Electrolyte-Induced Changes in Glass Transition Temperatures of Freeze-Concentrated Solutes

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Addition of electrolytes to solutions of non-crystallizing solutes can cause a significant decrease in the glass transition temperature (T_g') of the maximally freeze-concentrated solution. For example, addition of 2% sodium chloride to 10% solutions of dextran, PVP, lactose, and sucrose causes a decrease in T_g' of 14° to 18°C. Sodium phosphate has a smaller effect on T_g', and is unusual in that 1% to 2% sodium phosphate in 10% PVP causes a second glass transition to be observed in the low-temperature thermogram, indicating a phase separation in the freeze concentrate. Comparison of DSC thermograms of fast-frozen solutions of sucrose with and without added sodium chloride shows that electrolyte-induced reduction of T_g' is not caused by a direct plasticizing effect of the electrolyte on the freeze concentrate. Measurement of unfrozen water content as a function of temperature by a pulsed nmr method shows that the most likely mechanism for electrolyte-induced changes in T_g' is by increasing the quantity of unfrozen water in the freeze concentrate, where the unfrozen water acts as a plasticizer and decreases T_g'. The correlation time (τ_c) of water in the freeze concentrate is in the range of 10^{-7} to 10^{-8} seconds. The results underscore the importance of minimizing the amount of added salts to formulations intended for freeze drying.

KEY WORDS: freeze drying; lyophilization; thermal analysis; water mobility.

INTRODUCTION

Inorganic salts are often present in freeze-dried therapeutic and diagnostic formulations in order to maintain pH or isotonicity of the re-hydrated product. However, little attention is generally paid to the effect of salts on the physical properties of the frozen material. Of particular importance is the glass transition temperature of the freeze concentrated solution, T_g', which is closely related to the collapse temperature during freeze drying (1,2).

In a previous study, inorganic salts at a level of about 0.1% were used as "dopants" in order to enhance the sensitivity of electrical thermal analysis (ETA) for detection of T_g' (3). While it was observed that the presence of salts at this low level had minimum effect on T_g', higher concentrations of salt substantially reduce T_g'—in some cases, to a temperature low enough that freeze drying the formulation with most commercially available freeze drying equipment is not feasible.

The purpose of this investigation was to further examine the quantitative relationship between T_g' of freeze-concentrated solutions of selected non-crystallizing solutes and the quantity of salt present, to determine the most likely mechanism by which electrolytes decrease T_g', and to measure the mobility of water in freeze-concentrates with and without added salts at low temperature.

EXPERIMENTAL

Materials

The solutes used in this study were sorbitol, glucose, sucrose, lactose, mannitol, trehalose, Ficoll® 400K, dextran 70K, and polyvinylpyrrolidone (PVP) 40K. All organic solutes were reagent grade materials. Mannitol, sucrose, glucose, and sorbitol were used as received from Mallinckrodt, and trehalose was obtained from Aldrich Chemical Co. The inorganic salts were ACS reagent grade materials used as received from Mallinckrodt Co.

Methods

Differential scanning calorimetry was used for both T_g' and T_g measurement. The DSC method for T_g' measurement was described previously (2). Briefly, solutions of about 20 μl of the solute of interest with quantities of salt ranging from 0 to 3% were placed in aluminum sample pans and crimped. Samples were cooled at 10°C per minute to a temperature of about −50°C and warmed at a rate of 5° to 10°C/min. T_g' is reported as the midpoint of the glass transition region. T_g of rapidly frozen samples was carred out according to the method described by Roos and Karel (4). Solutions of 50 to 92% sucrose were obtained by adding the appropriate amount of water to sucrose or sucrose/salt mixtures. Samples were heated until a solution was obtained. The amount of water was measured gravimetrically before transferring approximately 20 μl of the solution into an aluminum sample pan, crimping, and immersing the sample in liquid nitrogen for about 2 minutes. The sample was quickly transferred to the DSC sample holder which had been previously cooled to −150°C. The thermogram was obtained at a heating rate of 10°C/min after the sample was held at −150°C for 5 minutes for thermal equilibration. T_g is reported as the midpoint temperature of the glass transition.

