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Glycine Crystallization During Freezing: The Effects of Salt Form, pH, and Ionic Strength

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Purpose. The purpose of the study is to characterize glycine crystallization during freezing of aqueous solutions as a function of the glycine salt form (i.e., neutral glycine, glycine hydrochloride, and sodium glycinate), pH, and ionic strength.

Methods. Crystallization was studied by thermal analysis, microscopy, x-ray diffraction, and pulsed Fourier transform nmr spectroscopy.

Results. A solution of neutral glycine with no additives undergoes rapid secondary crystallization during freezing, forming the β polymorph, with a eutectic melting temperature of −3.4°C. Glycine hydrochloride solutions undergo secondary crystallization relatively slowly, and the eutectic melting temperature is −28°C. Sodium glycinate crystallizes from frozen solution at an intermediate rate, forming a eutectic mixture with a melting temperature of −17.8°C. Where secondary crystallization does not occur rapidly, a complex glass transition is observed in the −70° to −85°C temperature range in the DSC thermograms of all systems studied. Rates of secondary crystallization and the type of crystal formed are influenced by solution pH relative the the pKs of glycine, and also by the change in ionic strength caused by adjustment of pH. Increased ionic strength significantly slows the crystallization of neutral glycine and promotes formation of the γ polymorph. Thermal treatment or extended holding times during the freezing process may be necessary in order to promote secondary crystallization and prevent collapse during freeze drying.

Conclusions. The results underscore the importance of recognizing that seemingly minor changes in formulation conditions can have profound effects on the physical chemistry of freezing and freeze drying.

KEY WORDS: freeze drying; lyophilization; thermal analysis; x-ray diffraction.

INTRODUCTION

Glycine is a common bulking agent for freeze dried formulations—particularly proteins—because it is non-toxic, highly soluble, and has a high eutectic temperature when it crystallizes from frozen solution, which promotes efficient freeze drying. Glycine has also been shown to inhibit aggregation of certain proteins upon freezing (1,2). However, the ability of a solute to function as a cryoprotectant or lyoprotectant requires that the compound remain amorphous (3–5), which may not be consistent with favorable freeze drying characteristics.

Understanding the physical state of a solute, whether excipient or drug, is important to prediction of freeze drying behavior, development of optimum processing conditions, and assessment of final product quality. Seemingly subtle changes in solution composition such as changes in pH—particularly when the solute contains one or more ionizable groups—can significantly change the physical chemistry of the freezing process. The purpose of this study was to determine how formulation conditions such as pH, ionic strength, and the starting form of glycine (i.e., neutral glycine, glycine hydrochloride, or sodium glycinate) affect both the rate and extent of crystallization as well as the polymorph formed.

EXPERIMENTAL

Materials

Glycine (Sigma Ultra, >99% pure), glycine hydrochloride (at least 99% pure by TLC), and sodium glycine (at least 98% pure by TLC) were used as received from Sigma Chemical Co. Sodium chloride was analytical grade (Mallinckrodt, Inc.). All water was purified by reverse osmosis, followed by distillation.

Methods

Differential scanning calorimetry of frozen solutions was carried out with a Perkin-Elmer Series 7 DSC as described previously, except that liquid nitrogen cooling was used instead of a mechanical cooling accessory (6). Briefly, samples of approximately 20 μl of solution were placed in aluminum sample pans and the pans were crimped. Helium was used as the purge gas at a rate of 40 ml/min. The DSC was calibrated using indium and mercury as standards, and the calibration was checked with the eutectic melting of a 5% (w/v) solution of sodium chloride in water. Samples were frozen at a controlled rate (usually 20°C/min) to −100°C, and the thermograms were recorded at a warming rate of 5°C/min except where noted.

