Effects of Sugars and Polymers on Crystallization of Poly(ethylene glycol) in Frozen Solutions: Phase Separation Between Incompatible Polymers

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Received March 18, 1996; accepted June 17, 1996

Purpose. This study examined the effect of third components (low-molecular-weight saccharides and polymers) on the crystallization of poly(ethylene) glycol (PEG) in frozen solutions, focusing on the relationship between their crystallization-inhibiting ability and molecular compatibility.

Methods. Effects of sugars and polymers on the crystallization of PEG 3000 in frozen solution were monitored by differential scanning calorimetry (DSC). Pulsed-NMR was employed to monitor the molecular mobility of water and solutes in the frozen solutions. Miscibility between PEG and third components in aqueous solution was estimated from the lowering of cloud point of PEG 20,000. Thermal analysis of frozen solutions containing some non-crystallizing solutes was used to examine the possibility of phase separation in frozen solutions.

Results. Some sugars and polymers inhibited the crystallization of PEG and formed practically stable amorphous phases among ice crystals. The mobility of solute molecules in the amorphous phase increased above the softening temperature of maximally concentrated solutions (T_s), whereas that of water molecules appeared at a lower temperature. Mono- and disaccharides that are relatively less miscible with PEG in solution inhibit PEG crystallization to a lesser degree. Two T_s regions were observed in frozen solutions containing both polyvinylpyrrolidone (PVP) and dextran, at much lower concentrations than those causing aqueous two-phase separation at ambient temperatures.

Conclusions. Ice crystallization raises the concentration of solutes in the remaining solution, which can lead to phase separation in the amorphous phase. Molecular compatibility between components is an important factor determining their propensity to phase separate and crystallize.

KEY WORDS: frozen solution; phase separation; poly(ethylene glycol); crystallization; molecular interaction.

INTRODUCTION

Freezing and freeze-drying are popular methods for stabilizing pharmaceuticals. Some components crystallize in frozen solution, whereas others remain in a supercooled solution (amorphous phase) that contains solute and unfrozen water among ice crystals. Often a given component can be distributed between both crystalline and amorphous phases. The physical states of the "active" component are an important factor determining the quality of pharmaceutical products, such as appearance, solubility and stability (1). Crystallinity of other components in the formulation is also important since it alters physico-chemical states surrounding the "main" component and molecular interactions between them (2). For example, selective crystallization of buffer salts during freezing can result in change in solution pH. The molecular interactions among solutes during freezing can be especially important for protein formulations. Interaction of the protein with an amorphous solute (usually a sugar) is needed both for protection during drying and during subsequent long-term storage in the dried solid. Poly(ethylene glycol) (PEG) is a potent protectant of proteins during freezing, but its crystallization during freeze-drying is associated with its inability to protect proteins during the dehydration step of the freeze-drying (3–5). Understanding the partitioning of solutes between crystal and amorphous phases in frozen solutions and controlling this distribution are essential for developing acceptable pharmaceutical formulations.

The physical characteristics of frozen solutions have been studied mainly with aqueous single solutes (6). Frozen solutions containing multiple components have properties that are different from the individual solutions. The complexities of solutions used for pharmaceutical formulations require information on the physical characteristics of multiple component solutions (7–9). For example, polyols are reported to inhibit crystallization of other components in frozen solutions. Glycerol, sugars and hydroxyethylstarch (HES) have been shown to inhibit the crystallization of NaCl/water systems (8–11). Without the crystallization of components, the freeze-concentrated solutions remain amorphous, maintaining the intermolecular hydrogen-bonding network (7,8).

Supercooled solutions formed among ice crystals have usually been treated theoretically as single uniform amorphous phase. However, Her *et al.* recently reported the existence of multiple glass transition temperatures (T_g') of maximally freeze-concentrated solution in frozen solutions containing both polyvinylpyrrolidone (PVP) and phosphate buffer components (12). This suggests the presence of multiple amorphous phases in the freeze-concentrate, although the mechanism of phase separation during freezing is unclear. Understanding of the phase separation phenomena is crucial for the design of formulations and processes for freeze-dried pharmaceuticals. Aqueous solutions containing high concentrations of PVP and phosphate buffer form two liquid phases at room temperature (13). Aqueous liquid-liquid phase separation also occurs between incompatible polymers such as dextran and PVP or PEG (13,14). It appears plausible that freezing-induced increases in solute concentrations can lead to such phase separation during the freezing process.

To gain further insight into how solution components alter phase behavior during freezing, we investigated the effects of low-molecular weight saccharides and polymers on the crystallization of PEG in frozen solutions. PEG crystallization was studied by thermal analysis of frozen solutions, and by measurement of molecular mobility using pulsed-NMR. In addition to the information obtained by the DSC study, the pulsed-NMR

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0724-8741/96/0900-1393$09.50/0

study provides data on the mobility of both water and solute molecules at each temperature. The effect of third components in aqueous solution on the cloud point of PEG was used as a parameter to quantify molecular interactions between solutes. The cloud point provides information on compatibility between PEG and various molecules whose interaction is not repulsive enough to form aqueous two-phase systems in low and moderate temperatures (15). PEG is a good model for studying these relationships because of the availability of data on its physical state in frozen solution and on its phase behavior in liquid solutions. The possibility of phase separation in frozen solutions was also examined using some non-crystallizing polymers that often form aqueous two-phase systems at room temperature.