The unfrozen water content of the freeze concentrate, W_u, was measured by proton nmr. The method reported here was adapted from methods described by Hanafusa (5), Andrewartha, et.al. (6), Litowska and Dohler (7), and Hays et.al. (8). The standard solution was 24% LiCl with 0.1% $MnCl_2$ in 90% D_2O/10% H_2O, where D_2O was used to decrease the signal from H_2O and allow the same receiver gain for both samples and the standard solution.

Sample solutions included 30% sucrose containing 0, 1.0, 1.5, 2.0, and 3.0% NaCl. The densities of the LiCl/$MnCl_2$ and sucrose solutions were determined gravimetrically relative to the same volume of distilled water. Samples were placed in teflon sample tube liners in order to avoid breaking the sample tube during freezing and thawing, then placed in a 5 mm diameter sample tube.

A Bruker ARX 300 nmr spectrometer (300 MHz) was

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used in this study. The sample tube was placed in a ceramic spinner and lowered into the center of the magnet. The sample was cooled at a rate of 2°C/min to −50°C and held for 30 minutes. Sample spin rate was 20 rpm. Instrument parameters used in this study were as follows: spectrum width (sw), 416.67 ppm (125000 Hz); pulse width (pl), 11–12.2 μs; receiver gain (rg), 1430; delay time (d1), 10 s; total data points (td), 256k; data points used (si), 128k; line broadening, 60 Hz; number of scans, 16; number of dummy scans, 4.

Spectra were collected in 5°C increments from −50°C and −25°C, holding at each temperature for 20 minutes to assure thermal equilibrium. The amount of unfrozen water was calculated by the following equation:

$$W_u = (0.076 \times A_2 \times \rho_1)/(0.30 \times A_1 \times \rho_2)$$

where W_u is in grams water per gram of solute, A_1 and A_2 are the peak areas for unfrozen water in the standard and sample solutions, respectively, and ρ_1 and ρ_2 are the densities of the standard solution and the sample, respectively. Densities of the frozen solutions were determined relative to the gravimetrically measured density at room temperature. The solutions were frozen in an isopropanol bath to the measurement temperature, and height of the frozen solution in the nmr tubes was measured. The density of the frozen solution was then calculated by the ratio of heights of the column at the intended measurement temperature to the room temperature height.

The longitudinal relaxation time, T_1, was measured by the progressive saturation technique. The pulse sequence $(\pi/2\text{-acquire-d1})_n$ was used with delay times (d1) of 0.5, 1.0, and 10 seconds. Because the signal intensity did not change when d1 was longer than 10 seconds, the signal intensity at d1 = 10 seconds was assigned as A_0. T_1 was then calculated from the equation

$$A_t = A_0[1 - \exp(-D_t/T_1)]$$

where A_t is the signal intensity measured at the corrected delay time D_t, the actual time interval between each scan. Because the acquisition time (AQ) had to be included when using the progressive saturation method, the actual time interval between each scan was 2.049 sec for temperatures of −35, −40, −45, and −50°C (d1 = 1;td = 256k;AQ = 1.049 sec) and 0.566 sec for temperatures of −25 and −30°C (d1 = 0.5;td = 16k;AQ = 0.066 sec) for calculating T_1 values.

RESULTS AND DISCUSSION

Thermal Analysis

Figure 1 illustrates the DSC thermograms of 10% lactose containing various concentrations of sodium chloride. T_g' decreases by about 4°C for each 0.5% NaCl added. Figure 2 summarizes the effect of adding sodium chloride on T_g' of several non-crystallizing solutes. A continuous decrease in T_g' is observed as the concentration of salt is increased. Addition of 2% sodium chloride results in a decrease in T_g' of 14° to 18°C for dextran, PVP, lactose, and sucrose. Other electrolytes have similar effects on T_g'. For example, addition of ammonium acetate to the same solutes causes a decrease in T_g' of from 14° to 24°C, with T_g' of PVP affected most dramatically.

