

# High Critical Temperature above $T_g$ May Contribute to the Stability of Biological Systems

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**ABSTRACT** In this study, we characterized the molecular mobility around  $T_g$  in sugars, poly-L-lysine and dry desiccation-tolerant biological systems, using ST-EPR, <sup>1</sup>H-NMR, and FTIR spectroscopy, to understand the nature and composition of biological glasses. Two distinct changes in the temperature dependence of the rotational correlation time ( $\tau_R$ ) of the spin probe 3-carboxy-proxyl or the second moment ( $M_2$ ) were measured in sugars and poly-L-lysine. With heating, the first change was associated with the melting of the glassy state ( $T_g$ ). The second change ( $T_c$ ), at which  $\tau_R$  abruptly decreased over several orders of magnitude, was found to correspond with the so-called cross-over temperature, where the dynamics changed from solid-like to liquid-like. The temperature interval between  $T_g$  and  $T_c$  increased in the order of sucrose < trehalose < raffinose  $\leq$  stachyose < poly-L-lysine < biological tissues, from 17 to >50°C, implying that the stability above  $T_g$  improved in the same order. These differences in temperature-dependent mobilities above  $T_g$  suggest that proteins rather than sugars play an important role in the intracellular glass formation. The exceptionally high  $T_c$  of intracellular glasses is expected to provide excellent long-term stability to dry organisms, maintaining a slow molecular motion in the cytoplasm even at temperatures far above  $T_g$ .

## INTRODUCTION

The ability of sugars to preserve biomolecules has been recognized for years in food, pharmaceutical, and biological sciences (Burke, 1986; Leopold et al., 1994; Roos, 1995; Hancock et al., 1995; Hancock and Zografis, 1997; Crowe et al., 1996, 1998). Apart from a direct interaction of the sugars with the biomolecules, the ability of these sugars to form glasses is thought to be a major factor in long-term preservation (Levine and Slade, 1988; Roos, 1995; Leopold et al., 1994; Crowe et al., 1998). The high viscosity of the glass and its concomitant low molecular mobility are known to preserve stability of proteins (Chang et al., 1996), to prevent fusion of liposomes (Sun et al., 1996), or to slow down reaction rates, such as browning reactions (Karmas et al., 1992; Roos, 1995).

Nature, too, makes use of glasses to preserve biological tissues in the dehydrated state. Some well-known examples are seeds (Williams and Leopold, 1989; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995), pollen (Buitink et al., 1996), prokaryotes (Potts, 1994), and the so-called resurrection plants (Ghasempour et al., 1998). These tissues are capable of resisting extreme dehydration and are able to resume metabolic activities after the addition of water. All these tissues accumulate, to a large extent, soluble sugars, predominantly sucrose, trehalose, and, in the case of seeds, oligosaccharides such as raffinose and stachyose (Amuti

and Pollard, 1977; Hoekstra et al., 1992; Ghasempour et al., 1998). Because of the reputation of these sugars to be excellent glass formers, it has been suggested that these sugars play an important role in the glass formation in these biological tissues (Hirsh, 1987; Koster, 1991).

The glass transition temperature ( $T_g$ ) varies with molecular weight of the polyols in a characteristic and predictable manner, almost increasing linearly with the molecular weight (Slade and Levine, 1991). The higher the  $T_g$  of the polyol, the more advantageous it will be in preserving biomolecules (Slade and Levine, 1991). For example, trehalose, a sugar with a high  $T_g$ , has often been reported to be remarkably effective in stabilizing dry or frozen biomolecules, cells, and tissues (Crowe et al., 1996, 1998). Oligosaccharides, such as raffinose and stachyose, have high  $T_g$  values, rendering them effective protectants (Levine and Slade, 1988). These sugars are also capable of forming crystalline hydrates (Aldous et al., 1995). It has been suggested that formation of some crystalline hydrate results in keeping the remaining amorphous sugar dry, thereby maintaining a high  $T_g$  (Aldous et al., 1995; Crowe et al., 1998).

Despite the frequent use of sugars as protecting agents, little is known about the molecular mobility in sugar glasses around their  $T_g$ . The melting of the glassy state of sugars has been implied to result in a dramatic decrease in viscosity and increase in molecular mobility (Williams et al., 1955; Soesanto and Williams, 1981; Roos, 1995). An understanding of how molecular mobility changes upon melting of the glassy state in the different sugars might give an alternative clue as to why trehalose and oligosaccharides are such effective stabilizers.

Biological organisms can survive in the dry state for extremely long periods, even at ambient temperatures

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(Priestley et al., 1985; Shen-Miller et al., 1995; Steiner and Ruckebauer, 1995). Sucrose and oligosaccharides comprise over 10 to 20% of the dry weight of seeds, and mixtures of these sugars were found to inhibit crystallization (Caffrey et al., 1988), which could explain the absence of crystallization in seeds (Sun and Leopold, 1993). Nonetheless, it has been suggested that these sugars are not the only components that participate in the intracellular glass formation (Hirsh, 1987; Sun and Leopold, 1993, 1997; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Wolkers et al., 1998a; Buitink et al., 1999). The change in molecular mobility in the cytoplasm of seeds with temperature, as measured by spin probe saturation transfer electron paramagnetic resonance (ST-EPR) spectroscopy, was found to be quite different compared with that of pure sugars (Buitink et al., 1999). Upon melting of a pure sucrose glass, a large increase in mobility was found within the first 30°C above  $T_g$ , encompassing four orders of magnitude (Roozen and Hemminga, 1990), whereas in biological tissues, molecular mobility increased only a factor 7 upon melting of the intracellular glass over the same temperature range (Buitink et al., 1999). Besides sugars, another major compound present in the cytoplasm is protein. Polypeptides have been shown to alter the glass properties of sugars significantly (Kalichevsky et al., 1993; Wolkers et al., 1998a). Characterization of the molecular mobility of compounds that could be involved in intracellular glass formation might lead to a better understanding of how these biological tissues can survive for such long periods. Ultimately, this might aid in the preservation of biomolecules in the pharmaceutical and food sciences.

