

A Calorimetric Study of the Glass Transition Behaviors in Axes of Bean Seeds with Relevance to Storage Stability

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Although the presence of intracellular aqueous glasses has been established in seeds, their physiological role in storage stability is still conjectural. Therefore, we examined, using differential scanning calorimetry, the thermal behavior of glass transitions in axes of bean (*Phaseolus vulgaris* L.) with water contents (WC) between 0 and 1 g H₂O/g dry weight (g/g) and temperatures between -120 and +120°C. Three types of thermal behaviors associated with the glass transition were observed. The appearance, the glass → liquid transition temperature, and the amount of energy released during these transitions were dependent on the tissue WC. No glass transitions were observed at WC lower than 0.03 and higher than 0.45 g/g. A brief exposure to 100°C altered the glass properties of tissues with WC between 0.03 and 0.08 g/g but did not affect the thermal behavior of glasses with higher WC, demonstrating that thermal history is important to the intracellular glass behavior at lower WC. Correspondence of data from bean to models predicting the effects of glass components on the glass → liquid transition temperature suggests that the intracellular glasses are composed of a highly complex sugar matrix, in which sugar and water molecules interact together and influence the glass properties. Our data provide evidence that additional glass properties must be characterized to understand the implications of a glassy state in storage stability of seeds.

Water and temperature play a significant and fundamental role in determining the storage longevity of orthodox seeds. Early models developed from storage experiments suggested that water and temperature were independent variables (Justice and Bass, 1978; Roberts and Ellis, 1989). However, theoretical considerations (Vertucci, 1992; Vertucci and Roos, 1993) and experimental data (Vertucci and Roos, 1993; Vertucci et al., 1994a, 1994b) have demonstrated that the effects of water and temperature on seed aging are interdependent. Conceptual models have been introduced to describe the interdependencies of WC and temperature on seed longevity. They rely on the findings that the nature and kinetics of degradative reactions in seeds change according to the physical properties of water (Roberts and Ellis, 1989; Vertucci and Leopold, 1989; Vertucci, 1992; Vertucci and Roos, 1993; Leopold et al., 1994).

The motional and thermal properties of intracellular water in seeds change as a function of the WC and temperature of the tissue (Vertucci, 1990; Bruni and Leopold, 1992b). The WC/temperature combinations at which these changes occur have been described using equilibrium thermodynamic principles assessed by measurements of water sorption and chemical potential (Vertucci and Roos, 1993; Vertucci et al., 1994a). Alternatively, the WC/temperature combinations at which changes in water properties are detected can be described using nonequilibrium thermodynamic principles by invoking the concept of glassy states of water in seeds (Burke, 1986; Williams and Leopold, 1989; Bruni and Leopold, 1991, 1992a, 1992b; Williams et al., 1993; Leopold et al., 1994).

A glass is defined as an amorphous metastable state that resembles a solid, brittle material but retains the disorder and physical properties of the liquid state (Burke, 1986; Yonezawa, 1991; Franks, 1994; Perez, 1994; Slade and Levine, 1994). A glass is usually identified by its high, but arbitrary, value of viscosity (approximately 10¹² to 10¹⁴ Pa s⁻¹ [Angell, 1988; Angell et al., 1994]). An extremely high viscosity is probably a common denominator between a glassy state and the state of water in dried organisms. Burke (1986) and Green and Angell (1989) suggested that the formation of a glassy matrix (i.e. vitrification) in dried plant and animal systems could represent a strategy for desiccation tolerance, quiescence, and storage stability. Vitrification during drying would achieve a high viscosity without a great deal of molecular reorganization (Angell, 1988; Slade and Levine, 1991; Yonezawa, 1991; Angell et al., 1994; Perez, 1994) and, therefore, limit major changes in the cellular structures (Burke, 1986). The high intracellular viscosity that is typical of glasses would severely slow molecular diffusion and decrease the probability of chemical reactions. In consequence, storage of frozen and/or dried seed tissues below the T_g would ensure survival for long periods, since tissues are protected from deleterious changes in structure and chemical composition (Burke, 1986; Green and Angell, 1989; Vertucci and Roos, 1990; Williams et al., 1993).

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Abbreviations: ΔC_p, specific heat capacity; DSC, differential scanning calorimetry; g/g, g H₂O/g dry weight; HTP, high-temperature peak; T_{cr}, temperature of ice crystallization peak; T_g, glass → liquid transition temperature; T_g', glass → liquid transition of the freeze-concentrated matrix; WC, water content(s).

Because glass theory predicts eventual change in the system, it is an attractive principle to model the slow but inevitable changes in vigor and viability associated with seed aging. The concept of glassy states in designing storage protocols has been applied to cryopreservation of hydrated tissues (Wesley-Smith et al., 1992; Williams et al., 1993; Pritchard, 1995) and dried and frozen food stuff (Levine and Slade, 1988; Noel et al., 1990; Slade and Levine, 1991, 1994). Research on aqueous glasses in relatively dry seeds has focused mainly on methods for detecting their presence in embryonic tissues of corn (defatted embryos, Williams and Leopold, 1989; intact axes, Bruni and Leopold, 1992a), soybean (Bruni and Leopold, 1992b), and red oak (Sun et al., 1994). The experimental evidence gathered from different techniques (reviewed by Williams, 1994) suggests that water in seed tissues with a WC >0.05 g/g exhibits some of the thermodynamic and mechanical properties representative of a glassy state at <30°C. However, despite these studies, the evidence for a physiological role of intracellular glass in both storage stability and desiccation tolerance is still conjectural (Williams et al., 1993; Sun and Leopold 1994; Vertucci et al., 1994a, 1994b). For example, the WC/temperature combinations that give a glass transition do not always correlate with the WC/temperature combinations that induce the physiological changes associated with desiccation tolerance (Vertucci and Farrant, 1994) and seed aging (Vertucci, 1992; Vertucci and Roos, 1993; Sun and Leopold, 1994). A better understanding of the water properties in relevance with the nature of an intracellular glassy matrix in seeds is needed before applying glass principles to the storage technology of orthodox seeds.

To achieve this objective, we investigated the nature of the intracellular glasses in seeds by exploiting the theoretical and experimental considerations of glasses in food, polymeric, and inorganic materials. The kinetic stability of glasses is of fundamental importance when studying the effects of time and external conditions (temperature, pressure, and humidity) on glassy behaviors (Moynihan et al., 1976; Tant and Wilkes, 1981; Berens and Hodge, 1982; Eisenberg, 1984; Jewell and Shelby, 1990; Slade and Levine, 1991, 1994; Angell et al., 1994; Franks, 1994). The concept of kinetic stability originates from the way a glass is formed and can be explained using cryopreservation principles. Vitrification consists of rapidly bringing the original liquid system out of reach of its equilibrium state using a fast-cooling treatment, which induces a rapid increase in viscosity. Because of the high viscosity, the glass system is inhibited from rapidly reaching its equilibrium state. Nevertheless, there is still a "driving force" for the glass to achieve equilibrium (Tant and Wilkes, 1981; Franks, 1994). For that reason, the glass is described as kinetically stable but not thermodynamically stable. With respect to storage stability, the kinetic stability is an important factor because it dictates, over long periods, both the changes in the structure and properties of a glass (also referred as relaxation or physical aging) and the rates at which these changes occur (Tant and Wilkes, 1981; Jewell and Shelby, 1990; Williams et al., 1993; Angell et al., 1994; Franks, 1994;

Slade and Levine, 1994). We speculate that the changes in seed viability and/or vigor during long-term storage may be associated with certain time-dependent changes in the thermal properties of intracellular water. Therefore, adapting the concept of kinetic stability to intracellular glasses in seeds may lead to insights of a cause-effect relationship between the occurrence of a glassy state and storage stability.

