of conventional heat flow as well as the reversible and nonreversible heat flow, while Figure 2 shows the specific heat.

For each sample, at least three MDSC runs were carried out with the same modulation ($\pm 0.5\text{ }^{\circ}\text{C}$ over 30 s) but different cooling/heating rates, to extrapolate to the relevant values at

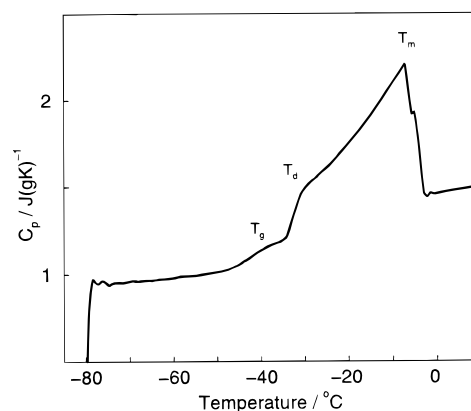


Figure 2. Specific heat (C_p) measurement from a typical MDSC run for 50 wt % trehalose solution with cooling/heating rate of $3\text{ }^{\circ}\text{C min}^{-1}$: glass transition (T_g), melting peak (T_m), and ice dissolution (T_d).

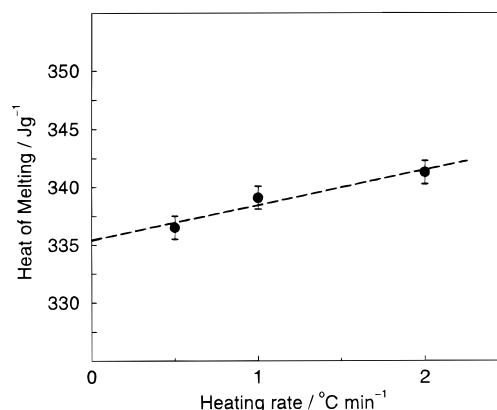


Figure 3. Extrapolation of H_m^{ne} to zero heating rate for pure water: experimental data (dot) and extrapolation (dashed line).

zero cooling/heating rate. Figure 3 is an example of extrapolation of H_m^{ne} for pure water. Three MDSC runs with cooling/heating rates of 0.5 , 1.0 , and $2.0\text{ }^{\circ}\text{C min}^{-1}$ were performed. The quantities of H_m^{ne} obtained with these three different heating rates reveal a basically linear relationship with heating rate and were fitted with standard least-squares fitting. From this fitting, we extrapolate H_m^{ne} to 335.4 J g^{-1} at zero heating rate, which is very close to the reported value (335 J g^{-1}).²⁶

The uncertainty of the measured values of H_m^{ne} and H_f^{ne} is estimated on the basis of the following factors. For each individual MDSC run, the uncertainty of measured H_m^{ne} and H_f^{ne} values is primarily related to the selection of the starting and ending point of the melting/freezing peak. Since the measured base line is not exactly zero, selection of different starting and ending points will result in slightly different peak areas, which yields error in the corresponding heat. In general, this uncertainty is approximately $\pm 1.5\text{ J g}^{-1}$. With $\pm 1.5\text{ J g}^{-1}$ uncertainty in each individual measurement and with the fact that each measured H_m^{ne} and H_f^{ne} can sometimes deviate from the linear relationship with the heating/cooling rate, our extrapolated values have an overall estimated uncertainty of $\pm 5\text{ J g}^{-1}$. Note that dissolution can clearly be distinguished from melting in the MDSC heat flow measurements, and hence there is no need to correct the above data for this effect.

3. Results and Discussion

A. Heat of Melting (H_m^{ne}). The values of H_m^{ne} for various solutions are plotted in Figure 4 as a function of sugar mole

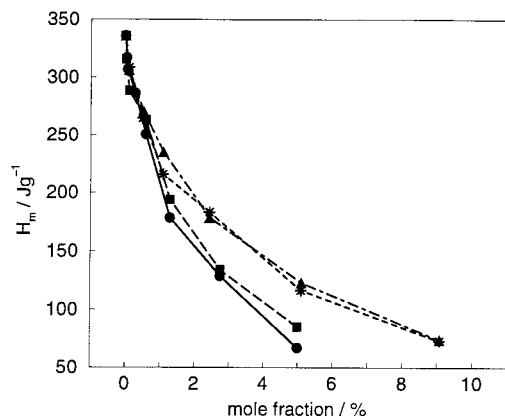


Figure 4. Heat of melting as a function of sugar mole fraction: trehalose (filled circle), sucrose (filled square), fructose (filled triangle), and glucose (star).

