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

Julia Buitink,*† Ivon J. van den Dries,‡ Folkert A. Hoekstra,* Mark Alberda,* and Marcus A. Hemminga†
Wageningen University, *Department of Plant Sciences, Laboratory of Plant Physiology, 6703 BD Wageningen, †Department of Biomolecular Sciences, Laboratory of Molecular Physics, 6703 HA Wageningen, and ‡Department of Food Science, Food Physics Group, 6703 HD Wageningen, The Netherlands

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, ^{1}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 (τ_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 τ_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 $\leq$ staychose $<$ 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 Zografi, 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

Received for publication 10 January 2000 and in final form 30 April 2000.
Address reprint requests to Julia Buitink, Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands. Tel.: +31-317-482452; Fax: +31-317-484740; E-mail: julia.buitink@algem.pf.wau.nl.

0006-3495/00/08/1119/10 $2.00

(Priestley et al., 1985; Shen-Miller et al., 1995; Steiner and Ruckenbauer, 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 τ_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}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}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 μl droplets of 0.1 g 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 $MgCl_2$ (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 ~0.7 g H_2O g 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 H_2O g dw^{-1}. After drying, pollen was stored over a saturated solution of $MgCl_2$ (33% RH) for 7 days. For each EPR measurement, ~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 $MgCl_2$ (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 τ_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 τ_R becomes longer than 10^{-8} s, τ_R cannot be calculated according to Eq. 1 because of the appearance of a powder spectrum. ST-EPR was used to determine τ_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, τ_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 τ_R values (for details, see Buitink et al., 1999). From this calibration curve, the values of τ_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.

^{1}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 $MgCl_2$ (33% RH) at room temperature. Samples were loaded in a 5-mm NMR tube that was sealed to prevent water evaporation.

^{1}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 μs. 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),

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

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 0.01g ml^{-1} sucrose (Pfahnstiel), amorphous trehalose (Pfanstiel), raffinose-pentahydrate (Sigma), and amorphous stachyose (Sigma) on circular CaF_2 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^{-1}. 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_2 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_2-free air (Balston, Maidstone, Kent, UK) at a flow rate of 25 liter min^{-1}. The acquisition parameters were 4 cm^{-1} resolution, 32 co-added interferograms, 3600–900 cm^{-1} 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^{-1} was monitored by observing the position of the band around 3300 cm^{-1} (OH-stretching vibration, υ_{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 u_{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-μl droplets of 0.1 g ml^{-1} 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^{-1}). 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^{-1}. 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 (τ_R) of CP in the sugars around T_g. For all sugar glasses, the τ_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 τ_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 τ_R values depicted in Fig. 1 were from trehalose and raffinose glasses that were equilibrated to 33% RH. The 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 τ_R curve because of a technical limitation of 400 K for the EPR measurements. Because sucrose crystallized at 33% RH, τ_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, τ_R had the same temperature dependence (data not shown), indicating that the measured τ_R was not dependent on the thermal history of the sugar samples.

The range of molecular motions for which τ_R could not be determined using our protocols is from 10^{-6} to 10^{-8} s. For τ_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 τ_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 τ_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 τ_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 τ_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

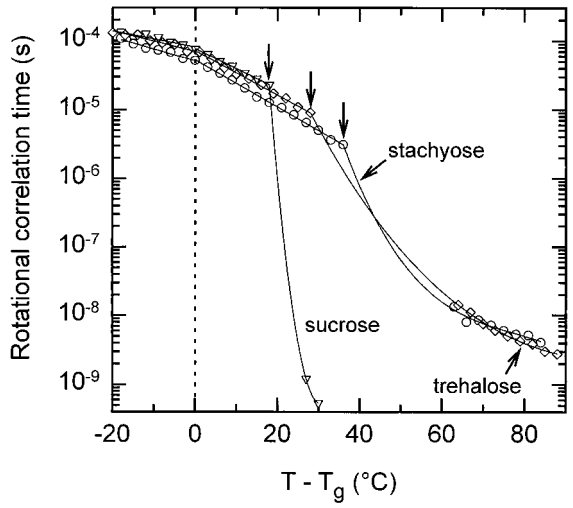

FIGURE 1 Rotational correlation time (τ_R, s) of CP in different sugars as a function of $T - T_g$. *Dashed line*, T_g; *arrows*, the critical temperature T_c; *inverted triangles*, sucrose equilibrated to 3% RH; *diamonds*, trehalose equilibrated to 33% RH; *circles*, stachyose equilibrated to 33% RH.

