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Fundamentals of Freeze-Drying

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1. INTRODUCTION

The development of freeze-dried injectable pharmaceutical products has traditionally been a process of trial and error, both with respect to the composition of the formulation and the process conditions used during freeze-drying. Although this approach ultimately may result in an acceptable product, it is a time-consuming and labor-intensive process, and is unlikely to result in the highest quality product attainable or in a freeze-dry process which is optimized.

The increased importance of freeze-drying as a pharmaceutical unit operation, in large part caused by widespread development of protein therapeutic agents, has spurred interest in an analytical approach to formulation and process development based on a sound understanding of the physical chemistry of freezing and freeze-drying, the material science of viscoelastic systems, and fundamentals of heat and mass transfer. A growing

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body of scientific literature has demonstrated that the scientific approach can result in improved product quality with minimum trial and error empiricism.

In addition to the need for rapid development of new pharmaceutical products, growing scrutiny of health care costs has focused more attention on minimizing cost of goods by pharmaceutical manufacturers. Among pharmaceutical unit operations, drying operations are, in general, the most expensive. Of these, freeze-drying is generally the most expensive, not only in operating cost, but also in cost of equipment. Development of overly conservative freeze-drying conditions results in long cycle times, added cost of production, and unnecessary consumption of drying capacity. Optimization of the freeze-drying cycle—minimizing drying time without a measurable adverse effect on product quality—should be integrated into the product development plan.

Freeze-drying of proteins often presents a greater challenge to the formulation scientist than that of more traditional, low molecular weight drug compounds, for several reasons. First, proteins are, in general, more susceptible to damage by the stresses involved in freezing and freeze-drying than low molecular weight drugs. Successful freeze-drying commonly requires the use of solutes intended to stabilize the protein against damage by these stresses. Second, the conventional wisdom that "the dryer, the better" with respect to both acute damage caused by freeze-drying and long-term stability does not apply to some proteins. Avoiding overdrying places added importance on careful monitoring of the process, particularly when maintenance of product quality demands keeping the dried product within a "window" of residual moisture activity. Third, the stability of protein formulations as freeze-dried solids is more uncertain than that of most low molecular weight drugs. Freeze-dried protein pharmaceutical products usually require refrigerated storage, and stability of the freeze-dried protein is more affected by seemingly subtle changes in the formulation and in processing conditions than low molecular weight drugs. Finally, the frequent high cost of bulk active material provides added incentive to maximize the yield of pharmaceutically acceptable product.

The purpose of this chapter is to review the basic physical chemistry and material science of freeze-drying, with particular reference to protein formulations, and is geared toward readers unfamiliar with the subject. We also attempt to highlight areas in need of further research. The mechanism of stabilization of proteins against freezing- and drying-induced loss of activity by cosolutes has been thoroughly reviewed by Carpenter *et al.* (1994) and Arakawa *et al.* (1993), and this body of information will not be covered in detail.

2. OVERVIEW OF THE FREEZE-DRYING PROCESS

A schematic diagram showing the major components of a modern, production-scale pharmaceutical freeze-dryer is shown in Fig. 1. The chamber contains hollow shelves through which a heat transfer fluid is circulated (usually silicone oil). This allows control of the shelf temperature from about −50°C to perhaps +50°C. A condenser, operating at temperatures from about −60°C to −75°C, collects water vapor from the product. The condenser is usually located externally to the chamber, as shown in Fig. 1, or it may be of an internal design, consisting of plates located along the side walls of the chamber. Vacuum pumps for evacuating the system are usually of the rotary, oil-sealed design, and are usually capable of maintaining a system pressure of 50 mTorr or less. Redundant compressors for the refrigeration system as well as redundant vacuum pumps are used on production freeze-dryers, and are easily justified based on the high value of the product. A valve is located between the chamber and the condenser, and the stack of shelves is hydraulically movable to allow the stack to be compressed in order to fully insert stoppers into vials after freeze-drying.

Operationally, freeze-drying of a final dosage form usually consists of filling glass vials with an aqueous solution of the solutes to be freeze-dried,

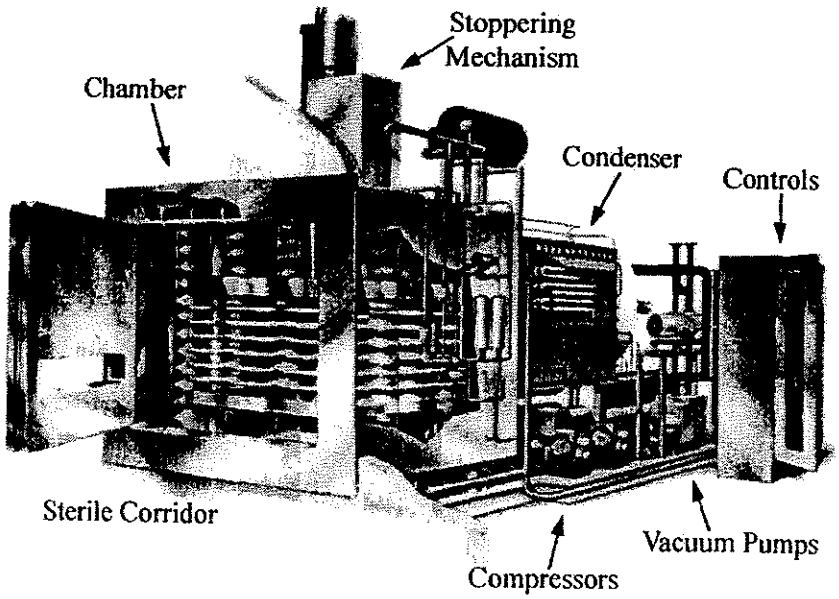

Figure 1. Schematic of a production-scale pharmaceutical freeze-dryer. Photograph courtesy of BOC Edwards Calumatic, Tonawanda, NY.

partially inserting a special rubber stopper (called a lyostopper, see Fig. 2), which allows water vapor to flow through slots in the stopper when in the partially inserted position, and transferring the vials to the shelves of the freeze-dryer.

Temperature sensors are placed in a few vials to monitor product temperature during the freeze-drying process (see Fig. 2). The shelves are then cooled to a temperature in the range of −40° to −50°C. The vials are held long enough to approach thermal equilibrium with the shelves, usually for a minimum of 4 hr. Pressure in the freeze-dryer is then reduced to a level which is less than the vapor pressure of ice at the temperature of the product (see Table I). For example, for a product temperature of −40°C, the pressure in the system would be reduced to less than 0.096 mm Hg (96 μm Hg). In freeze-drying, removal of ice takes place by a process of sublimation, or a phase change from a solid to a vapor without passing through the liquid state. Ideally, this results in retention of the microstructure of the frozen material and a high specific surface area of the freeze-dried solid. This promotes rapid, complete rehydration of the solid.

In order for sublimation to take place, energy must be provided in a quantity equal to the heat of sublimation of ice ΔH_s, which is approximately 670 cal/g. This is accomplished by heating the transfer fluid and warming the

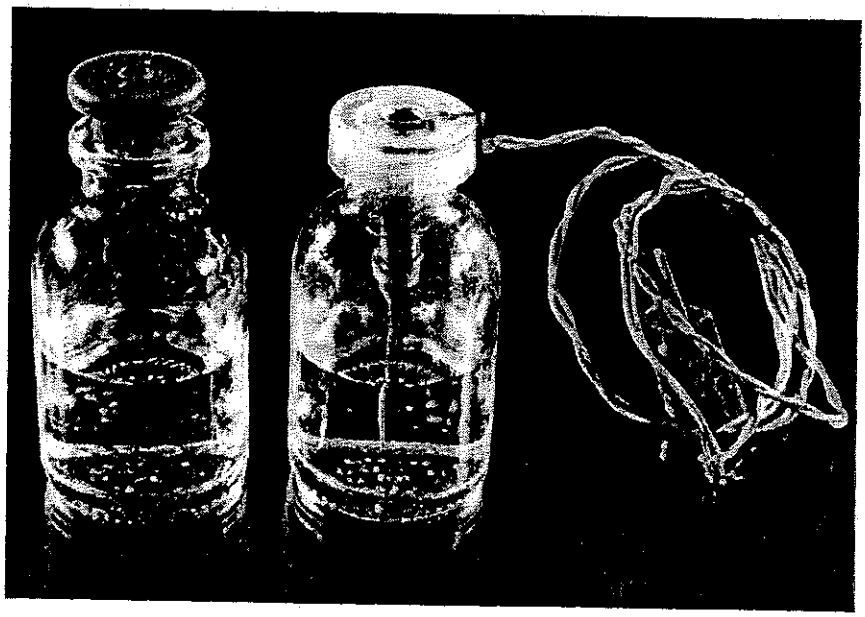

Figure 2. Filled vials with partially inserted lyostopper and a product temperature thermocouple.

Table I
Vapor Pressure of Ice

Temperature (°C)	P (mm Hg)	Temperature (°C)	P (mm Hg)
−80	0.0004	−30	0.285
−70	0.0019	−25	0.476
−60	0.0080	−20	0.776
−50	0.0295	−15	1.241
−45	0.054	−10	1.950
−40	0.096	−5	3.013
−35	0.164	0	4.579

shelves to a temperature high enough to effect sublimation, but not so high as to melt the frozen material in contact with the bottom of the vial or to collapse the partially dried product. The partial pressure of water vapor in the chamber is maintained at a low level by the condenser, and water vapor is removed from the product primarily by a process of *bulk flow* from a region of relatively high pressure (the sublimation front) to low pressure (the condenser). This phase of the process, called *primary drying*, is characterized by a visible receding boundary from the top of the frozen layer. When the ice has sublimed, the heat of sublimation is no longer needed, and the product temperature usually increases sharply toward the shelf temperature.

In general, not all of the water initially present in the product is converted to ice during freezing. The quantity of unfrozen water is a function primarily of the composition of the formulation and, to a lesser extent, the thermal history of the freezing process. Removal of this "unfrozen" water, which may be 20% or more of the weight of dry solids, is called *secondary drying*. During secondary drying, the shelf temperature is usually increased, since ice is no longer present. In contrast to primary drying, where water vapor is removed by bulk flow, water vapor removal during secondary drying is largely by a process of diffusion, or flow by molecular motion from a region of high concentration to a region of lower concentration. Because secondary drying is generally slow relative to primary drying, it often represents a larger total portion of drying time than primary drying, even though the quantity of water removed is less. At the end of secondary drying, the shelf stack is compressed together, causing insertion of the lyostoppers to the fully stoppered position. This may be done under full or partial vacuum or at atmospheric pressure. For proteins that are sensitive to oxidation in the solid state, the composition of gas in the headspace of the vial may have a critical impact on product stability (Townsend *et al.*, 1990).

Since the driving force for freeze-drying is the vapor pressure of ice, it is important from the standpoint of process efficiency to keep the product temperature as high as practical during primary drying. However, the

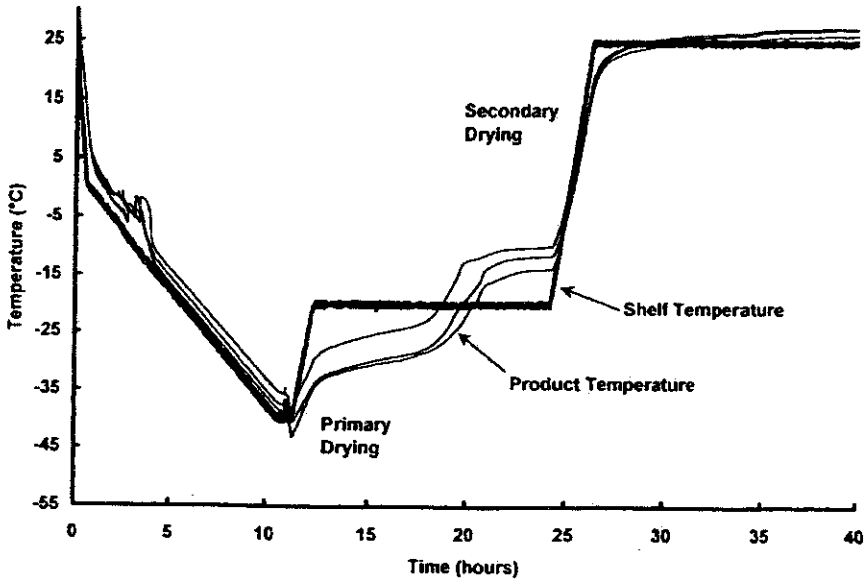

Figure 3. Plot of process variables versus time during the freeze-dry process, including shelf temperature, product temperature, and chamber pressure.

product temperature must be maintained below the maximum allowable product temperature, which is either a eutectic melting temperature or a collapse temperature (see discussion below). Monitoring of product temperature is important for establishing optimum process conditions. A typical plot of process variables is shown in Fig. 3, where product temperature increases gradually during primary drying, followed by a more rapid increase at the beginning of secondary drying.

3. THE FREEZING PROCESS

An understanding of the physical chemistry of freezing is essential for understanding how both formulation composition and process conditions influence the quality of the freeze-dried product. A schematic diagram of the physical events occurring during freezing is shown in Fig. 4. Supercooling, or retention of the liquid state below the equilibrium freezing point of the solution, always occurs to some extent: It is not uncommon for aqueous solutions to supercool by 12°C or more. This is seen in Fig. 3 as a sudden "jump" in temperature during the freezing process ~~(the smaller "jumps" after the main exotherm are caused by ice nucleation in neighboring vials)~~. The

effect of supercooling is to limit the ability to control the freezing rate by manipulation of shelf temperature, because the greater the degree of supercooling, the faster is the effective rate of freezing once ice crystals nucleate.