fraction. Several conclusions can be drawn from this figure. Firstly, H_m^{ne} decreases with increasing sugar concentration, but not in a linear way. Since we conclude that the release of H_m^{ne} is associated primarily with the melting of ice, the nonlinear relationships shown in Figure 4 indicate that all sugar solutions are not ideal solutions. Furthermore, it can be calculated that the reduction in the magnitude of H_m^{ne} is far greater than that due to the decreasing water percentage in the solution. Secondly, for the four sugars, the corresponding values of H_m^{ne} generally show a similar decreasing trend with sugar concentration, but decreasing at different rates. Briefly, this decreasing trend can be interpreted according to the usual thermodynamic rules.²⁷ However, the different decreasing rates are certainly associated with the intrinsic properties of each individual sugar. Furthermore, it was noted from Figure 4 that the isomer sugars (trehalose and sucrose, fructose and glucose) show a more similar decreasing trend in H_m^{ne} . This may be linked with the fact that isomer sugars have the identical number of OH groups.²⁸

Thirdly, for the same sugar concentration, trehalose solution shows the lowest H_m^{ne} among the four sugars, followed by sucrose, glucose, and fructose. As mentioned above, the release of H_m^{ne} is considered to be associated with the melting of ice. The smaller H_m^{ne} , the less ice melted. From this point of view, it seems that during heating the amount of ice melted in the trehalose solution is less than that in the sucrose solution, and far less than that in glucose and fructose solutions. Since the ice was formed during cooling, the above result is consistent with the deduction that less water was frozen in the trehalose solution. This finding is consistent with the report that trehalose is more efficient in cryopreservation than sucrose and fructose,⁵ but at variance with studies which found⁶ that all sugar–water solution have the same freeze-concentration upon slow cooling.²⁹

B. Heat of Freezing (H_f^{ne}). The heat of freezing (H_f^{ne}) has also been obtained, since the DSC 2920 instrument also records the heat changes associated with the events occurring during the cooling process (see Figure 5). Basically, H_f^{ne} as a function of sugar concentration shows a similar trend as H_m^{ne} , that is trehalose solution exhibits the lowest H_f^{ne} among the four sugars. As the MDSC runs were performed under same protocol for the four sugars and the values of H_f^{ne} as shown in Figure 5 are extrapolated ones at zero cooling rate, the prediction that more water molecules were unfrozen in the trehalose solution is directly confirmed.

It is also noted from Figures 4 and 5 that H_m^{ne} and H_f^{ne} are not equal for the same solution. This is likely due to the strong

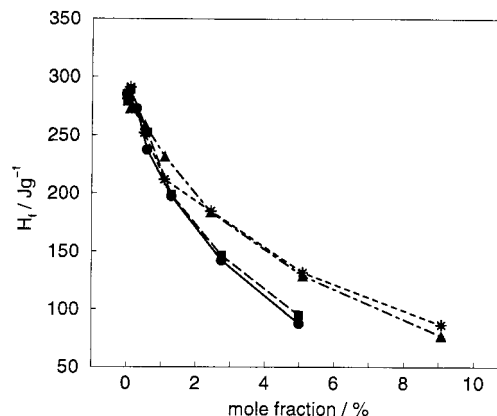


Figure 5. Heat of freezing as a function of sugar mole fraction: trehalose (filled circle), sucrose (filled square), fructose (filled triangle), and glucose (star).