DSC heating scans of frozen solutions containing a single amorphous phase show two discontinuities. The transition that occurs at a lower temperature is considered to be the glass transition of the amorphous phase (T_g)(16–18). Many researchers, including ourselves, have referred the other transition as "glass transition temperature of maximally freeze-concentrated solution" or "T_g'" (6,12,19). More correctly, T_g' is not a glass transition process of the supercooled solution phase but a relaxation phenomenon that occurs between the glass phase and surrounding ice crystals (16,17). This relaxation temperature has been called the ante-melting temperature (16) or softening temperature of maximally concentrated solution (17). Softening of the system is observed at this temperature in thermomechanical analysis (TMA). Our NMR data and earlier NMR studies support this conclusion (20,21). Therefore, in this paper we use the term "softening temperature (T_s)" to refer to the higher temperature transition.

MATERIALS AND METHODS

Dextran (from *Leuconostoc mesenteroides,* average MW: 11,000), Ficoll (Type 400, approximate MW: 400,000) and polyvinylpyrrolidone (PVP, average MW: 10,000) were purchased from Sigma Chemical Co. (St. Louis, MO). D-Cellobiose was obtained from ICN Biochemicals Inc. (Aurora, OH). Deuterium oxide (100.00% atom % D) and (α-D-melibiose hydrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Poly(ethylene glycol)s (PEG 3000, PEG 20,000) and other excipients were obtained from Wako Pure Chemical Co. (Osaka, Japan). All chemicals were used without further purification. All solutions were prepared in doubly distilled water.

Thermal analysis of frozen solutions was completed with a differential scanning calorimeter (DSC 2920 system of TA Instruments, New Castle, DE). A sample solution (10 μl) in a hermetic aluminum cell was placed in the apparatus and cooled to −70°C with liquid nitrogen. Thermal transitions were recorded as the sample was warmed at 2°C/min. The enthalpy of the endothermic peak at around −20 to −15°C due to melting of the PEG 3000/water complexes was obtained from the thermal data. The effect of another solute on the crystallization was expressed as the ratio (%) to that of a solution containing only PEG. Softening temperatures of the maximally freeze-concentrated solutions (T_s) were obtained from the midpoint of the region at which there was a second order shift in the baseline (12,22).

The proportion of liquid-phase protons present in each solution was measured using a broadline pulsed NMR spectrometer with a Lamor frequency of 25 MHz (JNM-MU25, JEOL, Tokyo, Japan), by a modification of the method originally developed to measure the amount of unfrozen water in biological systems (11,20,21,23). Aqueous sample solutions (in H_2O or D_2O, 1 ml) were placed in glass tubes (10 mm in diameter) and frozen by immersion in liquid nitrogen. Measurements were made every 2°C from −70°C by rewarming at 0.5°C/min. In experiments using D_2O as the solvent, samples were stored overnight at room temperature, prior to NMR spectroscopic analysis. Free induction decay (FID) signals were sampled every 0.2 sec after 90° pulses and used to construct an FID curve. This was resolved into two components indicating long and short spin-spin relaxation times (T_2), which were considered to represent liquid and solid-state protons. The proportions of liquid-state protons in the samples were determined by extrapolating the portions of the FID curve between 40 and 120 seconds back to zero time. The values were corrected to that of 25°C according to Curie's law to eliminate temperature dependence of the sensitivity of FID signals. The relative amounts of liquid-phase protons were expressed as the proportion (%) in pure H_2O at 25°C, using a calibration curve obtained from various concentrations of H_2O/D_2O solutions.

The phase transition due to the cloud point of 10% PEG 20,000 (w/w), and the effect of sugars and polyols (10%, w/w) on the cloud point were detected visually in a closed glass tube immersed in an oil bath. The temperature of the oil bath was measured by a Yokogawa model 2455 digital thermometer (Tokyo). The temperature at which the last trace of cloudiness disappeared while lowering the temperature at 0.2°C/min was considered to be the cloud point.

RESULTS AND DISCUSSION

Effects of Third Components on Crystallization of PEG 3000 in Frozen Solution

Figure 1 shows the thermal behavior of frozen PEG 3000 solutions. The solution without sucrose shows two apparent