TABLE 1 Rotational correlation time (τ_R) and wavenumber position (υ_{OH}) of sugar glasses at T_g

	ESR			FTIR		
Sugar	WC (g/g dw)	T_g (°C)	τ_R at T_g (μs)	WC (g/g dw)	T_g (°C)	υ_{OH} at T_g (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 τ_R or υ_{OH} as a function of temperature. WC, water content, g H_2O g 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}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}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 τ_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).

To understand why the τ_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 (υ_{OH}) reveals information about the strength and length of hydrogen bonding (Jeffrey, 1997). Figure 3 shows the υ_{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 υ_{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 υ_{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 τ_R of CP in *T. latifolia* pollen, equilibrated to 33% RH (T_g = 38°C), was determined. The predominant sugar in the pollen is sucrose (23%

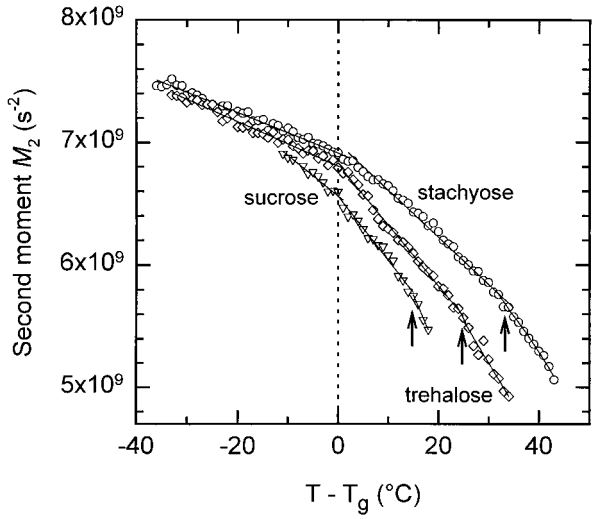

FIGURE 2 The second moment (M_2, s^{-2}) of immobile protons of different sugars as a function of $T - T_g$. *Dashed line*, T_g; *arrows*, the critical temperature; *inverted triangles*, sucrose equilibrated to 3% RH; *diamonds*, trehalose equilibrated to 33% RH; *circles*, stachyose equilibrated to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

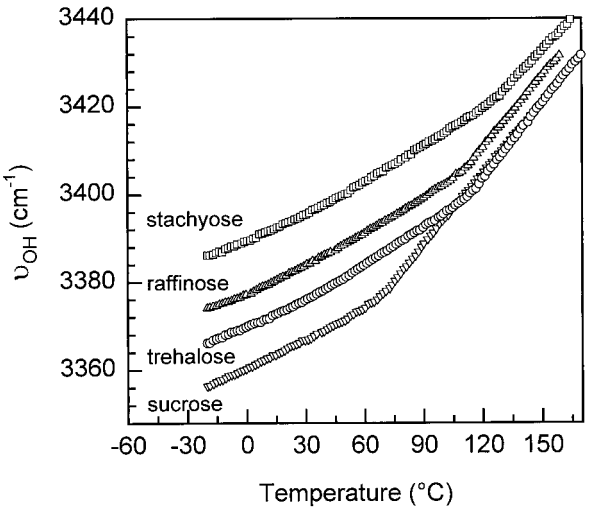

FIGURE 3 The wavenumber of the OH stretching vibration (υ_{OH}, cm^{-1}) for different sugars as a function of temperature. Sugar glasses were first heated to remove residual water. *Inverted triangles*, sucrose; *circles*, trehalose; *triangles*, raffinose; *squares*, stachyose.