Another aspect that has received extensive consideration from the polymer and material science concerns the molecular interactions of the components that form glassy matrices (Kelley et al., 1987; Orford et al., 1989; Schneider, 1989; Jewell and Shelby, 1990; Kalichevsky et al., 1993). Both the relative proportions of the components and the resulting interactions between them influence the properties of the glassy matrix and the storage stability (Levine and Slade, 1988; Schneider, 1989; Jewell and Shelby, 1990). The food science literature dealing with storage of low-moisture stuffs has used several models adapted from the polymer science literature to explain the influence of water on the properties of aqueous glass-forming materials (Levine and Slade, 1988; Roos and Karel, 1991a, 1991c; Slade and Levine, 1994).

In this paper, we report the glass transition behavior in embryonic axes of bean (*Phaseolus vulgaris* L.) seeds with varying WC. According to glass science, a prior understanding of the glass transition behavior is necessary to provide an understanding of the properties of the glass such as its kinetic stability (Tant and Wilkes, 1981; Angell, 1988; Jewell and Shelby, 1990; Angell et al., 1994). Experimental treatments and models developed in food, polymer, and materials science disciplines were adapted to the seed system to gain insights into the complexity and the kinetic stability of the intracellular glassy matrices.

MATERIALS AND METHODS

Plant Material and Sample Preparation

Axes of bean (*Phaseolus vulgaris* cv Kentucky wonder L.) seeds were used to study the relationship among WC, temperature, and characteristics of different thermal transitions. Bean axes contain about 10% lipid, and there appears to be no interference between the water-dependent thermal transitions and those associated with lipids (Williams and Leopold, 1989; Vertucci, 1990). Embryonic axes were dissected and placed in jars over salt-saturated solutions at room temperature for 4 to 7 d. The WC after equilibration ranged from 0 to 0.45 g/g. To achieve WC >0.45 g/g, known quantities of water were added to pre-weighed samples. Two to four axes (4–6 mg of dry weight) were packed and hermetically sealed into DSC aluminum pans. After the samples were sealed, fresh weight was measured and the pans were subjected to DSC analysis. Afterward, pans were punctured and dry weights were measured after a 36-h incubation at 96°C. WC are expressed on the dry weight basis.

DSC

Thermal transitions were measured in axes with WC between 0 and 1.1 g/g at temperatures between -120 and

+120°C using a Perkin-Elmer differential scanning calorimeter DSC-7 interfaced with a thermal analysis data station. Helium was used as the purge gas. The calorimeter was calibrated for temperature using methylene chloride (-95.32°C) and indium (156°C) standards and for energy using indium (28.496 J/g). The thermal behavior of samples was studied during heating in a range starting at least 30°C below the T_g . Unless otherwise stated, cooling and heating rates were set at 20 and 10°C/min, respectively. To study some effects of thermal history, we repeated the scans after heating the tissues to 100°C. Before samples were heated the second time, they were cooled from 100 to 25°C at 200°C/min and then cooled to the desired temperature at 20°C/min. Samples were then rescanned at 10°C/min as described above.

Thermal Analysis

Measurements of thermal events were obtained during heating runs. Baseline curvatures were minimized by subtracting a quadratic equation from the heat-flow data points. To standardize for the dry mass, heat-flow data were divided by the sample dry weight. The energy of first-order transitions (melting and crystallization events) were measured from the area of the endothermic or exothermic peak above or below the baseline as shown in Figure 2A. The melting and crystallization temperatures were taken as the onset temperature of the peak (Vertucci, 1990). The amount of unfrozen water was determined by the x intercept of a linear regression of the melting enthalpies against sample WC (Vertucci, 1990). The glass transition is considered as transition between the nonequilibrium state and equilibrium state of the same liquid rather than a phase transition (solid versus liquid). However, it will be referred to in this paper as a glass \rightarrow liquid transition. The glass \rightarrow liquid transition characteristics were quantified by measuring the T_g , the associated release of ΔC_p , and time lapse of the transition (relaxation time). Measurements of these parameters were performed using the calorimetric interpretation given by the DSC-7 Perkin-Elmer software. Such interpretation is shown in Figure 2, where the dashed lines represent the baselines at the onset and the end of the transition. T_g is reported as the temperature at which the increase in heat capacity is one-half of the total ΔC_p . ΔC_p corresponds to the amplitude of the baseline shift between the glass and "true" liquid states (Eisenberg, 1984; Perez, 1994). The relaxation time was estimated by dividing the difference between the starting and ending temperatures of the transition by 10°C/min, which was the heating rate used in the experiments.

RESULTS

DSC Interpretation of Thermal Events Associated with Water Transitions

Thermal events associated with glass and phase transitions of water were recorded in intact bean axes with a WC between 0.03 and 1.1 g/g and temperatures between -120 and +120°C (Fig. 1). At a WC >0.30 g/g (Fig. 1A, curves 1-4), a broad endothermic peak centered at approximately

-15°C constituted the prevalent event; its size and onset temperature were dependent on the WC and corresponded to the melting of ice (Franks, 1982; Vertucci, 1990). This peak disappeared in samples containing <0.25 g/g (Fig. 1A, curve 5, and B). This WC therefore corresponds to the amount of unfrozen water as described by Vertucci (1990).

In contrast to the familiar first-order phase changes, evidence for glass transitions in DSC studies is usually based on the observation of an apparent endothermic shift of the heat flow, which yield a discontinuity in the baseline (Atkins, 1982; Franks, 1982). However, the clearcut behavior observed on DSC scans of model systems does not generally occur in complex systems, such as synthetic polymers (Eisenberg, 1984), low-moisture foods (Noel et al., 1990), and biological systems (Williams et al., 1993; Williams, 1994). Abrupt shifts in power were also not observed in bean axes used in this study. Therefore, the analysis and calorimetric interpretation of thermal events considered in this study are described in Figure 2 together with the methods of quantification. Thermal events that can be attributed to glass \rightarrow liquid transitions were ob-