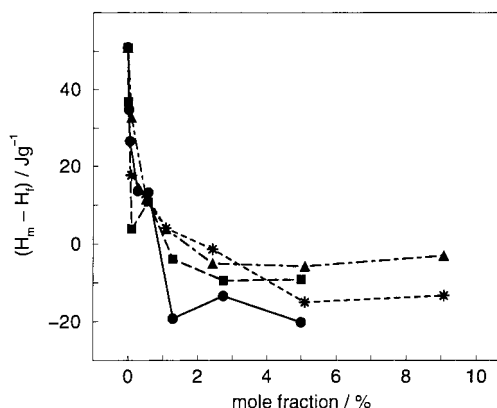


Figure 6. The difference between H_m^{ne} and H_f^{ne} as a function of sugar concentration: trehalose (filled circle), sucrose (filled square), fructose (filled triangle), and glucose (star).

dependence of H_f^{ne} on the cooling process as well as the possible nonequilibrium nature of both H_f^{ne} and H_m^{ne} . In principle, if nothing happened to the ice, H_f^{ne} should be equal to the value of H_m^{ne} , but in practice this is not the case. Taking the deionized distilled water as an example, H_m^{ne} (see Figure 4) matches quite well with the reported value of 335 J g^{-1} , but H_f^{ne} has a considerably lower value of 285 J g^{-1} . For distilled water, this result seems to suggest either (a) some exothermic events (such as ice recrystallization and/or structural relaxation) have taken place during cooling, leading to a further gradual release of heat which cannot be detected by this DSC apparatus but results in a large H_m^{ne} upon heating, or (b) the extraction of H_f^{ne} is not as physically meaningful as H_m^{ne} due to strong dependence on thermal history. Nevertheless, one of the interesting features of the four sugar solutions is that H_f^{ne} tends to have a larger value than the corresponding H_m^{ne} when the sugar concentration is higher than 1% mole fraction.

The difference between H_m^{ne} and H_f^{ne} is plotted in Figure 6 as a function of mole fraction of sugar. Since solutions of all four sugars follow a similar trend, this feature cannot be simply explained by the thermal-history-dependence of H_f^{ne} . Rather, we believe it indicates that some endothermal events have occurred within the high concentration solutions at temperatures below freezing point during cooling and heating. Indeed, given that an endothermal event was observed before melting in the solutions with sugar concentration higher than 1% mole fraction, it is believed that the above feature is associated with an event whose true nature is still in doubt but which some reports call

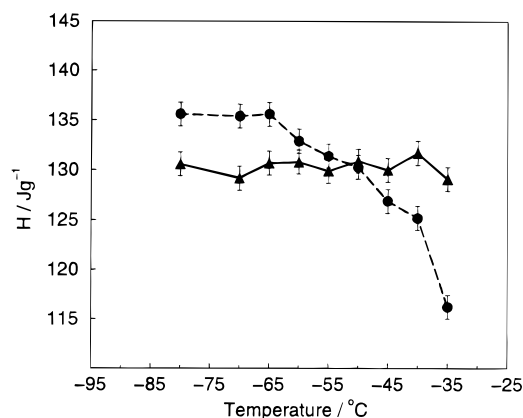


Figure 7. Dependence of H_m^{ne} and H_f^{ne} upon T_{low} for 35 wt % fructose solution: H_m^{ne} (filled circle) and H_f^{ne} (filled triangle).

ice dissolution or devitrification.^{30,31} If ice dissolution occurs before melting, it normally produces heat absorption and most probably causes a larger block of ice to melt into smaller ones. As a result, it leads to less heat absorption during melting. From this point of view, our result seems to support the conclusion that the endothermal event that occurred just before melting is ice dissolution. As can be seen from Figure 6, trehalose solutions show the largest difference between H_m^{ne} and the corresponding H_f^{ne} . This large difference likely indicates a relatively large ice dissolution. It is not yet clear if the difference between H_m^{ne} and H_f^{ne} saturates when the mole fraction of the sugar is larger than 1%.

C. Temperature and Annealing Time Dependence of H_m^{ne} and H_f^{ne} . To explore the possible changes in ice when the sugar solution was held at a temperature below the freezing point, two sets of MDSC runs were carried out on selected solutions. One set of runs involved cooling the sample to different low temperatures (T_{low} , well below the freezing point), with constant cooling rate (3.0 °C min⁻¹), to observe how H_m^{ne} and H_f^{ne} vary with T_{low} . Since cooling to different T_{low} also corresponds to different times the sample stayed below the freezing point, any changes to H_m^{ne} and H_f^{ne} with T_{low} may also involve time effects. To distinguish between these two effects, a second set of MDSC runs were performed to measure the dependence of H_m^{ne} and H_f^{ne} on the annealing times. This was done by cooling the sample to the same temperature as before, and holding the sample at this temperature for a certain time (annealing).