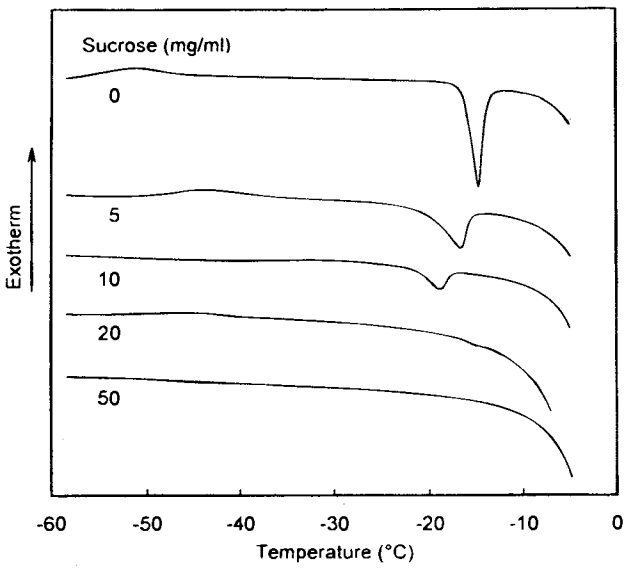

Fig. 1. Effect of sucrose concentration (0–50 mg/ml) on the thermal profiles of frozen 20 mg/ml PEG 3000 solution studied by DSC. Sample solutions (10 μl) were scanned by heating at 2°C/min.

peaks during the rewarming of the frozen solution. The exothermic peak at around −50°C and endothermic peak at around −15°C, respectively, have been reported to be the formation (crystallization) and melting of PEG/water complexes (containing two or three water molecules on the average per PEG monomer) (3).

The effect of several low-molecular-weight saccharides and polymers on the thermal behavior of PEG 3000 was next examined. Thermograms in Fig. 1 show the effect of varying sucrose concentration. The peaks due to crystallization and melting of the PEG/water complexes are reduced in area as the concentration of sucrose is increased. PEG crystallization is completely inhibited with 20 mg/ml sucrose.

Table I shows the effects of various low-molecular-weight saccharides and polymers on the crystallization of PEG 3000, based on the enthalpy of the melting endotherm. The concentration of the third components required to suppress the crystallization varies considerably. The crystallization of PEG 3000 is completely inhibited by addition of 20 mg/ml glucose, fructose, sucrose, palatinose and maltose. In contrast, the crystallization of the PEG is not fully inhibited by up to 200 mg/ml of melibiose, Ficoll and dextran. The variation was much larger than that of the concentration required to inhibit crystallization of NaCl/water systems (11). A difference in the effect was also observed between the third components of similar molecular weights. Among monosaccharides, a greater concentration was required to inhibit the crystallization of PEG 3000 using galactose compared to that for fructose and glucose. The order of the effect among disaccharides was sucrose > maltose, palatinose, cellobiose > trehalose > lactose > melibiose. Addition of 100 mg/ml PVP (average MW: 10,000) completely inhibited the crystallization of PEG 3000, but dextran (average MW: 11,000) showed little effect.

The low-molecular-weight saccharides and polymers are considered to inhibit crystallization by forming an amorphous phase with water and maintaining an intermolecular hydrogen-bonding network in the freeze-condensed amorphous phase (7,8). Inhibition of crystallization by third components results in the absence of PEG crystals in freeze-dried cakes (13). Descriptions of the ternary frozen solutions by equilibrium phase diagrams (24) and "dynamic phase diagrams" (8) shows how components in ternary systems are kept amorphous in frozen solutions. "Dynamic phase diagrams" describe practical nonequilibrium cooling and heating conditions. In many cases, only ice crystallization occurs during cooling. The crystallization of PEG during cooling occurs only at very slow cooling rate, since crystallization of PEG is kinetically inhibited.

Frozen solutions containing low-molecular-weight saccharides and polymers as single solutes often form an amorphous phase among ice crystals. But no apparent relationship was observed between ability of these solutes to inhibit the crystallization of PEG and physical properties of the amorphous phase such as T_s (referred as T_g' in original report) and the amount of unfrozen water at the T_s(W'g)(18,19,22). T_s of monosaccharides and disaccharides in the literature (19) are, cellobiose: −29.0°C, sucrose: −32°C, melibiose: −30.5°C, maltose: −29.5°C, trehalose: −29.5°C, lactose: −28.0°C.

Proportion of Liquid-phase Protons

The proportions of liquid-phase protons in frozen solutions containing PEG 3000 and third components were obtained using pulsed-NMR. Aqueous solutions in H_2O and D_2O were studied to give the molecular mobility of water and solute molecules separately. The method is based on the difference in spin-spin relaxation time (T_2) between solid and liquid molecules due to the changes in rotational and translational molecular motion. The "liquid-phase protons" consist of protons of mobile molecules. Those of molecules that lose mobility by crystallization and glass formation are excluded.