Electron paramagnetic resonance (EPR) spectroscopy is a powerful technique to obtain information about the rotational mobility of spin-labeled molecules, because a very broad range of molecular motions can be covered by this technique (Hemminga and Van den Dries, 1998). Conventional EPR is sensitive in the motional region for  $\tau_R$  values between  $10^{-11}$  and  $10^{-7}$  s, whereas ST-EPR is sensitive to molecular motions between  $10^{-6}$  and  $10^{-3}$  s (Hemminga and Van den Dries, 1998; Buitink et al., 1999). However, a drawback of the use of spin probe EPR spectroscopy is that one measures the molecular motion of a foreign molecule (spin probe) introduced into a glass, and not the molecular mobility of the molecules that form the glass themselves. A technique that avoids this is  $^1\text{H}$ -nuclear magnetic resonance (NMR) spectroscopy, that can be used to determine the second moment of the NMR lineshape,  $M_2$ , which provides information on the mobility of the immobile proton fraction around  $T_g$  (Van den Dries et al., 1998). Alternatively, Fourier transform infrared (FTIR) spectroscopy can be used to assess the properties of the hydrogen-bonding network of the sugar glasses around  $T_g$  (Wolkers et al., 1998b).

In this study, we characterized the molecular mobility around  $T_g$  in sugars that are thought to be involved in biological glass formation, using ST-EPR,  $^1\text{H}$ -NMR, and

FTIR. Also, the molecular mobility of a polar spin probe present in the cytoplasm of pollen and seed was measured and compared with sugar and protein glasses to understand the nature and composition of biological glasses. The high stability of biological tissues is discussed in relation to the composition of the intracellular glass.

## MATERIALS AND METHODS

### EPR spectroscopy

Glasses were prepared by rapidly drying 10  $\mu\text{l}$  droplets of 0.1 g  $\text{ml}^{-1}$  sucrose (Pfahnstiel, Waukegan, IL), amorphous trehalose (Pfahnstiel), raffinose-pentahydrate (Sigma, St. Louis, MO), amorphous stachyose (Sigma), and poly-L-lysine (PolyLys, Sigma,  $M_w$  36 kDa), containing 0.1 mM 3-carboxy-proxyl (CP, Sigma), on a glass plate in a box with an airflow of 3% relative humidity (RH) at room temperature. In a similar way, glasses were formed of a 1:1 (w/w) sucrose:stachyose mixture, and a 1:1 (w/w) sucrose:PolyLys mixture. Glasses were formed within 5 min (Wolkers et al., 1998a). After 3 days of drying, the material was removed from the glass plate and equilibrated at 3% RH in the box or over a saturated salt solution of  $\text{MgCl}_2$  (33% RH) at 10°C for at least 7 days. Subsequently, the glasses were loaded into EPR capillaries under the respective RH conditions, sealed, and measured directly.

*Typha latifolia* L. pollen was harvested in The Netherlands in 1997. Labeling of the pollen with the spin probe was performed according to Buitink et al. (1998a). Briefly, 3 g dry pollen was prehydrated in water vapor for 16 h at 5°C to  $\sim 0.7$  g  $\text{H}_2\text{O}$  g  $\text{dw}^{-1}$  and then mixed at 25°C with 6 ml liquid germination medium containing 2.5 mM CP. After a few minutes, an additional 20 ml germination medium was added, and the pollen was recovered by filtration. The pollen was then mixed with a 20-ml solution of 1 mM CP and 120 mM potassium ferricyanide. The ferricyanide was added to broaden the signal of CP outside of the cells. Because ferricyanide cannot penetrate intact cells, the signal obtained is exclusively derived from the cytoplasm. After an additional 5 min, the pollen was recovered by filtration, spread out in a large petri dish, and rapidly dried in a flow of dry air (3% RH) to a water content of  $< 0.05$  g  $\text{H}_2\text{O}$  g  $\text{dw}^{-1}$ . After drying, pollen was stored over a saturated solution of  $\text{MgCl}_2$  (33% RH) for 7 days. For each EPR measurement,  $\sim 20$  mg pollen was loaded at 33% RH and sealed in a 2-mm diameter EPR capillary. After the measurements, the pollen was removed from the capillaries and water content was determined. Water content was analyzed by weighing the samples before and after heating at 96°C for 36–48 h.

Dry bean embryonic axes (*Phaseolus vulgaris* L.) were excised from the seeds, and allowed to imbibe in water for 2 h at 15°C, after which the axes were incubated in a 10-ml solution of 1 mM CP. After 45 min, potassium ferricyanide was added to a final concentration of 200 mM, and the axes were incubated for another 15 min. Subsequently, the bean embryonic axes were blotted dry on filter paper, and dried in dry air (3% RH) for 24 h. After drying, axes were stored over a saturated solution of  $\text{MgCl}_2$  (33% RH) for two weeks. Four axes were loaded at 33% RH and sealed in a 2-mm diameter capillary for EPR measurements. After the measurements, the axes were removed from the capillaries and water content was determined. Water content was analyzed by weighing the samples before and after heating at 96°C for 36–48 h.

EPR spectra were recorded with a Bruker X-band EPR spectrometer (model 300E, Bruker Analytik, Rheinstetten, Germany). The  $\tau_R$  in the fast motional region was determined from the lineshapes of EPR spectra according to the method of Knowles et al. (1976) for isotropic tumbling:

$$\tau_R = 6.5 \times 10^{-6} \Delta B_0 \{ (h_C/h_H)^{1/2} - 1 \}, \quad (1)$$

where  $B_0$  is the width of the center field component in Tesla, and  $h_C$  and  $h_H$  are the amplitudes of the central and high field components of the three-line nitroxide radical spectrum, respectively.

At low water contents and temperatures, when  $\tau_R$  becomes longer than  $10^{-8}$  s,  $\tau_R$  cannot be calculated according to Eq. 1 because of the appearance of a powder spectrum. ST-EPR was used to determine  $\tau_R$  between  $10^{-6}$  and  $10^{-3}$  s. ST-EPR is based on the diffusion and recovery of saturation between different parts of the powder spectrum in competition with field modulation (Hemminga, 1983). For ST-EPR measurements, the second harmonic quadrature absorption signal was detected under the following conditions: field modulation amplitude, 0.5 mT; microwave power, 100 mW; and field modulation frequency, 50 kHz. The phase was set with the self-null method (Thomas et al., 1976).

ST-EPR spectra can be characterized by independent lineshape parameters, such as the line-height ratio  $L'/L$ . Using reference material with known viscosity,  $\tau_R$  values can be obtained in an empirical way. Here we used spectra of CP in anhydrous glycerol to construct a calibration curve (Hemminga and Van den Dries, 1998) of the lineshape parameter  $L'/L$  versus  $\tau_R$  values (for details, see Buitink et al., 1999). From this calibration curve, the values of  $\tau_R$  of CP in the sugar, polypeptide, and biological material were obtained by interpolation of the corresponding lineshape parameters. For all samples, EPR measurements were performed at least twice to check for reproducibility.