Figure 7 shows the results obtained from 35 wt % fructose solution. Since the MDSC runs were performed using the same sample and the same cooling/heating rate, it is understandable that H_f^{ne} is unrelated to T_{low} , given that T_{low} is below the freezing point. Indeed, Figure 7 shows H_f^{ne} is independent of T_{low} within the experimental accuracy (± 1.5 J g⁻¹). However, H_m^{ne} is found to increase with T_{low} and reaches a constant value of ~ 135 J g⁻¹ when T_{low} is below -65 °C. Since the experimental accuracy of H_m^{ne} is about ± 1.5 J g⁻¹, the changes of H_m^{ne} with T_{low} cannot be attributed to experimental uncertainty. In other words, something real happens to the ice which results in an increase of H_m^{ne} .

Two factors may be associated with this increase: temperature and time. To clarify these two factors, the same sample was also annealed at -45 °C for various times up to 1 h (7.5 min, 15 min, 30 min, 45 min, and 1 h). As shown by Figure 8, H_m^{ne} increases from ~ 127 to ~ 132 J g⁻¹ when annealed for 7.5 min and no further increase was observed by increasing the

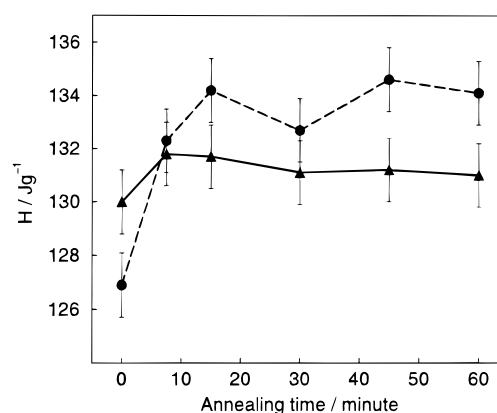


Figure 8. Dependence of H_m^{ne} and H_f^{ne} upon annealing time for 35 wt % fructose solution at -45 °C: H_m^{ne} (filled circle) and H_f^{ne} (filled triangle).

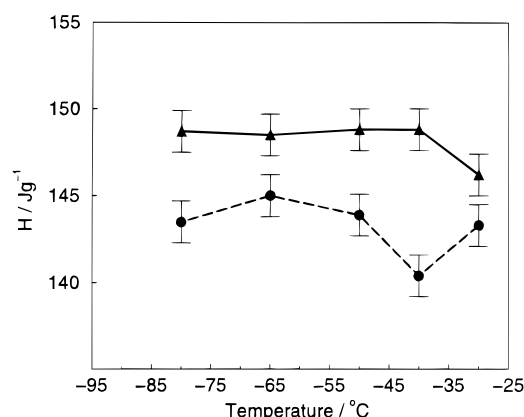


Figure 9. Dependence of H_m^{ne} and H_f^{ne} upon T_{low} for 35 wt % trehalose solution: H_m^{ne} (filled circle) and H_f^{ne} (filled triangle).

annealing time up to 1 h. Compared with Figure 7, Figure 8 indicates that the increase of H_m^{ne} is primarily due to the time effect. In other words, given sufficient time, changes (presumably structural changes) will occur in the ice, which lead to a gradual heat release. This heat release results in a large H_m^{ne} upon melting.

A trehalose solution with the same weight percent concentration was also tested under the same experimental protocol. However, within experimental uncertainty, no clear increase of H_m^{ne} and H_f^{ne} was observed when the solution was cooled to different T_{low} or when annealing was performed, as shown by Figures 9 and 10, respectively. In comparison with the fructose solution, these results suggest that freezing is more or less completed in this trehalose solution and nothing happened to the ice when the solution was kept below the freezing point. However, on the other hand, as we noted that the mole fraction of trehalose in the 35 wt % trehalose solution (2.75% mole fraction) is much less than that of fructose in 35 wt % fructose solution (5.1% mole fraction), the relatively fewer trehalose molecules in the 35 wt % solution may be another factor contributing to the almost unchanged H_m^{ne} and H_f^{ne} . To clarify this, 50 wt % trehalose solution was chosen to carry out the same MDSC runs as were performed on 35 wt % trehalose solution, because the mole fraction of trehalose in 50 wt % solution (5.0% mole fraction) is quite close to that of fructose in 35 wt % solution. As shown in Figure 11, H_m^{ne} increases with T_{low} initially and then stabilizes below -70 °C, a similar trend as observed in 35 wt % fructose solution. However, when annealing the 50 wt % trehalose solution at -50 °C for various times up to 30 min, no clear increase of H_m^{ne} was observed.