of the dry weight), present for ~97% of the total sugars, the remaining few percent being monosaccharides like glucose and fructose (Hoekstra et al., 1992). The logarithm of τ_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 τ_R in the pollen was quite different from τ_R of CP in the sucrose glass (Fig. 4, *triangles*). In the sucrose matrix, τ_R strongly decreased at ~18°C above T_g, corresponding to the T_c, whereas the τ_R in the pollen did not appear to exhibit a T_c within the range of the measurements. Instead, the logarithm of τ_R continued to decrease linearly with temperature for at least 45°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 τ_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°C. The τ_R of CP in PolyLys below T_g decreased linearly on a logarithmic scale with increasing temperature (Fig. 4, *inverted triangles*). Above T_g, τ_R decreased more steeply with increasing temperature until T_c was reached at ~50°C above T_g. At T_c, there was a sharp decrease in τ_R, reaching a value of 10^{-9} s at 40°C above T_c (data not shown). Also depicted in Fig. 4 is the τ_R of CP in a 1:1 (w/w) mixture of sucrose and PolyLys, equilibrated to 3% RH (*open circles*, T_g = 69°C). For this mixture, T_c was observed at 33°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°C) with the glass mixture of 1:1 w/w sucrose and stachyose (Fig. 5, *circles*; T_g = 33°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°C, but only 33°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 raffinose (a trisaccharide) and stachy-

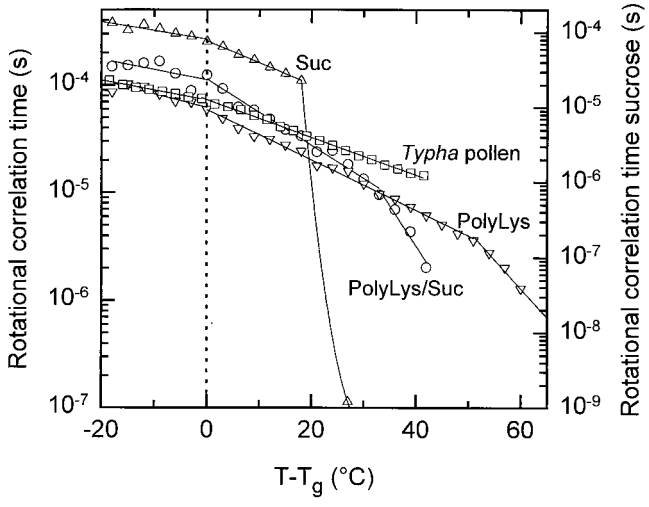

FIGURE 4 Rotational correlation time (τ_R, s) of CP in the cytoplasm of *Typha latifolia* pollen (*squares*), and in sucrose (*triangles*), PolyLys (*inverted triangles*) and a 1:1 (w/w) mixture of PolyLys and sucrose (*circles*) as a function of $T - T_g$. Dashed line indicates T_g. All samples were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

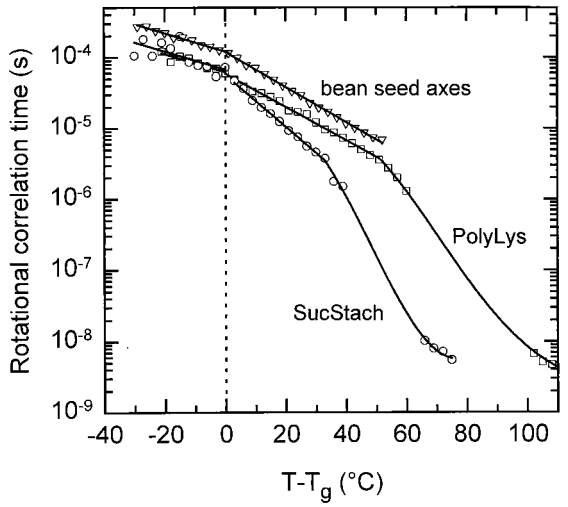

FIGURE 5 Rotational correlation time (τ_R, s) of CP in the cytoplasm of bean seed axes (*Phaseolus vulgaris*) (*inverted triangles*), and in a 1:1 (w/w) mixture of sucrose and stachyose (*circles*), and PolyLys (*squares*) as a function of $T - T_g$. Dashed line indicates T_g. All samples were equilibrated 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 τ_R of the nitroxide spin probe CP, measured by EPR, and the second moment M_2, measured by ^{1}H-NMR. FTIR spectroscopy provided information on the hydrogen-bonding network of the sugars around T_g.