Freeze dry microscopy was carried out using a specially constructed stage for observation of materials during freezing and freeze drying (7). Approximately 10 μl of sample was placed between two quartz cover slips (quartz was used to minimize vertical temperature gradients), and the cover slips were placed over a viewing hole in the second stage of a two-stage thermoelectric cooling assembly. The low temperature limit of this apparatus is about −50°C. Samples were observed using crossed polars with a first order red compensator in the light path. Observations were recorded on videotape for fast crystallization and on 35 mm slide film for samples which crystallized slowly during isothermal experiments.

Freeze drying was carried out using a laboratory model freeze dryer (Dura-Stop μP, FTS Systems, Stone Ridge, New York). Shelf temperature was controlled at various temperatures depending on the characteristics of the material being freeze dried. Chamber pressure was controlled at 100 μ Hg for most experiments.

X-ray powder diffraction patterns of freeze dried samples were measured with a Siemens Krystalloflex diffracto-

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meter, Cu K_α radiation at a voltage of 40 kV and a current of 20 ma. Powder specimens were prepared by gently breaking up the freeze dried cakes and placing in an aluminum powder mount. Samples were scanned from 2°to 40° 2Θ at 0.1° per second.

The quantity of unfrozen water in a frozen solutions of 5% neutral glycine and 5% glycine HCl neutralized to pH 7 was measured by a pulsed nmr technique (8,9). A Bruker ARX 300 MHz nmr spectrometer was used with the following instrument parameters: spectrum width, 100 ppm; pulse width, 17.7 μs (90°); receiver gain, 2040; delay time, 3 sec. A standard solution of 24% $LiCl/MnCl_2$ was prepared using 90% D_2O in order to decrease the signal from H_2O and allow the same receiver gain for both samples and standard. Sample solutions were placed in a teflon tube liner filled to a height of 5.5 cm. Samples were cooled at a rate of 2°C/min to −35°C and held for 30 minutes to assure thermal equilibrium. Sample spin rate was 20 rpm. The quantity of unfrozen water was calculated as follows:

$$W_u = (0.076 \times A_2 \times d_1)/(0.05 \times A_1 \times d_2), \qquad (1)$$

where A_1 and A_2 are the integrated area of the peak attributed to the unfrozen water in the standard solution and the sample, respectively; d_1 and d_2 are the densities of the standard solution and sample solution in the liners, respectively, at the measurement temperature of −35°C. Densities were determined by measuring the change in height of a column of both the standard and the sample in a 5 mm diameter nmr tube as a function of temperature. Difference in density between the standard and the sample was about 2 percent.

RESULTS AND DISCUSSION

Freezing Characteristics of Glycine Salt Forms

The DSC thermogram of a 0.5% (w/v) solution of neutral glycine (no pH adjustment), scanned at 1°C per minute, is shown in Figure 1. The low concentration and slow scan rate was used in order to clearly resolve the eutectic melting peak from the ice melting endotherm. Neutral glycine crystallizes readily upon freezing with eutectic melting at approximately −3.4°C. No glass transition was detected under the conditions used for thermal analysis. Other investigators, studying the ternary system glycine-sucrose-water, have reported melting temperatures of the glycine-water eutectic of −3.6 ± 0.1°C (10) and −4.7°C (11). Microscopic observation during freezing demonstrated that secondary crystallization of glycine was complete within a matter of seconds.

Thermograms of 10% glycine hydrochloride are shown in Figure 2. A complex glass transition in the −70° to −90°C temperature range (curve A), which appears to be two glass transitions with midpoint temperatures of −88°C and −72°C. A small endotherm is present at about −27°C. Annealing at −40°C for two hours (curve B) causes the glass transition to disappear, and a well defined eutectic melting endotherm is seen with an onset temperature of −28°C.

The glass transition region was examined further in order to distinguish between two glass transitions and a single transition with a superimposed exotherm. These interpretations can be distinguished by heating through the glass transition region, recooling, and scanning through the glass transition region again. An irreversible thermal event, such as a crystallization exotherm, would not be observed in the second scan, whereas multiple glass transitions would be expected to be reversible. The thermogram of the second scan through the glass transition region was superimposable on the first scan, which supports the interpretation of multiple glass transitions.