As indicated in Fig. 4, ice may not be the first component of the solution to crystallize. As the temperature is decreased, the equilibrium solubility of one or more solutes may be exceeded, allowing nucleation and crystal growth. An example of this behavior is illustrated by the drug pentamidine isethionate, where, depending on the thermal history of freezing, either drug or ice may nucleate first (see below). Process validation studies should include determination of whether the thermal history of freezing (for example, placing vials on precooled shelves versus slowly decreasing the shelf temperature during freezing) has a measurable impact on product quality, including such factors as activity, residual moisture, appearance of solids, and reconstitution time.

(Chong present, et al. 1997)

As ice crystals grow in the freezing system, the solutes are concentrated. Increased ionic strength accompanying the freezing process is often an important consideration in freeze-drying of proteins as well as other biological materials, since high ionic strength may promote denaturation of proteins as well as osmotic dehydration of microorganisms. In addition, rates of some chemical reactions, particularly second-order reactions, may be accelerated by freezing through this freeze-concentration effect. Examples include reduction of potassium ferricyanide by potassium cyanide

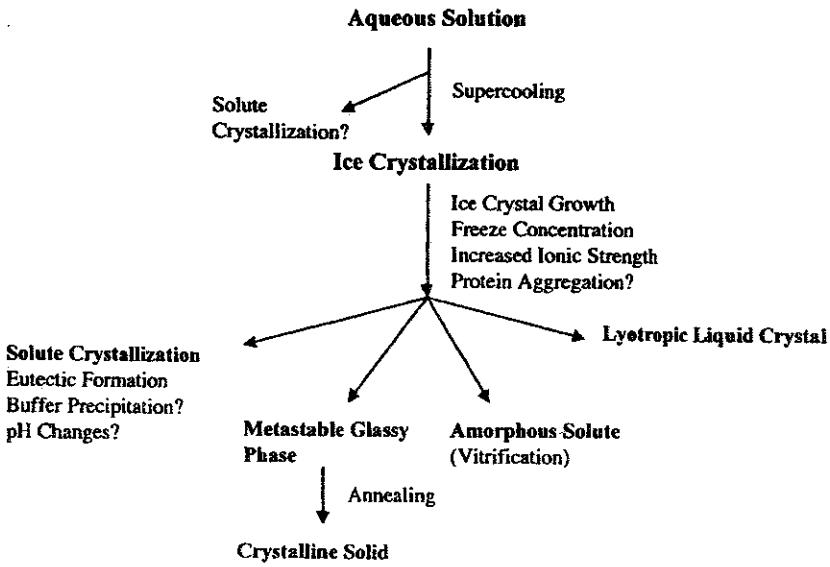

Figure 4. Physical events occurring during freezing of an aqueous solution.

(Hatley *et al.*, 1986a), oxidation of ascorbic acid (Hatley *et al.*, 1986b), and polypeptide synthesis (Schuster *et al.*, 1991). The kinetics of reactions in frozen systems has been reviewed by Pincock and Kiovsky (1966).

3.1. Solute Crystallization

3.1.1. EUTECTIC SYSTEMS AND THE PHASE DIAGRAM

The fate of the solute in the freeze-concentrated solution is a key concept in understanding the material science of freeze-drying. As indicated in Fig. 4, there are several possibilities, the simplest of which is crystallization of solute from freeze-concentrated solution to form a simple eutectic ~~system~~ mixture. A pure binary eutectic system, illustrated in Fig. 5 for the system sodium chloride/water, is not often encountered in practice, but this system is useful for a conceptual understanding of the relevance of eutectic mixtures to freeze-drying. The line *ab* represents the freezing point depression curve of water in the presence of sodium chloride, and the line *bc* represents the solubility of sodium chloride in water. The intersection of these lines is the eutectic temperature, which for sodium chloride/ice is −21.5°C, and the eutectic composition is about 23.3% (w/w) sodium chloride. The freezing of a 5% solution of sodium chloride in water is represented by the line *defgh* (called an *isopleth*). At room temperature (point *d*), the system is entirely liquid. As the solution cools, ice appears at point *e* (in the absence of supercooling). As the system cools, ice continues to crystallize and the remaining solution becomes more concentrated in sodium chloride. At point *f*, two phases are present, ice and a freeze concentrated solution of sodium chloride in water. The freeze concentrate has the composition given by point *i*, which is in equilibrium with ice. At point *g*, the freeze concentrate is saturated with respect to sodium chloride, and (again, in the absence of supercooling) sodium chloride begins to precipitate. It is only below the eutectic temperature that the system is completely solidified. The relevance of the eutectic temperature to freeze-drying is that it represents the maximum allowable product temperature during primary drying, because eutectic melting would introduce liquid water and destroy the desirable properties of a freeze-dried solid. Melting of the frozen system during primary drying is sometimes called *meltback*.

The phase diagram illustrates the degree of concentration of sodium chloride caused by the freezing process. For example, if a solution of normal saline (0.9% w/v, or 0.15 *N*) is frozen, the concentration reaches 23.3%

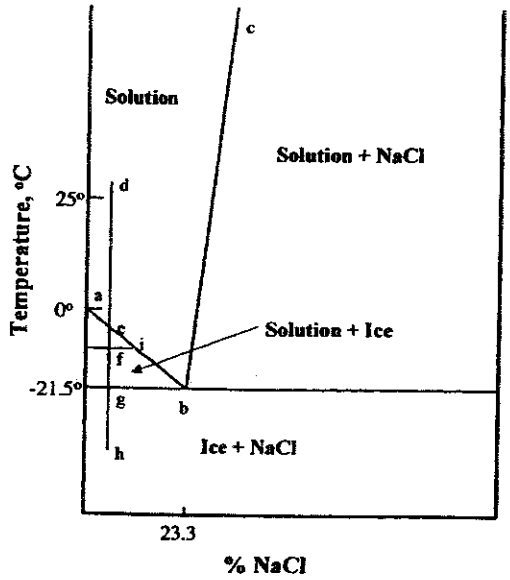

Figure 5. Phase diagram of sodium chloride/water.

before sodium chloride crystallizes and forms a eutectic mixture, or just over 4.0 *N*. This increase in ionic strength associated with freezing of salt solutions is a contributing factor in osmotic dehydration of cells during freezing and has been thought to contribute to protein denaturation during freezing.

The phase diagram of glycine/water, illustrated in Fig. 6, is more representative of the freezing characteristics of aqueous solutions of organic compounds that crystallize from a freezing system. The eutectic melting temperature of the glycine/ice eutectic mixture, −3.6°C, is much closer to the melting temperature of ice than is that of the sodium chloride/ice eutectic mixture, and the amount of glycine present in the eutectic mixture is lower, about 12%. In general, the lower the solubility of a given solute in water, the higher is the eutectic melting temperature. Figure 6 is probably an oversimplification, however, since glycine is known to exist in three polymorphic forms. Since different polymorphs of a compound have different physical properties, it might be expected that the solubility curve would be different. This, in turn, would change the eutectic melting temperature. This aspect of the physical chemistry of freeze-drying has not been investigated in depth, and more work is needed to better understand the impact of polymorphism on the physical chemistry of freeze-drying.

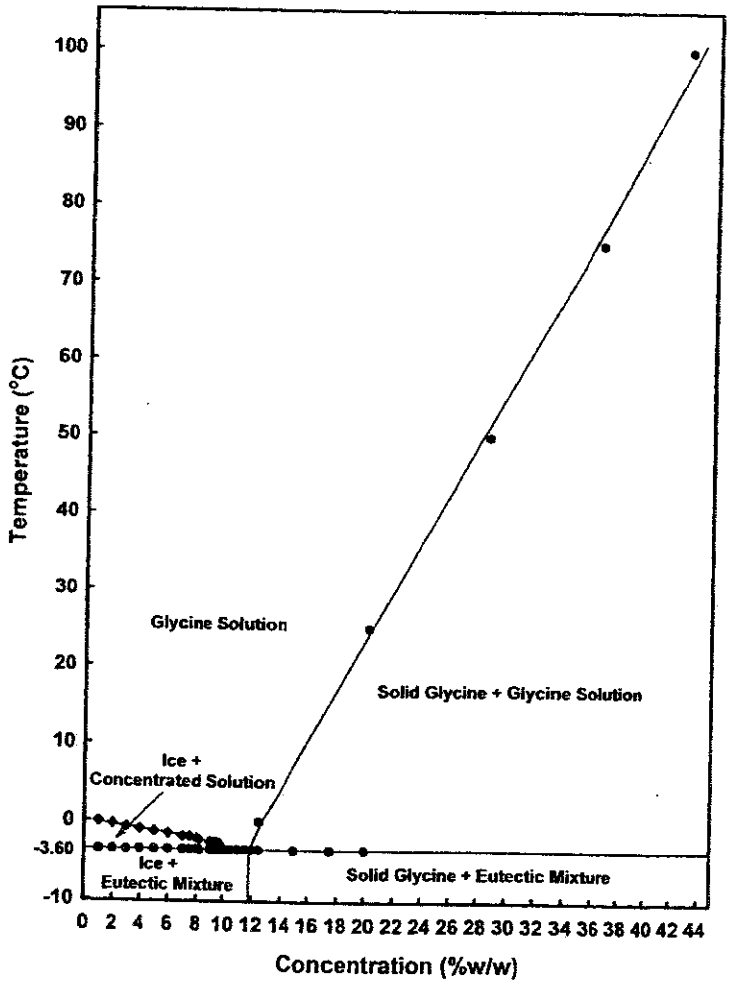

Figure 6. Phase diagram of glycine/water.

3.1.2. pH SHIFTS DURING FREEZING

Crystallization of phosphate buffers during freezing is a special case of eutectic mixture formation during freezing, and is worthy of mention not only because phosphate salts are the most common buffers used in protein formulations, but also because the crystallization process can cause significant shifts in pH during freezing. Such shifts are an illustration of LeChâtelier's principle, which states that, when a disturbance is imposed on a system at equilibrium, the equilibrium shifts so as to compensate for the

disturbance. If a component precipitates from solution, the equilibrium shifts so as to compensate, as follows:

$$H_2PO_4^- + H_2O \leftrightarrow HPO_4^{2-} + H_3O^+$$

The effect of freezing on the pH of sodium and potassium phosphate buffers has been extensively studied by van den Berg and co-workers (van den Berg, 1959; van den Berg and Rose, 1959). These investigators used a technique where precipitated solids were removed from unfrozen solution by a sintered glass filter, and the pH was measured above 0°C. The pH was found to decrease with temperature during the precipitation of ice alone, as would be expected simply from freeze concentration. The pH shift was shown to depend on the salt used. The monobasic salt of potassium phosphate, being less soluble than the dibasic salt, tends to precipitate during freezing and causes an increase in pH. For sodium phosphate, the dibasic salt is less soluble than the monobasic salt, and the opposite effect is observed. Changes in pH of as much as three units toward the acid side have been reported for the sodium phosphate system. Changes toward more alkaline pH for the potassium phosphate system are less pronounced. Eutectic temperatures of the phosphate buffer salts are listed in Table II, including ternary eutectic mixtures consisting of ice and monobasic and dibasic salts. It is important to remember, however, that a eutectic mixture is only formed as a result of crystallization of one or more solutes, and the presence of a phosphate buffer in a formulation does not mean that the pH will shift during freezing, other than the small (about 0.5 unit) shift which would result from freeze concentration of the solution.

Later work by van den Berg reported development of a calomel and glass electrode modified to make it suitable for use at low temperatures, allowing direct measurement of pH in frozen systems (van den Berg, 1960). This was accomplished by use of reference solutions containing roughly equal weight proportions of water and ethylene glycol or glycerol, saturated

Table II
Eutectic Temperatures of Phosphate Buffers[a]

Solute	T_e (°C)
Na_2HPO_4	−0.5
NaH_2PO_4	−9.7
NaH_2PO_4–Na_2HPO_4	−9.9[b]
K_2HPO_4	−13.7
KH_2PO_4	−2.7
KH_2PO_4–K_2HPO_4	−16.7[b]

[a] From Murase and Franks (1989).
[b] Ternary eutectic.

with potassium chloride. A glass membrane with a high electrical conductivity was also used to decrease the electrode response time (electrodes suitable for this type of measurement are available from Mettler-Toledo, Inc., Wilmington, MA). This technique was used by Gomez *et al.* (2001) in a further study of crystallization during freezing of aqueous solutions of sodium phosphate buffers. This work extended previous work by Larsen (1973) by examining the influences of initial buffer solution pH and concentration on subsequent pH changes during freezing, as well as the influence of other species in solution on buffer salt crystallization. Gomez *et al.* (2001) reported that the pH changes associated with crystallization of a sodium phosphate buffer solution initially at pH 7.4 are directly related to the initial concentration of buffer in the range of 8–100 m*M*. Further, the lower the initial pH of the buffer, the higher is the observed pH at −10°C. These investigators further reported that addition of NaCl increases the ion product of dibasic sodium phosphate, thereby leading to larger pH changes. Solutes such as sucrose and mannitol inhibited crystallization of buffer species, resulting in smaller pH shifts upon freezing. The presence of sucrose and mannitol at concentrations above 3 mol per mole of dibasic sodium phosphate completely prevented salt crystallization. In this case, the pH change upon freezing was only 0.5 unit, which was attributed to the effect of freeze concentration.