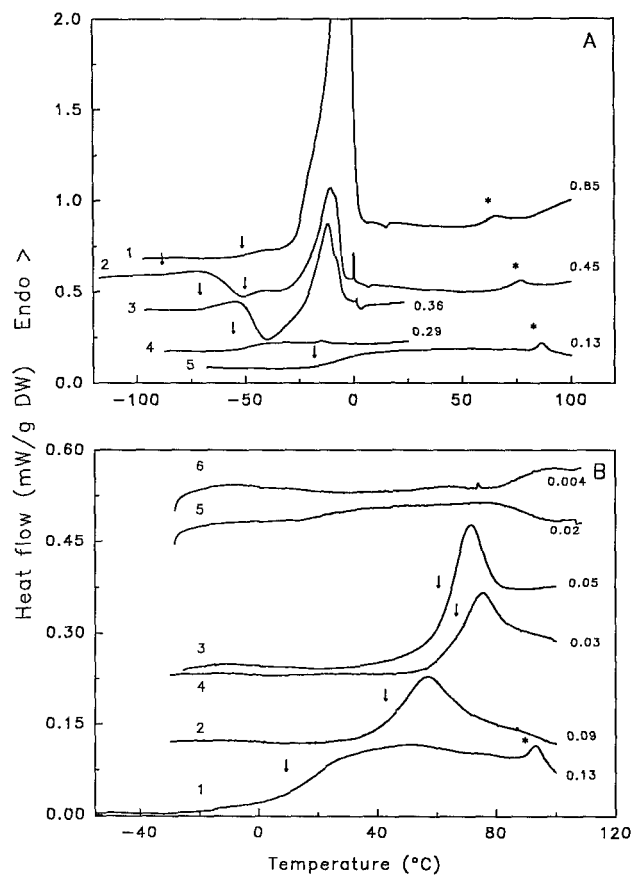


Figure 1. Heating DSC thermograms of bean axes at WC between 0.85 and 0.13 g/g (A) and between 0.13 and 0.005 g/g (B). The arrows mark the onset of the glass \rightarrow liquid transitions. The asterisk (*) indicates the presence of the high-temperature-melting peak. Thermograms are normalized with respect to the sample dry weight (DW) and corrected for the curvature. The temperature axis is not corrected for the thermal lag of the instrument. Endo, Endothermic.

served at decreasing WC from 0.45 to 0.03 g/g. They exhibited several types of thermal behaviors depending on the WC (Figs. 1 and 2). For samples with a WC between 0.45 and 0.29 g/g (Fig. 1A, curves 2 and 3; Fig. 2A, curve 1), the heating scans successively exhibited an endothermic shift starting at approximately -75°C (labeled in Fig. 1A with arrows and in Fig. 2A as T_g), immediately followed by a pronounced exothermic event centered at approximately -56°C (Fig. 2A, T_{cr}), and finally a second endothermic shift (T_g') occurring at -45°C prior to the melting of ice (T_m).

Based on DSC data from the literature (Franks, 1982; Levine and Slade, 1988; Vertucci, 1990; Williams et al., 1993), both the endothermic shift (T_g) and the second endothermic shoulder (T_g') are believed to be evidence for glass \rightarrow liquid transitions. T_{cr} corresponds to a crystallization event that resulted from the first glass \rightarrow liquid transition. The formation of ice crystals leads to the concentration of solutes trapped in the unfrozen matrix and this

solution supersaturates. Such freeze-induced concentration provokes an increase in local viscosity, which allows the unfrozen matrix to temporarily form a secondary glass (Roos and Karel, 1991b; Slade and Levine, 1991) that relaxes at T_g' during further heating (Fig. 2A). The temperature described by T_g and T_{cr} increased with decreasing WC (Fig. 1A, curves 2 and 3). In contrast, T_g' was always observed in samples with WC between 1.1 and 0.36 g/g at about -45°C . At WC < 0.36 g/g this event was not observed, probably because of the overlap with the devitrification event (Fig. 1A, curve 3). There was no evidence of a transition at T_g' for samples containing < 0.29 g/g (Fig. 1B, curves 1–5).

A second type of thermal behavior attributable to a glass \rightarrow liquid transition was observed in thermograms of axes between 0.29 and 0.09 g/g (Figs. 1A, curves 4 and 5, 1B, curve 1, and 2B, curve 1). The heat flow in these thermograms exhibited an endothermic shift, the onset temperature of which progressively increased from -54 to $+15^{\circ}\text{C}$ with decreasing WC. Such behavior, which can be thermodynamically ascribed to a second-order transition, is very similar to glass \rightarrow liquid transitions found in model systems (Atkins, 1982; Perez, 1994) and saccharide or protein solutions (Levine and Slade, 1988; Koster, 1991). A third type of thermal glass behavior appeared in samples with WC between 0.09 and 0.03 g/g, as an endothermic peak resembling a first-order transition at about 50°C (Fig. 2B, curve 2). The onset temperature of this apparent peak increased with decreasing WC (Fig. 1B), whereas the amplitude first increased to a maximum corresponding to a water content of ± 0.05 g/g (curve 3) and then decreased at lower WC. Such thermal behavior has also been observed at the glass \rightarrow liquid transition of a wide range of amorphous glassy materials, including synthetic polymers (Berens and Hodge, 1982; Eisenberg, 1984), saccharide mixtures (Green and Angell, 1989; Noel et al., 1990), and biopolymers, such as hemicellulose and lignin (Kelley et al., 1987). In the polymer and materials science, similar endothermic peaks have been ascertained as a glass \rightarrow liquid transition and are called "overshoot" (Moynihan et al., 1976; Tant and Wilkes, 1981; Berens and Hodge, 1982; Eisenberg, 1984; Jewell and Shelby, 1990; Angell et al., 1994; Perez, 1994). These studies support our interpretation of the endothermic peak in bean axes as a glass \rightarrow liquid transition.

Another thermal event that also appeared as a small endothermic peak (Fig. 1, *, thereafter referred to as HTP) occurred after the glass transition, at 60°C in the wettest samples (Fig. 1A, curve 1), and then shifted to higher temperatures with decreasing WC (Fig. 1A, curves 2 and 5, and 1B, curve 1). HTPs were not observed at temperatures $> 115^{\circ}\text{C}$ and in samples with WC < 0.04 g/g (data not shown). The amplitude of the HTP also appeared to be associated with the sample WC (data not shown). The origin of the HTP is likely to be associated with thermal denaturation of protein. We ruled out the possibility that the HTP is associated with a melting of sugar crystals, since the melting enthalpy values (0.1 – 3.0 J/g dry weight, which are equivalent to 0.8 – 25 J/g of sugars, assuming that em-

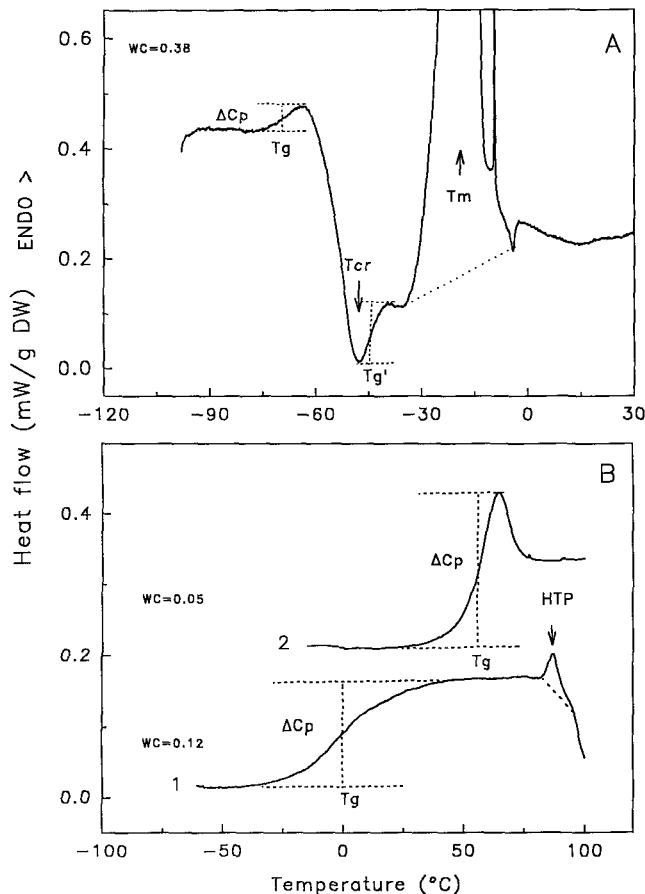


Figure 2. Labeling and analysis of the different thermal events occurring in bean axes heated at $10^{\circ}\text{C}/\text{min}$. The dashed lines correspond to the interpretation of the theoretical baselines of equal heat capacity. ΔC_p is calculated from the amplitude of the baseline shift at the T_g . T_g' results from the prior devitrification. T_m , Temperature of the ice melting peak. Thermograms are normalized with respect to the sample dry weight (DW) and corrected for the curvature. The temperature axis is not corrected for the thermal lag of the instrument. Endo, Endothermic.

bryonic axes contains 12% sugar/g dry weight) and onset temperatures (60–110°C) are not compatible with those for different sugar crystals (e.g. melting enthalpies and onset temperature for Suc are 118–120 J/g and 160–190°C, respectively [Levine and Slade, 1988]).