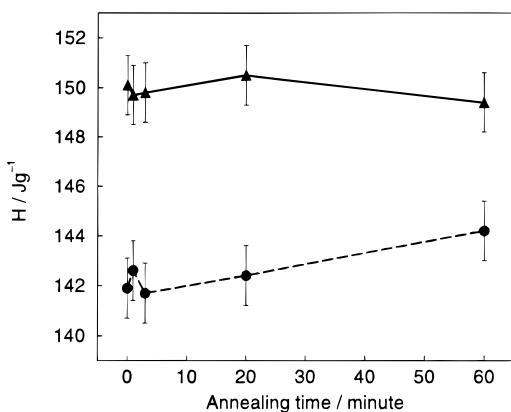


Figure 10. H_m^{ne} and H_f^{ne} as a function of annealing time for 35 wt % trehalose solution at -45 °C: H_m^{ne} (filled circle) and H_f^{ne} (filled triangle).

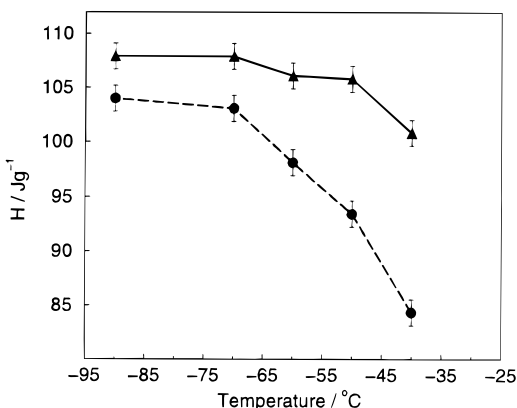


Figure 11. Dependence of H_m^{ne} and H_f^{ne} upon T_{low} for 50 wt % trehalose solution: H_m^{ne} (filled circle) and H_f^{ne} (filled triangle).

It is interesting to note the above changes of H_m^{ne} with annealing time and with T_{low} for the selected trehalose and fructose solutions, and these changes demonstrate following points.

The Nonequilibrium Nature of Measured H_m^{ne} . The equilibrium value of heat of melting is independent both of (i) the time the sample has been kept below the freezing point and (ii) the temperature to which the sample was cooled, given that no irreversible phase transition takes place. The above observed increase of H_m^{ne} with annealing time and T_{low} not only indicates that the measured H_m^{ne} is not the equilibrium value but also suggests that some structural evolution or relaxation has happened to the ice during annealing or cooling to T_{low} . In other words, freezing occurs at the freezing point, but the ice formed has not yet reached its final equilibrium state. When the time is increased or the temperature is further decreased, gradual structural evolution or relaxation takes place, leading the ice to a more stable structure. Although no MDSC evidence exists of what sort of structural evolution or relaxation has occurred in the ice, it is reasonable to consider events such as the gradual growth of a small block of ice and the local rearrangements of hydrogen and oxygen atoms within the ice. In addition, given the presence of sugar in the solution, some of the structural changes may also be associated with the sugar molecules. Further investigation is needed in order to clarify this matter.

Concentration Dependence of the Changes in H_m^{ne} . The measured H_m^{ne} values for 35 and 50 wt % trehalose solutions during cooling to different T_{low} clearly reveal that the change in H_m^{ne} is linked with the sugar concentration: the higher the

sugar concentration, the more likely H_m^{ne} will change with T_{low} . We believe that this result is an indirect indication of interactions between water and sugar molecules. For example, one sugar molecule (trehalose, for example) may cause a certain number of water molecules to remain unfrozen or make them stay in metastable states by interacting with them when approaching the freezing point. By decreasing T_{low} , which also means decreasing the internal energy of the solution, this sort of interaction may be weakened or may not be able to be sustained. As a result, those water molecules which did not freeze previously may relax to a more stable state by freezing or by structural relaxation. However, only in a concentrated solution (50 wt % for trehalose) is the collapse of the interaction between sugar and water molecules severe enough to cause the H_m^{ne} change.