Murase and Franks (1989) used differential scanning calorimetry to study freezing of phosphate buffer solutions. This study differed from previous work in that precipitation was studied under conditions far from equilibrium using small volumes of solution. Precipitation was found to be both concentration- and freezing-rate-dependent, with fast freezing rates inhibiting crystallization. Whereas dibasic sodium phosphate was found to precipitate readily, monobasic sodium phosphate did not precipitate at all under the experimental conditions examined. Monobasic potassium phosphate was found to precipitate readily. Dibasic potassium phosphate also precipitated readily and had the lowest eutectic point of any of the phosphate salts. Ternary eutectics were not observed in any of the systems studied.

Other pharmaceutically relevant buffer systems have not been as well characterized as phosphate with respect to pH changes accompanying freezing. Larsen (1973) reported that acetate, citrate, glycine, and Tris show only small pH shifts upon freezing.

Orii and Morita (1977) reported the use of pH indicators such as phthaleins, sulfonaphthaleins, and universal pH indicators to study pH changes with freezing. Although this method is subject to errors caused by changes in p*K* of indicators with temperature, as well as effects of increased ionic strength on dissociation constant, it can provide a convenient method of estimation of pH changes in a semiquantitative way. Of the 30 buffers

they studied, Orii and Morita reported that over half exhibited pH changes with freezing.

There are very few published studies which examine the practical significance of pH shifts on the integrity of pharmaceutically relevant proteins, primarily because very few studies of the relationship between formulation or processing conditions and protein integrity after freeze-drying attempt to measure pH changes with freezing. The most direct evidence of a pH shift-induced loss of protein activity during freeze-drying was reported by Anchordoquy and Carpenter (1996) for lactate dehydrogenase (LDH) in a sodium phosphate buffer system. Dissociation of LDH was attributed to a large decrease in pH in the frozen state, from 7.5 to 4.5. Addition of polymers such as bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) to the solution inhibited crystallization of buffers, thereby attenuating the pH shift and stabilizing LDH against dissociation. Stabilization could not be attributed solely to prevention of buffer crystallization, however, since stabilization of the protein via Timasheff's preferential exclusion hypothesis (Carpenter *et al.*, 1994) would also be expected.

Williams-Smith *et al.* (1977) detected pH changes in phosphate, pyrophosphate, and Tris buffers containing three different metalloenzymes, using visual pH indicators, and measured pH-dependent changes in the electron paramagnetic resonance spectra of these proteins. Zwitterionic buffers resulted in relatively small pH changes upon freezing. Lam *et al.* (1996) used Fourier-transform infrared (FTIR) spectroscopy and circular dichroism to study the influence of succinate and glycolate buffers on stability of freeze-dried interferon γ. It was hypothesized, based on electrical resistance measurements, that the monosodium form of succinate crystallized during freezing, resulting in an effective pH in the freeze concentrate of 1–2 pH units lower than the original solution pH of 5.0 (the pH during freezing was not actually measured). This appeared to promote unfolding of the protein and cause loss of bioactivity. Use of glycolate buffer instead of succinate minimized the pH shift during freezing. The freezing-associated conformational change was also reflected in the solid-state stability of the lyophilized protein, with the glycolate formulation showing superior stability after 4 weeks of storage at 25°C.

Although it was not directly related to buffer-induced pH changes accompanying freezing, Costantino *et al.* (1997b) reported that lyophilized organic compounds containing protein functional groups (amino, carboxylic, and phenolic) exhibit "pH memory", that is, the ionization state of the solid, as reflected by the FTIR spectrum, is similar to that of the aqueous solution from which the compound was freeze-dried. Tarelli and Wheeler (1994) reported that freeze-drying from aqueous phosphate buffers can promote O-phosphorylation of hydroxyl groups on serine and threonine as well as saccharides

and alditols. Primary hydroxyl groups are more reactive than secondary hydroxyl groups, and the reaction occurs more readily at acid pH. Residual moisture in the dried solid also affects this reaction, with the most rapid reaction occuring in the range of 3–10% residual moisture. The potential for pH shifts with freezing of phosphate buffers was not mentioned in this study.

Sarciaux *et al.* (1999) reported substantially higher levels of insoluble aggregates in formulations of bovine immunoglobulin G (IgG)-containing sodium phosphate as compared with potassium phosphate at the same concentration. This effect was not due to a pH shift upon freezing, however. It was hypothesized that the difference in degree of aggregation observed for the two buffer systems was attributable to Hofmeister series effects.

Regardless of the relative scarcity of published data linking pH changes with freezing to loss of protein integrity, the potential for significant changes exists, particularly with phosphate buffers. Given the impact of pH on many mechanisms for loss of protein activity, both chemical and physical, the formulation scientist who is alert to the potential effects of these changes is wise.

The list of buffers used in approved products is relatively small and includes, in addition to sodium phosphate, citrate (pK of 3.15, 4.8, and 6.4), Tris-hydroxymethylaminomethane (THAM, pK 8.1), succinate (pK of 4.2 and 5.6), histidine (pK 1.8, 6.0, and 9.0), glycine (pK 2.35 and 9.8), and arginine (pK 2.18 and 9.1). It should be noted that glycine and arginine, although often listed as buffers, would only be effective well above or well below neutral pH. The physical state of L-histidine after freeze-drying and long-term storage was studied by Osteberg and Wadsten (1999). The tendency of L-histidine to crystallize from frozen solutions was dependent upon the pH of the formulation, and was found to be maximum at pH 6.0, corresponding to the pK of the imidazoline function. Histidine was also reported to crystallize from the amorphous solid state upon exposure to water vapor.

As a general formulation guideline, it is good practice to keep the concentration of buffers to a minimum. This minimizes the chance of precipitation during freezing, thereby minimizing the potential for pH shifts.

3.2. Vitrification during Freezing

Many solutes do not crystallize from a freeze-concentrated aqueous solution. In this case, the freezing behavior is illustrated by Fig. 7, which describes sucrose, a common lyoprotective solute, which remains amorphous during freezing and freeze-drying. This type of representation was first suggested by MacKenzie (1977) and termed a *supplemented* phase

diagram, whereas other investigators have used the term *state* diagram (Blond *et al.*, 1997). The freezing curve and the solubility curve are the same as presented in Fig. 5 for sodium chloride/water, and the composition of the freeze concentrate is given by the freezing curve; however, sucrose is kinetically inhibited from crystallizing at T_{eut}. Instead, the composition of the freeze concentrate continues to be represented by the dashed line, which represents a supercooled liquid. Ice crystallization continues, and the freeze-concentrated system becomes more viscous, until ice crystal growth (on a practical time scale) ceases and the degree of freeze concentration of the solute reaches a maximum. The glass transition of the maximally freeze-concentrated solution is designated as T'_g. Note that T'_g is a point on the *glass transition curve*, which represents a reversible change of state between a viscous liquid and a rigid, glassy system. The viscosity of this freeze concentrate changes by three or four orders of magnitude over a temperature range of a few degrees in the region of the glass transition. The point T_{gs} represents the glass transition of amorphous sucrose (about 65°C) and the ~~point~~ T_{gw} represents the glass transition temperature of

Tg

intersection of the glass transition curve with the y-axis

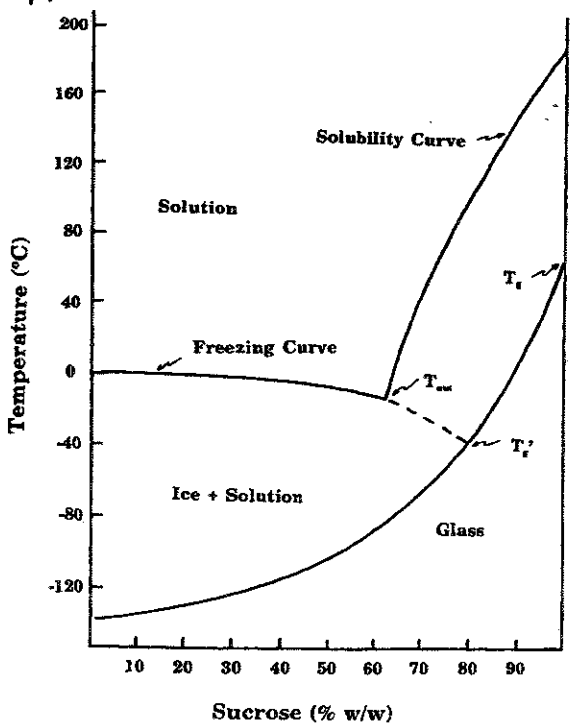

Figure 7. State diagram of sucrose/water.

amorphous solid water, at about −135°C. Extremely fast freezing rates, on the order of 10^5 K/min, are required to form solid amorphous water when starting with pure water (Johari *et al.*, 1987).

The glass transition curve in Fig. 7 illustrates that only a small amount of water in amorphous sucrose causes a sharp drop in the glass transition temperature of the solid. Thus, water is acting as a *plasticizer*. The point designated as T_g', the glass transition temperature of the maximally freeze-concentrated solute, corresponds to about 20% water/80% sucrose. This glass transition is generally accepted as the physical basis for *collapse* in freeze-drying. Low-temperature thermal analysis of the sucrose/water system and the uncertainty concerning the relationship between T_g' and collapse are discussed below. Collapse is caused by viscous flow of the freeze-concentrated solute on the time scale of freeze-drying, with resultant loss of the microstructure that was established by freezing, decreased surface area of the freeze-dried solid, and loss of pharmaceutical elegance. Thus, the onset of collapse represents the maximum allowable product temperature during freeze-drying of systems in which the solute(s) remain amorphous during freezing.

3.3. Differences in Freeze-Drying Behavior between Crystalline and Amorphous Solutes

If the solute crystallizes completely during freezing, the microstructure of the system is illustrated by the sketch in Fig. 8, where the interstitial material between the ice crystals consists of an intimate mixture of small crystals of ice and solute. For an amorphous solute, the interstitial material consists of a solid solution of solute and unfrozen water. For a eutectic-forming system, the upper product temperature limit during primary drying is the eutectic melting temperature, whereas for an amorphous system the upper temperature limit is the collapse temperature. Although eutectic melting temperatures of ice/inorganic salt mixtures can be quite low (the eutectic melting temperature of calcium chloride/water is about −52°C), eutectic melting temperatures of ice and most organic compounds are in the range of just below 0°C to perhaps −12°C. These relatively high melting temperatures allow maintenance of high product temperatures during primary drying, resulting in a more efficient drying process. Collapse temperatures are generally much lower than eutectic melting temperatures. Collapse temperatures below −40°C are not uncommon, particularly for freeze-dried protein formulations containing sugars as protective additives. These formulations often contain added salts, which further decrease the

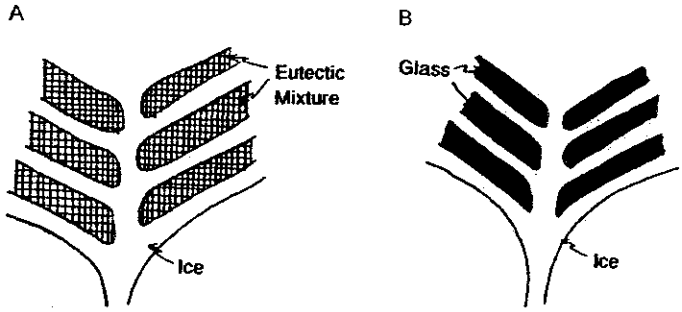

Figure 8. Microstructure of frozen system when (A) solute crystallizes versus (B) when the solute remains amorphous upon freezing.

collapse temperature (Her *et al.*, 1995). Given that most production-scale freeze-dryers are unable to consistently maintain product temperatures below about −40°C, freeze-drying is not feasible for all formulations. Even if the product temperature can be controlled at temperatures below −40°C, the vapor pressure of ice at such low temperatures, and therefore the driving force for freeze drying, are relatively low. Because the vapor pressure of ice at −40°C is only 0.096 Torr, the system pressure must be kept well below this pressure, which can be a significant demand on the vacuum system. As a general formulation guideline, formulations with collapse temperatures of −40°C or lower should be avoided if possible.

As illustrated in Fig. 8, nearly all water is present as ice (either pre-eutectic or eutectic) when the solute crystallizes. As the sublimation front moves through the frozen system, all of the ice can be removed by a process of *bulk flow*, or transport of water vapor from a region of high pressure to lower pressure, through the porous bed of dried solid. There is essentially no secondary drying. For the sucrose/water system, representing an amorphous solute, there is 20% water associated with sucrose, which must be removed during secondary drying. This water must be removed by a process of *diffusion*, or flow by molecular motion from a region of high concentration to a region of lower concentration. This combination of a significant amount of water to be removed by secondary drying and the slower transport mechanism often means that secondary drying is a significant part of the total drying time needed.

In addition to these influences on the drying process itself, the physical state of a drug as a freeze-dried solid may be important with respect to solid-state stability of the compound. It is well known that freeze-drying of some cephalosporins is not feasible because the drug does not crystallize during freeze-drying, and the amorphous form of the drug is not sufficiently stable,

despite being thoroughly dried, to support a reasonable shelf life of a freeze-dried product at room temperature (Pikal *et al.*, 1978).

Of course, it is common (and often desirable) to have both amorphous and crystalline phases present in a freeze-dried formulation. This is particularly relevant to freeze-dried proteins, where the lyoprotectant is present in the amorphous state and another component, such as glycine, is present as a crystalline solid in order to impart mechanical integrity and pharmaceutical elegance to the lyophilized solid.