No thermal events were observed in embryos with a WC < 0.03 g/g between –100 and 120°C (Fig. 1B, curves 5 and 6). Discontinuities in the baseline shown in curves 5 and 6 in Figure 1B were not reproducible and were attributed to variations in the background heat flow.

Relationship between WC and Characteristics of the Glass → Liquid Transition Behavior

The relationship between WC and the thermal properties (T_g , ΔC_p , and relaxation times) of the glass → liquid transition were quantified in bean axes containing 0 to 0.6 g/g. As WC increased from 0.03 and 0.13 g/g, T_g decreased sharply from 70 to –5°C by about 7°C per 0.01 g/g of water gained (Fig. 3A). For samples with WC from 0.13 to 0.28 g/g, T_g decreased more gradually to –55°C at a rate of about 3°C per 0.01 g/g. With WC increasing up to 0.45 g/g, the decrease in T_g was about 1°C per 0.01 g/g. The above pattern, which describes a depressing effect of water on T_g , is very similar to that reported for other seeds (Williams and Leopold, 1989; Bruni and Leopold, 1992a, 1992b) and many glass-forming polysaccharides and proteins (Levine and Slade, 1988; Slade and Levine, 1994). This is an important observation because it will be used to study both how water plays a significant role in the structure and properties of glasses and how the depressing effect of water is linked to viscosity.

The relationship between WC and ΔC_p was complex and appeared to define at least five water regions (Fig. 3B). The first region (WC between 0 and 0.03 g/g) corresponds to the absence of a glass transition ($\Delta C_p = 0$). In the second region (WC = 0.03–0.095 g/g) the WC/ ΔC_p relationship featured a sharp peak with maximum amplitude in samples with a WC between 0.05 and 0.06 g/g. The third region was delimited by a short range of WC (0.095–0.12 g/g), where ΔC_p values were minimum (between 0.15 and 0.28 J/°C g dry weight). The fourth region (0.12–0.6 g/g) was represented by a broad peak centered at approximately 0.25 g/g. The fifth region was related to the WC above which no glass was observed. The range of ΔC_p 's presented here is in good agreement with several aqueous glasses made from biopolymer or saccharide mixtures (Orford et al., 1989; Roos and Karel, 1991a; Slade and Levine, 1991). It is noteworthy that the WC/ ΔC_p relationship (Fig. 3B) gives a better reflection of the various water-dependent behaviors of the glass transition rather than the WC/ T_g relationship (Fig. 3A).

Relaxation times (i.e. time lapse of the transition) were calculated as 120 ± 5.6 s and appeared to be independent of the WC of the samples (data not shown). This value represents the mean relaxation time of a macroscopic observation. Accurate measurements were not possible because the relatively poor thermal conductivity of biological materials interferes with the relaxation time measurements. However, the value reported here is of the same

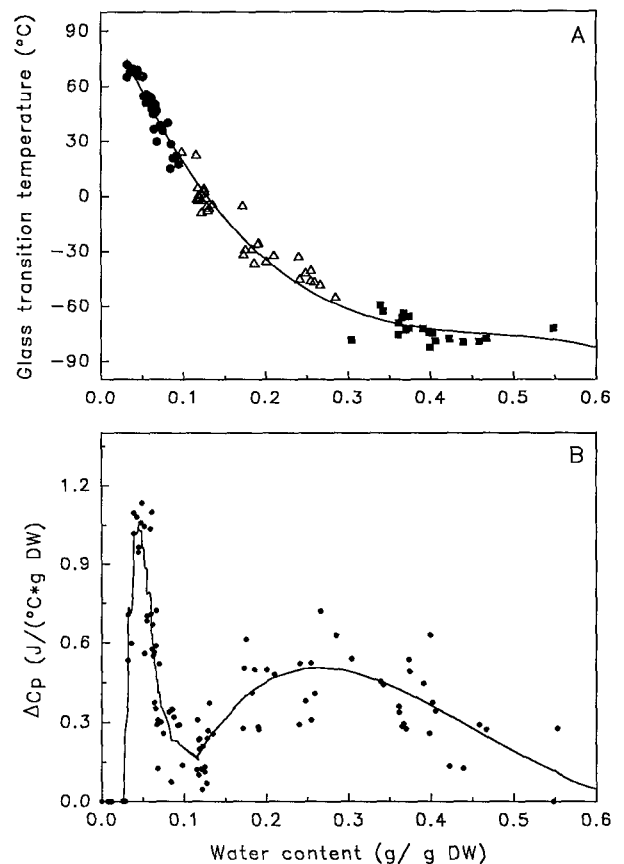


Figure 3. A, The relationship between the T_g and WC in embryonic beans axes. The different symbols reflect the different thermal behaviors encountered at T_g (second-order-like transitions with \blacksquare or without \triangle) a devitrification event or endothermic overshoot (\bullet). A third-order polynomial regression is shown as an aid to the eye. B, The relationship between the ΔC_p released at the glass → liquid transition and WC of bean axes. Two third-order polynomial regressions were fitted to the data (for WC above and below 0.13 g/g) as an aid to the eye. DW, Dry weight.

order of magnitude as relaxation times (20–200 s) that were obtained in amorphous salts and saccharides solutions using DSC at 10°C/min (Moynihan et al., 1976; Angell and Torell, 1983; Slade and Levine 1991).

The Glass → Liquid Transition Behavior in Relation to the Thermal History

The concept of a nonequilibrium state in aqueous glasses is of fundamental importance for the study of storage stability (Tant and Wilkes, 1981; Jewell and Shelby, 1990) and may be applicable to the study of seed aging. The nonequilibrium nature of glasses can be studied by investigating the thermal history of glasses prior to and during their formation (Tant and Wilkes, 1981; Berens and Hodge, 1982; Eisenberg, 1984). This was examined in bean axes by measuring the effect of a brief exposure to 100°C on the thermal behavior of the different water transitions described above. Thermal events were recorded in samples containing 0.04 to 0.45 g/g during a first heating run to

100°C and then during a second heating run after the samples were rapidly cooled to the desired temperature (Figs. 4 and 5).

The glass transition behaviors (Fig. 4A, curve 1), T_g , and ΔC_p (Fig. 5) in samples with WC > 0.10 g/g were similar in the first and second heating runs. This suggests that at these WC the glass-forming substances and/or their ability to form a glass are resistant to the heating treatment. In spite of this, embryos with WC of ± 0.22 and 0.13 g/g could not survive a 2-min exposure at 80°C (data not presented).

