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VITRIFICATION OF TREHALOSE BY WATER LOSS FROM ITS CRYSTALLINE DIHYDRATE

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Abstract

Trehalose dihydrate, on careful dehydration below its fusion point, retains its original crystal facets but becomes X-ray amorphous, an unusual example of direct crystal-to-glass transformation. From DSC studies, the glass obtained by this route seems to be of abnormally low enthalpy, but after an initial scan, the normal form of glass transition is exhibited, with $T_g=115°C$, a higher value than previously reported. We give a preliminary thermal and mechanical characterization of this material and find it to be a very fragile liquid. The high T_g is shown to rationalize the exceptionally high water content of the trehalose+water solution that vitrifies at ambient temperature (i.e. $T_g=298$ K), and hence helps explain its use by Nature as a desiccation protectant. The spontaneous vitrification of crystalline materials during desolvation is related to the phenomenology of pressure-induced or decompression-induced vitrification of crystals via the concept of limiting metastability.

Keywords: bioprotection, biostabilization, crystal stability limit, desolvation amorphization, fragility of saccharides, trehalose

Introduction

Trehalose is a non-reducing disaccharide that is frequently synthesized in living organisms as an intracellular protectant or co-protectant against harsh conditions, permitting organisms to survive dehydration or freezing [1–3]. The origin of these favorable properties is controversial [3–7]. Trehalose has been the subject of a large number of recent studies on biopreservation and is much used in the handling of proteins, because of the stabilization it provides [7, 8]. In this note, we report an aspect of trehalose phenomenology that is not necessarily connected to its biological usefulness, but which is rather interesting from a materials science/glass science viewpoint. It provides an example of an unusual type of direct transition from ordered crystal to glass.

Amorphization of crystalline substances by compression of ambient-pressure stable crystals has evoked great interest in the materials science community, since the amorphization of ice was reported in 1984 [9], and vitrification by low

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temperature reaction of crystalline components, one of which is mobile in the product, has also been intensively studied [10–12]. The process of vitrification by removal of a mobile component from a crystal has not been generally recognized as a specific route to the glassy state [13, 14]. There are, of course, very many cases of X-ray-amorphous products from desolvation (drying) or degasing procedures, including removal of hydrogen from metallic hydrides (as a referee pointed out); however, vitrification requires that the product be a material with a glass transition, and we are only aware of two cases in which such distinction between glassy and nanocrystalline products has been made. Seki and co-workers [15] long ago reported the preparation of an amorphous material, which exhibited a glass transition, by the careful dehydration of magnesium acetate tetrahydrate, but no detailed reports followed. Then, very recently, Zografi and co-workers [16] reported a glassy form of raffinose obtained directly from the crystalline pentahydrate by vacuum-dehydration at 100°C. The amorphous state was characterized by X-ray diffraction studies, and the glassy state was confirmed by measurement of a glass transition (at 103°C), which coincided with that obtained for a glassy state produced by a quite different route, freeze-drying. Freeze-drying commences with the generation of a vitreous state of a sugar–water concentrated solution (T_g=–26°C) in mixture with ice crystals, from which water is removed by gradual heating under vacuum. In this case, the process depends on the mobility of water within the glassy structure.

We have now found that trehalose dihydrate behaves, under vacuum-drying, in a similar manner and here report our results, with an emphasis on their interest from a materials science viewpoint. It is to be noted that this route to the glassy state was completely omitted from a recent summary of the known possibilities, which was intended to be comprehensive [14]. Amorphization, direct from the crystal state in this manner, must involve bringing the crystal to a point of mechanical instability compositionally. The route of destabilization of crystals mechanically by isotropic compression (or its converse, decompression) has recently been examined in some detail using the methods of molecular dynamics [17], and interesting instability precursor phenomena, predictable from mean field theories, were observed. The related experiments are not easy to perform. The prospect of being able to study comparable destabilization phenomena, by the simple procedure of isothermally removing a structurally significant component of a crystalline compound under vacuum-heating, is a very attractive one [18].

Trehalose, which occurs in αα– and ββ– forms, is an unusual sugar. Most saccharides crystallize only with difficulty from aqueous solutions, and few hydrates have been characterized. In the sucrose case, many have been said to exist, but only two have been confirmed by X-ray studies. A summary is given in Ref. [19]. The crystallization of α-trehalose dihydrate, however, occurs readily from even quite complex solutions, e.g. 1:1 w/w insulin-trehalose melts containing only a few wt% H_2O [20]. The crystal structure is well known [21] and, according to computer simulation studies [22], the location of the two water molecules remains unchanged, apart from statistical smearing, in the aqueous solution. However, the water molecules are quite easily removed from the crystal structure. Vacuum-dehydration of the brightly faceted crystals is completed in less than an hour when carried out at temperatures close to the melting point, no change in crystal form being evident to microscopic examination. As we will describe below, however, the interior structure of the crystal must become dramatically altered during the water-evacuation process. The β form of trehalose forms a tetrahydrate [7]. Whether or not it can also be amorphized by crystal desolvation remains to be seen.