The thermogram of 10% sodium glycine, shown in Figure 3 (curve A), is characterized by a low temperature glass transition (midpoint about −80°C), an exotherm with a peak temperature of just above −50°C, and a eutectic melting endotherm at −17.8°C. Minimal thermal treatment (1 minute at −50°C, curve B) is needed in order to cause the glass transition and the crystallization exotherm to disappear, leaving only the eutectic melting and ice melting endotherms.

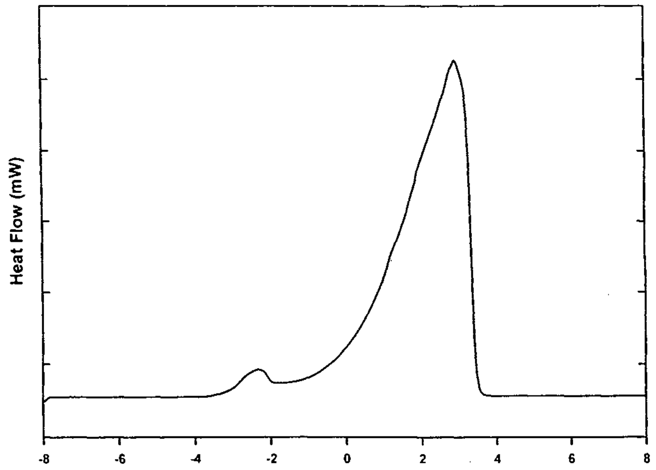

Fig. 1. DSC thermogram of 0.5% (w/v) neutral glycine solution. Heating rate was 1.0°C per minute.

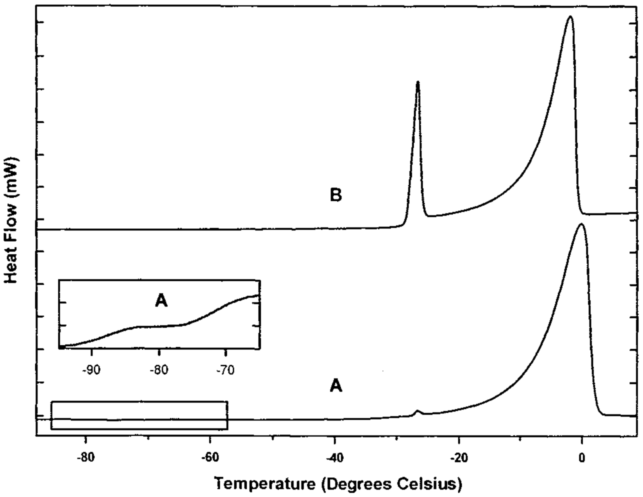

Fig. 2. DSC thermograms of 10% (w/v) glycine hydrochloride solution immediately after freezing (A) and after annealing at −40°C for 2 hours. Inset shows glass transition region, where each division on the y-axis represents approximately 1 mw.

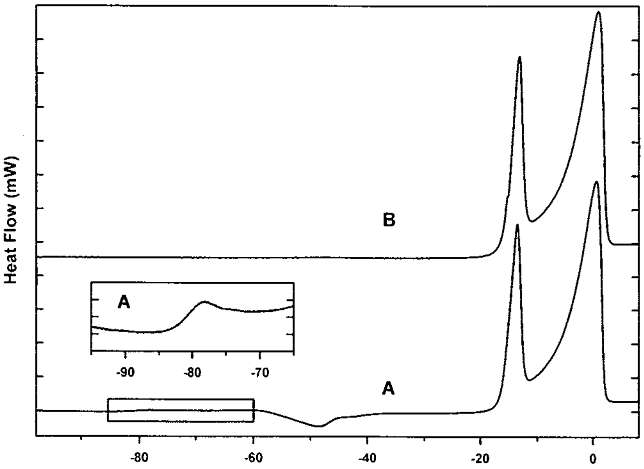

Fig. 3. DSC thermograms of 10% (w/v) sodium glycinate immediately after freezing (A) and after annealing at −50°C for 1 minute. Inset shows glass transition region, where each division on the y-axis represents approximately 1 mw.