Figure 2 shows the proportions of liquid-phase protons in various aqueous (H_2O) solutions, and indicates the sum of the protons of unfrozen water and mobile solute molecules (11,20). The proportion of liquid-phase protons in frozen 20 mg/ml PEG

Table 1. Effect of Third Components on the Crystallization in 20 mg/ml PEG 3000 Frozen Solutions

	Crystallinity of PEG/water Systems (%)							
	Third Components (mg/ml)							
	5	10	15	20	50	100	150	200
Glucose	62.6	43.5	1.5	0	0	0	0	0
Fructose	78	45.9	5.2	0	0	0	0	0
Galactose	79.6	59.5	6.4	5.8	9.6	2.8	0	0
Sucrose	71.7	42.2	0.7	0	0	0	0	0
Palatinose	72.9	60.5	12.3	0	0	0	0	0
Maltose	80.4	73	14.2	0.3	0	0	0	0
Cellobiose	80.6	63.4	4	0.7	0	0	0	0
Trehalose	82.2	82.1	55	14.5	0	0	0	0
Lactose	87.6	60.5	41.5	74.6	65.1	47.3	2.4	0
Melibiose	89	89.5	91.6	90.7	85.5	76	57.1	57.5
Raffinose	90.4	83.2	34.8	4.8	0.9	0	0	0
PVP10000	86.4	80.3	75.5	75.5	2.7	0	0	0
Ficoll	98.5	96.2	94.8	90.1	84.5	67.3	53.9	49.7
Dextran	94.7	92.7	98.6	94.5	92.5	93.1	85.1	87.2

Note: Crystallinity of a PEG/water system was described as the ratio (%) of that 20 mg/ml PEG3000 solution. All values were expressed as means of two experiments.

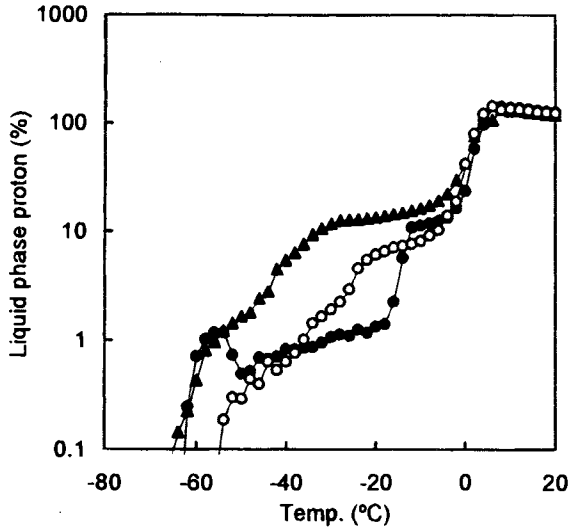

Fig. 2. Proportions of liquid-phase protons in various aqueous (H_2O) solutions. Temperature dependence of the liquid-phase protons in ●: 20 mg/ml PEG 3000, ○: 20 mg/ml sucrose, ▲: 20 mg/ml PEG 3000 and 20 mg/ml sucrose were determined by pulsed-NMR every 2°C by rewarming at 0.5°C/min, and expressed as the ratio (%) to that of pure water at 25°C.

3000 solution increases significantly at around −15°C and 0°C due to the melting of the PEG/water complex and ice crystals, respectively. A small drop in liquid-phase protons at −55°C indicates the reduction in mobility of unfrozen water molecules that crystallize as a PEG/water complex above that temperature (3).

Liquid-phase protons in frozen 20 mg/ml sucrose solution are first evident (more than 0.1% liquid-phase proton) at −54°C, and increase gradually until the melting of the ice crystals. The temperature was close to the reported glass transition temperature of frozen sucrose solutions (−50°C, 18). A frozen solution containing both sucrose and PEG 3000 has a greater proportion of liquid-phase protons than the sum of the individual solutions, especially at temperatures between −50 to −20°C. The lack of an apparent decrease in liquid-phase protons at around −55°C indicates that PEG crystallization was inhibited by sucrose. Similarly the lack of any apparent change at −15°C indicates that melting of the PEG does not occur at this temperature. These observations are consistent with the results obtained in the DSC study (Fig. 1), and indicate that a freeze-concentrated amorphous phase consisting of PEG, sucrose and unfrozen water forms among ice crystals in the frozen solution, in which crystallization of PEG is inhibited.

The proportions of liquid-phase protons in D_2O solutions were also studied to clarify the mobility of solute molecules (11,20). Figures 3A to 3D show the liquid-phase protons of PEG 3000 in D_2O solutions and effects of third components (low-molecular-weight saccharides and polymers) on them. The proportions of liquid-phase protons in D_2O solution reflect mainly protons of mobile solute molecules. Loss of solute mobility by crystallization and glass formation in frozen solution results in a decrease in liquid-phase protons. The proportion of liquid-phase protons in PEG 3000 solution (● in Fig. 3B) increases significantly at around −10°C, where melting of PEG/D_2O complex is observed by DSC (−9.5°C). Protons in PEG molecules show some mobility even below the melting temperature, which may due to a small amount of uncrystallized PEG.

Disaccharides (sucrose, lactose, melibiose) exhibit significant increases in the proportions of the liquid-phase protons in narrow temperature ranges between −25 and −15°C. Thermal analysis of the 20 mg/ml sucrose solution in H_2O and D_2O shows T_s at −36 and −32°C, respectively. Thus, the mobile sugar molecule appears about 10°C above the T_s of the equivalent solution in D_2O. The liquid-phase proton of PVP and dextran molecules, which appear at −28°C and −14°C respectively, increase rather gradually compared to those of sugar molecules. The T_s of the 20 mg/ml PVP and dextran solutions (in D_2O) were observed with DSC at −27°C and −12°C, respectively. Thus, liquid-phase protons in PVP and dextran become mobile at temperatures close to their T_s.