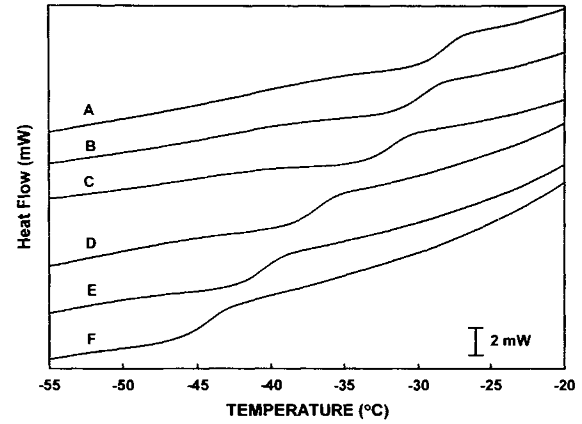

Fig. 1. DSC thermograms of 10% lactose (a) and 10% lactose containing b) 0.2%, c) 0.5%, d) 1.0%, e) 1.5%, and f) 2.0% NaCl.

The effect of sodium phosphate buffer at pH 7.4 on T_g' of 10% solutions of dextran 70K, lactose, and sucrose is small compared with salts such as sodium chloride. Addition of up to 2.0 percent sodium phosphate decreased T_g' by only about 1–2°C. The effect of adding sodium phosphate buffer to PVP solutions (Figure 3) is noteworthy in that concentrations of buffer in the 1 to 2% range cause a second glass transition to appear at a lower temperature. As the concentration of buffer is increased, the heat capacity change of the lower glass transition increases, and that of the higher glass transition decreases. The presence of multiple glass transitions in frozen solutions has not been observed in previous work. More careful examination will be required, but the data suggest a phase separation upon freezing.

Figure 4 demonstrates that a constant ratio of sodium chloride to sucrose will result in a constant value of T_g', within experimental error. It follows that a given concentration of salt will have a significantly greater effect on T_g' as the concentration of the solute is decreased. For example, the T_g' of 20% sucrose containing 0.5% NaCl is about −34°C, whereas the T_g' of 5% sucrose containing 0.5% NaCl is about −41°C (data not shown).

When concentrated solutions of sucrose—higher than

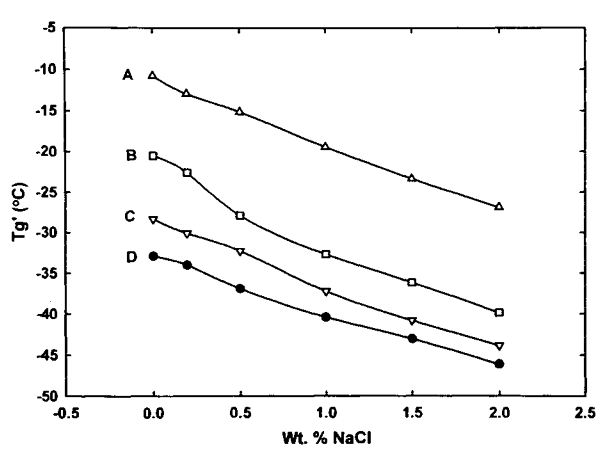

Fig. 2. T_g' values of 10% a) dextran 70K, b) PVP 40K, c) lactose, and d) sucrose as a function of NaCl concentration.