Characterization of Freeze Dried Solids

Solutions of neutral glycine, glycine hydrochloride, and sodium glycinate, all at a concentration of 10%, were freeze dried after overnight freezing at −45°C and drying at product temperatures below the respective eutectic melting temperatures. X-ray powder diffractograms, along with reference diffractograms (12), are shown in Figure 4. Neutral glycine forms essentially the pure β polymorph. This is consistent with data reported by Shalaev and Kanev (10) and Suzuki and Franks (11). The β polymorph has been reported to be unstable, readily forming a mixture of α and γ glycine upon exposure to air (13). This is also consistent with observations in our laboratory (data not shown). Glycine hydrochloride crystallizes readily provided that either an annealing treatment or a slow freezing process is carried out. Only one polymorph of glycine hydrochloride is reported in the powder diffraction file (12). The powder diffractogram of freeze dried sodium glycinate is different from the diffractogram of the starting material (data not shown), and is consistent with the starting material being a mixture of at least two polymorphs. We have been unable to find reference diffractograms of sodium glycinate.

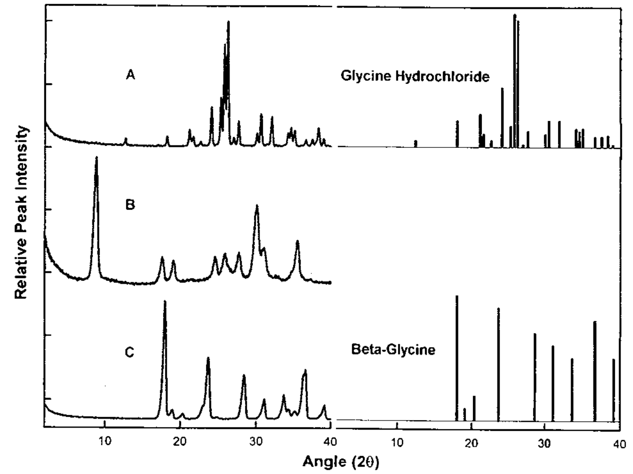

Fig. 4. X-ray diffractograms of freeze-dried 10% (w/v) glycine hydrochloride (A), sodium glycinate (B), and neutral glycine (C) solutions. Reference diffractograms are shown in the right panel.

Effects of pH Adjustment and Ionic Strength

Microscopic observations demonstrated that solutions of neutral glycine adjusted to pH 3 and pH 10 crystallize much more slowly than a solution with no pH adjustment (about pH 6.5). The thermogram of neutral glycine solution adjusted to pH 3 comprises a complex glass transition region with two apparent glass transitions at −70°C and −57°C, an exotherm at −32°C, and the eutectic melting endotherm at −3.5°C (Figure 5). Annealing this solution briefly (−30°C for one minute) results in a thermogram containing only the eutectic melting endotherm for neutral glycine. The thermogram of neutral glycine adjusted to pH 10 also has a glass transition region consisting of two apparent transitions, at −64° and −80°. When the sample is annealed at −45°C for 90 minutes, a melting endotherm is detected at approximately −18°C, which corresponds to the eutectic melting endotherm for sodium glycinate.

X-ray powder diffractograms of freeze dried solids prepared from these solutions, also shown in Figure 5, demonstrate the effect of pH adjustment on the character of the lyophilized product. Neutral glycine, adjusted to pH 3 and freeze dried, produces predominantly γ glycine. When adjusted to pH 10, the resulting solid appears to be a mixture of sodium glycinate and β glycine. Glycine has pK values of 2.35 and 9.78, and exists as a zwitterion in solution between these pH values. At pH 10, a mixture of salt forms would be expected. At pH 3, glycine would exist predominantly as the zwitterion, and a neutral glycine polymorph is expected. Neutral glycine solution which has been adjusted to pH 3 crystallizes more slowly than a solution adjusted to pH 7. We have observed that slow rates of crystallization tend to favor formation of γ glycine, whereas rapid crystallization from solution seems to favor formation of the β polymorph.