3.4. Other Types of Freezing Behavior

3.4.1. METASTABLE GLASS FORMATION

In addition to eutectic crystallization and glass formation (vitrification), other types of freezing behavior may be observed (see Fig. 4). A metastable glass may form, which, with subsequent heating, undergoes crystallization. The most common example of metastable glass formation is mannitol, which crystallizes when heated above its T_g' (see discussion of thermal analysis below). This type of behavior is the basis for *annealing* during freeze-drying (see below), which refers to warming the product after an initial freezing step, generally to a temperature above T_g', but below the onset of melting, holding for a period of perhaps 1–4 hr, then cooling the material again before starting drying.

Mannitol is one of the most commonly used excipients in freeze-dried formulations; however, the formulation scientist should be aware of problems associated with the use of mannitol. It is well known that mannitol is associated with vial breakage during freeze-drying. This appears to be related to crystallization of mannitol from the metastable amorphous state. Vial breakage associated with mannitol can be minimized by keeping the depth of fill in a vial to no more than about 30% of the overflow capacity of the vial. The thermal expansion coefficient of the glass and the thermal history of freezing may also be significant factors affecting vial breakage.

3.4.2. LYOTROPIC LIQUID CRYSTAL FORMATION

States of matter which have degrees of order intermediate between amorphous and crystalline are called liquid crystals. Liquid crystals are broadly categorized as *thermotropic*, which are formed by heating, and *lyotropic*, which are formed by addition of solvent to a solid. Compounds

which form liquid crystals are generally surface-active, and the liquid crystal represents a more ordered structure than a micelle. These higher ordered structures are a result of freeze concentration, and may be either lamellar or rod-shaped.

There have been few reports of lyotropic liquid crystal formation in aqueous solutions of drugs, and even fewer which are relevant to freeze-drying. Powell *et al.* (1994) reported peptide liquid crystal formation by the luteinizing-hormone-releasing hormone deterelix and the effect of added salts on thermodynamic stability of the liquid crystal phase. Vadas *et al.* (1991) reported that a leukotriene D_4 receptor antagonist forms lyotropic liquid crystalline phases when lyophilized from aqueous solution. Bogardus (1982) studied the phase equilibria of nafcillin sodium–water and reported a lamellar mesophase in aqueous solutions containing more than 55% nafcillin sodium. Milton and Nail (1996) extended this work by characterizing the low-temperature differential scanning calorimetry (DSC) thermogram of frozen aqueous solutions of nafcillin as well as the freeze-dried solid. Freeze-drying of frozen systems containing lyotropic mesophases appears to result in a unique X-ray diffractogram consisting of a single sharp peak at low angle (less than about 5° 2θ) in addition to the "halo" characteristic of amorphous solids. Herman *et al.* (1994) reported a similar X-ray powder diffraction pattern in methylprednisolone sodium succinate. The influence of liquid crystal formation during freezing on critical quality attributes of freeze-dried products is a subject which remains largely unexplored.

3.4.3. LIQUID–LIQUID PHASE SEPARATION DURING FREEZING

Crystallization of one or more solutes during freezing is the most extreme form of phase separation. However, another type of phase separation is possible: liquid–liquid phase separation into phases which are enriched in one component. This type of behavior has been reported for aqueous solutions of polymers such as poly(ethylene glycol)/dextran, PVP/dextran, and Ficol/dextran as well as polymer/phosphate buffer systems such as poly(ethylene glycol) (PEG)/potassium phosphate and PVP/sodium phosphate (Walter *et al.*, 1991). This type of behavior arises when enthalpically unfavorable interactions between solutes overcome the entropy gained by complete miscibility. A critical concentration of the solutes must be reached in order for the enthalpic contribution to be dominant. For example, the critical concentration of PEG 3350 and dextran T500 to form a two-phase system at 0°C is about 5% for each component (Heller *et al.*, 1997). Alternatively, phase separation has been proposed to arise because of differing effects of solutes on water structure (Zaslavsky *et al.*, 1989).

Izutsu *et al.* (1996) studied the effect of selected sugars and polymers on crystallization of PEG in frozen solutions by differential scanning calorimetry and pulsed NMR. Whereas some solutes are effective inhibitors of PEG crystallization, certain polymers, such as PVP and dextran, have virtually no influence on PEG crystallization. Izutsu and co-workers concluded that this is caused by phase separation between the incompatible polymers during freezing. Some possible consequences of liquid–liquid phase separation with respect to protein stability have been the subject of a recent review by Randolph (1997), and are discussed further below.

3.5. Effect of Freezing Rate on Recovery of Protein Activity

Many biochemists involved in protein purification hold the view that rapid freezing promotes high recovery of biological activity. There are scientifically sound reasons for this point of view, including minimization of buffer crystallization with resulting pH shifts, minimization of exposure of the protein to the high ionic strength which results from freeze concentration if salts are present, and reduction of the time during which cold denaturation could take place. Although the body of published literature on the subject is not large, there is growing evidence that rapid freezing adversely affects recovery of protein activity after freeze-thawing and freeze-drying, and may adversely affect the stability of the lyophilized solid during storage. Eckhardt *et al.* (1991) studied the effect of freezing rate (from 0.5 to about 50°C/min) on the formation of soluble and insoluble aggregates of human growth hormone using combinations of phosphate buffer and mannitol as an excipient system in the pH range of 7.4–7.8. Formation of soluble aggregates was not measureably affected by freezing rate; however, the effect of freezing rate on formation of insoluble aggregates (measured turbidimetrically) was dramatic, with considerably more aggregation at higher freezing rates. The effect of the excipient system was not clear in this study, except that formation of insoluble aggregates seemed to be promoted by a lower pH prior to freezing. pH of the system during freezing was not measured, and the effect of freeze-drying on aggregation was not studied.

Chang *et al.* (1996) reported that there is a strong correlation between the tendency of a protein to denature at surfaces and its tendency to denature during freezing, and their data support the conclusion that the ice–water interface can play a significant role in protein denaturation during freezing. Proteins examined were phosphofructokinase, lactate dehydrogenase, glutamate dehydrogenase, interleukin-1 receptor antagonist, tumor-necrosis-factor-binding protein, and ciliary neurotropic factor. Slow freezing

(3°C/hr) resulted in markedly less turbidity after freezing than dipping vials in liquid nitrogen (quench-freezing), presumably because the relative area of the ice–liquid interface is smaller after slow freezing. Furthermore, addition of 0.01% Tween 80 resulted in essentially complete protection of all of the model proteins after freeze-thawing, presumably because surfactants can compete with proteins for the available ice surface. These findings are consistent with a report by Strambini and Gabellieri (1996), where measurements of phosphorescence lifetime of tryptophan residues demonstrated that freezing is accompanied by loosening of the native fold and loss of secondary and tertiary structure. Although this process is largely reversible, a fraction of the protein molecules do not recover either the original phosphorescence or the original activity. Proteins examined were alcohol dehydrogenase, alkaline phosphatase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase, ribonuclease T_1, and apoazurin. Cryoprotectants such as sucrose and glycerol were found to reduce or eliminate the perturbation caused by freezing. Neither freezing rate effects nor surfactant effects were reported by Strambini and Gabellieri.

Jiang and Nail (1998) measured recovery of enzymatic activity of lactate dehydrogenase and β-galactosidase as a function of freezing rate, and found that rapid freezing of solutions of these proteins by placing vials in liquid nitrogen consistently resulted in decreased recovery of activity relative to slower freezing methods, and that this effect was more pronounced at low protein concentration. The effects of shelf freezing methodology were examined by comparing recovery of activity after (1) "slow" freezing by placing vials on the shelf of the freeze-dryer and slowly decreasing the shelf temperature at a rate of 0.5°C/min to −45°C versus (2) "fast" freezing by placing vials on shelves which had been precooled to −45°C. Recovery of activity of the model proteins was significantly higher when vials were placed on precooled shelves compared to the "slow" freezing method. Although further study is needed, these results are not necessarily inconsistent with faster freezing rates resulting in loss of protein integrity after freezing, because the degree of supercooling and, consequently, the effective freezing rate actually may be faster when more time is allowed for thermal equilibrium to be established throughout the volume of the supercooled solution.

Sarciaux *et al.* (1999) reported lower levels of insoluble aggregate in bovine IgG after freeze-drying following slow cooling relative to quench cooling in liquid nitrogen. Annealing of quench-cooled samples resulted in a significant reduction in the level of insoluble aggregates after freeze-drying, along with a corresponding decrease in the specific surface area of the freeze-dried solids. The data in this study are consistent with a mechanism for aggregation involving denaturation of the protein at the

ice/freeze-concentrate interface which is reversible upon freeze-thawing, but becomes irreversible after freeze-drying and reconstitution.

Although not widely investigated, freezing rate may affect the storage stability of freeze-dried protein formulations. Hsu *et al.* (1995) reported that the rate of increase in turbidity during storage of reconstituted formulations of recombinant tissue-type plasminogen activator is correlated with the specific surface area of the lyophilized solid. This finding was the result of investigation into the reason for the observation that fast freezing consistently produced a less stable product than slow freezing. Hsu *et al.* hypothesized that denaturation takes place at the solid–void interface during storage, which is not reversible upon reconstitution of the solid.

Co-workers

Milliqvist-Fureby and *et al.* (1999) used electron spectroscopy for chemical analysis (ESCA) to examine freeze-drying of proteins with various carbohydrates by measuring the surface composition of the amorphous solid. The proteins were found to be enriched at the solid surface relative to the bulk concentration of protein in the powders, and the mechanism was believed to be preferential adsorption of the proteins to the ice/freeze-concentrate interface, where the degree of surface accumulation depends on the carbohydrate used. The surface accumulation of protein was increased in annealed samples compared with nonannealed controls.

Regardless of the mechanism of loss of protein integrity after freezing and freeze-drying, the published literature points to the need to consider the thermal history of freezing when identifying critical process variables during validation studies.

4. MATERIAL CHARACTERIZATION

Minimizing empiricism in formulation and process development for freeze-dried products requires characterization of the formulation intended to be freeze-dried. The result of such characterization should be information on the upper product temperature limit during the primary drying stage of freeze-drying, knowledge of the physical state of the solute(s), and an assessment of the degree to which the characteristics of the frozen system are affected by changes in composition of the formulation. Characterization of the freezing behavior of a formulation is an important part of process validation for a freeze-dried product. Failure to generate this information systematically results in an approach to both formulation and process development which is strictly trial and error. Besides being inefficient, this approach has come under increased regulatory scrutiny, particularly since the advent of preapproval inspections for new pharmaceutical products. Characterization

of the formulation is also needed for process optimization. For the sake of this discussion, an optimized freeze-drying process is one in which freeze-drying is carried out in the minimum amount of time while meeting all specifications for quality attributes of the final product.

A frequently overlooked aspect of development of freeze-dried products is characterization of the freeze-dried product. This includes determination of the physical state of the dried product, that is, crystalline, partially crystalline, or amorphous. It may also include identification of the polymorph of a crystallizing component which exhibits polymorphism and determination of whether the crystal form observed is affected by changes in formulation and processing conditions. For amorphous systems, the glass transition temperature of the amorphous solid as well as the extent to which T_g changes with residual moisture may be critical attributes of the product with regard to both physical and chemical stability, both of which are discussed in more detail below.

Because the methods used for characterization of frozen systems and freeze-dried solids are similar, both will be discussed together. Aspects unique to either frozen systems or freeze-dried solids will be pointed out as needed.

4.1. Thermal Analysis

Changes in any material which accompany a change in temperature are almost always accompanied by absorption or release of energy in the form of heat. Thermal analysis is used to measure the temperatures at which these changes occur, determine whether the transitions are endothermic (absorption of heat) or exothermic (release of heat), and quantitate the amount of energy involved in the transition. Experimentally, a small quantity (typically 5–20 mg) of sample is placed in an aluminum pan and a lid is crimped into place. An empty pan and lid is generally used as a thermally inert reference. Subambient capability is needed for analysis of frozen solutions, which is accomplished either by using a mechanically refrigerated cooling accessory or by liquid nitrogen cooling.

There are two types of differential scanning calorimetry instruments. In *power compensation* DSC (for example, Perkin-Elmer, Norwalk, CT) the sample and reference pans are placed in isolated furnaces. The sample and reference are maintained at the same temperature, and the power required to do so is measured. In *heat flux* DSC (for example, TA Instruments, Newcastle, DE) the sample and reference are placed in the same furnace and heated from the same source. The temperature difference between the sample (T_s) and reference (T_r) is measured and converted to heat flow (ΔQ) using the relationship

$$\Delta Q = (T_s - T_r)/R_T \quad (1)$$

where R_T is the thermal resistance of the cell. In either case, the result of the experiment is a plot of heat flow versus temperature. Thermal transitions which are commonly observed in frozen systems are illustrated in Fig. 9, where, for the sake of this discussion, a deflection upward indicates an endothermic transition. The glass transition is seen as a shift in the baseline toward higher heat capacity. Crystallization during the DSC experiment is observed as an exothermic event, and eutectic melting is an endothermic transition which precedes melting of ice. The convention for assignment of onset temperature is the intersection of the extrapolated baseline with the tangent at the steepest slope of a transition. Glass transitions are commonly reported as either the onset temperature or the midpoint of the transition.