Experimental

αα–Trehalose dihydrate (from Sigma Chem. Co., T_m=95.7°C) was dehydrated under both ambient and vacuum conditions at temperatures between 95°C (ambient, 48 h, or ~5 torr, 1 h) and 70°C (~5 torr, 3 days). The final mass corresponded to that expected for quantitative loss of the two waters of hydration.

For thermal studies, the crystalline-appearing product of dehydration was sealed into aluminum DSC pans and subjected to repeated scans, using a Perkin-Elmer DSC-7 differential scanning calorimeter and scan speeds of 10 K min^{-1}. A quantitative determination of the heat capacity was made using a Setaram Model 121 DSC and scanning at 5 K min^{-1}.

For X-ray diffraction studies, the anhydrous crystals were powdered and smeared onto aluminum plates, and diffraction patterns were then obtained using a Siemens 5000 X-ray diffractometer.

For mechanical studies, the crystals were heated at 125°C, where they slowly deform and contract to a compact state. The compacted phase was opaque, due to many small vesicles that are probably trapped water-vapor bubbles, because they could be made to disappear by compression. A portion of the compacted material was placed into the outer part of a double-sandwich cell for shear relaxation studies [23] (form factor b=0.20 m), and at 125°C, the tongue that forms the inner part of the sandwich was slowly pushed into place as the sample deformed around it. Extruded parts were removed, and any gaps between the plates were closed up by careful additions of sample to completely fill the cell. The shear modulus was determined at several temperatures between 115 and 125°C, using a Toyo-Instruments Rheovibron model DDVII dynamic mechanical analyzer adapted for time-domain measurements [24]. The strain was stepped in 5 μm increments with a stepper motor, and the resulting stress was recorded. Above ~120°C, the modulus becomes a function of time. In this high range, the decay of stress with time was monitored and subsequently analyzed to obtain the average relaxation time and characteristic parameters of the relaxation function.

Results

The thermal behavior of the (crystal-faceted) anhydrous material, as obtained from the vacuum oven, is shown in Fig. 1 (thick scan).

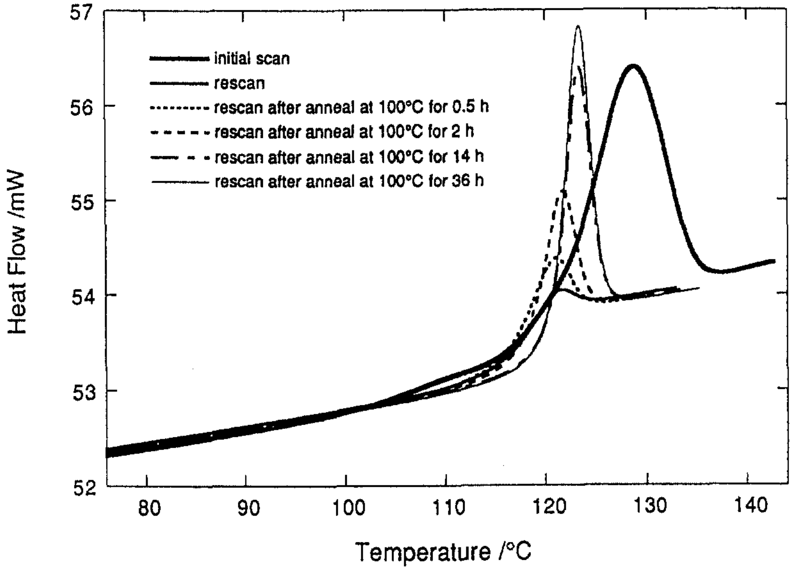

Fig. 1 Perkin-Elmer DSC-7 scans of vacuum-dehydrated trehalose crystals after initial dehydration at 70°C for 30 h, and after subsequent heat treatments as listed in the figure legend. Plots suggest that the initial material was obtained in a glassy state of unusually low enthalpy (and consequent long relaxation time) [25]. Note that the low enthalpy state could only be partly restored by subsequent annealing heat treatments of long duration. The rescan gives the T_g at 115°C

The shape of the peak suggests a broad fusion, but the area under the curve is too small (only ~13.0 J mol^{-1}) to be a normal fusion. A repeat scan yields a trace with displacements of high from low temperature regimes, which are similar to those in the initial scan and which is clearly a glass transition. Repeating the scan after different annealing periods at a fixed temperature, 70°C, below T_g produces the series of dashed and dotted curves of Fig. 1, in which the overshoot characteristic of annealed-glass DSC scans [25] increases with increasing annealing time. This suggests that the initial scan is best explained as the re-equilibration above T_g of a glass of unusually low enthalpy relative to the enthalpy of the rate-cooled glass at its T_g. However, the possibility that the exceptional enthalpy absorption above 125 K of the initial scan is the terminal, weakly enthalpic collapse of a highly defective, almost-vitreous crystalline phase, or of pockets of unvitrified metastable anhydrous crystalline trehalose, cannot be excluded. The issue is discussed in the next section.