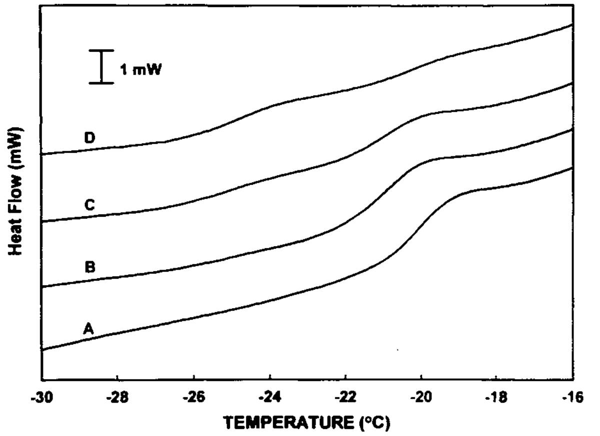

Fig. 3. DSC thermograms of 10% PVP (a) and 10% PVP containing b) 0.2%, c) 1.0%, and d) 2.0% sodium phosphate buffer at pH 7.0

about 70%—are quickly frozen with liquid nitrogen, the rapid rate of freezing prevents crystallization of ice, and no ice melting endotherm is observed in the thermogram. Instead, a uniform glassy phase is produced where the glass transition temperature (T_g in this case, not T_g') decreases as the water content increases. For concentrations of about 60% sucrose, crystallization of ice occurs during the warming process as indicated by a crystallization exotherm following a glass transition at about −80°C, and an ice melting endotherm with an onset teperature of about −25°C. As the concentration of sucrose in the starting solution is reduced to about 50%, ice apparently forms during the rapid freezing process, since no crystallization exotherm is observed in the subsequent thermogram. In this case, a T_g' transition would be expected at about −33°C, but this transition is apparently obscured by the onset of the ice melting endotherm caused by the high concentration of sucrose. These observations are in general agreement with the results reported by Luyet and Rasmussen (9).

Analysis of thermograms following rapid freezing of sucrose solutions with and without added electrolyte allows insight into the mechanism by which electrolytes decrease T_g'. Figure 5 is a plot of T_g values of rapidly frozen sucrose solutions (concentrations higher than about 60%) with and without added sodium chloride. Glass transitions for water (10) and amorphous sucrose (11) are included in Figure 5. If electrolyte-induced changes in T_g' were caused by a direct plasticizing effect of electrolyte, then T_g of fast-frozen solutions would be expected to be lower in solutions containing electrolyte than those without. The two glass transitions curves in Figure 5 are superimposable, which indicates that added electrolyte has no effect on T_g of rapidly frozen sucrose solutions. These data support the conclusion that added electrolyte does not exert a plasticizing effect directly.

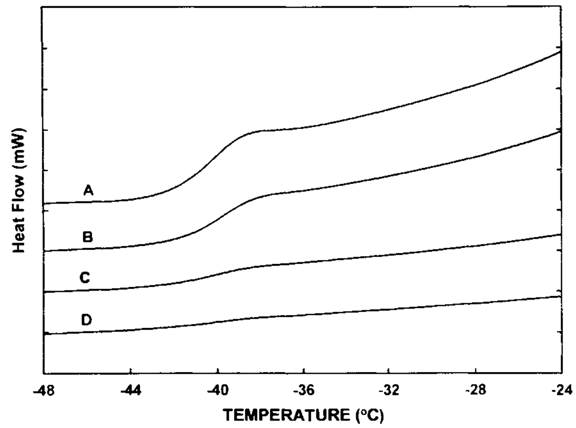

Fig. 4. DSC thermograms of sucrose/NaCl solutions at a constant ratio of sucrose to NaCl: a) 30% sucrose + 3% NaCl, b) 20% sucrose + 2% NaCl, c) 10% sucrose + 1% NaCl, and d) 5% sucrose + 0.5% NaCl.