## <sup>1</sup>H-NMR spectroscopy

Sugar glasses were prepared by lyophilizing 20% (w/w) trehalose-dihydrate (Sigma), amorphous stachyose (Sigma), and sucrose (Merck, Darmstadt, Germany). Subsequently, powders were stored for 3 weeks over phosphorus pentoxide or a saturated solution of MgCl<sub>2</sub> (33% RH) at room temperature. Samples were loaded in a 5-mm NMR tube that was sealed to prevent water evaporation.

<sup>1</sup>H-NMR measurements were performed on a Bruker AMX 300 spectrometer equipped with a Bruker 5-mm proton probe operating at a resonance frequency of 300.13 MHz. The temperature was regulated with a liquid nitrogen temperature control. In this way, stability was within 0.5°C. A spectral width of 500 kHz was used. The duration of the pulse was 6–7  $\mu$ s. The presented free induction decays are averages of 128 or 256 scans having 2048 data points. For the analysis of the NMR, the free induction decays (FiDs)  $F(t)$  were fitted to the equation (Van den Dries et al., 1998),

$$F(t) = A \exp[(a^2 t^2)/2](\sin bt)/bt + B \exp[-t/T_{2m}]. \quad (2)$$

In this equation, the parameters  $A$  and  $B$  represent the contributions of the immobile and mobile protons and  $T_{2m}$  is the spin-spin relaxation time of the mobile proton fraction. The NMR spectrum of the immobile proton fraction is assumed to be a rectangular lineshape with a total width  $2b$ , convoluted with a gaussian lineshape with a standard deviation given by  $a$  (Van den Dries et al., 1998). The second moment  $M_2$  of the broad lineshape, which is a measure of the strength of the dipolar interactions, is calculated from the fit parameters  $a$  and  $b$  by the equation,

$$M_2 = a^2 + b^2/3. \quad (3)$$

A reduction of  $M_2$  takes place if the proton density in the samples is lowered, because dipolar interactions decrease with the sixth power of the distance between protons. Furthermore,  $M_2$  will be reduced by anisotropic mobility or slow isotropic rotations that partly average out the dipolar interactions (Van den Dries et al., 1998).

## FTIR spectroscopy

Glasses were prepared by rapid air drying (at 3% RH) of a droplet of 0.01 g ml<sup>-1</sup> sucrose (Pfanstiel), amorphous trehalose (Pfanstiel), raffinose-pentahydrate (Sigma), and amorphous stachyose (Sigma) on circular CaF<sub>2</sub> windows of 13-mm diameter, width 2 mm. Glasses were formed within 5 min (Wolkers et al., 1998a). The absence of crystallization was confirmed by the absence of sharp bands in the FTIR spectra (Wolkers et

al., 1998b). Residual water in the sugar glasses was removed by heating the sample for 5 min to 70°C in the case of sucrose, and to 100°C for the other sugars. The absence of water in the glasses was verified by the absence of the water band around 1650 cm<sup>-1</sup>. FTIR spectra were recorded on a Perkin-Elmer 1725 spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope interfaced to a personal computer as described previously (Wolkers et al., 1998a,b). Each sample was hermetically sealed between two CaF<sub>2</sub> IR-windows, using a rubber O-ring, and mounted into a temperature-controlled brass cell that was cooled by a liquid nitrogen source. The temperature was regulated by a computer-controlled device that activated a liquid nitrogen pump, in conjunction with a power supply for heating of the cell. The temperature of the sample was recorded separately using a PT-100 element that was located very close to the sample windows. The optical bench was purged with dry CO<sub>2</sub>-free air (Balston, Maidstone, Kent, UK) at a flow rate of 25 liter min<sup>-1</sup>. The acquisition parameters were 4 cm<sup>-1</sup> resolution, 32 co-added interferograms, 3600–900 cm<sup>-1</sup> wavenumber range.

Spectral analysis and display were carried out using the Infrared Manager Analytical Software, version 3.5 (Perkin-Elmer). The melting of glasses during heating of the sample at 1.5°C min<sup>-1</sup> was monitored by observing the position of the band around 3300 cm<sup>-1</sup> (OH-stretching vibration,  $\nu_{OH}$ ). The band position was calculated as the average of the spectral positions at 80% of the total peak height.  $T_g$  was determined by the intersection of linear regressions of the  $\nu_{OH}$  at low and high temperatures. Temperature scans for each sugar sample were performed in triplicate.

## Differential scanning calorimetry

Dry glasses were prepared by rapidly drying 10- $\mu$ l droplets of 0.1 g ml<sup>-1</sup> of different sugars, PolyLys or sucrose:PolyLys and sucrose:stachyose mixtures. After drying for 3 days in an airflow of 3% RH, glasses were removed from the glass plate and equilibrated for 7 days at 3% or 33% RH, after which the material was loaded under the respective RH conditions and sealed in hermetically sealed differential scanning calorimetry pans. Second-order transitions of the samples were determined using a Perkin-Elmer (Norwalk, CT) Pyris-1 differential scanning calorimetry, calibrated for temperature with indium (156.6°C) and methylene chloride (–95°C) standards and for energy with indium (28.54 J g<sup>-1</sup>). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. Heating thermograms were obtained at a scanning rate of 10°C min<sup>-1</sup>. The  $T_g$  values were determined as the onset of the temperature range over which the change in specific heat occurred.

Samples prepared for EPR and NMR measurements were checked for crystallization. Sucrose glasses equilibrated to 33% RH were found to be completely crystallized. Therefore, EPR and NMR measurements of sucrose equilibrated to 33% RH were omitted from the analyses. For the other sugar samples, not more than 2% hydrate formation was observed, by comparison of the melting enthalpies of pure trehalose-dihydrate, raffinose-pentahydrate, and stachyose-dihydrate (Sigma) to the melting enthalpies of the prepared amorphous sugars. All analyses were performed with Perkin-Elmer software.

## Sugar determination

The soluble sugar content in bean embryonic axes was determined by DIONEX-HPLC (Tetteroo et al., 1994). Excised dry bean axes were ground in a mortar in the presence of 3 ml 80% methanol containing lactose as the internal sugar standard. The suspension was removed from the mortar with 80% methanol and heated in a water bath at 76°C for 15 min. The liquid was evaporated under vacuum in a Speedvac AES1010 (Savant Instruments). The residue was dissolved in distilled water, and after appropriate dilution, sugars were analyzed by high performance liquid chromatography on a CarboPac PA-1 column (Dionex Corp., Sunnyvale,

CA) using pulsed amperometric detection, as described by Tetteroo et al. (1994). Data are the average of three extractions, each extraction containing 10 axes.