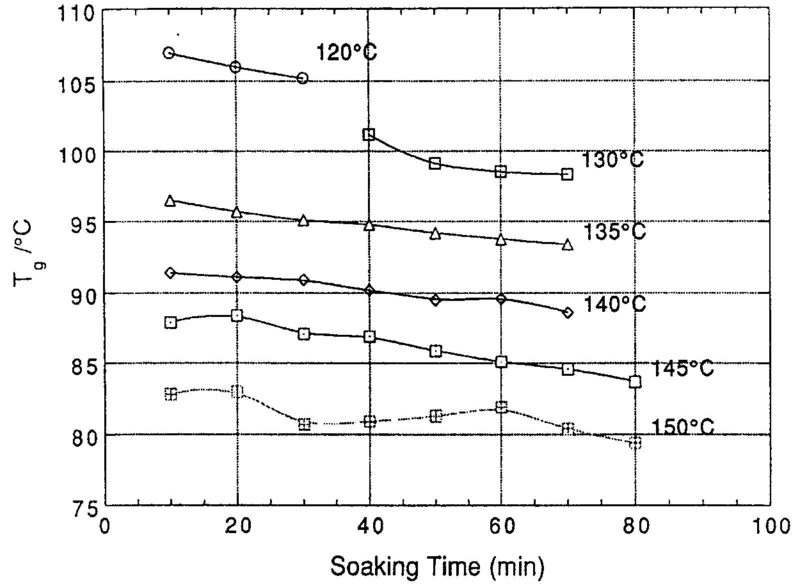

Fig. 2 Decrease of T_g of originally anhydrous trehalose sample with time of exposure to high temperatures

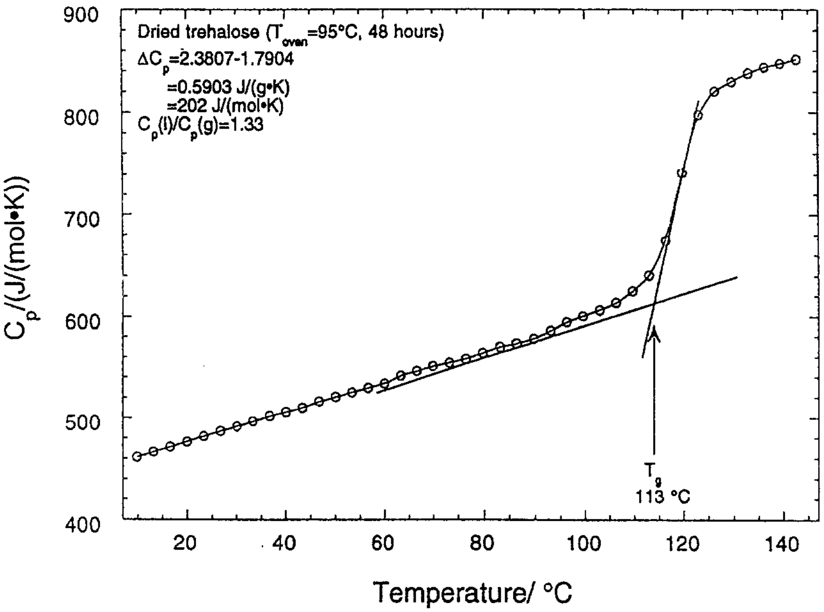

Fig. 3 Quantitative heat capacity *vs.* T relationship obtained with a Setaram model 111 DSC. The change of C_p at T_g is obtained as 202 J mol K^{-1}, which is an increase of 33% over the glass value at T_g=113°C

Figure 2 shows that under some circumstances, T_g can decrease systematically with exposure to higher temperatures, presumably explaining the lower values reported in some previous studies [4, 26]. The slow decomposition of anhydrous sugar melts is well documented, particularly for sucrose [27], and the depressive effect on T_g was monitored in a recent study of molten fructose held at T~120°C [28]. Such effects are catalyzed by acids and impurities, and therefore tend to be irreproducible.

Figure 3 shows the quantitatively determined heat capacity *vs*. temperature relationship obtained with the Setaram DSC 121, after an initial scan to 148°C (total exposure time to $T > T_g$ was very short) had been made to remove all effects of the preparation route seen in Fig. 1.