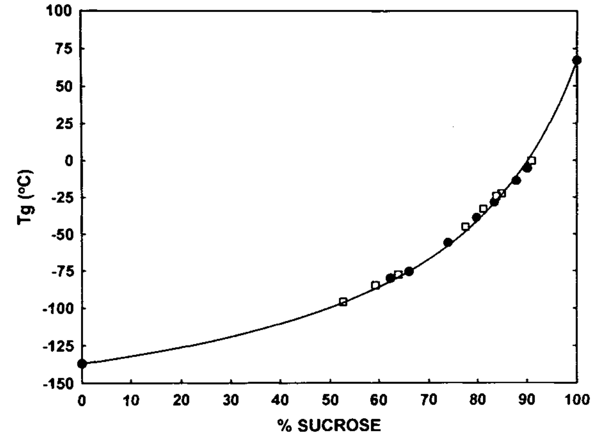

Fig. 5. Glass transition curve for sucrose/water solutions (closed circles) and sucrose/water containing NaCl at a ratio of 10:1 sucrose to NaCl (open squares).

The data in Figure 5 were fitted to the Gordon-Taylor equation (4),

$$T_g = (w_1T_{g1} + kw_2T_{g2})/(w_1 + kw_2)$$

where T_g is the glass transition temperature of a mixture, w_1 and w_2 are weight fractions of each component, T_{g1} and T_{g2} are the glass transition temperatures of the individual components, and k is an empirical constant. The value of k obtained from curve fitting was 4.48, which is in good agreement with the value reported by Roos and Karel (4).

Sugars and sugar alcohols are known to form complexes with metal ions through the hydroxyl groups of the sugars (12). The possibility of such direct interaction between electrolyte and solute causing altered physical properties of the frozen solution was considered, but other factors in addition to the T_g data in Figure 5 argue against this. First, the presence of electrolytes has been reported to increase the viscosity of aqueous solutions of sugars (11). This should cause an increase in T_g', not a decrease. In polymer electrolyte systems, the addition of inorganic salts tends to increase the glass transition temperature by complex formation, which restricts molecular mobility. Second, as indicated in Figure 2, the influence of added electrolyte is fairly consistent, irrespective of the nature of the solute. Given the structural diversity of the solutes examined, a mechanism involving such direct interaction between solute and electrolyte is unlikely, particularly since PVP contains no hydroxyl groups.

NMR Studies

Figure 6 is the ^1H nmr spectrum of a 30% sucrose solution at −35°C. The spectrum is approximated by the superposition of two Lorentzian lines: a narrow line (L1) with a line width of about 60 ppm, and a broad line (L2) with a line width of about 350 ppm. The narrow line arises from unfrozen water, where the water is mobile enough to provide a relatively sharp nmr signal. The broader line arises from the less mobile protons of the solute. A signal for the protons of ice is not seen, since the signal has a line width on the order of 10^5 Hz (7). Similar spectra were obtained from hydrated polypropylene phosphate (PPP) by Litowska and Dohler (7) with wide line, 60 NMz pulsed nmr at low temperature, and by Kuntz and Kauzman (13) for aqueous solutions of proteins. Similar studies have also been done on hydrated amphiphiles (14) and on biopolymers and polysaccharides (6).

T_1 values of ice protons measured with 60 MHz nmr have been reported to be longer than 1 minute, as compared with T_1 for unfrozen water of about 0.2 seconds (7). Thus, a short repetition time of $\pi/2$ pulses discriminates between ice and unfrozen water by only allowing the magnetization of proton nuclear spins of the unfrozen water to completely relax back to their equilibrium state between pulses. In our study with 300 MHz nmr, a delay time of 10 seconds was used, which is slightly longer than $5T_1$ of the unfrozen water protons.

Figure 7 illustrates the relationship between W_u vs. temperature for 30% sucrose and 30% sucrose with 3% sodium chloride. The W_u value of 30% sucrose at −35°C is 0.205 g H_2O/g sucrose, which is in good agreement with a value of 0.211 g H_2O/g sucrose reported by Franks, et.al. using DSC (15). The W_u value for 30% sucrose/3% sodium chloride at −35°C is about twice that of sucrose alone, and W_u increases continuously with increased NaCl concentration. Below about −45°C, the value of W_u is approximately the same for both samples. Figure 7 also demonstrates that nmr appears to be a sensitive method for determining T_g'. There is an abrupt change in the unfrozen water content at T_g', with W_u nearly constant below T_g' and increasing dramatically above T_g'. Values of T_g' for 30% sucrose and 30% sucrose/3% NaCl are −33°C and −40°C, respectively, which are in good agreement with T_g' values measured by DSC.