## RESULTS

### Molecular characterization of sucrose, trehalose, and oligosaccharide glasses

We investigated the molecular mobility of the polar nitroxide spin probe 3-carboxy-proxyl (CP) in glasses of the various biologically relevant sugars, sucrose, trehalose and oligosaccharides, using ST-EPR and conventional EPR spectroscopy. Figure 1 shows the rotational correlation time ( $\tau_R$ ) of CP in the sugars around  $T_g$ . For all sugar glasses, the  $\tau_R$  became shorter below  $T_g$  with increasing temperature, indicating a faster rotational motion of the spin probe in the sugar glasses. Around  $T_g$ , a break in the linear relationship was observed (Fig. 1). Above  $T_g$ , the logarithm of the rotational motion increased linearly but more strongly with an increase in temperature compared with below  $T_g$ . Remarkably, a second break in the relationship between  $\tau_R$  and temperature was observed, hereafter referred to as the critical temperature ( $T_c$ ) (Fig. 1, arrows). For sucrose, this  $T_c$  occurred at 18°C above  $T_g$ , whereas for the trehalose sample, this  $T_c$  occurred at 25°C above  $T_g$ . For raffinose,  $T_c$  occurred 35°C above  $T_g$  (data not shown), and for stachyose  $T_c$  occurred 37°C above  $T_g$  (Fig. 1). Above  $T_c$ , rotational motion increased abruptly with an increase in temperature over several orders of magnitude for all sugars, until the environment of the spin probe had become completely fluid (indicated by the presence of a three-line EPR spectrum). The  $\tau_R$  values depicted in Fig. 1 were from trehalose and raffinose glasses that were equilibrated to 33% RH. The

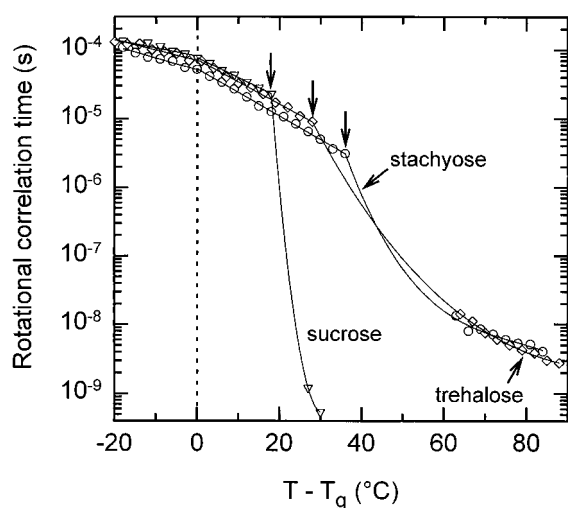


FIGURE 1 Rotational correlation time ( $\tau_R$ , s) of CP in different sugars as a function of  $T - T_g$ . Dashed line,  $T_g$ ; arrows, the critical temperature  $T_c$ ; inverted triangles, sucrose equilibrated to 3% RH; diamonds, trehalose equilibrated to 33% RH; circles, stachyose equilibrated to 33% RH.

reason for using this elevated RH was the following: the high  $T_g$  for these sugars when equilibrated to 3% RH prevented the determination of the entire  $\tau_R$  curve because of a technical limitation of 400 K for the EPR measurements. Because sucrose crystallized at 33% RH,  $\tau_R$  was only determined for sucrose samples equilibrated to 3% RH. The temperature interval between  $T_g$  and  $T_c$  in dry trehalose, raffinose, and stachyose, all equilibrated to 3% RH, was 26, 34, and 34°C, respectively (data not shown). Comparison of these temperature intervals with those of sugars equilibrated to 33% RH indicated that a change in water content of the sugar glasses did not affect these temperature intervals significantly. When a second scan of the sugar samples was performed,  $\tau_R$  had the same temperature dependence (data not shown), indicating that the measured  $\tau_R$  was not dependent on the thermal history of the sugar samples.

The range of molecular motions for which  $\tau_R$  could not be determined using our protocols is from  $10^{-6}$  to  $10^{-8}$  s. For  $\tau_R$  longer than  $10^{-8}$  s, the EPR spectrum takes the shape of a powder spectrum, which makes it impossible to use Eq. 1. Furthermore, the  $L''/L$  ratio of the ST-EPR spectra is insensitive when  $\tau_R$  is shorter than  $10^{-6}$  s. The curves drawn through the data points suggest that there is an abrupt increase in rotational motion at  $T_c$  (Fig. 1). This abrupt increase is derived from the shape of the EPR and ST-EPR spectra of CP recorded at the temperature just above the last data point that could be calculated by ST-EPR (3°C higher). The insensitivity of the lineshape of the ST-EPR spectrum indicated that the  $\tau_R$  was shorter than  $10^{-6}$  s. In addition, the conventional EPR spectrum showed that there was a decrease in the principal values of the hyperfine interaction of CP ( $2A_{zz}$ ), indicating faster rotational motion (Buitink et al., 1999).

The  $\tau_R$  at  $T_g$  for sugars equilibrated to 3% RH had the following order: sucrose > trehalose > raffinose > stachyose (Table 1). For sugars equilibrated to 33% RH, the  $\tau_R$  at  $T_g$  changed in the same order. As can be seen from Table 1, the  $T_g$  values obtained by FTIR measurements are higher

TABLE 1 Rotational correlation time ( $\tau_R$ ) and wavenumber position ( $\nu_{OH}$ ) of sugar glasses at  $T_g$

Sugar	ESR			FTIR		
	WC (g/g dw)	$T_g$ (°C)	$\tau_R$ at $T_g$ ( $\mu$ s)	WC (g/g dw)	$T_g$ (°C)	$\nu_{OH}$ at $T_g$ ( $\text{cm}^{-1}$ )
Sucrose	0.027	43	80.1	<0.01	69	3373
Trehalose	0.037	63	78.6	<0.01	107	3392
Raffinose	0.041	72	58.1	<0.01	106	3401
Stachyose	0.025	81	36.0	<0.01	123	3411
Trehalose 33% RH	0.072	23	85.0	nd	nd	nd
Raffinose 33% RH	0.077	29	80.0	nd	nd	nd
Stachyose 33% RH	0.060	39	52.0	nd	nd	nd

Sugar glasses were equilibrated to 3% or 33% RH (ST-EPR) or first heated to remove the residual water (FTIR). The  $T_g$  values were taken as the break in the relationship of the  $\tau_R$  or  $\nu_{OH}$  as a function of temperature. WC, water content, g  $\text{H}_2\text{O}$  g  $\text{dw}^{-1}$ ; nd, not determined.

than those obtained by EPR measurements. This can be explained by the different methods of sample preparation. With FTIR, samples were first heated to 70°C to remove the residual water. Therefore, the  $T_g$  values found with this technique are close to the literature values (Slade and Levine, 1991; Roos, 1995). For the EPR measurements, samples were equilibrated to 3% RH for 7 days, which was not sufficient to remove all the residual water (see water contents in Table 1). A remarkable observation was that an increase in water content in the sugar glasses resulted in slower rotational motion at  $T_g$  (Table 1).