The Unique Effect of Different Sugars on the Changes of H_m^{ne} . The responses of H_m^{ne} to annealing time and T_{low} for the fructose and trehalose solutions reveal how different sugars affect the changes of H_m^{ne} . For 35 wt % fructose solution, the experimental results (see Figures 7 and 8) indicate that H_m^{ne} increases with both annealing time and decreasing T_{low} . In other words, without decreasing T_{low} or reducing the internal energy of the solution, gradual structural evolution or relaxation spontaneously takes place within the ice, which leads to an increased H_m^{ne} . However, this is not true for the 50 wt % trehalose solution, in which reduction of the internal energy of the solution by external effort (decreasing T_{low}) is required in order to induce the structural evolution or relaxation of ice. Given the same mole fraction of fructose in 35 wt % fructose solution and trehalose in 50 wt % trehalose solution, the above different responses of H_m^{ne} to annealing time and T_{low} for different sugars are most likely caused by the unique interaction between the individual sugar and water molecules. Further, this interaction is very likely to be associated with the unique properties or structure of the individual sugar.

D. Depression of the Freezing and Melting Temperature. The depression of the freezing temperature (T_f) and melting temperature (T_m) by solutes in a dilute solution of nonelectrolytes is determined thermodynamically by Van't Hoff's law.²⁷ For an infinitely dilute solution, T_m or T_f follows the equation:

$$(\Delta H/R)(1/T_0 - 1/T_m) = x \quad (1)$$

where ΔH is the molar enthalpy of the transition from pure solid to pure liquid, T_0 is the freezing point of pure solvent, and x is the mole fraction. From this equation, generalized approximately to noninfinite dilution, it can be seen that $1/T_m$ will show a linear relationship with mole fraction, assuming that ΔH is constant, independent of temperature, between the temperatures T_0 and T_m .

As shown in Figure 12, the values of the reciprocal peak temperature of melting for the fructose and glucose solutions show an approximate linear relationships with mole fraction. Sucrose and trehalose solutions show a similar linear trend, but with larger variation. This suggests that within the investigated range of mole fraction, the four sugars depress the melting point in a similar way as described by Van't Hoff's law²⁷ with evidence that fructose and glucose solutions follow Van't Hoff's law more closely. In our experiments, the temperature of onset of freezing is highly dependent on the history of the sample, and from run to run, as expected from studies of nucleation,³² and a plot similar to Figure 12 for freezing cannot be made from our data.

To test quantitatively the linear relationship between the reciprocal values of peak temperature of melting and the sugar

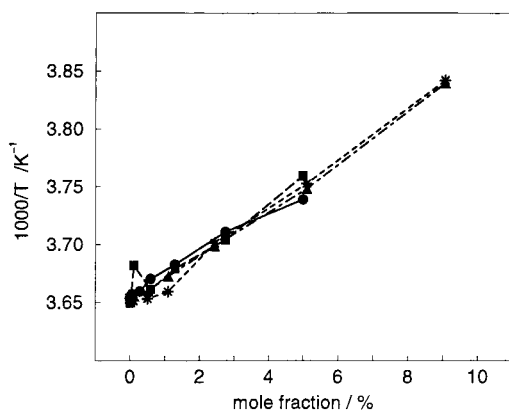


Figure 12. Values of the reciprocal melting temperature as a function of sugar mole fraction: trehalose (filled circle), sucrose (filled square), fructose (filled triangle), and glucose (star).

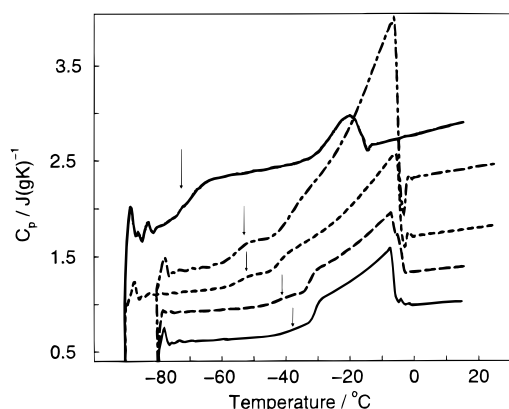


Figure 13. Specific heat curves which show the glass transition for 5% mole fraction trehalose (bottom solid line), sucrose (long dashed line), fructose (dash-dot line), glucose (dashed line), and 10.9% mole fraction sucrose (top solid line) solutions. The curves for trehalose, glucose, and 10.9% mole fraction sucrose solutions were shifted in order to avoid overlapping.