We examined the hypothesis that the effect of pH adjustment on crystallization of glycine from 'frozen' solution

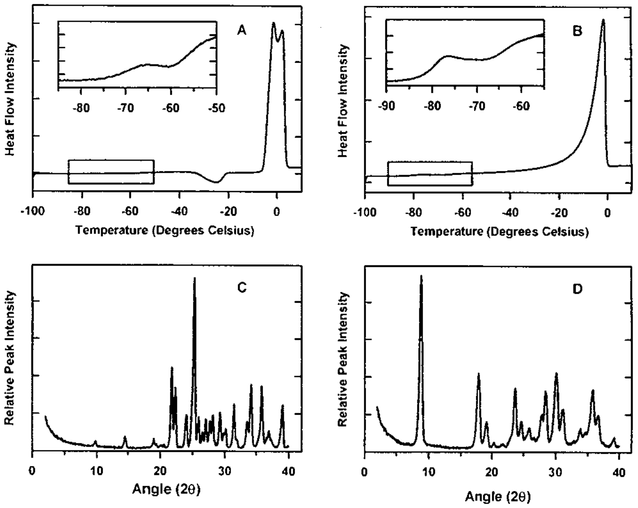

Fig. 5. DSC thermograms and x-ray diffractograms of 10% (w/v) neutral glycine adjusted to pH 3 (A and C) and pH 10 (B and D), respectively.

can be accounted for by both a direct effect of pH on glycine ionization and by the presence of sodium chloride resulting from pH adjustment with either sodium hydroxide or hydrochloric acid, where the rate of crystallization is inversely related to sodium chloride concentration. Solutions of 10% neutral glycine containing sodium chloride:glycine molar ratios between 0.08 and 1.0 were prepared. DSC thermograms of the frozen solutions with no thermal treatment are shown in the left panel of Figure 6. As the molar ratio of sodium chloride to glycine increases, the midpoint temperature of the glass transition region decreases, which is consistent with a higher concentration of unfrozen water in the freeze concentrate. At molar ratios less than about 0.2, two separate crystallization exotherms, arising from glycine and sodium chloride, are observed in the thermogram. The peak of the first crystallization exotherm occurs at higher temperature as the relative amount of sodium chloride increases, with the second exotherm peak occuring at a roughly constant temperature. These events merge into a single exotherm at molar ratios between 0.38 and 0.45. At higher ratios, glycine crystallization is prevented, at least within the time frame of the DSC experiment, and no crystallization exotherm is observed in the thermogram.

The right-hand panel in Figure 6 shows the effect of annealing at −45°C for one hour on the DSC thermograms as the ratio of sodium chloride to glycine increases. Thermograms A, B, and C show one endotherm in addition to the ice melting endotherm. This appears to be the sodium chloride/ice eutectic. The sodium chloride eutectic melts at about −22°C in the binary sodium chloride/water system, but is observed at about −25°C when glycine is present. At higher molar ratios, another endotherm is observed with an onset temperature of about −42°C. The origin of this endotherm is unknown at present. Further work is necessary in order to characterize this material.