A comparable pattern of the temperature dependence of the mobility in the sugar glasses was observed with  $^1\text{H-NMR}$  (Fig. 2). With this technique, one can measure the second moment ( $M_2$ ), which provides information on the mobility of the immobile proton fraction around the  $T_g$  (Van den Dries et al., 1998). For all sugars,  $M_2$  decreased with increasing temperature below  $T_g$ , indicating an increase of the mobility of immobile proton fraction of the sugar glasses. Around  $T_g$ , a break in the linear relationship occurred, after which  $M_2$  decreased more strongly with an increase in temperature. Also with  $^1\text{H-NMR}$ , a second break in the relationship between  $M_2$  and temperature was observed for the various sugars (Fig. 2, arrows). At temperatures above this second transition, the protons of the immobile fraction became so mobile that they corresponded to the mobile proton fraction and could no longer be characterized by  $M_2$  (Van den Dries et al., 1998). This explains the occurrence of only a few data points above  $T_c$  (Fig. 2). Similar to  $\tau_R$  measurements, the temperature interval between  $T_g$  and  $T_c$  was smallest for sucrose (17°C) and largest for the oligosaccharide stachyose (32°C) (Fig. 2).

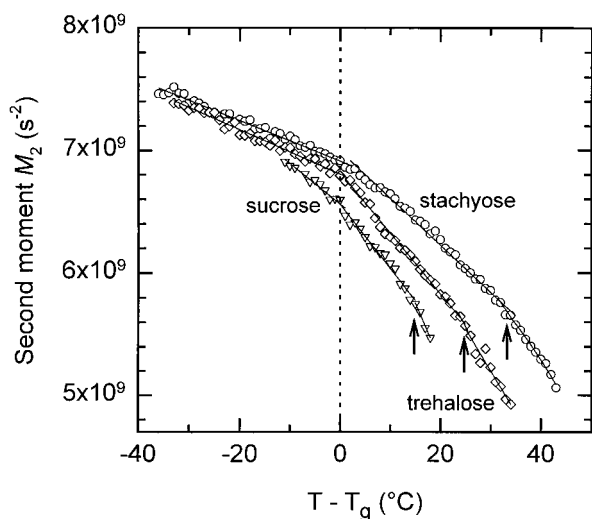


FIGURE 2 The second moment ( $M_2$ ,  $\text{s}^{-2}$ ) of immobile protons of different sugars as a function of  $T - T_g$ . Dashed line,  $T_g$ ; arrows, the critical temperature; inverted triangles, sucrose equilibrated to 3% RH; diamonds, trehalose equilibrated to 33% RH; circles, stachyose equilibrated to 33% RH.

To understand why the  $\tau_R$  at  $T_g$  of the spin probe became shorter with increasing size of the sugar molecules, information on the packing of the sugar molecules at  $T_g$  is necessary. FTIR spectroscopy was applied to obtain information on the hydrogen-bonding properties and molecular packing of the sugars. The wave number position of OH-stretching vibrations ( $\nu_{\text{OH}}$ ) reveals information about the strength and length of hydrogen bonding (Jeffrey, 1997). Figure 3 shows the  $\nu_{\text{OH}}$  of the sugar samples as a function of temperature. A break in the relationship corresponded to the  $T_g$  (Wolkers et al., 1998b), which is confirmed by molecular dynamics simulations, where it was found that the number and lifetime of hydrogen bonds increases below  $T_g$  (Caffarena and Grigera, 1996, 1999). The  $\nu_{\text{OH}}$  over the whole temperature range had the following order: sucrose < trehalose < raffinose < stachyose. This indicates that, for larger sugars, the hydrogen bond length increased (Jeffrey, 1997), implying a less dense hydrogen-bonding network for larger sugars. Note that, although the  $M_w$  of sucrose and trehalose is equal, the latter had a less dense hydrogen-bonding network. The  $T_g$  values and  $\nu_{\text{OH}}$  at  $T_g$  found with this technique are presented in Table 1.

#### Intracellular glass formation in seed and pollen in relation to sugars and proteins

Sugars, being present in high amounts in desiccation-tolerant tissues such as seeds and pollen, have been suggested to play a pivotal role in intracellular glass formation (Hirsh, 1987; Koster, 1991). To investigate the role of sugars in biological glass formation, the  $\tau_R$  of CP in *T. latifolia* pollen, equilibrated to 33% RH ( $T_g = 38^\circ\text{C}$ ), was determined. The predominant sugar in the pollen is sucrose (23%

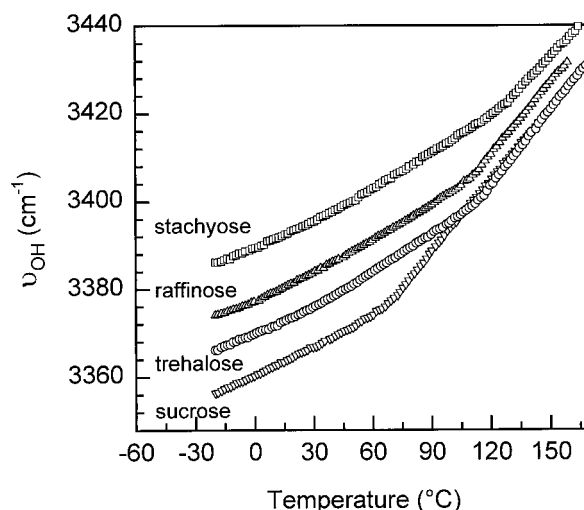


FIGURE 3 The wavenumber of the OH stretching vibration ( $\nu_{\text{OH}}$ ,  $\text{cm}^{-1}$ ) for different sugars as a function of temperature. Sugar glasses were first heated to remove residual water. Inverted triangles, sucrose; circles, trehalose; triangles, raffinose; squares, stachyose.

of the dry weight), present for  $\sim 97\%$  of the total sugars, the remaining few percent being monosaccharides like glucose and fructose (Hoekstra et al., 1992). The logarithm of  $\tau_R$  of CP in the pollen showed a linear decrease with increasing temperature, with a break around  $T_g$  (Fig. 4, *squares*). The temperature dependence of  $\tau_R$  in the pollen was quite different from  $\tau_R$  of CP in the sucrose glass (Fig. 4, *triangles*). In the sucrose matrix,  $\tau_R$  strongly decreased at  $\sim 18^\circ\text{C}$  above  $T_g$ , corresponding to the  $T_c$ , whereas the  $\tau_R$  in the pollen did not appear to exhibit a  $T_c$  within the range of the measurements. Instead, the logarithm of  $\tau_R$  continued to decrease linearly with temperature for at least  $45^\circ\text{C}$  above  $T_g$ .