Several qualitative (Fig. 4) and quantitative (Fig. 5) differences between the first and second heating runs were observed in the glass \rightarrow liquid behavior of samples with a WC < 0.08 g/g (Fig. 4C, curve 2). The initial endothermic overshoot at T_g observed during the first heating run to 100°C disappeared when samples were cooled and reheated. Instead, a second-order-like transition occurred that was similar to that observed in samples with a WC between 0.09 and 0.29 g/g (Figs. 1B, curve 1, and Fig. 5B).

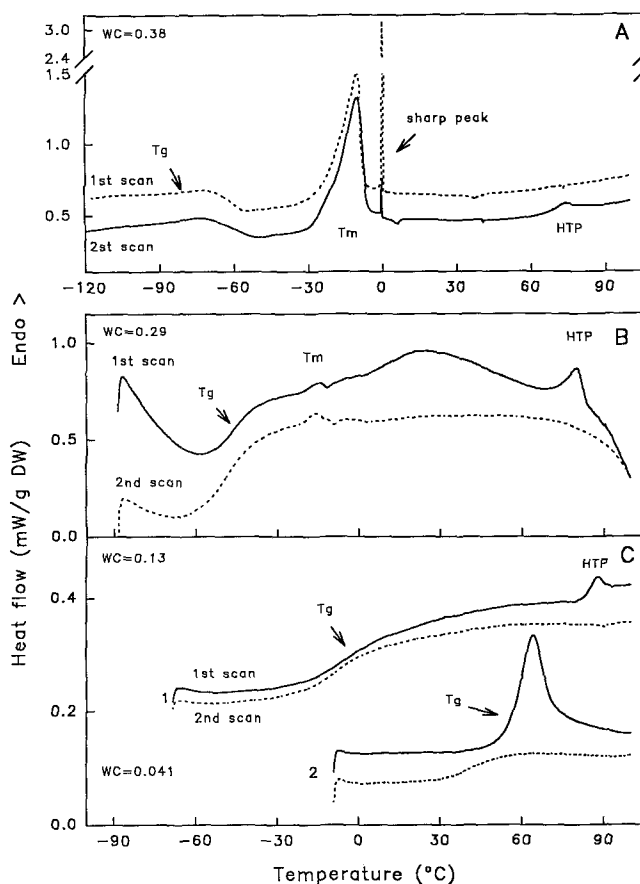


Figure 4. DSC heating thermograms describing the effect of a 100°C exposure on the glass \rightarrow liquid transition. The first scan (solid line) corresponds to the first heating, which was obtained with the same conditions as for Figure 1. The run was stopped immediately at 100°C, and samples were cooled at 200°C/min and then rescanned at 10°C/min (dashed line). Thermograms are normalized with respect to the sample dry weight (DW) and corrected for the curvature. The temperature axis is not corrected for the thermal lag of the instrument. The HTP in C, scan 2, is not shown. Endo, Endothermic.

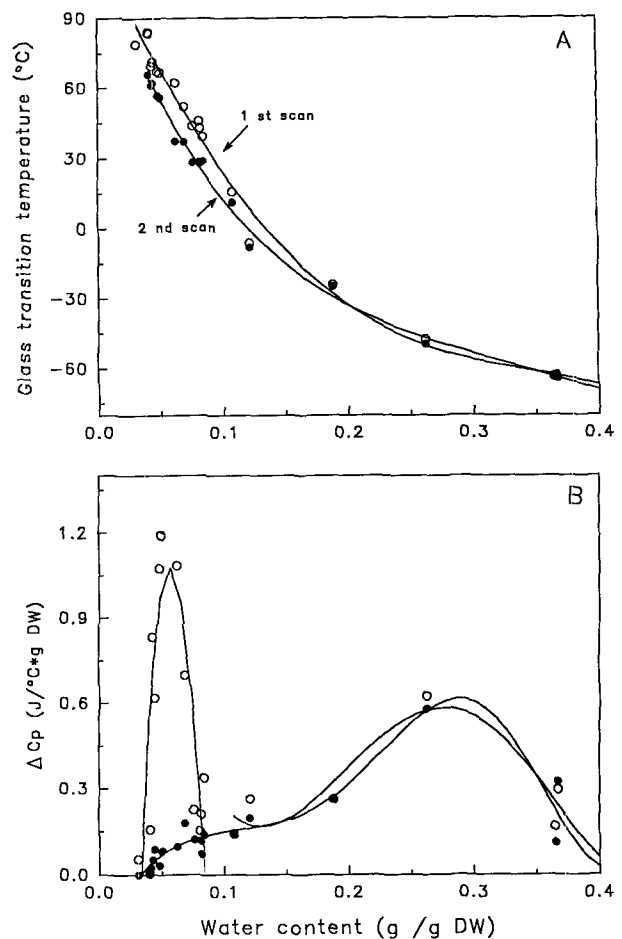


Figure 5. A, Relationship between WC and glass \rightarrow liquid transition occurring during a first heating (\circ) to 100°C and an immediate second heating (\bullet) obtained following the procedure described for Figure 4. B, The relationship between WC and ΔC_p released at T_g during the first (\circ) and second (\bullet) heating runs. Data are fitted with a third-order polynomial regression as an aid to the eye. DW, Dry weight.

The T_g and ΔC_p values were also significantly reduced following the second heating run (Fig. 5). The WC/ ΔC_p relationship featured a broad peak similar to that obtained after the first heating but did not exhibit a sharp peak at about 0.05 g/g observed with the first heating run (Fig. 5B). Furthermore, the WC limit for detecting T_g increased from 0.026 g/g during the first heating scan to 0.038 g/g during the second one. These results indicate that the glass-forming substances or their ability to form a glass were increasingly heat labile with WC decreasing < 0.08 g/g. In contrast to samples with WC at which the glass-forming properties were not heat labile but viability was, embryos with 0.07 g/g had a germination of 91% after a 2-min exposure at 80°C (data not presented). Since the sample WC did not change during heating and cooling, we suggest that the difference in transition behaviors at T_g resulted from changes in the conformation of components of the glass and/or redistribution of water molecules. A similar suggestion was made by Levine and Slade (1988) and Angell et

al. (1994), who showed that the structural properties of glasses made from protein or saccharide solutions can also be changed by a high-temperature ($T > T_g$) manipulation.

The idea that the 100°C exposure causes a redistribution of the components in the aqueous solution is substantiated by observations of how the heat treatment affected first-order transitions. Differences associated with the melting of ice were found in samples with 0.39 to 0.45 g/g that were heated to 100°C, cooled, and reheated (Fig. 4A, curve 1). After the second run, the sharp melting peak at 0°C increased 12-fold, compared to the first run, even though the overall melting enthalpy did not vary significantly between the two runs. In embryonic tissues, the presence of the sharp peak during heating runs is interpreted as a melting of relatively pure water resulting from a demixing event (Vertucci et al., 1991) and appears to be associated with freezing injury (Wesley-Smith et al., 1992). The HTP present during the first heating run disappeared in the second heating run (Fig. 4, A and B). In samples with a WC ± 0.061 g/g, the HTP that should occur at 109°C was not observed during the second heating of samples previously exposed to only 80°C (data not shown).