Figure 4, part (a) shows the X-ray diffractogram of the dihydrate before dehydration, while part (b) shows that of the anhydrous product of the initial dehydration process. The highly amorphous character of the latter is obvious. The diffractogram shown in part (c) is from the sample that was heated for some hours at 125°C, during which it compacted to a state suitable for determination of the shear modulus. A DTA scan of the compacted sample, taken at the end of the preparations for the relaxation studies discussed next, showed that the glass transition remained at 115°C, suggesting that the sample had not decomposed significantly during the exposure to the higher temperature. However, this sample was not hermetically sealed, and loss of some water of decomposition could have helped maintain a high T_g. This scan also showed an exotherm, indicative of α-trehalose crystallization, starting at 160°C, followed by a sharp endotherm, tentatively identified as fusion of α-trehalose, commencing at 188°C. The fusion was followed by decomposition.

Figure 5 shows the decay of stress, normalized by its initial value, after an instantaneous small strain was imposed at different constant temperatures. The solid lines through the data points are best fits to the stretched-exponential relaxation function [29].

$$\Theta(t) = \exp - \left[\left(\frac{t}{\tau} \right)^{\beta} \right] \tag{1}$$

Figure 6 shows, in Arrhenius form, the most probable relaxation times, τ, determined by fitting the data of Fig. 5. Both the slope and the pre-exponent are very high, typical of a fragile glass-former.

Discussion

From the scans of Fig. 1 and the diffraction patterns of Fig. 3, it is evident that the dehydration of crystalline trehalose dihydrate has produced the glassy state of this substance. Since the highest temperature to which the substance has been exposed is that of the glass transition, this is presumably a more accurate value of T_g than those reported in several previous studies [4, 26], including one of our own [4]. A comparable value (107°C) has been reported by Roos [30]. The value we find here, 115°C, is the highest value of T_g recorded for any disaccharide. A T_g of 109°C for anhydrous ββ-trehalose has recently been reported by Roberts and Franks [31].

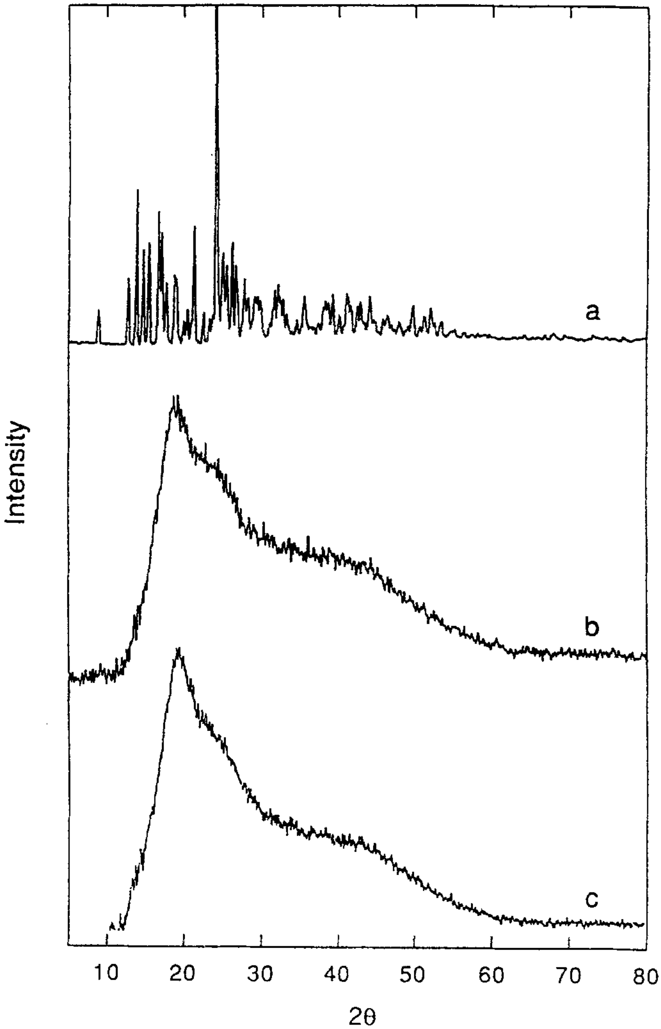

Fig. 4 X-ray diffractograms of (a) trehalose dihydrate, (b) anhydrous trehalose after vacuum-dehydration at 90°C for 30 h, and (c) after subsequent soaking with mechanical deformation at 125°C for 5 h. During a subsequent DTA scan to 200°C, the latter sample showed a heat absorption starting at 160°C and a well-defined melting point starting at 188°C. Melting was followed by foaming decomposition