Proteins are likely candidates to participate in intracellular glass formation because of their high abundance in the cytoplasm. Therefore, we also investigated the  $\tau_R$  of CP in PolyLys. Although PolyLys is a synthetic polypeptide, it was used to obtain information of how a spin probe behaves in high  $M_w$  proteins. Also, the interaction of this polypeptide with sugars has been investigated previously using FTIR spectroscopy (Wolkers et al., 1998a), enabling comparison of these results to the molecular mobility of CP in the polypeptide. The  $T_g$  of PolyLys, equilibrated at 33% RH, was  $29^\circ\text{C}$ . The  $\tau_R$  of CP in PolyLys below  $T_g$  decreased linearly on a logarithmic scale with increasing temperature (Fig. 4, *inverted triangles*). Above  $T_g$ ,  $\tau_R$  decreased more steeply with increasing temperature until  $T_c$  was reached at  $\sim 50^\circ\text{C}$  above  $T_g$ . At  $T_c$ , there was a sharp decrease in  $\tau_R$ , reaching a value of  $10^{-9}$  s at  $40^\circ\text{C}$  above  $T_c$  (data not shown). Also depicted in Fig. 4 is the  $\tau_R$  of CP in a 1:1 (w/w) mixture of sucrose and PolyLys, equilibrated to 3%

RH (*open circles*,  $T_g = 69^\circ\text{C}$ ). For this mixture,  $T_c$  was observed at  $33^\circ\text{C}$  above  $T_g$ , intermediate to the  $T_c$  of sucrose and PolyLys alone.

Seeds contain a large amount of oligosaccharides such as raffinose and stachyose (Amuti and Pollard, 1977). Soluble sugar analysis using DIONEX-HPLC revealed that embryonic axes of bean seeds contain in total 20% of their dry weight in soluble sugars. The composition of the soluble sugars was 43% sucrose, 7% raffinose, 49% stachyose and 1% verbascose. To understand the role of these sugars in glass formation in the cytoplasm of seeds, we compared the rotational motion of CP in bean embryonic axes (Fig. 5, *inverted triangles*;  $T_g = 25^\circ\text{C}$ ) with the glass mixture of 1:1 w/w sucrose and stachyose (Fig. 5, *circles*;  $T_g = 33^\circ\text{C}$ ), both equilibrated to 33% RH. The slope of the relationship between the logarithm of rotational motion and temperature was steeper above  $T_g$  for the sugar mixture than for bean axes. Furthermore, the temperature interval between  $T_g$  and  $T_c$  for bean axes was at least  $>50^\circ\text{C}$ , but only  $33^\circ\text{C}$  for the sugar mixture. The rotational motion of CP in the axes resembled that of CP in PolyLys glasses (Fig. 5, *squares*). A clear difference between the two glasses was the overall faster rotational motion of CP in the PolyLys as compared with the intracellular glass at similar temperatures.

## DISCUSSION

### Characterization of molecular mobility around $T_g$

Sugars that are abundantly present in desiccation-tolerant tissues are trehalose, sucrose, and, particularly in seeds, oligosaccharides such as raffinose (a trisaccharide) and stachy-

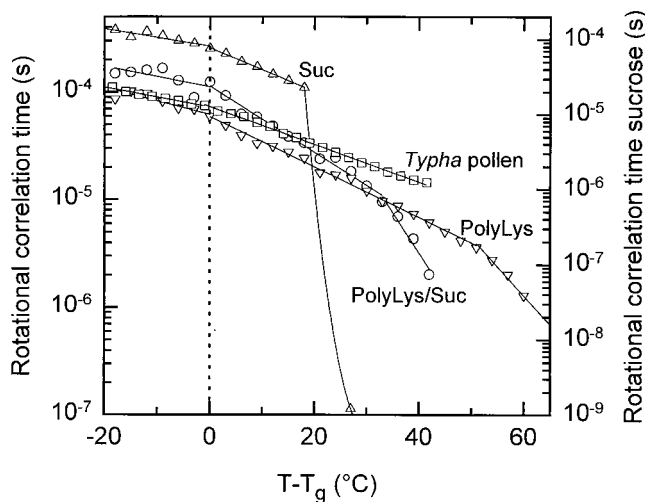


FIGURE 4 Rotational correlation time ( $\tau_R$ , s) of CP in the cytoplasm of *Typha latifolia* pollen (*squares*), and in sucrose (*triangles*), PolyLys (*inverted triangles*) and a 1:1 (w/w) mixture of PolyLys and sucrose (*circles*) as a function of  $T - T_g$ . Dashed line indicates  $T_g$ . All samples were equilibrated to 33% RH, except for sucrose and the mixture of PolyLys and sucrose (3% RH).

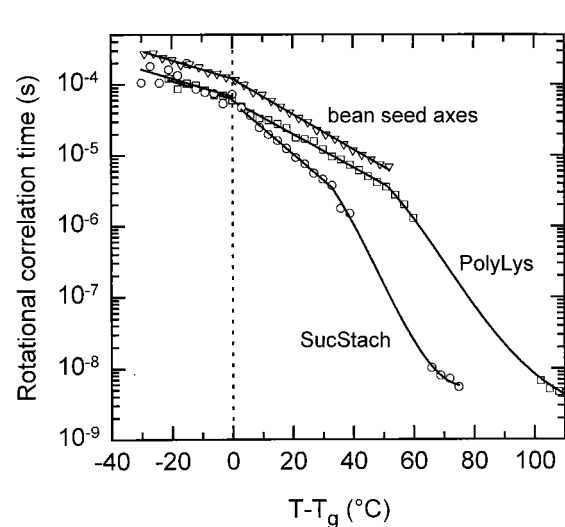


FIGURE 5 Rotational correlation time ( $\tau_R$ , s) of CP in the cytoplasm of bean seed axes (*Phaseolus vulgaris*) (*inverted triangles*), and in a 1:1 (w/w) mixture of sucrose and stachyose (*circles*), and PolyLys (*squares*) as a function of  $T - T_g$ . Dashed line indicates  $T_g$ . All samples were equilibrated to 33% RH.

ose (a tetrasaccharide) (Amuti and Pollard, 1977; Hoekstra et al., 1992; Crowe et al., 1998; Ghasempour et al., 1998). These sugars have been suggested to play a pivotal role in the glass formation of desiccation-tolerant biological tissues (Hirsh, 1987; Koster, 1991). Determination of the molecular mobility in these sugar glasses and comparison with that in intracellular glasses was expected to reveal what role these sugars play in intracellular glass formation. Here, the molecular mobility around  $T_g$  in the sugar glasses was characterized by  $\tau_R$  of the nitroxide spin probe CP, measured by EPR, and the second moment  $M_2$ , measured by  $^1\text{H-NMR}$ . FTIR spectroscopy provided information on the hydrogen-bonding network of the sugars around  $T_g$ .