Based on the data in Figure 7, the mechanism by which addition of sodium chloride decreases the T_g' of sucrose solutions appears to be by increasing the amount of unfrozen water in the freeze concentrate. After ice nucleates during the freezing process, crystal growth decreases the quantity of unfrozen water in the freeze concentrated phase. This process continues until T_g' is reached, where further ice crystal growth ceases—at least on a practical time scale. Since the quantity of unfrozen water is significantly higher in the sucrose/sodium chloride system, a lower temperature is required before the amount of unfrozen water reaches a plateau value at T_g'. This mechanism is also consistent with the state diagram, which supports the conclusion that sodium chloride does not act as a plasticizer directly.

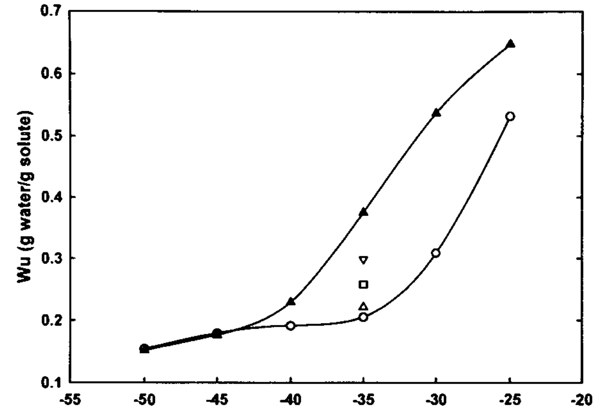

Fig. 7. Unfrozen water vs. temperature for 30% sucrose (open circles) and 30% sucrose + 3% NaCl (closed triangles). The open triangle (up), open square, and open triangle (down) represent W_u of 30% sucrose with 1%, 1.5%, and 2% NaCl, respectively.

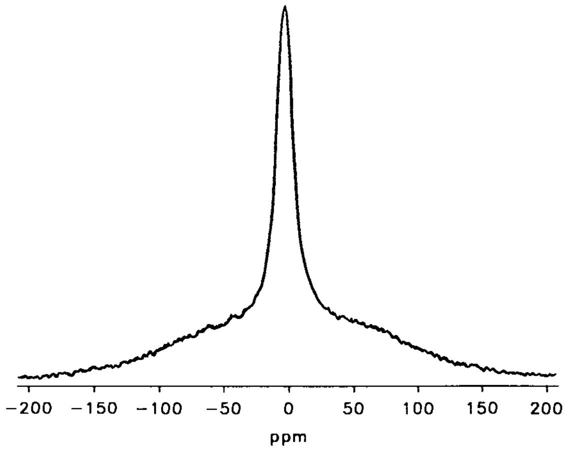

Fig. 6. NMR spectrum of 30% sucrose at −35°C.

At −35°C, the quantity of unfrozen water in solutions of biomacromolecules, including proteins, DNA, RNA, and ribosomes range from 0.24 g H_2O/g solute to as high as 1.7 g H_2O/g solute (6) and are, in general, significantly higher than the value of about 0.2 g H_2O/g solute reported for solutions of polysaccharides. Further study of the quantity and mobility of water in freeze concentrated solutions of biomolecules will result in better understanding of the mechanism of cryoprotective and lyoprotective effects of added solutes. For example, Hanafusa reported that the addition of as little as 0.001 M sucrose to a solution of 0.57% ovalbumin decreased the unfrozen water content from about 0.4 to about 0.2 g H_2O/g protein at −35°C, indicating that the sucrose displaces water in the microenvironment of the protein (5).