4.1.1. EXPERIMENTAL CONSIDERATIONS IN THERMAL ANALYSIS

Thermal analysis is a very valuable tool for development of freeze-dried formulations and processing conditions, yet it is too often regarded as a "quick and dirty" screening technique. As a result, valuable information about the system under study is too often lost. The development scientist

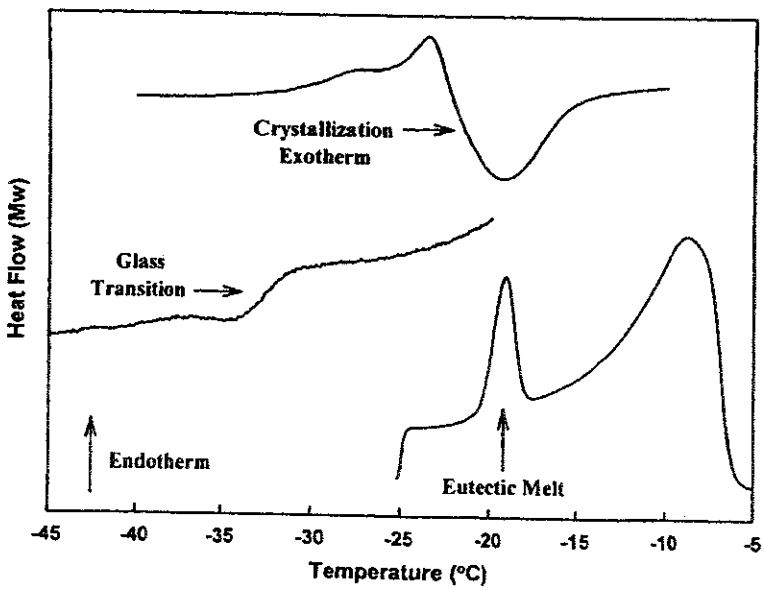

Figure 9. Typical thermal transitions in frozen solution, including eutectic melting, glass transitions, and crystallization exotherms.

should recognize that the results obtained by thermal analysis reflect the experimental conditions used for the measurement, and useful information can be gained by using more than one experimental condition, such as sample concentration, cooling rate, and heating rate.

A purge gas should always be used when carrying out a DSC experiment. For subambient thermal analysis, it is important to avoid condensation of atmospheric moisture between the sample and the surface of the sample well. The purge gas also helps to transfer heat from the sensor to the sample pan. Either helium or nitrogen is usually acceptable as purge gas. Nitrogen has a wider operating temperature range, and helium has a higher thermal conductivity and usually reduces baseline curvature relative to nitrogen.

The calorimeter must be calibrated for temperature measurement, and it is often desirable to calibrate for enthalpy measurement. Common temperature calibration standards for low-temperature operation are listed in Table III.

Liquid sample sizes of 10–20 mg are typical. Larger sample sizes are not necessarily better, because thicker samples will have a larger temperature gradient across the sample, which can decrease the sharpness of observed transitions. This is particularly important for temperature calibration.

For analysis of freeze-dried solids, sample sizes of 5–10 mg are typical. A larger mass of sample is not necessarily better, for the reason mentioned above. We have found preparation of a thin powder compact using a punch-and-die with a diameter slightly smaller than the inside diameter of the sample pan to be useful for maximizing the quantity of material in the sample pan while minimizing sample thickness. A representative thermogram of freeze-dried maltodextrin as a powder compact versus the same quantity of sample as a loose powder is shown in Fig. 10, where the glass transition region is better defined when the sample is presented as a powder compact.

Because aqueous systems are prone to supercooling, the most common practice is to record the thermogram during the heating cycle after freezing of

Table III
Common Standards for Thermal Analysis

Material	Melting temperature (°C)	Heat of fusion (J/g)
Indium	156.6	28.71
Mercury	−38.8	11.44
n-Octane	−56.7	182
n-Decane	−29.6	202.1
n-Dodecane	−9.7	216.7

the sample. This avoids supercooling-related artifacts. However, the cooling rate of an aqueous solution can have a significant influence on the thermogram recorded during subsequent heating of the sample, particularly for solutes which tend to crystallize during the time course of freezing. The term "critical cooling rate" usually refers to a cooling rate above which crystallization of solute during freezing is prevented. For example, at cooling rates less than about 2°C/min, crystallization of mannitol from a freezing solution takes place during the cooling cycle, whereas, at faster cooling rates, crystallization is inhibited. In this case, complex thermal behavior is observed in the subsequent heating thermogram, which consists of a glass transition followed by a melting endotherm and an exotherm indicating crystallization of the solute (Her and Nail, 1994).

Heating rates used for thermal analysis are typically 5–10°C/min, but it is often useful to carry out experiments at lower rates in order to maximize resolution of thermal events taking place at nearly the same temperature. The choice of a heating rate for conventional DSC involves a trade-off between sensitivity and resolution, where sensitivity refers to the ability to detect a given transition, and resolution is the ability to separate two thermal events taking place at nearly the same temperature. In thermal analysis of frozen systems, particularly those in which the solute crystallizes, resolution is often a more important issue, because the eutectic melting temperature may be very close to the melting temperature of ice. An example of the use of slow heating rates to maximize resolution is shown in

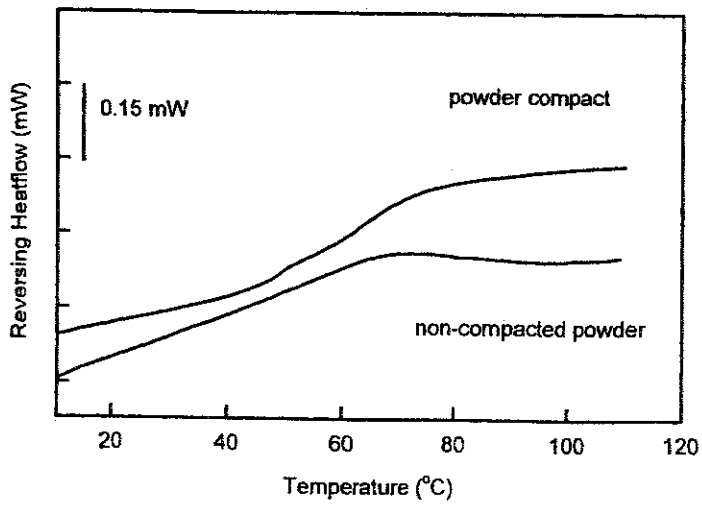

Figure 10. DSC trace of a freeze-dried maltodextrin using a loose powder versus a powder compact.

Fig. 11 for the glycine–water system. In this case, three thermal events—readily apparent at a very slow heating rate—could be interpreted as simply the melting of ice if only rapid heating rates are used. In the glycine–water system, the complex thermal behavior in the region of the melting endotherm of ice arises from the polymorphism of glycine (Chongprasert et al., 2004).

For detection of glass transitions in both frozen systems and freeze-dried solids, sensitivity may be more important than resolution, because glass transitions are often difficult to detect. In this case, relatively rapid heating rates are recommended. However, it should be remembered that heating rate influences the observed glass transition temperature, where faster heating rates cause observation of the transition at higher temperatures. For example, for a frozen solution of 10% dextran, the observed

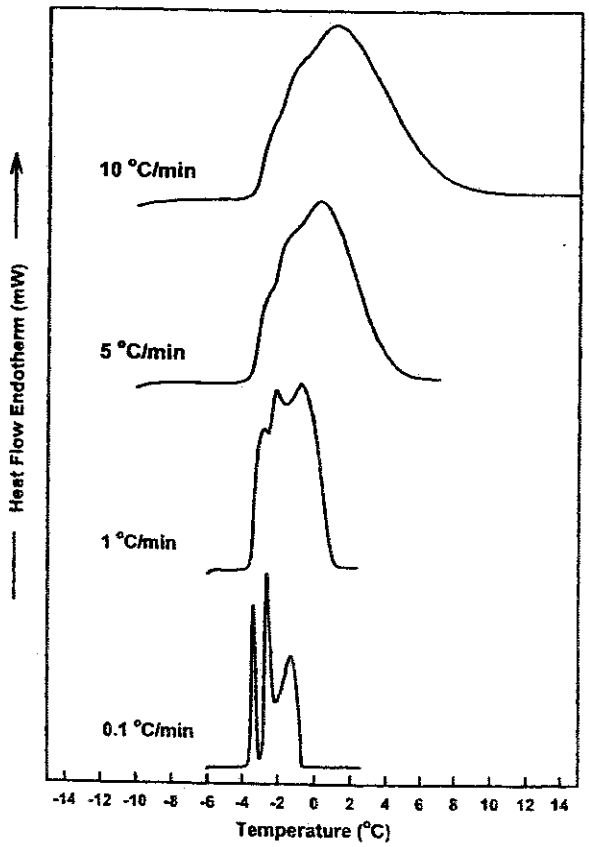

Figure 11. DSC trace of frozen glycine solution at different heating rates in the region of ice melting.

midpoint of the glass transition increases from about −13.5°C to about −9.5°C as the heating rate is increased from 2°C/min to 20°C/min.

Another reason to use more than one heating rate when characterizing frozen systems by DSC is that, depending on the heating rate, a solute may crystallize during the DSC experiment. This is illustrated in Fig. 12 for a sucrose/glycine system containing 10% (w/v) total solids at a 1:1 weight ratio of sucrose to glycine. At the fastest heating rate, the only notable feature of the thermogram other than melting of ice is the glass transition region in the temperature range of about −45°C to about −50°C. At slower heating rates, an exotherm arising from crystallization of glycine is observed (note also that the glass transition region is not apparent at very slow heating rates). This is important information, given that the time scales of thermal analysis and freeze-drying may be more than an order of magnitude different. Using a too-rapid heating rate can result in misleading information regarding the physical state of the formulation after freeze-drying.

For thermal analysis of frozen solutions, it may be useful to increase the solute concentration above the intended formulation concentration in order to maximize sensitivity, particularly for glass transitions. As the state diagram predicts, the glass transition temperature is essentially independent of solute concentration. Her and Nail (1994) demonstrated this effect for solutions of lactose and related compounds.

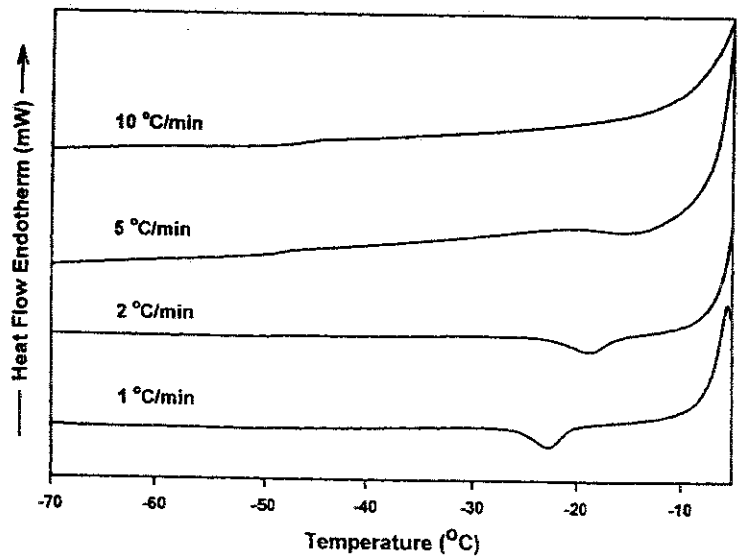

Figure 12. DSC thermograms of sucrose:glycine (7:3 weight ratio) at different heating rates.

4.1.2. SOURCES OF UNCERTAINTY IN INTERPRETATION OF DSC THERMOGRAMS

Although the transitions illustrated in Fig. 9 are easily interpreted, uncertainties often arise. For many organic compounds which crystallize from freezing aqueous solution, the eutectic melt occurs very close to the melting temperature of ice. In this case, the eutectic melting endotherm may be partially, or completely, obscured by the ice melting endotherm even at very slow heating rates. Examples are the ice/mannitol and ice/dibasic sodium phosphate eutectic melts at about −1°C and −0.5°C, respectively. Although resolution of such closely spaced endotherms is enhanced by slow heating rates, this is by no means assured.

Glass transitions, both in frozen systems and freeze-dried solids, can be difficult to detect. This may be caused by the small heat capacity change associated with the glass transition, a broad glass transition region, or both. Interpretation is made more uncertain by baseline drift or other noise. In addition, other thermal events at temperatures close to the glass transition, such as enthalpy recovery or crystallization, may disguise the heat capacity change associated with the glass transition. Sensitivity to such transitions is enhanced by rapid heating rates.