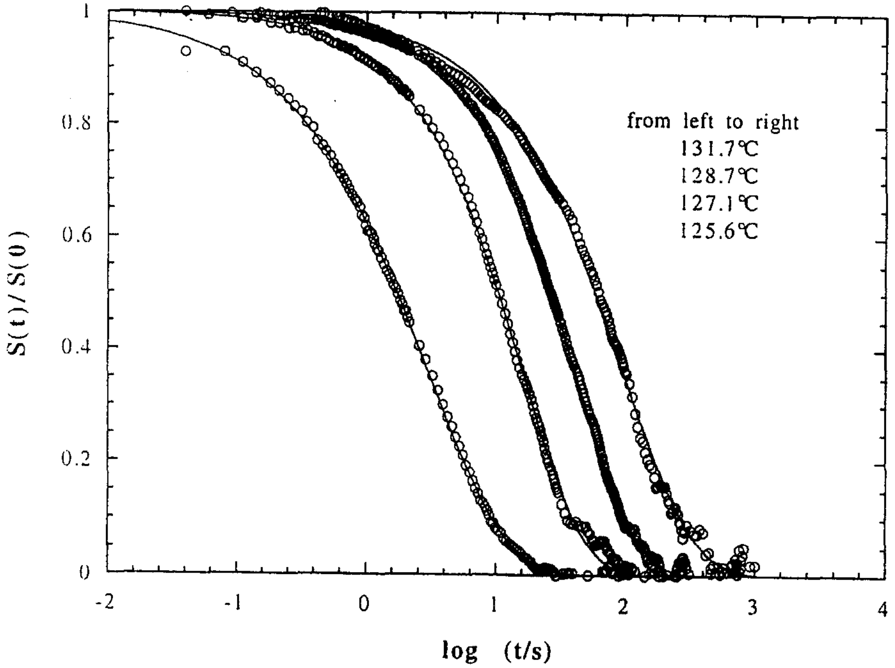

Fig. 5 Shear stress decay curves for compacted anhydrous trehalose in sandwich configuration at different temperatures as marked in the legend. Solid lines are best fits to the Kohlrausch decay function, $\Theta(t) = \exp-[(t/\tau)^{\beta}]$

We discuss three aspects of our findings. Firstly, we consider the relevance of the high T_g value we determined to the role of trehalose in biopreservation. Secondly, we consider the viscous relaxation behavior of trehalose in relation to that of other hydrogen-bonded glass-formers. Thirdly, we consider the spontaneous amorphization phenomenon in the light of Saleki-Gerhardt *et al.*'s more detailed study [16].

The value of T_g obtained for trehalose in this work, 115°C, is 43°C higher than a value attributed to the best-known of the sugars, sucrose: the latter T_g is also subject to some uncertainty, due to decomposition during melting. An immediate consequence of this finding is the rationalization of the exceptional value of w'_g (the water content of the solution for which the T_g and ice-supersaturation lines meet), which, as Slade and Levine have emphasized [6], is the clearly exceptional characteristic of the trehalose-water system, when compared with other disaccharides. To account for the small value of w'_g, it is only necessary to assume the same rate of plasticization of pure trehalose glass by water as for other sugars. The T_g line is then a curve parallel to the sucrose T_g line of Fig. 5 of Ref. 26 and Fig. 3 of Ref. 32. This figure is reproduced in Fig. 7 with the T_g curve for trehalose, based on the present datum and data at higher water