The solute molecules could exchange protons with deuterium in D_2O molecules, but the amount of protons in unfrozen water molecules should be small in the experiment. The increase in the liquid-phase proton at the temperature of ice melting shows the amount of protons in ice. Although most of the water molecules are crystallized in the frozen solutions, the increase in the liquid-phase proton at around 0°C is small compared to that in frozen solution, indicating that the contribution of protons in water molecules to the proportion of liquid-phase proton in frozen solutions observed in Fig 3 is not significant.

Figures 3C and 3D show the proportions of liquid-phase protons in frozen solutions containing both PEG 3000 and third components. The effects of the third components were divided into three categories. First, sucrose forms an amorphous phase with PEG 3000 and unfrozen water molecules. The temperature dependence of the liquid-phase proton in the mixture is obviously different from that of the individual solutions, and indicates that melting of PEG/water does not occur in the presence of sucrose. These results are consistent with the earlier observations that sucrose inhibites PEG crystallization. Solutes in frozen solutions containing both sucrose and PEG 3000 gradually become mobile above −40°C, a temperature close to the T_s of the solution (−44°C in H_2O).

Second, another group of the polyols (lactose and PVP) and PEG form a relatively unstable amorphous phase. The proportions of liquid-phase protons show peaks at −42°C (PVP and PEG 3000) and −36°C (lactose and PEG 3000). Exothermic peaks are observed during thermal analysis at these temperatures (data not shown). The peaks in the proportions of the liquid-phase protons indicate increasing molecular mobility in the amorphous mixture phase and subsequent crystallization of PEG/water complex. The increase in the devitrification (crystallization) temperature was reported in NaCl/polyol/water systems (8). Polyols kinetically restrain the crystallization of NaCl, but the ratio of polyols/NaCl is insufficient to form a practically stable amorphous phase. The liquid-phase protons in these solutions increase again at around −20 to −15°C, due to mobilization of lactose and PVP molecules above their T_s and melting of PEG. Smaller changes in the liquid-phase protons and smaller melting peak in DSC measurements at the melting temperature, relative to those noted with PEG alone, indicate the coexistence of amorphous and crystal phases in the mixtures.

The third group of polyols (melibiose, dextran, Ficoll) showed little effect on the crystallization of PEG. The proportion of liquid-phase protons in solution containing PEG 3000 and dextran was close to the sum of that of each solution. These

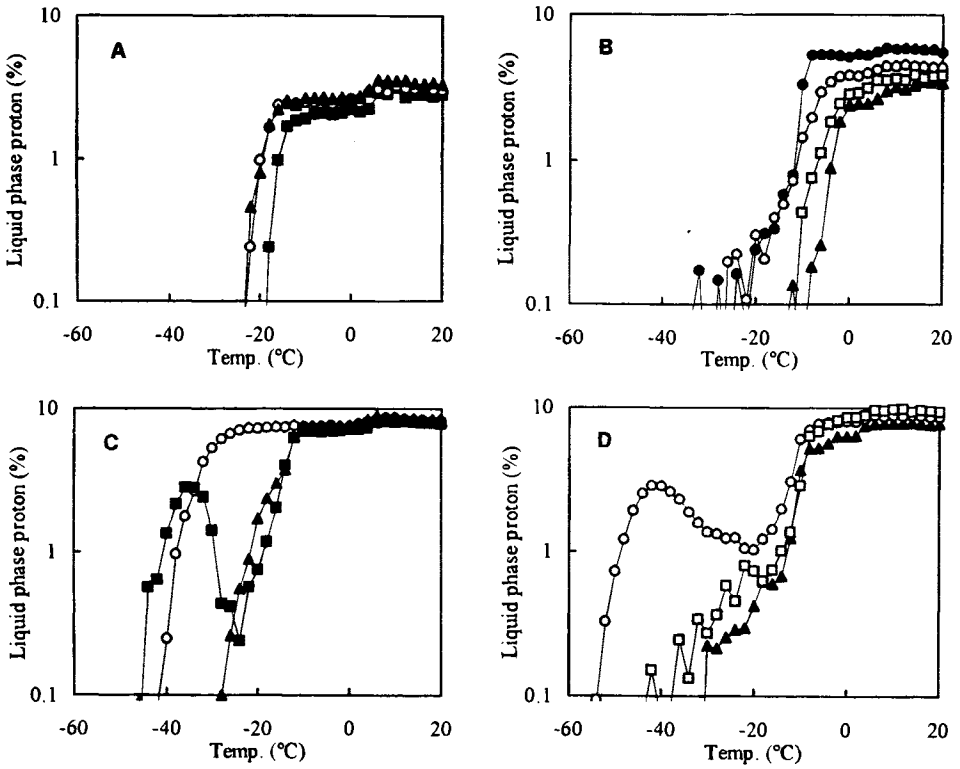

Fig. 3. Proportions of liquid-phase protons in various D_2O solutions expressed as the ratio (%) to that of pure water at 25°C. Figures A, B and C, D show liquid-phase proton of 20 mg/ml single solute solutions, and in the presence of 20 mg/ml PEG 3000, respectively. Each symbols shows disaccharide (○: sucrose, ■: lactose, ▲: melibiose in A and C) and polymer (●: PEG 3000, ▲: dextran, ○: PVP, –; Ficoll in B and D) solution.

results indicate that PEG and these third components maintain their independent physical characteristics in the frozen solutions. Thermal analysis of frozen solutions containing both PEG and dextran or Ficoll shows peaks of PEG crystallization and melting at temperatures identical to that of a solution containing only PEG (data not shown), further demonstrating the minimal interactions between PEG and dextran or Ficoll in frozen solutions.