Models Predicting the Effect of Water on the Glass Transition

Polymer and materials sciences have developed several theoretical models that predict the dependence of T_g on the glass composition and on the effects of specific interactions of the glass components. With regard to aqueous glasses, the models may be used to study the role of water in the glass properties (refs. in Levine and Slade, 1988; Schneider, 1989; Kalichevsky et al., 1993). To provide insight about the influence of water on the glass behavior in bean axes, we investigated whether our data fit two models developed, which receive theoretical support from calorimetric considerations (refs. in Schneider, 1989; Kalichevsky et al., 1993). The first model referred to as the Gordon-Taylor equation relates the dependence of the T_g of glassy polymeric mixtures on the weight fraction and T_g values of the glass component (Orford et al., 1989; Schneider, 1989; Kalichevsky et al., 1993). The second model referred to as the Schneider equation takes into account both the Gordon-Taylor equation and the complexity of interactions between the different glass components (Schneider, 1989).

The Gordon-Taylor equation (Eq. 1), which can be used to describe the WC/ T_g relationship, considers water as an ideal solvent. Increasing concentrations of solvent will decrease the viscosity of the system and consequently increase the opportunity for faster molecular rearrangement. This will result in a decrease in T_g values as a function of increasing concentration of solvent. This phenomenon corresponds to a "plasticization effect." The plasticization effect of water is important for designing storage protocols of low-moisture food stuff, because degradative reactions are believed to be dependent on the viscosity of the system (Levine and Slade, 1988; Roos and Karel, 1991a; Slade and Levine, 1991, 1994). Because we observed a depressing effect of water on T_g in bean axes (Fig. 3A), it was tempting to regard the depression pattern as a plasticizing effect of

water. With respect to seed storage for which viscosity is also considered a primary factor controlling seed aging (Vertucci, 1992; Vertucci and Roos, 1990, 1993; Williams et al., 1993; Sun and Leopold, 1994), the prediction of a T_g /WC relationship via the Gordon-Taylor equation may have some relevance to seed storage. The Gordon-Taylor equation can be formulated by its simplified expression as:

$$T_g = \frac{W_1 T_{g1} + kW_2 T_{g2}}{W_1 + kW_2}, \quad (1)$$

where k = an experimental coefficient specific to a mixture; T_g is the T_g of the mixture; W_1 and T_{g1} are, respectively, the weight (or mass) fraction (in percent) and the T_g (in K) of the component 1 (i.e. glass-forming solute); and W_2 and T_{g2} are the weight fraction (i.e. WC) and T_g of pure water, which equals 134 K (Angell, 1988; Angell et al., 1994). Therefore, the T_g /WC relationship and the subsequent storage strategy can be predicted by determining T_{g1} and k values, which are specific to the glass-forming solutes. Such an approach has been widely used in food science to describe the WC/ T_g relationship for solutions of saccharides (Levine and Slade, 1988; Orford et al., 1989; Roos and Karel, 1991a, 1991b; Kalichevsky et al., 1993) and biopolymers (protein [Slade and Levine, 1991] and lignin and hemicellulose complexes [Kelley et al., 1987]).

To obtain W_2 for our system, WC must be recalculated and expressed on a fresh weight basis rather than a dry weight basis. Since the nature of the glass-forming solutes (component 1) was not known for bean axes, we assumed that $W_1 = 1 - W_2$ as in Roos and Karel (1991a). T_{g1} and k were then calculated from the best fit to our phase diagram of bean T_g values ($r^2 = 0.975$, Fig. 6A). The values for T_{g1} and k were 415 K and 11.5, respectively, and corresponded to those of a complex oligomeric sugar, such as maltodextrin (Roos and Karel, 1991a, 1991b; Slade and Levine, 1991). However, as WC decreased below 0.05 g/g, the Gordon-Taylor equation gave a poor fit to our data when the experimentally derived T_{g1} and k values were used. When T_{g1} and k values for Suc (Roos and Karel, 1991a), which is speculated to be the major vitrifying agent in seeds (Burke, 1986; Williams and Leopold, 1989; Koster, 1991; Sun et al., 1994), were used in the Gordon-Taylor equation, there was a poor fit to the data (Fig. 6A). Correspondence of the equation to our data was also poor when coefficients for Glc (Roos and Karel, 1991a) were used. Although there is no direct evidence that sugars form the intracellular glasses in seeds, the better fit obtained with coefficients corresponding of a complex polysaccharide such as maltodextrin reinforces the idea that, if sugars are involved with glass formation, it is through a complex mixture of Suc and oligosaccharides (Koster, 1991; Williams et al., 1993; Vertucci and Farrant, 1994).

The Schneider equation (Eq. 2) was derived from the Gordon-Taylor equation and was designed to take into account two types of interactions that influence the glass transition behavior of polymer blends. This equation is interesting because it actually tests the applicability of the Gordon-Taylor equation, which assumes that water acts as an ideal solvent of the glassy matrix and affects only vis-

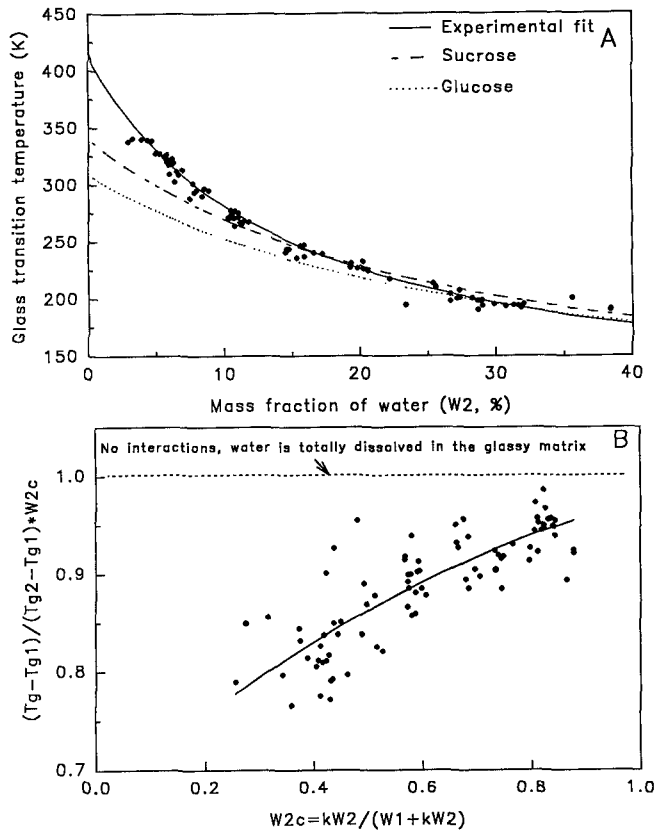


Figure 6. Study of fittings to the Gordon-Taylor (A) and Schneider equations (B), which relate T_g to the influence of the glass components (see "Results" for details). A, Relationships between the mass fraction of water (W_2 ; 0% = no water, 100% = pure water) and T_g s. Observed data (●) are compared with values obtained from an experimental fit ($r^2 = 0.975$) to the Gordon-Taylor equation and with the Gordon-Taylor equation for Suc and Glc (Roos and Karel, 1991a, 1991b). B, Schneider plot obtained from our experimental data (●) (Schneider, 1989). Both first- and second-order regressions (solid lines) show a significant deviation from the dashed line. The dashed line corresponds to the theoretical case when the Schneider plot gives a slope = 0 and a y intercept = 1. This dashed line represents the case for which interactions between the glass components do not influence T_g .