Thermal analysis of frozen solutions is a relatively small subset of the thermal analysis literature, most of which is directed toward thermal analysis of polymer systems. As a result, some phenomena which are commonly observed are poorly understood, including lyotropic liquid crystal formation during freezing and apparent multiple glass transitions in frozen systems. A pharmaceutically important and relatively widely studied example of uncertainty in interpretation of a DSC thermogram is the system sucrose/water, illustrated for six different concentrations of sucrose in Fig. 13. The glass transition which is commonly reported, illustrated by the thermogram of 5% sucrose, is in the range of −32°C to −35°C. Examination of higher concentrations, however, reveals another transition at a lower temperature. This apparent multiple glass transition has given rise to uncertainty regarding both the physical significance of both of these transitions and assignment of the T_g' temperature for the purpose of characterizing frozen solutions of amorphous solutes. Other investigators have reported results similar to the data in Fig. 13 for sucrose and related solutes, and the bulk of the evidence supports the conclusion that the lower temperature transition is the glass transition of the maximally freeze concentrated solution. Ablett *et al.* (1992a) reported that, in sucrose and glycerol solutions which are sufficiently concentrated that ice formation is inhibited (bottom thermogram in Fig. 13), a single glass transition is observed at the lower temperature. It is

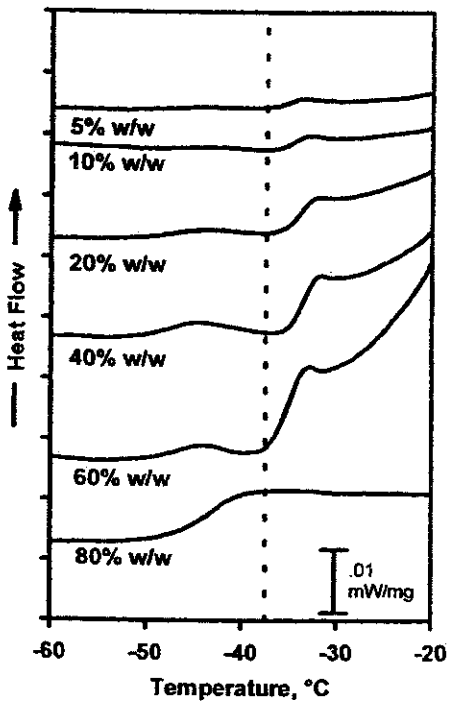

Figure 13. DSC thermograms of sucrose solutions in the glass transition region.

only when ice is present that the complex transition is observed. Ablett and co-workers argue convincingly that the higher temperature transition is not a glass transition at all, because (1) maximum freeze concentration was observed at −40°C, (2) the heat capacity change at the higher temperature transition is well in excess of the heat capacity change for totally vitrified samples, and (3) the higher temperature transition is considerably narrower than the glass transition region for totally vitrified samples. The physical significance of the second transition is interpreted by Ablett and co-workers as the temperature at which the mobility of the solute has increased to the point where ice dissolution can begin. Shalaev and Franks (1995) also reported a double transition in frozen solutions of sucrose/water and fructose/water. The lower temperature transition is attributed to the glass transition, whereas the higher transition is interpreted as a softening process. Izutsu *et al.* (1996) also refer to the higher temperature transition as a softening temperature, T_s, which is distinct from the "real" glass transition temperature. The physical significance of the higher temperature transition is attributed to a relaxation process between ice crystals and the freeze-concentrated solute phase.

From the formulation scientist's point of view, it is important to know which of these transitions, if either, is predictive of collapse in freeze-drying. Although a comprehensive answer is not available yet, a study by Knopp *et al.* (1998), using both thermal analysis and microscopic observation during freeze-drying of sucrose solutions, concluded that the onset of collapse occurs approximately midway between the end of the lower temperature transition and the onset of the higher temperature transition. Since the higher temperature transition is usually the only "glass transition" observed in thermal analysis of disaccharide-containing protein formulations, this transition is generally accepted as the thermal event that is predictive of collapse, and it is often very close to the observed collapse temperature. However, more data are needed for a wide variety of solutes in order to better determine the degree to which the higher temperature transition is predicitive of collapse during freeze-drying.

THIS is denoted by the vertical dashed line in Figure 13.

Another source of uncertainty in thermal analysis of frozen systems relates to glass transition temperatures of protein solutions. When the concentration of protein is low, the freeze-drying characteristics of the formulation are generally determined by added solutes, or excipients. When the protein is the predominant solute, as in the case of some immunoglobulin preparations, for example, it is common to observe no transitions other than the melting of ice. Although the reason for this is not clear, possible explanations include the following: (1) The transition temperature is too close to the onset of ice melting for the two events to be resolved, (2) the heat capacity change at the transition is too small to be measured by the instrumentation, or (3) the transition region is so broad that the transition is "lost" in baseline drift or noise in the thermal analysis experiment. This behavior is probably related to the type of glass formed, that is, strong versus fragile, which is discussed below. In any event, other methods are often needed in order to adequately characterize the system.

4.1.3. MODULATED-TEMPERATURE DSC

A recent advance in thermal analysis, first described by Reading *et al.* (1994), that is finding application in pharmaceutical research and development is modulated-temperature DSC (MTDSC). Briefly, the total heat flow observed in conventional DSC consists of the sum of two components, a heat capacity component, $C_p(dT/dt)$ and a kinetic component $f(T, t)$:

$$dH/dt = C_p(dT/dt) + f(T, t) \quad (1)$$

where the heat capacity component is heating-rate-dependent and the kinetic component is a function of time at constant temperature. In MTDSC a sine wave modulation is superimposed on the underlying linear temperature program, where the amplitude and the frequency of the modulation are controllable. A discrete Fourier transform algorithm is applied to the resulting sample response to deconvolute the signal into a heating-rate-dependent, or reversing, component and a time-dependent, or nonreversing, component. Examples of reversing transitions are the glass transition and some melting phenomena. Nonreversing transitions include enthalpy recovery, evaporation, crystallization, decomposition, curing of polymers, and some melting phenomena. Melting can be observed in both components to differing degrees, depending upon experimental conditions.

MTDSC offers a solution to the trade-off between sensitivity and resolution by providing, in principle, both the high resolution provided by a slow underlying heating rate and the high sensitivity provided by a high *instantaneous* heating rate from the modulation. Other advantages include the possibility of separating overlapping thermal phenomena, detection of metastable melting phenomena, and greater ease of measuring heat capacity. We have observed that conventional DSC is more suitable for detection of glass transitions in frozen solutions, because the upper limit of the heating rate for modulated DSC is about 3°C/min. However, modulated DSC appears to provide a significant improvement over conventional DSC for analysis of freeze-dried amorphous solids, where detection of the glass transition is often uncertain due to overlapping thermal events. An example is shown by the thermogram in Fig. 14 for a freeze-dried maltodextrin, where the dominant feature of the total heat flow thermogram is the enthalpy recovery endotherm, which is superimposed on the glass transition region.

Using modulated DSC, an experimental technique referred to as stepwise isothermal heat capacity measurement may provide added enhancement of sensitivity for glass transition measurement. With scanning DSC, even in the modulated mode, detection of glass transitions becomes more difficult as the glass transition region becomes broader and the heat capacity change at the glass transition becomes smaller. This is illustrated in Fig. 15 for maltose and a series of maltodextrins of increasing molecular weight. Note that the glass transition region is uncertain in curves *C* and *D*. In stepwise isothermal heat capacity measurement, the sample is held at a constant underlying temperature while the temperature is modulated for a period of about 5 to as much as 15 min. This allows calculation of the heat capacity of the sample. The temperature is then increased by perhaps 1°C, and the experiment is repeated. The use of stepwise isothermal heat capacity measurement is shown in Fig. 16 for a maltodextrin with an average molecular weight of 3600 after equilibration at 23% and 11% relative

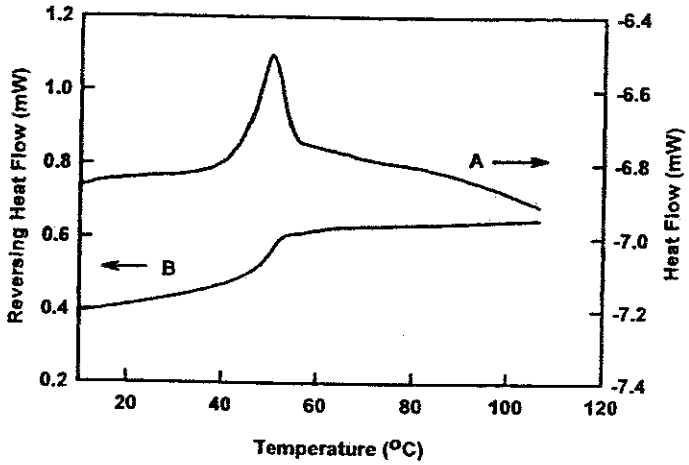

Figure 14. DSC thermograms of freeze-dried maltodextrin showing total heat flow (A) versus reversing heat flow (B) in the glass transition region.

humidity. The heat capacity change over the relatively broad glass transition region is apparent in both thermograms. Although stepwise isothermal heat capacity measurements are time-consuming, this technique appears to offer

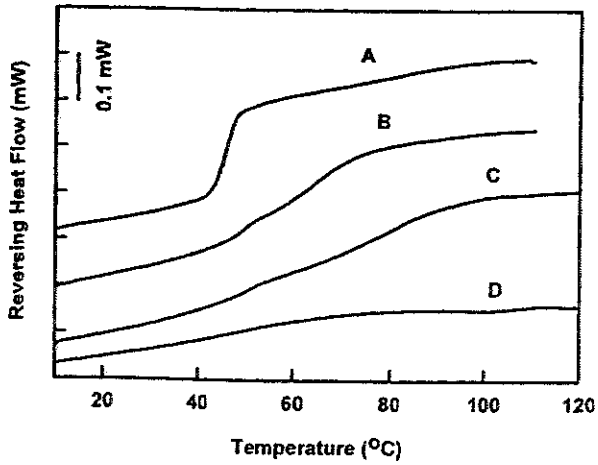

Figure 15. DSC thermograms of maltose and different molecular weights of freeze-dried maltodextrins in the glass transition region. As molecular weight increases, the heat capacity change at the glass transition decreases and the width of the glass transition increases. (A) Maltose; (B) maltodextrin, MW 900; (C) maltodextrin, MW 1800; (D) maltodextrin, MW 3600.

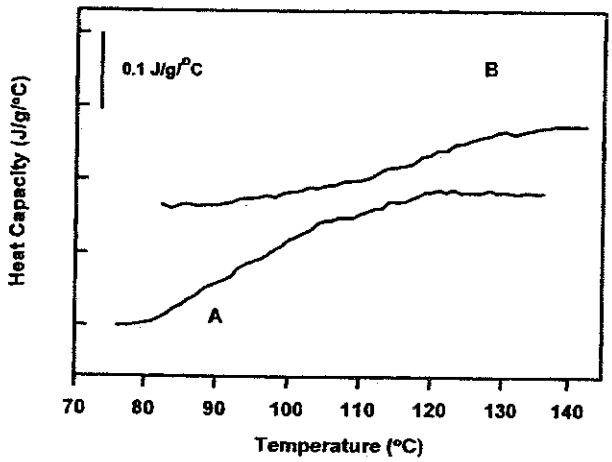

Figure 16. DSC thermograms of maltodextrin (MW 3600) in the glass transition region by stepwise isothermal heat capacity measurement. (A) Equilibrated at 23% relative humidity; (B) equilibrated at 11% relative humidity.

enhanced sensitivity for glass transition temperature measurement relative to scanning calorimetry.

4.2. Optical Microscopy

Optical microscopy using a special low-temperature stage which can be evacuated to operate at pressures representative of freeze-drying allows direct observation of materials during freezing and freeze-drying, generally under a low magnification of 50–100×. The technique is not new. Rey (1957) was perhaps the first to describe a cryomicroscope, to which he later added vacuum capability. MacKenzie (1964) later built a microscopy system suitable for the study of freeze-drying. Other devices for freeze-drying microscopy were subsequently described, and the designs varied widely (Flink and Gejl-Hansen, 1978; Flink *et al.*, 1973; Freedman *et al.*, 1972; Kochs *et al.*, 1989; Pikal *et al.*, 1983). However, growing interest in the technique has resulted in at least two types of commercially available instruments. One type uses a two-stage Peltier cooling device (Physi-Temp Instruments, Clifton, NJ) capable of cooling the top plate of the device to about −50°C. A second type of freeze-drying stage uses liquid nitrogen vapor circulated through a silver block along with a heater to control the surface temperature (Linkam Scientific Instruments, Tadworth, Surrey, UK). This allows cooling the sample to well below −150°C. Both instruments (see Fig. 17) offer the ability

to program the temperature profile, which allows studies such as the effect of thermal history of freezing as well as annealing on the morphology of the frozen and freeze-dried material.

It is important in freeze-drying microscopy to avoid vertical temperature gradients, because it is not practical to measure the sample temperature by placing a thermocouple directly in the sample. Calibration of temperature measurement is easily done by comparing observed transitions of standard materials with ~~measured~~ temperatures. In addition to the standards used for DSC listed above, eutectic melting temperatures for frozen aqueous solutions of inorganic salts can also be used, such as ice/NaCl (−21.5°C), ice/KCl (−11.1°C), ice/NaBr (−28.0°C), and ice/NaI (−31.1°C). Observed transitions should be within about 0.5°C of published values for standards. Temperature calibration should be checked both at atmospheric pressure and under vacuum. Temperature accuracy may be improved by using a heat sink compound to improve thermal contact between the sample and the surface of the stage.

In order to minimize vertical temperature gradients in the system, it is not appropriate to use traditional microscope slides. Cover slips should be used instead. Some investigators use quartz cover slips in order to further reduce vertical temperature gradients, because the thermal conductivity of quartz is significantly higher than that of glass. A disadvantage of quartz cover slips is the expense and fragility of the material.

Experimentally, freeze-drying microscopy is usually carried out by placing a small quantity, perhaps 10 µl, of sample on a cover slip, freezing the sample, evacuating the system, then systematically varying the sample temperature and observing the resulting morphology in the dried material. This technique is particularly useful for measuring the *collapse* temperature in freeze-drying, which may be different from glass transition temperatures measured by thermal analysis. Glass transitions are measured on closed systems, whereas collapse is a dynamic phenomenon taking place during freeze-drying. An example of collapse phenomena is shown in the photomicrograph in Fig. 18a. In the authors' experience, the correlation between collapse observed microscopically and collapse in a vial is within a few degrees Celsius, although several exceptions have been observed.