The τ_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 τ_R at T_g with the u_{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 υ_{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 τ_R of a spin probe and M_2 by ^{1}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}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 (α-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 (α-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 τ_R using ST-EPR or M_2 using ^{1}H NMR.

Material	RH (%)	$T_c - T_g$ τ_R (°C)	$T_c - T_g$ M_2 (°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
Typha 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°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°C above T_g (Buitink et al., 1998b) might be explained by the large temperature interval between T_g and T_c (>45°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 polypetide 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.

This work was financially supported by the Netherlands Technology Foundation (STW), and was coordinated by the Life Sciences Foundation. This work was partly supported by the European Community Contract, Molecular Mobility in Foods, FAIRCT96–1085.

REFERENCES

Aldous, B. J., A. D. Auffret, and F. Franks. 1995. The crystallisation of hydrates from amorphous carbohydrates. *Cryo-Letters* 16:181–186.

Amuti, K. S., and C. J. Pollard. 1977. Soluble carbohydrates of dry and developing seeds. *Phytochemistry*. 16:529–532.

Bartos, J. 1996. Free volume microstructure of amorphous polymers at glass transition temperatures from positron annihilation spectroscopy data. *Colloid Polym. Sci.* 274:14–19.

Buitink, J., M. M. A. E. Claessens, M. A. Hemminga, and F. A. Hoekstra. 1998a. Influence of water content and temperature on molecular mobility and intracellular glasses in seeds and pollen. *Plant Physiol.* 118: 531–541.

Buitink, J., M. A. Hemminga, and F. A. Hoekstra. 1999. Characterization of molecular mobility in seed tissues: an electron paramagnetic resonance spin probe study. *Biophys. J.* 76:3315–3322.

Buitink, J., C. Walters, F. A. Hoekstra, and J. Crane. 1998b. Storage behavior of *Typha latifolia* pollen at low water contents: interpretation on the basis of water activity and glass concepts. *Physiol. Plant.* 103: 145–153.

Buitink, J., C. Walters-Vertucci, F. A. Hoekstra, and O. Leprince. 1996. Calorimetric properties of dehydrating pollen: analysis of a desiccation-tolerant and an -intolerant species. *Plant Physiol.* 111:235–242.

Burke, M. J. 1986. The glassy state and survival of anhydrous biological systems. *In* Membranes, Metabolism and Dry Organisms. A. C. Leopold, editor. Cornell University Press, Ithaca, NY. 358–363.

Caffarena, E. R., and J. R. Grigera. 1996. Crystal, melted and glassy states of glucose—A molecular dynamics simulation. *J. Chem. Soc. Faraday Trans.* 92:2285–2289.

Caffarena, E. R., and J. R. Grigera. 1999. Hydration of glucose in the rubbery and glassy states studied by molecular dynamics simulation. *Carbohydr. Res.* 315:63–69.

Caffrey, M., V. Fonseca, and A. C. Leopold. 1988. Lipid–sugar interaction. *Plant Physiol.* 86:754–758.

Chang, B. S., R. M. Beauvais, A. Dong, and J. F. Carpenter. 1996. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. *Arch. Biochem. Biophys.* 331:249–258.

Crowe, J. H., A. E. Oliver, F. A. Hoekstra, and L. M. Crowe. 1997. Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: the role of vitrification. *Cryobiology*. 35:20–30.

Crowe, L. M., D. S. Reid, and J. H. Crowe. 1996. Is trehalose special for preserving dry biomaterials? *Biophys. J.* 71:2087–2093.

Crowe, J. H., J. F. Carpenter, and L. M. Crowe. 1998. The role of vitrification in anhydrobiosis. *Ann. Rev. Physiol.* 60:73–103.

Dure, L., III. 1997. LEA proteins and the desiccation tolerance of seeds. *In* Cellular and Molecular Biology of Plant Seed Development. B. A. Larkins, I. K. Vasil, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 525–544.