cosity. If this assumption is incorrect, the Schneider equation then provides an insight into the nature and intensity of interactions between the different glass-forming solutes and water. To account for specific interactions of the polymeric glass compounds and the fact that these compounds are not perfectly miscible, Schneider (1989) introduced two coefficients, K_1 and K_2 , into the Gordon-Taylor equation. The Schneider equation can be formulated as:

$$W_{2c} \frac{(T_g - T_{g1})}{(T_{g2} - T_{g1})} = (1 + K_1) - (1 + K_2)W_{2c} + K_2W_{2c}^2, \quad (2)$$

where $W_{2c} = kW_2 / (W_1 + kW_2)$ and T_{g1} , T_{g2} , W_1 , W_2 , and k are defined by the Gordon-Taylor equation. The objective of Equation 2 is to determine the values for K_1 and K_2 , which give some insight into the interactions of the glass components. If $K_1 = K_2 = 0$, the glass constituents do not

interact and are completely dissolved in the system (hence, an ideal solution). Equation 2 then reduces to a simpler form:

$$W_{2c} \frac{(T_g - T_{g1})}{(T_{g2} - T_{g1})} = 1. \quad (3)$$

Thus, for ideal glasses it follows that a plot of values obtained from W_{2c} versus values from Equation 3 (so-called "Schneider plot") gives a horizontal line = 1. This means that the depressing effect on T_g of increasing WC can be described solely as a result of changes in local viscosity. In this case, the Gordon-Taylor equation is entirely satisfactory for describing the depressing effect of T_g with increasing WC. When K_1 and/or $K_2 \neq 0$, the Schneider plot exhibits linear deviations of slope K_1 ($K_2 = 0$) or curved deviations (K_1 and $K_2 \neq 0$), indicating that various interactions within the glass-forming solutes affect the glass behavior (Schneider, 1989; Kalichevsky et al., 1993). The Schneider plot obtained for bean axes showed a strong deviation from a horizontal line = 1, which can be described by a significant negative second-order regression ($r^2 = 0.820$) with $K_1 = 0.667$ and $K_2 = 0.479$ (Fig. 6B). The positive values for K_1 and K_2 imply the existence of complex interactions between water molecules and the different components of the intracellular glass. It follows that water does not interact with the glassy matrix in a simple and homogenous manner as described by the Gordon-Taylor equation. The calculated values of K_1 and K_2 values are of questionable significance, since they were designed to account for two types of energetic perturbations resulting from interactions specific to synthetic polymers.

DISCUSSION

The glass model used to link the water properties with storage stability is not entirely satisfactory (Vertucci, 1992; Williams et al., 1993; Vertucci et al., 1994b). For example, such a relationship actually fails to predict aging data of soybean seeds at very low WC and pea seeds at high WC (Williams et al., 1993; Sun and Leopold, 1994). Several aging studies on seeds (Vertucci and Roos, 1990; Vertucci et al., 1994b, and refs. therein) and pollen (J. Buitink, C. Walters-Vertucci, F. Hoekstra, O. Leprince, unpublished data) have demonstrated that the removal of water from the intracellular glass results in increased aging rates and/or decreased storage stability. Here, we have identified at least four factors in addition to the WC/ T_g relationship that need to be taken into account prior to investigating correlations between the presence of an intracellular glass and seed deterioration. These factors, which were derived from the polymer and materials science principles on glasses, are the nonequilibrium nature of glasses, the thermal history prior to the glass formation, the complexity of the intracellular structures involved in the glassy matrix, and the absence of glass matrix at lower WC.

The nonequilibrium nature of the glass is directly reflected in the glass transition behavior (Tant and Wilkes, 1981; Eisenberg, 1984; Levine and Slade, 1988). In bean axes, this behavior is characterized by three different types

(types 1, 2, 3) of thermal events, which are dependent on temperature, WC, and thermal history. Thermal events diagnosed as a glass \rightarrow liquid transition appeared as second-order-like transitions in samples with WC between 0.45 and 0.09 g/g (type 1 and 2) and an endothermic overshoot between 0.09 and 0.03 g/g (type 3). The appearance of a devitrification event in samples containing 0.45 to 0.3 g/g distinguishes type 1 from type 2. Theoretical considerations of the glass transition behavior consider that the glass \rightarrow liquid transition involves a distribution of molecular rearrangements (relaxation processes) of different types and kinetics (Moynihan et al., 1976; Tant and Wilkes, 1981; Eisenberg, 1984; Yonezawa, 1991; Angell et al., 1994; Perez, 1994). In hydrated, inorganic glasses (Jewell and Shelby, 1990) and amorphous sugar solutions (Green and Angell, 1989), different types of glass transition behaviors exhibited by DSC thermograms were dependent on the glass WC. In these systems, relaxation processes over time also appeared to be controlled by the glass WC. Different relaxation processes are also controlled by water in embryonic axes of soybean and red oak (Bruni and Leopold, 1992b). It follows that the different glass behaviors observed in bean axes result from differences in the kinetics or types of molecular rearrangements, these being dependent on the sample WC. One may suggest that during long periods, the intracellular glass behavior, which depends on relaxation processes, will vary according to the initial WC of the intracellular glass in seed tissues. Glasses are thermodynamically driven to slowly approach equilibrium over long periods. The theory predicts that the approach to equilibrium conditions is associated with relaxation processes, such as demixing of the glass components, phase separation, and/or crystallization (Franks, 1982). For seed storage protocols, the question is whether the various kinetics of seed deterioration during storage can be attributed to the different relaxation processes or glass behaviors occurring when the intracellular aqueous glass moves toward equilibrium.

The nature of equilibrium of a system can be determined by designing state-phase diagrams, which combine both the equilibrium and nonequilibrium states, as a function of temperature and composition of the system (Atkins, 1982; Franks, 1982). For relatively dry or frozen food stuff, state-phase diagrams are often used to determine the critical variables of temperature and WC for optimum storage conditions and predict the thermodynamic paths leading to deterioration of the food system (e.g. formation of ice crystals in ice cream; Levine and Slade, 1988). Therefore, to obtain information concerning the nature of equilibrium conditions with respect to glasses in bean axes, we designed a state-phase diagram of water. This defines the temperature-WC boundaries of the solid and liquid phases and the nonequilibrium versus equilibrium states (Fig. 7). In bean axes, the demixing events associated with the approach to equilibrium conditions below 0°C correspond to the formation of ice crystals and the transient formation of secondary glass within ice crystals at Tg' (Fig. 7). Note that Tg' is not dependent on the WC, an expected consequence, since the secondary glass formation always occurs

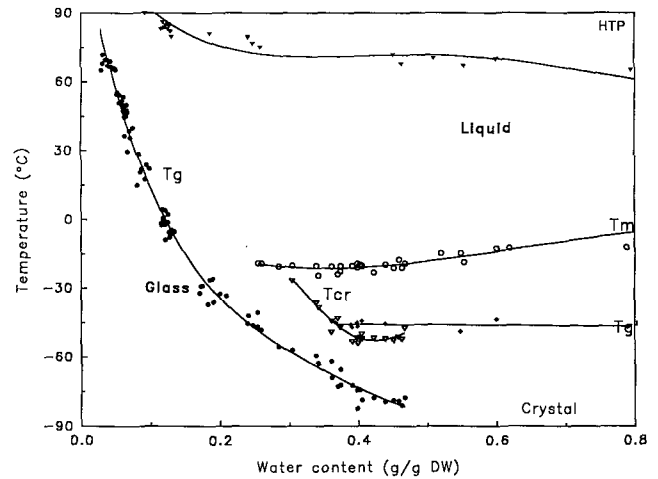


Figure 7. State-phase diagram of bean axes describing the relations between the water states and temperature. The labeling is as described for Figure 2. Tg is the glass \rightarrow liquid transition curve obtained from Figure 3. It delimits the regions of nonequilibrium conditions but kinetically stable and equilibrium conditions. All data points are fitted with a third- or fourth-order regression as an aid to the eye. DW, Dry weight.