This result suggests phase separation of components in some frozen solutions, as recently reported for solutions containing PVP and phosphate buffer components (12). The PEG/dextran system is a well known example that forms aqueous two-phase systems at high concentrations at room temperature (13,25). Though all the solutions studied above were single-phase at room temperature because of the low concentrations of components, increasing concentration due to ice crystallization (6) may induce a phase separation, resulting in absence of the inhibitory effect of dextran on the crystallization of PEG. The possibility of phase separation during freeze concentration is studied below.

Relationship Between Thermal Behavior and Mobility of Molecules in Frozen Solutions

The water molecules in the amorphous phase in frozen solutions gain mobility at a much lower temperature than that for solute molecules, which is consistent with behavior reported by Suzuki and Nagashima (20). They suggest that the molecular motions are activated in the following order: first the water molecules strongly associated with solute molecules, then the solute molecules, and last the ice-like or water molecules weakly associated with solutes. These changes in molecular motion occur in particular temperature regions that depend on solute molecules (20). Our results with DSC and pulsed-NMR show that water molecules become mobile at around the "real" glass transition temperature (T_g), and the solute molecules exhibit increased mobility at some temperature above the softening temperature (T_s). The relationship between the thermal behavior and molecular mobility is compatible with literature data on various frozen solutions including low-molecular-weight saccharides to polymers (18–21). The mobilization of solute molecule above the T_s explains why collapse of most of the amorphous phase occurs above T_s. Further these results agree with recent descriptions of physical states of amorphous phases in frozen solution (17).

The softening temperature (T_s) has been interpreted as glass transition temperature of maximally concentrated solution (T_g'). But a more apt description is not a glass transition but a relaxation phenomenon between the glass phase and surrounding ice. Melting of ice around the amorphous phase is also observed at T_s (16,17). Softening of the system is observed at T_s in thermomechanical analysis (TMA). In our study, subtraction of the proportion of liquid-phase protons of sucrose solution in D_2O from that in H_2O shows an apparent increase in the liquid-phase proton molecules at T_s (data not shown),

which is indicative of the melting of ice around the amorphous phase.

Phase Separation of Amorphous Polymers

Thermal analysis was performed to study separation of phases containing non-crystallizing polymer components in frozen solutions. Figure 4 shows DSC scans of the frozen solutions. All the solutions subjected to thermal analysis were in a single phase at room temperature. The amorphous phase in frozen solutions exhibits a T_s that is characteristic of the solutes (6,22). The T_s temperatures for sucrose, PVP, Ficoll and dextran solutions are consistent with those in other reports (22). Frozen solutions that contained both sucrose and polymers, and both PVP and Ficoll showed a single T_s region between their individual ones, indicating formation of a single amorphous phase in frozen solution.

On the other hand, frozen solutions that contained both dextran and Ficoll, or both dextran and PVP, showed two T_s regions that are close to those for their respective individual solutions. The two T_s regions indicate phase separation in the frozen solution (12). Concentrated solutions containing both dextran and PVP form aqueous two-phase systems at room temperature because of the molecular incompatibility between the polymers (13,25). Our results indicate that the phase containing two incompatible non-crystallizing polymers separates during freeze concentration. The two T_s values were not identical to those of the individual solutions. This is to be expected, because both of the separated phases should be rich in one polymer and contain a small amount of the other polymer. The "real" glass transition of the frozen solution (T_g) was not detectable.