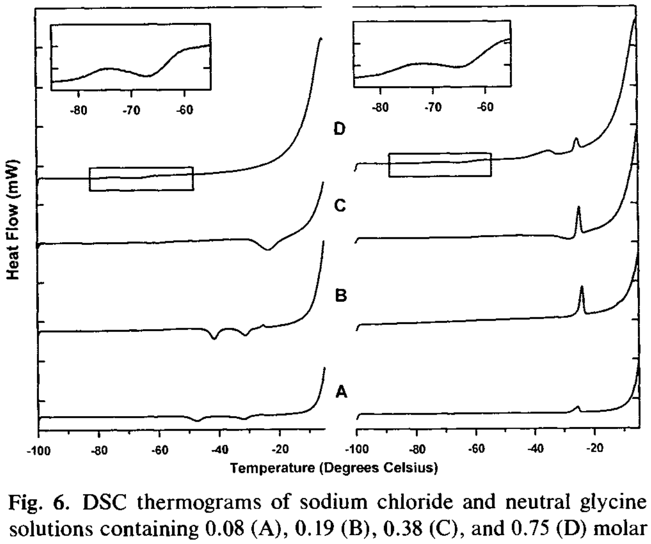

Fig. 6. DSC thermograms of sodium chloride and neutral glycine solutions containing 0.08 (A), 0.19 (B), 0.38 (C), and 0.75 (D) molar ratios of sodium chloride to neutral glycine. The panel on the left shows thermograms immediately after freezing and the panel on the right shows thermograms after annealing at −45°C for 1 hour.

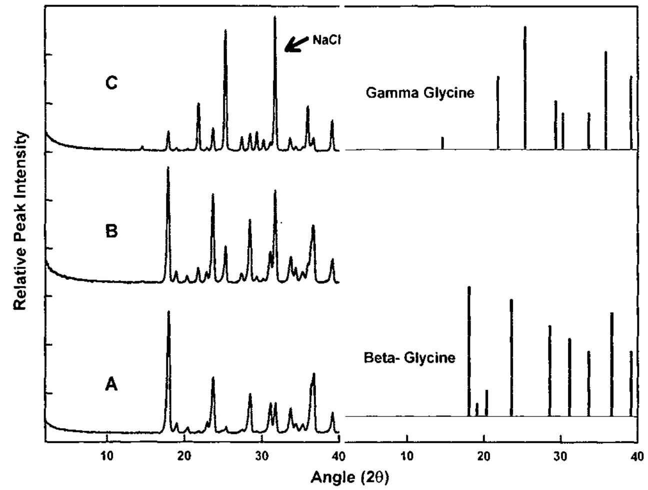

Fig. 7. X-ray diffractograms of freeze-dried sodium chloride and glycine mixtures containing 0.08 (A), 0.19 (B), and 0.38 (C) molar ratios of sodium chloride to glycine. The right panel represents the reference diffractograms for β-glycine and γ-glycine.

Figure 7 illustrates changes in the physical state of freeze dried powders of neutral glycine as the NaCl molar ratio increases from 0.08 to 0.38. As the concentration of NaCl increases, the relative amount of γ glycine in the freeze dried cake also increases. This is consistent with microscopic observation, which shows that the rate of crystallization decreases as the sodium chloride concentration increases, and that the crystal morphology changes as the rate of crystallization slows. Again, slower crystallization appears to favor the formation of γ glycine over β glycine. The presence of a peak corresponding to sodium chloride in the x-ray powder diffractogram of freeze dried solids confirms the presence of crystalline sodium chloride.

The freezing and freeze drying behavior of neutral glycine adjusted to pH 7 is dramatically different from that of glycine hydrochloride adjusted to pH 7. Adjusting the pH of 10% glycine hydrochloride to 7 with sodium hydroxide is equivalent to adding 0.8M NaCl to the solution. To determine whether the difference in behavior is explained by the high concentration of sodium chloride, the thermal behavior of 10% neutral glycine and 10% glycine hydrochloride, both adjusted to pH 7, was compared. Results are shown in Figure 8. The thermal behavior and the nature of the freeze dried solids are not distinguishable. Both systems show the complex glass transition at low temperature, and x-ray diffractograms of both freeze dried solids are consistent with a mixture of γ glycine and sodium chloride.

Unfrozen Water in the Freeze Concentrate

As discussed in a previous report (8), unfrozen water in 'frozen' systems gives rise to a narrow peak in the nmr spectrum superimposed on a wide peak arising from the less mobile protons of the solute. Because of the low mobility of the protons in ice, no peak is observed in the nmr spectrum.