Fox, T. G., and S. Loshaek. 1955. Influence of molecular weight and degree of crosslinking on the specific volume and glass temperatures of polymers. *J. Polym. Sci.* 15:371–390.

Ghasempour, H. R., D. F. Gaff, R. P. W. Williams, and R. D. Gianello. 1998. Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. *Plant Growth Reg.* 24:185–191.

Goff, H. D., K. B. Caldwell, and D. W. Stanley. 1993. The influence of polysaccharides on the glass transition in frozen sucrose solutions and ice cream. *J. Dairy Sci.* 76:1268–1277.

Hancock, B. C., S. L. Shamblin, and G. Zografi. 1995. Molecular mobility of amorphous pharmaceutical solids below their glass transition temperatures. *Pharm. Res.* 12:799–806.

Hancock, B. C., and G. Zografi. 1997. Characteristics and significance of the amorphous state in pharmaceutical systems. *J. Pharm. Sci.* 86:1–12.

Hemminga, M. A. 1983. Interpretation of ESR and saturation transfer ESR spectra of spin labeled lipids and membranes. *Chem. Phys. Lipids.* 32:323–383.

Hemminga, M. A., and I. J. van den Dries. 1998. Spin label applications to food science. *In* Biological Magnetic Resonance, Vol. 14: Spin Labeling: The Next Millennium. L. J. Berliner, editor. Plenum Publishing Corp., New York. 339–366.

Hirsh, A. G. 1987. Vitrification in plants as a natural form of cryoprotection. *Cryobiology*. 24:214–228.

Hoekstra, F. A., J. H. Crowe, L. M. Crowe, T. van Roekel, and E. Vermeer. 1992. Do phospholipids and sucrose determine membrane phase transitions in dehydrating pollen species? *Plant Cell Environ.* 15:601–606.

Jeffrey, G. A. 1997. An Introduction to Hydrogen Bonding, Oxford University Press, Oxford, UK.

Kalichevsky, M. T., J. M. V. Blanshard, and P. F. Tokarczuk. 1993. Effect of water content and sugars on the glass transition of casein and sodium caseinate. *Int. J. Food Sci. Technol.* 28:139–151.

Karmas, R., M. P. Buera, and M. Karel. 1992. Effect of glass transition on rates of nonenzymatic browning in food systems. *J. Agric. Food Chem.* 40:873–879.

Knowles, P. F., D. Marsh, and H. W. E. Rattle. 1976. Magnetic Resonance of Biomolecules: An Introduction to the Theory and Practice of NMR and ESR in Biological Systems. John Wiley and Sons, London, UK.

Koster, K. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiol.* 96:302–304.

Leopold, A. C., W. Q. Sun, and I. Bernal-Lugo. 1994. The glassy state in seeds: analysis and function. *Seed Sci. Res.* 4:267–274.

Leprince, O., and C. Walters-Vertucci. 1995. A calorimetric study of glass transition behaviors in axes of bean with relevance to storage stability. *Plant Physiol.* 109:1471–1481.

Levine, H., and L. Slade. 1988. Water as a plasticizer: physico-chemical aspects of low-moisture polymeric systems. *In* Water Science Reviews. F. Franks, editor. Vol. 3. Cambridge University Press, Cambridge, UK. 79–185.

Li, H. L., Y. Ujihira, A. Nanasawa, and Y. J. Jean. 1999. Estimation of free volume in polystyrene-polyphenylene ether blend probed by the positron annihilation lifetime technique. *Polymer.* 40:349–355.

Potts, M. 1994. Desiccation tolerance of prokaryotes. *Microbiol. Rev.* 58:755–805.

Prestrelski, S. J., N. Tedeschi, T. Arakawa, and J. F. Carpenter. 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* 65:661–671.

Priestley, D. A., V. I. Cullinan, and J. Wolfe. 1985. Differences in seed longevity at the species level. *Plant Cell Environ.* 8:557–562.

Rössler, E., K. U. Hess, and V. N. Novikov. 1998. Universal representation of viscosity in glass forming liquids. *J. Non-Cryst. Solids.* 223:207–222.