after the nonfrozen solution has reached its maximum concentration. Although Tg' is important in frozen foods, its physiological relevance in cryopreservation in biological material is unclear. Above the ice-melting curve (Fig. 7, Tm), our state-phase diagram is unable to define or predict the nature of the equilibrium state. Several studies (Green and Angell, 1989; Koster, 1991; Sun et al., 1994), together with the Gordon-Taylor fit to our data (Fig. 6A), suggest that a complex oligosaccharide mixture is the major component of aqueous intracellular glass in seed tissue. Therefore, the approach to equilibrium of these glasses might be demixing events involving the crystallization of sugars. However, the time scales of crystallization in amorphous sugar solutions (Roos and Karel, 1991c; Slade and Levine, 1991; Saleki-Gerhardt and Zograf, 1994) are far beyond the average longevity of seeds (Priestley, 1986). This indicates that sugar crystallization in intracellular glasses is probably not relevant biologically in seed storage. Therefore, one might hypothesize that the degradative reactions inside the glassy matrix might be faster than the relaxation processes occurring, while the intracellular glass is slowly approaching equilibrium.

The thermal behavior of the glass transition is also dependent on the thermal history of the vitrification process (Tant and Wilkes, 1981; Berens and Hodge, 1982; Eisenberg, 1984). This is also apparently the case in intracellular glasses in bean axes. The ability to exhibit an endothermic peak at Tg (overshoot) is strongly dependent on a combination of WC and heating treatments prior to inducing glass formation (Figs. 4 and 5). In addition, we showed that the high-temperature manipulation slightly depressed the WC/ Tg relationship (Fig. 5A) and induced the disappearance of the sharp peak depicted by the WC/ ΔC_p relationship (Fig. 5B). Thermal history would correspond to both the physiological and environmental conditions that lead

to the formation of the intracellular glass during the maturation drying of the embryo and to pre- and postharvest conditions such as priming. It has been shown that storage stability in seeds can be manipulated by priming procedures (e.g. Tarquis and Bradford, 1992). Therefore, it is reasonable to suppose that seed storage stability will depend on the thermal history during the intracellular glass formation.

The complexity of intracellular glasses in beans was studied using two models that relate T_g to the influence of water. The Schneider plot obtained for our data (Fig. 5B) indicates the presence of complex interactions between the water molecules and the glass-forming solutes within the intracellular glass. It is believed that these interactions are responsible for the differences in the glass transition behaviors (Schneider, 1989). The Schneider plot also suggests that water does not act solely as solvent that dilutes the glassy matrix and induces uniform changes in viscosity. The Gordon-Taylor equation, which describes a WC/ T_g relationship, models the effect of a solvent on the viscosity of the system. The poor fit of our data of the Gordon-Taylor equation at low WC, together with our Schneider plot, suggests that additional factors other than viscosity play a significant role in the dependence of T_g on water. Therefore, a WC/ T_g relationship is not entirely satisfactory to describe the different behaviors of water within the glassy matrix encountered at low WC.

Glass transitions were not observed at WC < 0.03 g/g. Loss of glassy structures at extremely low WC has also been reported in food systems (White and Cakebread, 1966; Noel et al., 1990). We speculate that at low WC the system might be more prone to denaturation processes during heating than at high WC. This might induce the collapse of the amorphous structure before detection. This interpretation is supported by our data (Fig. 5), showing that the glass-forming properties become increasingly heat labile with decreasing WC and by studies reporting a similar behavior in proteins and sugars solutions (Noel et al., 1990). This interpretation implies that the presence of glasses per se cannot prevent molecular rearrangements inside the glassy matrix. The absence of glass \rightarrow liquid transition at low WC suggests that the state of water on the left side of the WC/ T_g curve (Figs. 3A and 7) is complex and cannot be explained solely by an assignment of a glassy state. It might also explain the poor fit between T_g values and maximum temperatures for storage of soybean and corn seeds at very low WC (Sun and Leopold, 1994).

Intracellular glasses and hence the state of water in biological systems are somewhat more complex than protein or sugar mixtures (Levine and Slade, 1988; Green and Angell, 1989; Orford et al., 1989; Roos and Karel, 1991a, 1991b). In marked contrast to these systems, the cytoplasmic solution is a highly complex mixture of lipid, proteins, sugars, and salts with concentrations varying among organelles. The intracellular aqueous glass will also be constrained by interfaces such as lipid membranes and/or proteins. Water molecules that interact at these interfaces may have different glassy characteristics than the bulk amorphous matrix (Bruni and Leopold, 1992b). With de-

creasing WC, the contribution of interfacial interactions to the glass \rightarrow liquid transition behavior would become increasingly important within the glassy matrix and might explain both the result obtained from the Schneider plot and the disappearance of glass \rightarrow liquid transitions below 0.03 g/g. The presence of different types of water interacting within (or without) the glassy matrix is consistent with the five temperature-dependent hydration regions reported earlier (Vertucci, 1990, 1992). The type and kinetics of degradative reactions in seed tissues are believed to be different and specific to these hydration regions (Vertucci, 1992). The temperature-dependent boundary between regions 1 and 2 corresponds to the temperature/WC combination that gives maximum storage stability (e.g. for pea, 0.042 g/g, 65°C; 0.103 g/g, 5°C; 0.12 g/g, -20°C [Vertucci and Roos, 1993]). This boundary corresponds intriguingly to the T_g /WC curve described in Figures 3A and 7. This important observation makes the link between the hydration-level model based on equilibrium thermodynamics and the glass model based on nonequilibrium thermodynamics. The correspondence of both models suggests that, for the time considered by these studies, the distinction between equilibrium and nonequilibrium may not be relevant. It is noteworthy that the WC value corresponding to the maximum ΔC_p approximates that of the optimal WC for storage stability in various seeds (reference above). The WC/ ΔC_p relationship also shows the disappearance of evidence of a glassy state at WC < 0.03 g/g. We suggest that an additional hydration region exists inside region 1, where either the glassy characteristics disappear or the glassy matrix is rendered more fragile because of the removal of water molecules. This suggestion recently received support from a DSC study of water properties in *Typha* and corn pollen (J. Buitink, C. Walters-Vertucci, F. Hoekstra, O. Leprince, unpublished data).

We have shown that glasses are present in embryonic axes of beans. However, the properties of intracellular glasses in terms of their thermal behavior and thermal stability vary according to the WC of the tissue. At very low and very high WC, glasses were not detected in bean embryos, suggesting an inherent instability of the intracellular glassy matrices. Our data show that the interaction between water and the other glass-forming components is complex, indicating that water does not behave as a typical solvent in intracellular glasses. This discovery may make it difficult to apply theoretical considerations of thermal behavior of glasses to the storage stability of seeds.

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