Freeze-dry microscopy is useful for examining the influence of formulation composition and freezing history on the morphology of ice. Dendritic ice is normally observed (see Fig. 18b); however, at high solute concentrations, spherulitic ice can form (Fig. 18c). The significance of ice morphology is that, when dendritic ice forms, open channels are created by prior sublimation of ice, providing a relatively low resistance to transfer of water vapor through the partially dried solids. Spherulitic ice, on the other

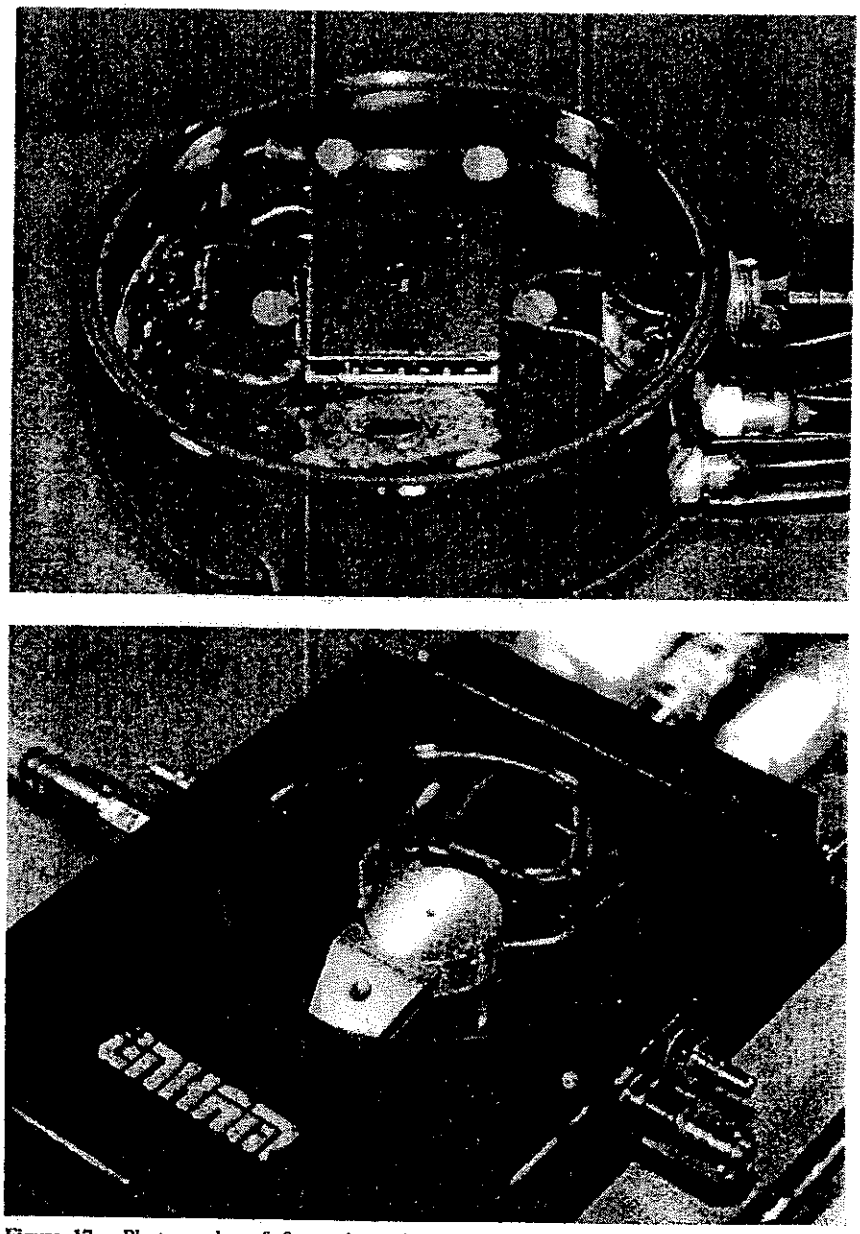

Figure 17. Photographs of freeze-dry microscopy stages. Top: PhysiTemp Instruments (Clifton, NJ); bottom: Linkam Scientific Instruments (Tadworth, Surrey, UK).

hand, is associated with a high resistance to mass transfer and inefficient freeze-drying.

Another application of freeze-drying microscopy is for observation of secondary, or solute, crystallization during freezing. Crystallization of nafcillin during annealing is an example, illustrated in Fig. 18d. Crystallization

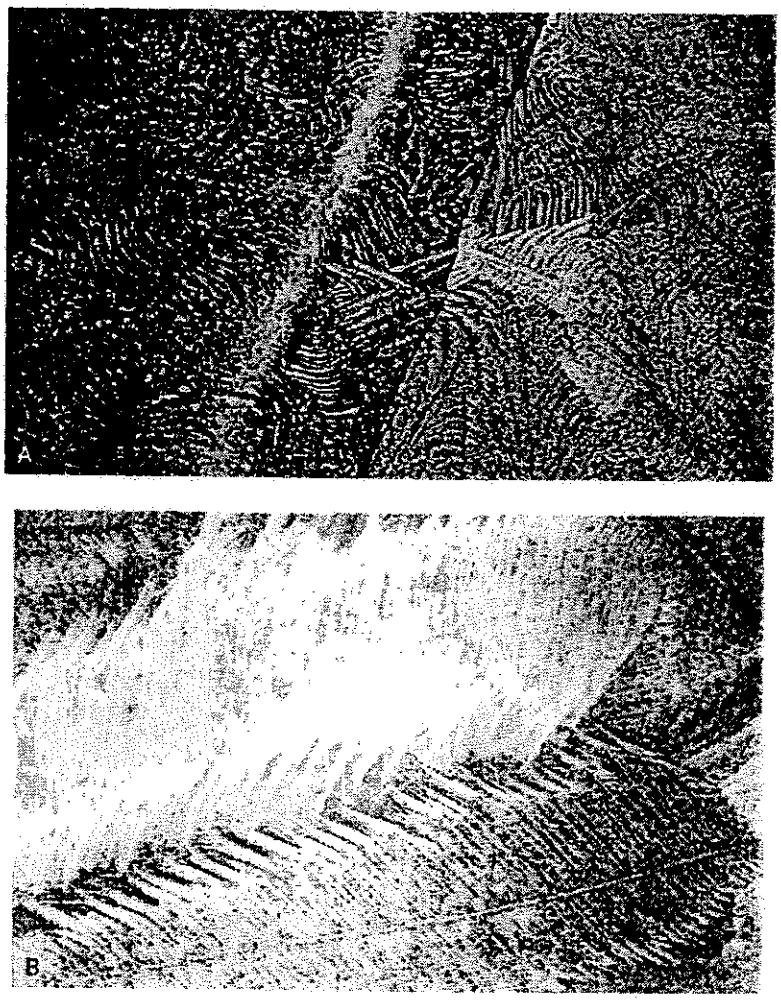

Figure 18. Freeze-drying photomicrographs illustrating (A) collapse versus retention behavior of recombinant human hemoglobin, (B) ~~spherulitic~~ ice morphology ~~at high solute concentration~~, (C) ~~dendritic~~ ice morphology, (D) crystallization of nafcillin sodium during annealing, and (E) liquid crystalline behavior of methylprednisolone sodium succinate.

dendritic

Spherulitic

at high solute concentration

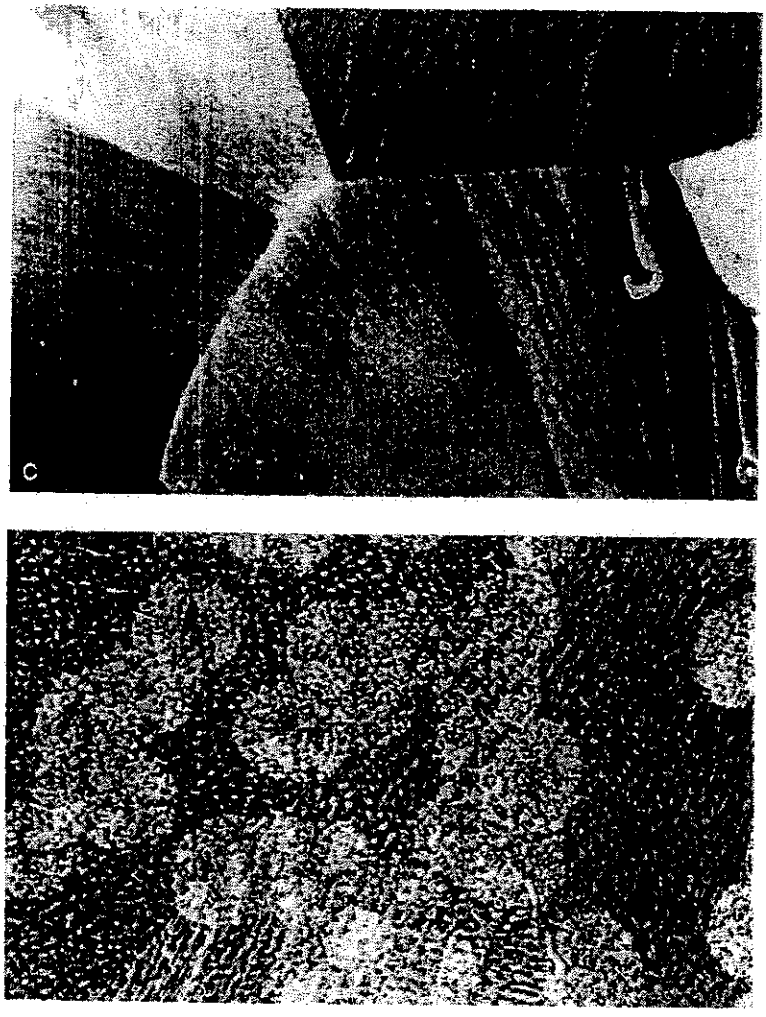

Figure 18. *(continued)*

of a solute may be essential in order to form a pharmaceutically acceptable cake. Crystallization is of particular interest for glycine, a commonly used formulation excipient, because the glass transition of amorphous glycine/water is well below the temperature range available in a commercial freeze-dryer, whereas the eutectic melting temperature of glycine/ice is about −3.6°C.

Eutectic melting and collapse phenomena are usually easy to observe microscopically and are the most common types of freeze-drying behavior.

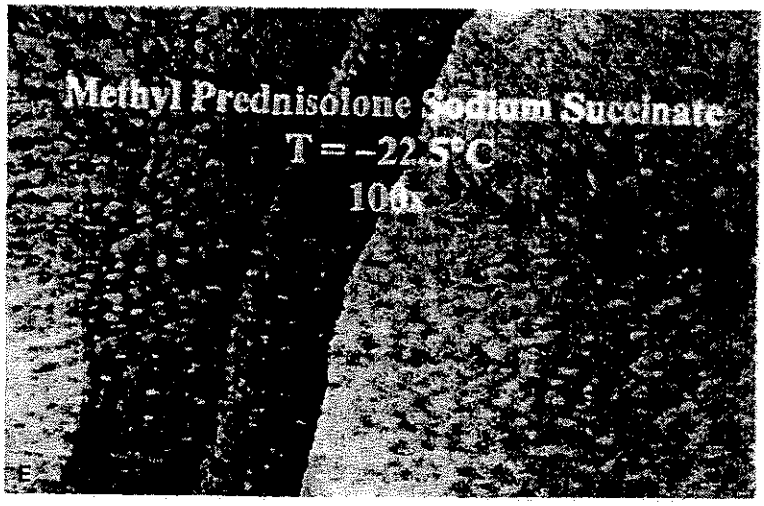

Figure 18. *(continued)*

However, another type of freeze-drying behavior, illustrated in for methylprednisolone sodium succinate in Fig. 18e, is not uncommon, yet is not well recognized. The freeze-dried material is highly birefringent, indicating order in at least two dimensions; however, this material appears to collapse as the product temperature exceeds a certain temperature. Microscopic observation suggests a crystalline material that collapses, which is not realistic for materials that are crystalline in the traditional sense. It is more likely that these compounds are examples of lyotropic liquid crystalline behavior. The significance of this behavior is that it makes determination of the maximum allowable operating temperature during primary drying uncertain. As stated above, the influence of lyotropic liquid crystal formation in freeze-drying on critical product attributes is not well understood.