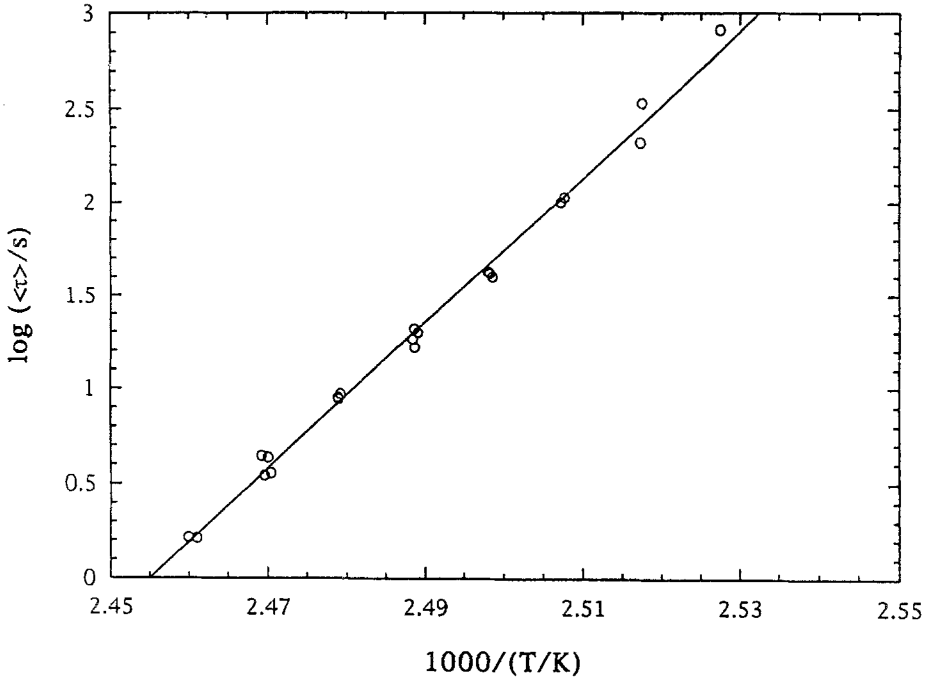

Fig. 6 Shear relaxation times for the viscous liquid phase of trehalose at different temperatures, from both single-slab and twin-slab configuration samples

contents from Ref. 4, added in. As a direct result of the high T_g of pure trehalose, therefore, the kinetics of processes in trehalose/water solutions can be brought to a halt by viscous slowdown, suspending life, while the water content remains quite high relative to that in other saccharide/water systems of the same T_g. Thus, the original Green-Angell interpretation [4] of trehalose efficacy is supported. (Of course, a comparable pure-sugar T_g can be obtained by using a trisaccharide, e.g. raffinose T_g=103°C [16], but the larger molecule may be disadvantageous for other reasons, and the T_g is, in any case, not as high.) On the other hand, we must note here a recent, novel interpretation of trehalose efficacy [7], which involves the dihydrate crystallizability as a key feature and the T_g of the residual glass only secondarily.

Trehalose can certainly be used to stabilize proteins against denaturation during hot desiccation. Figure 8 shows that cytochrome c, a protein recently investigated by us from a glassy-dynamics viewpoint [33, 34], resists denaturation up to 115°C, when dried at ambient temperature under vacuum to 6% residual water in the presence of 27 wt% trehalose, cf. normal denaturation at 65°C. Figure 8 also shows the increase in strength of the glass transition after unfolding, which was illustrated, using results from Sochava and Smirnova [35] on legumin, in a recent discussion of the fundamental nature of the unfolding transition [14].

What else can we say about pure trehalose? The relaxation data shown in Figs 5 and 6 may be used to argue that pure trehalose, like sucrose [36] is a

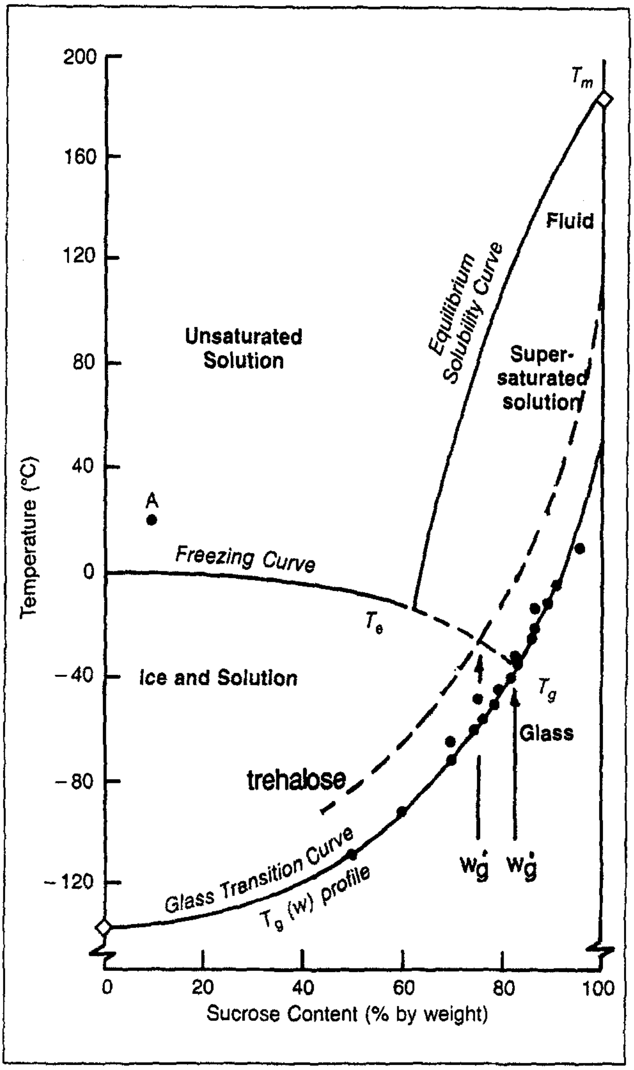

Fig. 7 Phase diagram for sucrose–water, from Ref. [31], including glass temperature *vs.* composition relationship. w'_g is the composition at which freezing-out of ice from metastable solutions becomes kinetically excluded, because the extrapolated ice liquidous line intersects the T_g curve. The corresponding T_g plot for trehalose-water, based on the new value of T_g for pure trehalose and older data for trehalose-water solutions from Ref. [4], is superimposed (see dashed line) to demonstrate the origin of the exceptional w'_g for the trehalose solutions. (By permission)