Rössler, E., J. Tauchert, and P. Eiermann. 1994. Cooperative reorientations, translational motions, and rotational jumps in viscous liquids. *J. Phys. Chem.* 98:8173–8180.

Roos, Y. 1995. Phase Transitions in Foods. Academic Press Ltd., London, UK.

Roozen, M. J. G. W., and M. A. Hemminga. 1990. Molecular motion in sucrose-water mixtures in the liquid and glassy state as studied by spin probe ESR. *J. Phys. Chem.* 94:7326–7329.

Slade, L., and H. Levine. 1991. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. *Crit. Rev. Food Sci. Nutr.* 30:115–360.

Soesanto, T., and M. C. Williams. 1981. Volumetric interpretation of viscosity for concentrated and dilute sugar solutions. *J. Phys. Chem.* 85:3338–3341.

Sokolov, A. P. 1996. Why the glass transition is still interesting. *Science.* 273:1675–1676.

Sokolov, A. P. 1997. The glass transition: new ideas in an age-old field. *Endeavour.* 21:109–113.

Sokolov, A. P. 1999. Glassy dynamics in DNA: ruled by water of hydration? *J. Chem. Phys.* 110:7053–7057.

Shen-Miller, J., M. B. Mudgett, J. W. Schopf, S. Clarke, and R. Berger. 1995. Exceptional seed longevity and robust growth: ancient sacred lotus from China. *Am. J. Bot.* 82:1367–1380.

Steiner, A. M., and P. Ruckenbauer. 1995. Germination of 110-year-old cereal and weed seeds, the Vienna sample of 1877. Verification of effective ultra-dry storage at ambient temperature. *Seed Sci. Res.* 5:195–199.

Sun, W. Q. 1997. Temperature and viscosity for structural collapse and crystallization of amorphous carbohydrate solutions. *Cryo-Letters.* 18: 99–106.

Sun, W. Q., and P. Davidson. 1998. Protein inactivation in amorphous sucrose and trehalose matrices: effects of phase separation and crystallization. *Biochim. Biophys. Acta.* 1425:235–244.

Sun, W. Q., and A. C. Leopold. 1993. The glassy state and accelerated aging of soybeans. *Physiol. Plant.* 89:767–774.

Sun, W. Q., and A. C. Leopold. 1997. Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comp. Biochem. Physiol.* 117A: 327–333.

Sun, W. Q., A. C. Leopold, L. M. Crowe, and J. H. Crowe. 1996. Stability of dry liposomes in sugar glasses. *Biophys. J.* 70:1769–1776.

Tetteroo, F. A. A., C. Bomal, F. A. Hoekstra, and C. M. Karssen. 1994. Effect of abscisic acid and slow drying on soluble carbohydrate contents of developing embroids of carrot (*Daucus carota* L.) and alfalfa (*Medicago sativa* L.). *Seed Sci. Res.* 4:203–210.

Thomas, D. D., L. R. Dalton, and J. S. Hyde. 1976. Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance. *J. Chem. Phys.* 65:3006–3024.

Van den Dries, I. J., D. van Dusschoten, and M. A. Hemminga. 1998. Mobility in maltose-water glasses studied with ^{1}H NMR. *J. Phys. Chem. B* 102:10483–10489.

Williams, R. J., and A. C. Leopold. 1989. The glassy state in corn embryos. *Plant Physiol.* 89:977–981.

Williams, M. L., R. F. Landel, and J. D. Ferry. 1955. The temperature dependence of relaxation mechanisms in amorphous polymers and other glass-forming liquids. *J. Am. Chem. Soc.* 77:3701–3707.

Wolkers, W. F., M. G. van Kilsdonk, and F. A. Hoekstra. 1998a. Dehydration-induced conformational changes of poly-L-lysine as influenced by drying rate and carbohydrates. *Biochim. Biophys. Acta.* 1425: 127–136.

Wolkers, W. F., H. Oldenhof, M. Alberda, and F. A. Hoekstra. 1998b. A Fourier transform infrared microspectroscopy study of sugar glasses: application to anhydrobiotic higher plant cells. *Biochim. Biophys. Acta.* 1379:83–96.