Water Mobility in the Freeze Concentrate

Molecular mobility is often expressed in terms of a correlation time, τ_c, which is roughly equivalent to the time required for a molecule to rotate through one radian or to move translationally through one molecular diameter. The correlation time is calculated from T_1 by the equation.

$$1/T_1 = (3\gamma^4 h^2/10r^6)\,[\tau_c/(1 + \omega^2\tau_c^2) + 4\tau_c/(1 + 4\omega^2\tau_c^2)]$$

where γ is the gyromagnetic ratio of a proton (2.67×10^4 radian gauss^{-1} sec^{-1}), h is Planck's constant, and ω is the Larmor frequency. For 300 MHz nmr, the Larmor frequency is 3×10^9 Hz, or 1.885×10^9 radians/sec. The published value for r, the internuclear distance for the protons in water is 1.58×10^{-8} cm (13).

Correlation time as a function of temperature for both 30% sucrose and 30% sucrose/3% NaCl is shown in Figure 8. Correlation times for the two materials are about equal at temperatures below −40°C, with values in the range of 4–5 $\times 10^{-7}$ seconds. Mobility at temperatures above −40°C is significantly higher for the sucrose/sodium chloride system. Typical values of τ_c for liquid water and for ice are on the order of 10^{-11} sec and 10^{-5} sec, respectively. As expected, the mobility of the unfrozen water is intermediate between these extremes, and a change in slope of the mobility versus temperature plot is observed at a temperature which is close to the observed value of T_g' as measured by DSC. The correlation times reported here are in reasonable agreement with τ_c of "bound" water on the order of 10^{-8} sec reported by Kuntz and Kauzman (13).

Practical Implications

It is important for formulation scientists involved with development of freeze dried dosage forms to recognize that interactions between components of a solution can substantially alter the physical properties of the frozen solution and subsequently affect the critical attributes of the freeze dried product. While eutectic temperatures of aqueous solutions of inorganic salts are well known, the eutectic temperature of the salt is of no significance in the studies reported here, because the salt does not crystallize from the freeze concentrated solution. Instead, the electrolyte causes more water to be present in the freeze concentrated solution, and this increased water content decreases T_g' because of the plasticizing effect of water. This effect is particularly important for formulations which employ a lyoprotectant such as glucose, sucrose, maltose, or lactose. Solutions of these sugars have rather low T_g' by themselves, and addition of salts can decrease T_g' to a point where a pharmaceutically acceptable freeze dried cake is not achievable with most commercial freeze drying equipment.

The results of this study demonstrate the utility of nmr measurement of unfrozen water content and molecular mobility in development of freeze dried formulations and processing conditions. Nmr may be a more sensitive method for measurement of T_g' than DSC, particularly for protein formulations. In addition, measurement of molecular mobility is an important tool for a better understanding on the molecular level of how protective solutes inhibit damage to biomolecules by the freeze drying process.

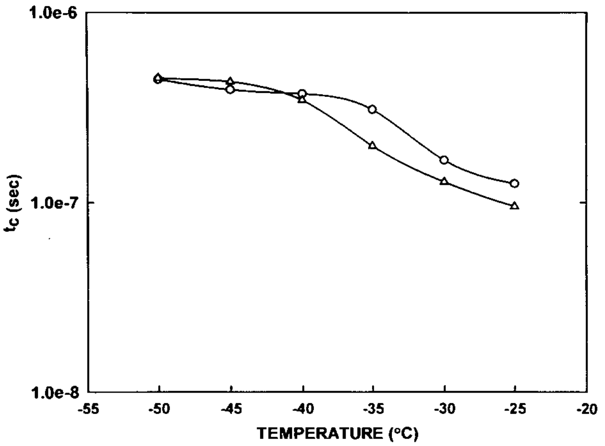

Fig. 8. τ_c vs. temperature for 30% sucrose (circles) and 30% sucrose + 3% NaCl (triangles).

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