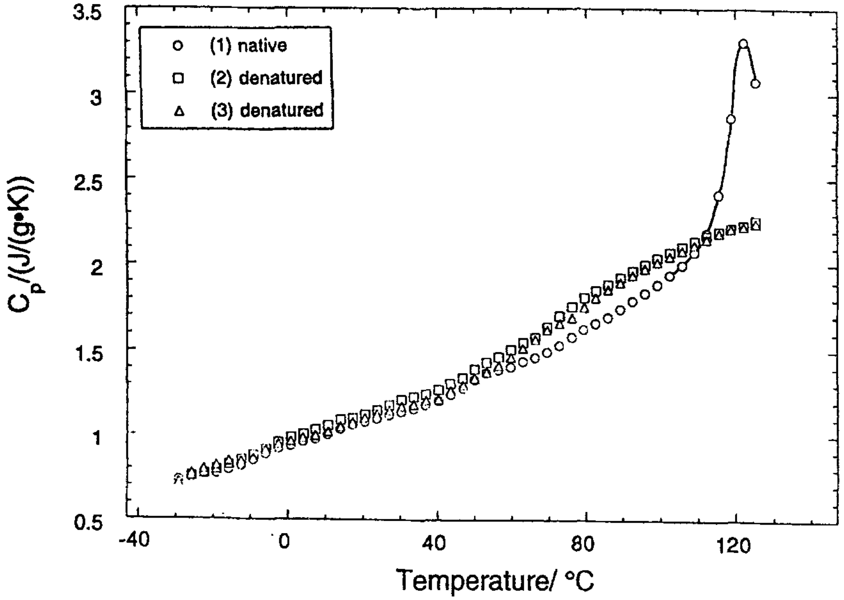

Fig. 8 DSC curves of low water-content (6 wt%) solutions of cytochrome c (67 wt%)-trehalose (27 wt%). These samples were produced by water removal from the initially fluid, aqueous solution by evaporation at 60°C. The protein clearly remained in the native state during the dehydration. The denaturation temperatures seen in Fig. 8 are increased by about 40°C over their normal values in water-saturated systems. Successive scans of the denatured solutions show the same behavior, within instrument reproducibility. Note the increase in configurational heat capacity consequent on the loss of tertiary structure

very fragile liquid [14, 37], despite its multi-lateral hydrogen-bonding nature [38] (sucrose has also been assigned high fragility on the basis of existing data [36]. To show this, we use the Maxwell equation

$$\eta_s = G_\infty \tau_{ss} \tag{2}$$

to convert our relaxation-time data to viscosities of trehalose at temperatures near but above T_g, for comparison with other representative liquids. In considering this conversion, we note that the apparently long values of τ obtained using the sandwich cell, convert to relatively small viscosities, because the value of the shear modulus whose relaxation is observed is rather small compared to the glassy moduli usually employed with Eq. (1). We measured an average G_∞ of $10^{5.6}$ Pa with the sandwich cell and use this value for all temperatures. While these values, plotted in Fig. 9, are subject to confirmation using direct measurements, we believe they serve to establish the fragile nature of liquid trehalose,

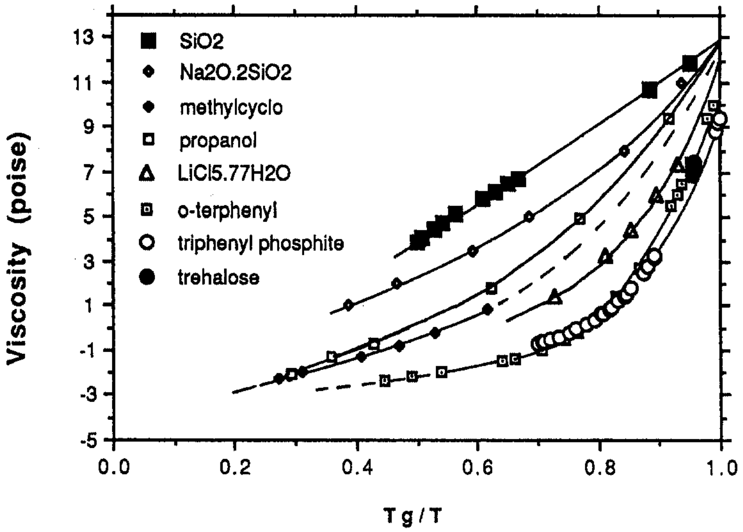

Fig. 9 Viscosities calculated from shear moduli and shear relaxation times, shown on expanded-scale, T_g-scaled Arrhenius plot, along with data for other glass-forming systems, to permit assessment of anhydrous trehalose fragility

via the comparison with other fragile liquids. A high fragility for trehalose+water solutions (i.e. trehalose plasticized by water) can also be inferred from the NMR relaxation data of Ludemann and co-workers [39].