The  $\tau_R$  of CP in the different sugars at  $T_g$  was dependent on the size of the sugars; a higher  $M_W$  of the sugar gave rise to faster rotational motion. Comparison of the  $\tau_R$  at  $T_g$  with the  $\nu_{\text{OH}}$  at  $T_g$  revealed that, in dry sugars, this faster rotational motion was accompanied by a less dense hydrogen-bonding network (Table 1). It has been reported that the molecular free volume at  $T_g$  increases with increasing  $M_W$  for small polymers/oligomers (Fox and Loshaek, 1955). Also, the average radius of molecular free volume holes at  $T_g$  was found to increase with  $T_g$  (Bartos, 1996; Li et al., 1999). Thus, the tetrasaccharide stachyose will form a glass matrix with a higher molecular free volume, in which the spin probe is less hindered in its rotation compared to a glass matrix formed by the disaccharide sucrose. PolyLys, with a  $M_W$  of 36 kDa, permitted an even faster rotational motion at  $T_g$  (Fig. 4), indicating an even larger molecular free volume in the PolyLys glass. Interestingly, although trehalose and sucrose have equal  $M_W$ , the faster rotational motion and higher  $\nu_{\text{OH}}$  at  $T_g$  indicate that the trehalose glass is less densely packed than a sucrose glass.

### Transition in molecular mobility above $T_g$ : Explanation of the critical temperature

The molecular mobility in the sugar glasses, measured by  $\tau_R$  of a spin probe and  $M_2$  by  $^1\text{H-NMR}$ , increased with increasing temperature (Figs. 1 and 2). The most characteristic feature that was observed in the temperature dependence of the molecular mobility was the presence of two transitions, the first one associated with melting of the glassy state, and the second one associated with the so-called critical temperature,  $T_c$ . Recently, this  $T_c$  was also found for glucose-water glasses, measured by  $^1\text{H-NMR}$ , and was interpreted as the so-called crossover temperature, where the dynamics changes from solid-like to liquid-like (Van den Dries et al., submitted for publication). This crossover temperature has been observed for many polymer glasses and can theoretically be predicted by the mode coupling theory (Sokolov, 1996, 1997; Rössler et al., 1994, 1998). Above the crossover temperature, a viscous solution behaves as a normal liquid, where diffusional motion dominates the dynamics ( $\alpha$ -relaxation). At the crossover temperature, a second relaxation,

the fast relaxation process, becomes temperature dependent and can be visualized as rattling of a molecule inside a cage formed by its neighbors (Sokolov, 1997). In the temperature range between  $T_g$  and the crossover temperature, these cages still undergo collective distortions leading to translational diffusion ( $\alpha$ -relaxation). At temperatures below  $T_g$ , only the motion of a molecule inside a cage persists. Additional evidence that this second transition can be explained by the concept of the crossover temperature comes from the similar time scale of the motion found at  $T_c$  and the crossover temperature (Van den Dries et al., 1998, Van den Dries et al., submitted for publication).

### Relation between the critical temperature and stability: Explanation for the stability of biological organisms?

The  $T_c$  or crossover temperature appears to be related to the stability of sugar glasses above their  $T_g$ . One of the characteristics of sugar glasses is that they exhibit a collapse at a temperature above the  $T_g$ , observed as a macroscopically visible change in physical properties. The collapse is generally attributed to a reduction in viscosity upon increasing temperature above the  $T_g$  such that flow on a practical time scale is observed, generally around  $10^8$  Pa s (for review see Roos, 1995). This is comparable to what happens at a molecular scale above the crossover temperature ( $T_c$ ), where the dynamics of the viscous matrix will be dominated by the diffusional motion. For various mono- and disaccharides, the temperature of structural collapse and glass softening occur 10–17°C above  $T_g$  (Levine and Slade, 1988; Roos, 1995; Sun, 1997). These values are comparable with those corresponding to the critical temperature ( $T_c$ ) found in our study for sucrose (Table 2). Apparently, the macroscopic collapse is a result of the change in molecular dynamics at  $T_c$ . The collapse phenomena results in a dramatic change in the stability of the matrices, such as loss and oxidation of encapsulated lipids and flavors, loss of enzymatic activity, nonenzymatic browning, stickiness and caking, or structural collapse (Levine and Slade, 1988; Slade and Levine, 1991; Roos, 1995).

The observation that  $T_c$  occurred at substantially higher temperatures above  $T_g$  in trehalose and oligosaccharides than in sucrose, implies that these glasses might be more stable above  $T_g$  (Table 2). The superior stability of trehalose over sucrose glasses could be related to their differences in  $T_c$  (Figs. 1 and 2 and Table 2). Some evidence for this conclusion comes from the observation that the protection of G6PDH was found to be better in glucose/trehalose than glucose/sucrose (1:4) systems during storage at similar temperatures above  $T_g$  (Sun and Davidson, 1998). The system containing trehalose could be heated 8°C more above  $T_g$  than the sucrose-containing system to preserve the protein to the same level. Crowe et al. (1998) also found that trehalose was better at protecting enzyme activity at high

**TABLE 2** The magnitude of the temperature interval between the glass transition temperature and critical temperature ( $T_g - T_c$ ) for various materials, measured by  $\tau_R$  using ST-EPR or  $M_2$  using  $^1\text{H}$  NMR.

Material	RH (%)	$T_c - T_g \tau_R$ ( $^{\circ}\text{C}$ )	$T_c - T_g M_2$ ( $^{\circ}\text{C}$ )
Sucrose	0	16/18	17
Trehalose	0	25/26	26
	33	30/31	23/26
Raffinose	0	33/35	nd
	33	34/36	nd
Stachyose	0	33/35	nd
	33	36/38	32
Sucrose/Stachyose (1:1 w/w)	33	33	nd
PolyLys	0	34/37	nd
	33	50/52	nd
PolyLys/Sucrose (1:1 w/w)	0	33	nd
<i>Typha</i> pollen	33	>45	nd
Bean seed axes	33	>50	nd
Pea seed axes	33	>60	nd

Samples that were performed in duplicate are indicated. nd: not determined.