mole fraction as shown in Figure 12, the data for fructose solutions were fitted with a straight line ($\chi = 1.4 \times 10^{-5}$). The intercept value of 3.6505 was obtained, leading to $T_0 = 273.9$ K, in good agreement with the reported equilibrium value of 273.15 K.²⁶ In general, this result confirms that the fructose solutions behave as dilute solutions as described by Van't Hoff's law.²⁷

E. Glass Transition. The glass transition temperature is an important parameter reflecting the stability of a material and the mobility of molecules within the material. Normally, a glass transition is indicated by a change in the specific heat in MDSC curves (as marked by T_g in Figure 2). For the four sugar solutions, it was found that, when the sugar concentration is less than 1% mole fraction, no glass transition is observed. Once the sugar concentration is larger than 1% mole fraction, the glass transition is observed with evidence that the higher the mole fraction, the more visible the glass transition. This observation is in good agreement with that reported by Angell et al.,²² Roos and Karel,³⁰ and Ablett et al.³¹ However, we wish to emphasize following points: (i) Trehalose solution has the highest glass transition temperature. As marked by arrows in Figure 13, with a heating rate of 3°C min^{-1} and a concentration of $\sim 5\%$ mole fraction, the glass transition temperatures (midpoint temperature) for four sugar solutions are -37.5°C (trehalose, solid line), -42.0°C (sucrose, long dashed line), -54.0°C (glucose, dashed

line) and -55.8°C (fructose, dot-dashed line). These values are consistent with those reported by Angell et al.²² and Roos and Karel.³⁰

(ii) With a fixed mole fraction (5%) and the same experimental protocol, it is noted from Figure 13 that the fructose solution shows the most clearly visible glass transition peak ($\Delta C_p \sim 0.14 \text{ J(g K)}^{-1}$), then glucose ($\Delta C_p \sim 0.10 \text{ J(g K)}^{-1}$), sucrose ($\Delta C_p \sim 0.09 \text{ J(g K)}^{-1}$), and trehalose ($\Delta C_p \sim 0.07 \text{ J(g K)}^{-1}$). Since the glass transition is associated with mobility of molecules within the materials, we believe that a weaker glass transition peak suggests less mobility of molecules within the solution. On the basis of this consideration, we further believe that the weakest glass transition peak shown by the trehalose solution indicates that the molecules (both water and trehalose) in the trehalose solution have the least mobility due to the strongest interaction/binding among them. This is consistent with the fact that the trehalose solution has the smallest H_m^{ne} and H_f^{ne} values.

(iii) When the sugar concentration is further increased to a certain value (10.9% mole fraction for sucrose for example, see the dotted line in Figure 13), no conventional freezing and melting peaks were observed in MDSC curves. Rather, a very clear glass transition was revealed at approximately -75°C . This is probably due to the fact that the sugar concentration is too high and the solution has a very high viscosity, which prevents water molecules from joining together to form ice. Similar results have been reported by Ablett et al.³¹

4. Conclusions

Through MDSC studies, it is revealed that trehalose in aqueous solution results in less freezing of water molecules compared with sucrose, fructose, and glucose solutions. This is reflected by the fact that the trehalose solution shows the lowest heat of freezing and heat of melting. On the other hand, both the high glass transition temperature and its behavior in depressing the melting temperature suggests that a strong interaction takes place between trehalose and water molecules. Since trehalose and sucrose are isomers, the fact that trehalose has a high melting point,²⁶ a higher glass transition temperature, and is effective in cryopreservation suggests that it is the unique structural features of trehalose that are responsible for all the above differences. It is worthwhile to focus future research on this feature.

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- (29) An anonymous referee suggests that the smaller amounts of ice which formed in the trehalose and sucrose solutions occur because the point at which ice first nucleates places the resulting solutions closer to the glass transition for the trehalose–water and sucrose–water systems than it does for the glucose–water and fructose–water systems. This would cause the disaccharide–water solutions which were in the process of freeze-concentrating to fall out of equilibrium with respect to the metastable ice–water liquidus at a higher temperature than the monosaccharide–water solutions, and this would account for the significantly different amounts of frozen water in the different solutions. We thank the referee for this suggestion.
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