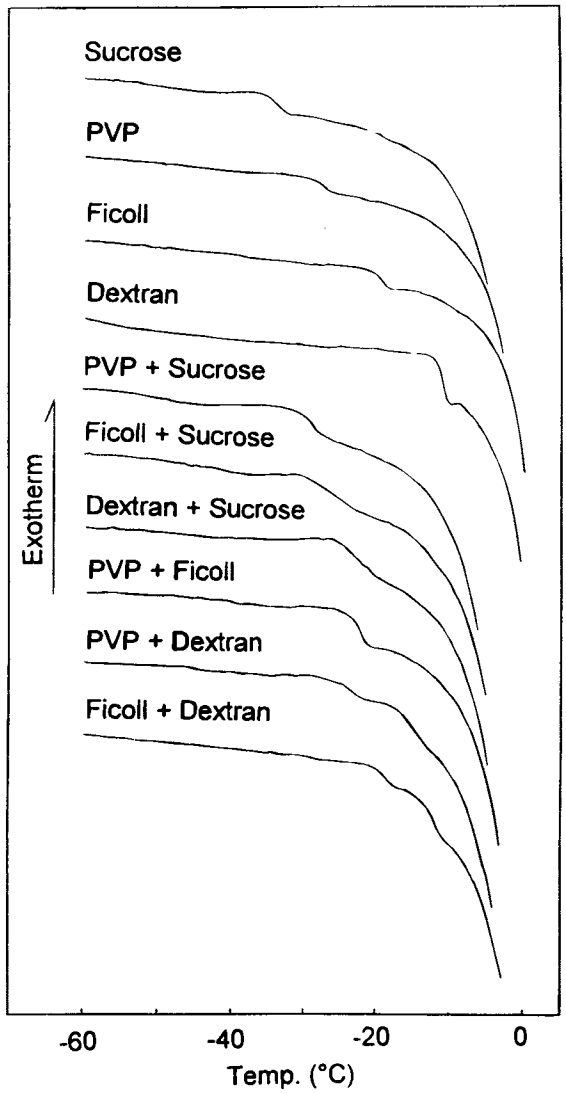

Fig. 4. DSC profiles of frozen solutions containing various sugars, polymers and their mixtures (50 mg/ml for each solute). Sample solutions (10 μl) were scanned by heating at 2°C/min. The T_s of each solution was observed at the following temperature: Sucrose, −33.8°C; PVP, −27.1°C; Ficoll, −19.5°C; dextran, −11.0°C; PVP + sucrose, −29.2°C; Ficoll + sucrose, −26.8°C; dextran + sucrose, −22.5°C; PVP + sucrose, −22.6°C; PVP + dextran, −23.2°C and −14.8°C; Ficoll + dextran, −18.7°C and −12.3°C.

Effect of Third Components on the Cloud Point of PEG 20,000

An aqueous solution of PEG has a lower critical solution temperature (LCST), in which liquids that are completely miscible at a lower temperature form a two-phase system at higher temperatures (15,26,27). The phenomenon is often called the cloud point. PEG separates as a precipitate from aqueous solution above the cloud point temperature because of self association and loss of water of hydration (15). The cloud point phenomena and the phase separation occurring in aqueous two-polymer solutions at room temperature can be described theoretically with a modified Flory-Huggins model (14,15). It shows that the basic mechanism for the phase separation is determined by both polymer-polymer segment interactions and polymer-water interactions. In addition to the contribution of PEG-polymer interactions at low and moderate temperatures, PEG-water interactions are important factors that induce phase separation. Changes in PEG conformation with increasing temperature make the surface of the molecule more hydrophobic, resulting in phase separation above the cloud point (15).

Many third components including low-molecular-weight saccharides decrease the cloud point of PEG (15,26). Since the low-molecular-weight saccharides are segments of polysaccharides that form aqueous two-phase system with PEG, the mechanism of the PEG cloud point lowering by these saccharides is fundamentally identical to formation of an aqueous two-phase system composed of PEG and dextran (15). The decrease in the cloud point is the result of interaction between PEG and sugar being more repulsive than the interaction between sugar and water (15). The data thus provides information on compatibility between PEG and low-molecular-weight saccharides in aqueous solution.

The cloud point of PEG depends on its concentration and degree of polymerization, decreasing with increasing molecular weight. In this experiment, we used PEG 20,000 because of the availability of cloud point data (14,15) and practical difficulties in handling the sample at the cloud point of PEG 3000 (above 120°C). The PEG 20,000 solution without third components showed a cloud point at 115.4°C. The difference in the temperature between this report and others (111.7°C, 15) is

likely due to differences both in cooling speed and in molecular weight distribution of PEG obtained from different suppliers.

Figure 5 shows the effects of third components (10%, w/w) on the cloud point of 10% (w/w) PEG 20,000 solution. Among the monosaccharides studied, galactose shows the greatest effect in lowering the cloud point, whereas fructose shows the smallest effect. The effect of disaccharides in depressing the cloud point occurs in the order sucrose < maltose, palatinose, cellobiose < trehalose < lactose < melibiose. Significant differences were observed in the effects of polymers. Addition of 10% (w/w) dextran or Ficoll results in phase separation at room temperature, whereas PVP has little effect on the cloud point of PEG 20,000.

The disaccharides employed for the experiment can be roughly divided into three groups. Disaccharides composed of glucose and galactose (lactose, melibiose) depress the cloud point of PEG solution more than those with glucose and fructose (sucrose, palatinose). Disaccharides composed of two glucose moieties (maltose, cellobiose, trehalose) fall in the middle of the groups above.

In the Flory-Huggins theory, energetically unfavorable interaction between the segments of two polymers is considered to be the reason for phase separation (13). Stronger repulsive interactions between the saccharides and PEG result in lower cloud points (15). The results in Fig. 5 indicate that interactions between PEG and some saccharides (melibiose, lactose, trehalose) are more repulsive than those of PEG and sucrose. It also suggests that the interaction between PEG and galactose moieties is more repulsive than that between PEG and glucose or fructose. From a thermodynamic aspect, the third components employed above depress the cloud point of PEG because they raise the chemical potential of PEG in aqueous solution (25). This is due to unfavorable interaction between PEG and the third components (e.g., preferential exclusion, 28). The saccharides that cause the largest depression of the cloud point of PEG should be excluded more from the surface of PEG molecules.