RH than sucrose. Another indication that the high  $T_c$  of polysaccharides might influence stability above  $T_g$  was the discovery that a frozen mixture of polysaccharides and sucrose resulted in a higher viscosity upon melting of the glass as compared with frozen sucrose alone (Goff et al., 1993). Apart from the beneficial property of trehalose, raffinose, and stachyose to form hydrates, their high  $T_c$  might also be responsible for the improved stability these sugars confer above  $T_g$ .

In biological tissues,  $T_c$  could not be measured, indicating that it likely occurs at temperatures  $>50^{\circ}\text{C}$  above  $T_g$  (Table 2). Considering that, above  $T_c$ , there is a strong reduction in viscosity, the absence of  $T_c$  in biological tissues implies that the system is very stable, still exhibiting a high viscosity ( $>10^8$  Pa s) far above  $T_g$ . This extremely high temperature interval between  $T_g$  and  $T_c$  could be an explanation for the long life span of dry biological tissues. In a natural environment, changes in environmental conditions to which seed and pollen are exposed are likely to bring the tissues above their  $T_g$ . Although resulting in a slight increase in the temperature dependence of the cytoplasmic molecular mobility, an increase of the temperature above  $T_g$  does not lead to an abrupt decrease in viscosity as seen for the sugar systems, that could lead to, for instance, crystallization or collapse. The observation that aging kinetics of *Typha* pollen changes only a factor 3 around  $T_g$ , and remains constant for  $>30^{\circ}\text{C}$  above  $T_g$  (Buitink et al., 1998b) might be explained by the large temperature interval between  $T_g$  and  $T_c$  ( $>45^{\circ}\text{C}$ ) (Fig. 4). Comparable high crossover temperatures were found for myoglobin or DNA (Sokolov, 1997, 1999). It was hypothesized (Sokolov, 1996) that the stabilization of biological objects below some  $T_c$  might be very important for their preservation and suppression of their degradation

with time. It was speculated that liquids below the crossover temperature can be stable against crystallization (Sokolov, 1996), which could explain the absence of crystallization found in biological tissues (Sun and Leopold, 1993).

### Composition of intracellular glasses: Role for sugars and proteins

The exceptionally large temperature interval between  $T_g$  and  $T_c$  found in biological tissues could be beneficial for a prolonged life span. A better understanding of the composition of intracellular glasses might benefit the preservation of biomolecules. It is clear from Figs. 4 and 5 that sugars are not the only molecules participating in the intracellular glass formation. Apart from the difference in slopes between sugar and intracellular glasses, the undetected  $T_c$  in the latter suggest that other molecules are equally important in the glass formation. The large temperature interval between  $T_g$  and  $T_c$  observed for the polypeptide PolyLys indicates that proteins might fulfill a role in intracellular glass formation (Table 2). Proteins comprise over 20% of the dry weight of the cytoplasm of seed and pollen. Comparison of the temperature dependence of mobility in PolyLys glasses with intracellular glasses of bean (Fig. 5) showed that rotational motion in the two glasses increased parallel in both systems. A clear difference between the two glasses was the overall faster rotational motion of CP in the PolyLys as compared with intracellular glass at similar temperatures, probably due to the larger molecular free volume present in the PolyLys matrix. The slower rotational motion in the biological tissues indicates that, if proteins or other high  $M_w$  molecules form intracellular glasses, the free volume between those molecules is filled up with small molecules such as small sugars or amino acids. Indeed, addition of sucrose to PolyLys decreased the rotational motion at  $T_g$  (Fig. 4). This is confirmed by an FTIR study on the interaction of sucrose with PolyLys, where addition of sugar increased the molecular packing density of the total matrix (Wolkers et al., 1998a).

Apparently, sugars are not the predominant molecules that are associated with glass formation in dry biological systems. However, they are still present in large quantities in cells, suggesting that they have an important function in biological systems. They could play a role in filling the molecular free volume between the large molecules to increase the density of glasses, allowing less molecular mobility in the matrix. The high  $T_c$  of oligosaccharides might indicate that these large sugars might help to increase the  $T_c$  of the total systems to high values. Another role for sugars in dry anhydrobiotic systems comes from the water replacement hypothesis (for review see Crowe et al., 1998). In the present study, proteins were rapidly dried to form glasses, but sugars were found to be important in preventing changes in protein conformation during slow drying in PolyLys (Wolkers et al., 1998a). This prevention of conformation



was the result of direct hydrogen-bonding interactions between the sugar and the protein. There is large body of evidence suggesting that direct interaction between sugars and membranes or proteins prevents membrane fusion or changes of protein conformation (Prestrelski et al., 1993; Sun et al., 1996; Crowe et al., 1997, 1998; Wolkers et al., 1998a). It was also demonstrated that small sugars are more capable of hydrogen bonding than large polymers such as hydroxyethyl starch (Crowe et al., 1997).

This study indicates that proteins could fulfill a role in intracellular glass formation, as suggested previously (Sun and Leopold, 1997; Wolkers et al., 1998a,b). It is likely that many different proteins participate in intracellular glass formation. Highly abundant proteins in the cytosol are those belonging to the family of the so-called LEA (late embryogenesis abundant) proteins (for review see Dure, 1997). Interestingly, these proteins have been associated with desiccation tolerance and longevity of seeds (Dure, 1997), and they have been suggested to participate in intracellular glass formation (Sun and Leopold, 1997).

## CONCLUSIONS

Here, we demonstrated the occurrence of a kinetic change in molecular mobility at a critical temperature ( $T_c$ ) above  $T_g$  in various glasses, measured by EPR and NMR spectroscopy. At  $T_c$ , the dynamics of the system changed from solid-like to liquid-like properties, accompanied by a large reduction in viscosity. The temperature interval between the  $T_g$  and  $T_c$  increased in the order of sucrose < trehalose < raffinose  $\leq$  staychose < PolyLys < biological tissues. The critical temperature in biological organisms was extremely high, at least 50°C above  $T_g$ . This large temperature interval is expected to give rise to very stable dry organisms, maintaining a slow cytoplasmic molecular motion even at temperatures far above  $T_g$ . Temperature-dependent changes in molecular mobility in intracellular glasses resembled that of protein glasses, with  $T_c$  50°C above  $T_g$ . Soluble sugars, present in large amounts in desiccation-tolerant organisms, exhibited a much smaller interval between  $T_g$  and  $T_c$  (between 17 and 35°C). Possibly, protein participates in the formation of intracellular glasses, in which small molecules are embedded that decrease the molecular free volume.

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