At −35°C, no unfrozen water could be detected in 5% neutral glycine. In contrast, the quantity of unfrozen water in 5% glycine hydrochloride adjusted to pH 7 at −35°C was 1.45 g H_2O per gram of glycine. These data are consistent with both the thermal data and microscopic observations of the freezing behavior. When the solute crystallizes from the frozen solution, all water should be present either as pre-

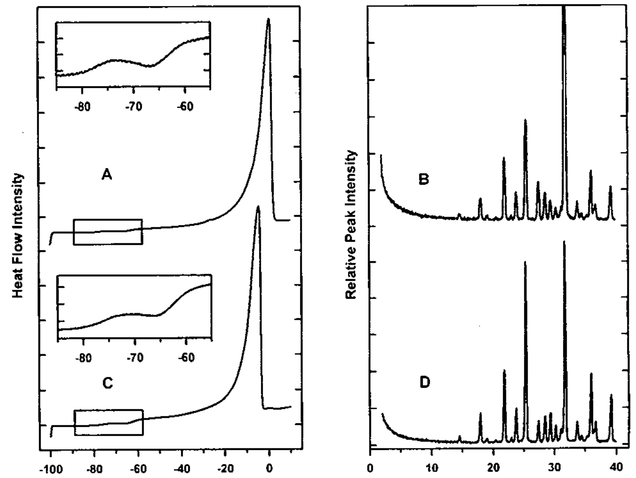

Fig. 8. DSC thermograms and x-ray diffractograms of 10% (w/v) glycine hydrochloride adjusted to pH 7 (A and B) and neutral glycine containing 0.8 M sodium chloride (C and D), respectively.

eutectic ice or as ice in the eutectic mixture. No glass transition would be expected in such a system, and none was observed in the thermogram of neutral glycine with no pH adjustment. The high concentration of unfrozen water in glycine hydrochloride adjusted to pH 7 is consistent with the low glass transition observed in such systems, where water acts as a plasticizer, lowering the glass transition temperature (14). Results of previous studies on sucrose/water (T_g' about $-32°C$) showed about 0.2 grams of unfrozen water per gram of sucrose, corresponding to about four molecules of water per molecule of solute (8). When glycine hydrochloride is neutralized, the freeze concentrate contains about six molecules of water per mole of solute. The lower T_g' value of glycine hydrochloride is consistent with its higher degree of hydration.

Practical Implications

The pharmaceutical scientist needs to be aware that neutral glycine, glycine hydrochloride, and sodium glycine are not equivalent when developing formulations intended to be freeze dried, even when solutions of different salts are adjusted to the same pH. For example, the freezing and freeze drying behavior of a solution of neutral glycine adjusted to pH 7.0 is much different than a solution of glycine hydrochloride adjusted to pH 7.0. Neutral glycine crystallizes rapidly, whereas glycine hydrochloride crystallizes slowly. If crystallization is not allowed to proceed to near completion, the amorphous phase may dominate the freeze drying behavior, causing collapse at temperatures below the lowest temperatures attainable with a commercial freeze dryer. Whether macroscopic collapse occurs depends on the relative amount of amorphous and crystalline phases. If the relative amount of amorphous phase is small, then the collapse associated with this material may not be detectable by visual observation of the final product. In addition, the amorphous fraction of glycine may be associated with another component of the formulation, and the glass transition of this mixture may be considerably higher than that of amorphous glycine alone. The presence of sodium chloride resulting from pH adjustment of glycine hydrochloride with sodium hydroxide should not be overlooked, especially with proteins, since the presence of sodium chloride may adversely affect stability of the protein against damage caused by freezing and freeze drying.

Finally, the thermal history of freezing has been shown to influence the rate and extent of crystallization of glycine. Allowing sufficient hold time in the frozen state or including a thermal treatment step may substantially affect the quality of the dried product. Further work is under way to better understand the temperature dependence of crystallization from frozen solutions using glycine as a model system.

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