The structural difference between these monosaccharides moieties is in the relative position of OH(4) in conjunction with OH(2), which determines hydration state of sugar molecules (15,29,30). Galactose and disaccharides that contain galactose subunits have relatively poor compatibility with the three-dimensional hydrogen-bonded structure of water relative to that of fructose, glucose, and their respective disaccharides (29). That is, galactose has a larger number of hydration waters per molecule, and exhibits more repulsion from PEG. The basis for this relationship is obscure. However, Sjöberg et al. (15) speculated that saccharides with more water of hydration are more hydrophilic, and interact less favorably with nonpolar PEG molecules.

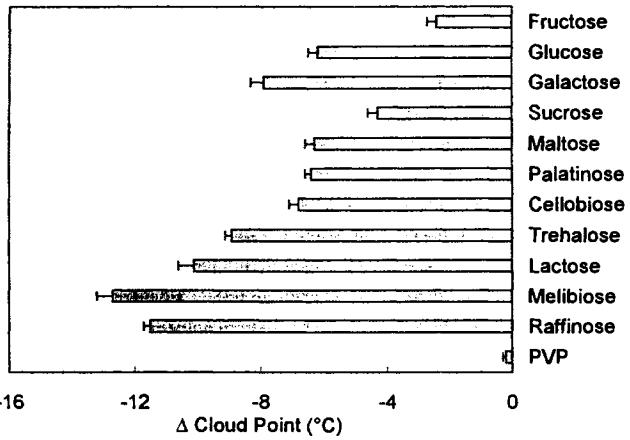

Fig. 5. Effect of third components (10% w/w) on the cloud point of 10% (w/w) PEG 20,000. Values are expressed as means and SD of three experiments. Cloud point of the 10% (w/w) PEG 20,000 solution was observed at 115.4°C.

Formation of two phases in aqueous PEG/dextran and PEG/Ficoll systems at room temperature means their interaction is much more repulsive than those of PEG and low-molecular-weight saccharides. This is expected, because the entropy of mixing of the polymer molecules decreases as the degree of polymerization increases (25).

The results in Table I and Figure 5 indicate that the sugars and polyols effective for depressing the cloud point of PEG have little effect in inhibiting the crystallization of PEG. As was observed in aqueous solution of non-crystallizing polymers such as PVP and dextran, the solution containing PEG and dextran or Ficoll should phase separate during freeze concentration. Crystallization and melting of PEG is not affected by dextran, suggesting the phase separation occurs during the cooling process. Ice crystallization raises the concentration of PEG and dextran in the remaining solution above that required for separation into different phases.

The effect of low-molecular-weight saccharides on PEG crystallization depends on their interaction strengths. In contrast to the PEG/dextran system, amorphous phases containing PEG and low-molecular-weight saccharides apparently do not separate during freeze concentration. The increase in PEG crystallization temperature in solutions containing PEG and lactose indicates that they are in the same phase, but the interaction is insufficient to maintain the amorphous mixture in the frozen solution. Similarly, lactose is less effective than other disaccharides at inhibiting PEG crystallization during freeze-drying (5). Intermolecular hydrogen bonding between components is thought to maintain the amorphous phases (7). This suggests that saccharides such as glucose, fructose and sucrose provide a sufficient hydrogen bonding network in amorphous PEG/sugar/water systems to keep PEG from crystallizing in frozen solutions. Increased repulsive interactions between components (e.g., PEG-PVP, PEG-lactose) result in failure of the hydrogen bonding network and subsequent crystallization of PEG, thus raising the saccharide/PEG ratio required to inhibit crystallization of PEG. Although the effect of PVP on the cloud point of PEG was smaller than that of mono- and disaccharides, it had a smaller effect on the crystallization of PEG. PVP is the only non-saccharide third component employed for the study. The cloud point does not reflect the interaction between PEG and PVP at low temperatures, probably because the change in hydrophobicity of PEG with temperature and the hydrophobic nature of PVP masks the effect. In addition, molecular size may also affect the ability to inhibit PEG crystallization.

Molecular interactions between components is an important factor that determines their physical state in frozen solution. Incompatibility between components deprives them of the intermolecular hydrogen bonding that maintains the amorphous mixture phase in freeze-concentrates, and leads to phase separation and crystallization. Though the molecular interaction and conformation of components depend on temper-

ature (2), parameters reflecting molecular interactions such as cloud point depression observed in solution help to estimate the interaction in freeze-concentrates. The stereochemical structure of hydrated molecules appears to be key to determining their molecular interaction.

ACKNOWLEDGMENTS

We gratefully acknowledge support from National Science Foundation Grant BES9505301 to T. W. R. and J. F. C., and a Japan Health Sciences Foundation Grant to K. I.

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