Finally, we return to the starting point of this paper to consider the spontaneous vitrification of the trehalose dihydrate crystal during dehydration. This phenomenon will warrant detailed study in future work, but some worthwhile comments on the phenomenology can be made, by combining our observations with those of Saleki-Gerhardt *et al.*on vitrification of raffinose hydrates under dehydration [16] and comparing them with what is known about spontaneous vitrification of anhydrous crystals under decompression [17, 40, 41].

The first observation of spontaneous vitrification of a crystalline material was made by Skinner and Fahy [41]. These workers found that when the metastable, high-pressure, rutile form of SiO_2, containing Si in 6-coordination, is heated above 300°C, it collapses to a glassy state with Si 4-coordinated as in silica glass. [The rutile form, known as Stishovite, is formed at high pressure (often during meteorite impact) and may be recovered in a metastable state at ambient.] Essentially the same observation is reported by Saleki-Gerhardt *et al.* [16], with the difference that the metastable crystal state is obtained by dehydration rather than by decompression. They report that raffinose pentahydrate can be deydrated at 30°C to the trihydrate without loss of crystal structure ("same integrated peak intensities" [16]), but that a pentahydrate heated at 100°C retains three waters of hydration, and the crystal morphology, but becomes amorphous. Evidently, the crystalline trihydrate at 30°C is in a condition of metastability analogous to that of SiO_2 stishovite at ambient pressure and, as in that case, heating to a higher temperature (between 30°C and 100°C in the raffinose trihydrate case) must permit the release of metastability by vitrification, i.e. the glass constitutes a lower free-energy state of the system than does the crystal derived from the pentahydrate by loss of two waters. In principle, if there were no kinetic barrier to water migration, the removal of water could bring the crystal to a state of absolute mechanical instability, in which spontaneous collapse to some other phase would be inevitable. This phenomenon has been discussed in detail, based on molecular dynamics and lattice dynamics studies, for the case of decompression amorphization of silicate perovskites [40]. In practice, however, the vitrification occurs by nucleation and growth of the amorphous phase, in an intriguing inversion of the familiar process of crystallization of metastable liquids. A similar scenario must apply to the trehalose case of this paper, except that the conditions for obtaining the metastable, fully-anhydrous crystal with the dihydrate structure were not met. (By use of lower temperatures and powdered crystals, it may be possible to generate a metastable crystalline anhydrate, and this will be sought in future work.)

Following the above discussion, a plausible origin for the large and irrecoverable endothermic overshoot in the first scan of Fig. 1 can be recognized, if, during the dehydration process, amorphous regions nucleate and grow until they overlap. Then, residual pockets of untransformed crystalline material, which could contain transformation-concentrated residual water (inverse to the normal concentration of residual water in interstitial liquid pockets during crystallization), could remain in the nominally anhydrous material. Only when the amorphous phase starts to relax at T_g can these remaining pockets of metastable crystal transform to the amorphous phase, with the absorption of heat as they disorder; hence, the additional endothermic strength of the glass transition seen in the first scan of Fig. 1.

What is not known in either case is the extent of water loss from the parent crystal which can be tolerated before the crystal becomes metastable with respect to the glass of the same composition, and this will be pursued in future work. It may be concluded that crystal desolvation studies offer a new area for investigation of spontaneous vitrification processes, which is now becoming a significant field of research (see Ref. [17] for references to this literature). The need for out-migration of the small molecule species, before crystal instability occurs, means, unfortunately, that the vitrification could never be studied as close to the limit of metastability of the crystalline phase (the spinodal boundary, [17, 40]) as in the case of decompression studies, because the low temperatures needed to repress homogeneous nucleation would also repress the small molecule out-migration.

Concluding remarks

The migration of water molecules in sugar hydrates, both crystalline and amorphous, appears to be opposed by relatively low barriers [39]. In the liquid and glassy states, this would be described as a decoupled relaxation mode, in the sense of being only weakly controlled by the viscosity, in violation of the Stokes-Einstein equation. It will be interesting in future studies to compare such decoupled motion with the more familiar cases of decoupled motion of small ions in "superionic" glasses [42], and to determine whether water mobility differs significantly among different sugars at the same water content and reduced temperature, T/T_g.

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