4.3. Scanning Electron Microscopy

Scanning electron microscopy (SEM), either conventional SEM or environmental SEM, can be particularly useful in characterization of freeze-dried solids. Two examples are illustrated in Fig. 19. Figures 19A and 19B are examples of two different polymorphs of pentamidine isethionate formed under different freezing conditions (Chongprasert *et al.*, 1998). Figures 19C and 19D illustrate an amorphous material (lactose) dried under conditions of retention of microstructure (Fig. 19C) and after the onset of

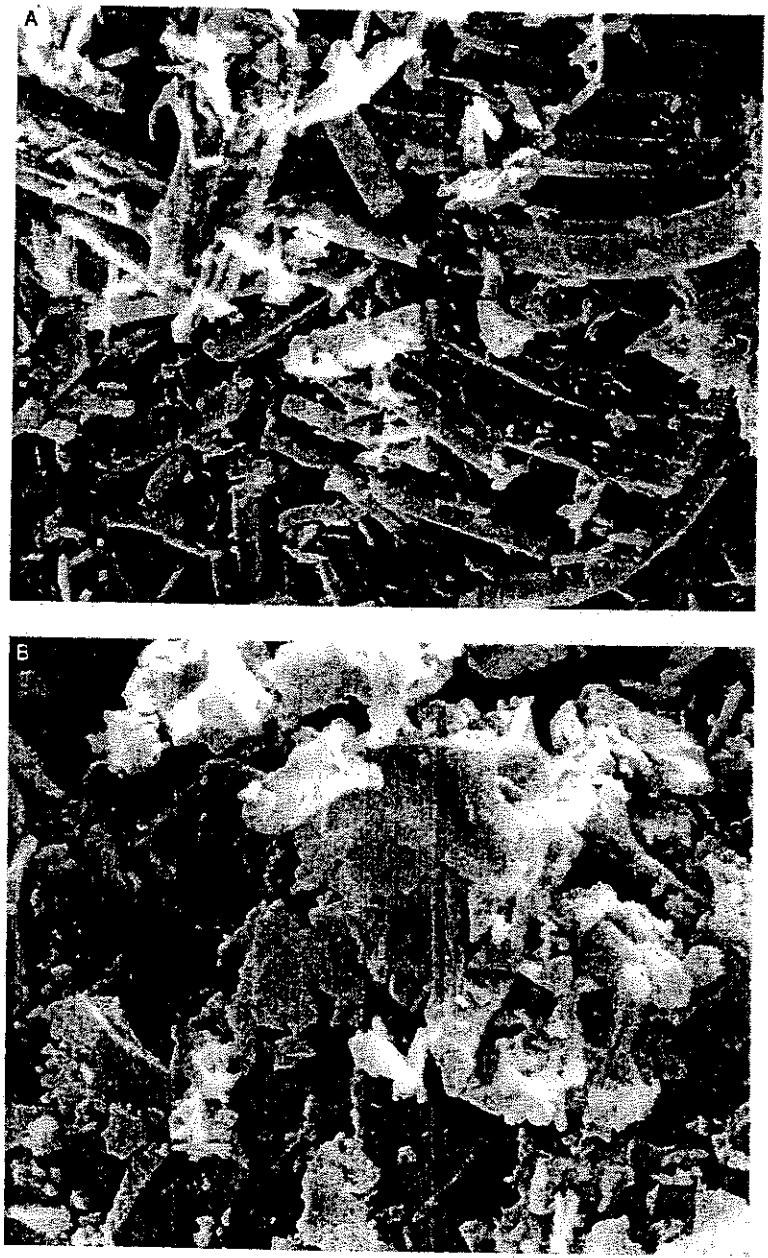

Figure 19. SEM photomicrographs illustrating different polymorphs of pentamidine isethionate (A, B) observed after different cooling histories and freeze-drying with retention (C) versus freeze-drying at the onset of structural collapse (D) of lactose.

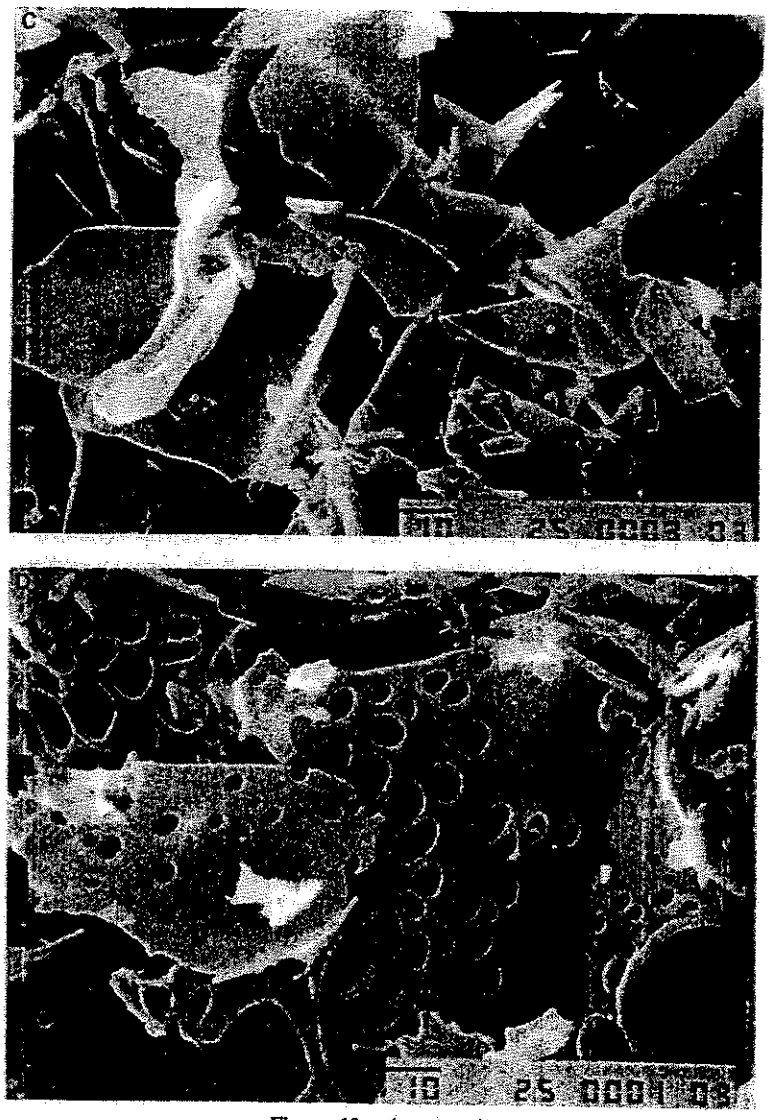

Figure 19. *(continued).*

collapse (Fig. 19D). The characteristic "Swiss cheese" morphology can be observed before macroscopic collapse is apparent macroscopically in the freeze-dried solid, and is associated with decreased resistance of the dried

material to flow of water vapor during the primary drying phase of freeze-drying (Milton *et al.*, 1997).

4.4. X-Ray Powder Diffraction

X-ray powder diffraction is the method of choice for characterization of the physical state of a freeze-dried solid. Documenting the physical state of a product both immediately after freeze-drying and periodically during stability studies is often overlooked during product and process development, but it can provide valuable insight into the processes underlying physical or chemical stability problems.

For protein formulations requiring a lyoprotectant such as a disaccharide, the freeze-dried solid will be at least partially amorphous, because it is generally accepted that such lyoprotectants must be amorphous in order to be effective. However, it is common practice to include a crystallizing component such as glycine or mannitol in order to promote a physically stable freeze-dried cake, that is, one that retains the size and shape of the original frozen solution (see stability discussion below) throughout the shelf life of the product. A particular concern is crystallization of a component of the formulation from the solid state during storage, illustrated for a mannitol-containing formulation in Fig. 20. This can lead to a redistribution of water within the solid, and the increased water activity in the microenvironment of the drug can lead to increased rates of degradation either through the participation of water as a

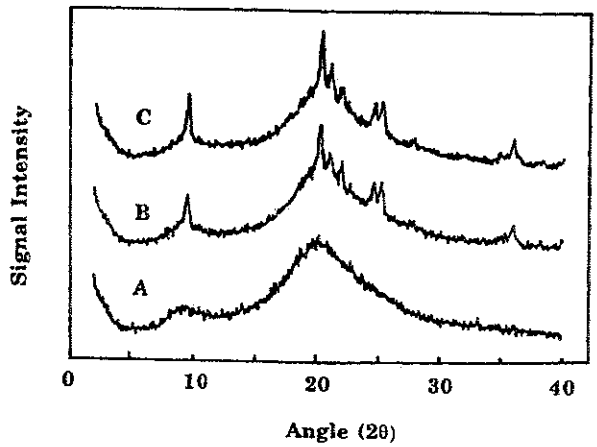

Figure 20. X-ray powder diffractograms showing crystallization of mannitol during storage at 40°C. (A) Initial; (B) 3 weeks of storage; (C) 5 weeks of storage.

Table IV
Characteristic Amide I Absorption Frequencies for Common Secondary Structures[a]

Structure	Amide I Frequency (cm^{-1})
Antiparallel β sheet/aggregated strands	1675–1695
3_{10} helix	1660–1670
α-helix	1648–1660
Unordered	1640–1648
β Sheet	1625–1640
Aggregated strands	1610–1628

[a] From Jackson and Mantsch (1995).

Move Table IV to next page

reactant or through the effect of water on glass transition-associated mobility (Herman *et al.*, 1994).

Polymorphs of crystalline materials have different physical properties, such as differences in melting point, dissolution rate, and solubility. As mentioned above, the influence of polymorphism of both drugs and excipients that crystallize during freezing or during subsequent annealing processes (see below) has not been investigated extensively, but there is a large enough body of literature to support the idea that formulation scientists should be aware of the effects of seemingly subtle changes in formulation and processing conditions on the crystal form in the freeze-dried solid. Kim *et al.* (1998) demonstrated that different polymorphs of mannitol form during freezing depending both on the initial concentration of mannitol and the rate of freezing. Chongprasert *et al.* (1998) studied the effects of freezing conditions on crystallization of pentamidine isethionate and established that at least two polymorphs are observed in the freeze-dried solid, depending on the thermal history of freezing. In this example, different photostability is observed in the freeze-dried solid for product frozen under different conditions.

X-ray powder diffraction can also be used to study crystallization processes directly in frozen systems by using a low-temperature stage. Cavatur and Suryanarayanan (1998) applied this technique to the *in situ* crystallization of nafcillin sodium and sodium phosphate dodecahydrate from frozen solutions.

For a comprehensive treatment of the theory and practice of X-ray diffraction, the reader is referred to Klug and Alexander (1976).

4.5. Infrared Spectroscopy

It has been known ~~because~~ since the early 1950s that a correlation exists between absorption frequencies in the amide regions of the infrared

spectrum of proteins and the predominant secondary structural motif (α helix, β sheet, and so forth) of proteins as determined by other methods (Elliot and Ambrose, 1950). Advances in instrumentation and data processing software as well as the relative ease of experimental procedures, small quantity of protein required (about 100 μg), and moderate cost of the instrumentation have spurred interest in the application of infrared spectroscopy to characterization of freezing and drying effects on protein structure.

The amide I absorption, from about 1600 to about 1700 cm^{-1}, arises primarily (about 80%) from C=O stretching, with a smaller contribution (about 20%) from C–N stretching. The amide II absorption (about 1600–1500 cm^{-1}) is due to both N–H bending vibrations and C–N stretching vibrations in roughly equal proportions, and the amide III region (about 1330–1220 cm^{-1}) consists of C–N stretching vibrations coupled to N–H in-plane bending vibrations with contributions from C–C stretching and C=O in-plane bending (Jackson and Mantsch, 1995). Several other amide absorption regions are discussed by Dong *et al.* (1995). Most investigators using IR spectroscopy to examine protein structure use the amide I region, probably because most of the absorption arises from one functional group. However, Costantino *et al.* (1996a, 1996b, 1997a) have used the amide III region in several studies, pointing out that, unlike the amide I frequency range, water absorption does not interfere in the amide III region.

The utility of infrared spectroscopy for the study of protein structure results from the fact that each type of secondary structure has a characteristic hydrogen bonding pattern between the amide C=O and N–H groups. The strength of hydrogen bonding influences the absorption frequency, where stronger hydrogen bonding causes decreased electron density around the carbonyl group, resulting in absorption at a lower frequency than for a carbonyl group which is not hydrogen-bonded. Table IV summarizes approximate frequency assignments for different secondary structures.

Of course, proteins generally contain more than one type of secondary structure, where the width of individual absorptions is greater that the separation between bands, often giving rise to featureless spectra in the range of interest. Several techniques for processing of the data in order to enhance resolution are available, including Fourier self-deconvolution and calculation of derivative spectra. A detailed discussion of these techniques is beyond the scope of this chapter, and the reader is referred to reviews by Jackson and Mantsch (1995), Surewicz *et al.* (1993), and Dong *et al.* (1995). All of these reviews advise caution when attempting to apply FTIR quantitatively to determine the amounts of different types of secondary structure within a protein. Most pharmaceutically relevant

studies use FTIR to compare the effects of different stabilizers on the spectrum of the dried protein relative to the same protein in aqueous solution. In this case, calculation of a spectral correlation coefficient between second derivative spectra has been reported (Dong *et al.*, 1995; Prestrelski *et al.*, 1995), which provides a quantitative estimate of the relative effects of formulation and processing parameters on protein structure.

The O–H bending absorption of water at 1644 cm^{-1} will obscure the amide I region of the spectrum. Most modern instruments have software for digital subtraction of the water or buffer spectrum from that of a protein solution. However, problems associated with the high molar absorptivity of water can cause problems with detector linearity. As a result, very short pathlength cells (not more than about 10 μm) are required for aqueous solutions of proteins, which means that relatively high concentrations of protein are needed (10–50 mg/ml). Alternatively, the protein can be dissolved in D_2O, because the O–D bending absorption is shifted to a frequency below the amide I region. Longer pathlength cells and lower concentrations of protein can then be used. Deuterons will exchange with the amide nitrogen, but this phenomenon can be exploited to gather information concerning the degree of solvent exposure within secondary structures. Residual water in dried proteins does not seem to be a significant source of interference in second derivative spectra up to about 10% residual water (Dong *et al.*, 1995), because the water absorbance is broad relative to the protein absorbances. This reduces the intensity of water absorbance in the second derivative spectra.

The flexibility of infrared spectroscopy with regard to the physical state of the sample makes it well suited to the characterization of freeze-dried proteins. In addition to examining the protein as a solution in water or D_2O, solutions in organic solvents can be used as well as colloidal dispersions and dried solids, either as a powder or as a film. Typically, freeze-dried powders are pressed into KBr pellets. General features of drying-induced changes in the amide I region include broadening of individual bands as a result of a general disordering of secondary structure (Prestrelski *et al.*, 1994). However, changes between the spectrum of a dried protein relative to the same protein in solution are very protein-specific. In general, drying-induced changes fall into three broad categories: (1) essentially no change in the spectrum upon drying, (2) conformational changes which are restored to the original solution conformation upon rehydration, and (3) conformational changes which are not restored upon hydration. A new band near 1615 cm^{-1} is commonly observed upon drying, which is consistent with intra- or intermolecular β sheets resulting from unfolding and aggregation (Prestrelski *et al.*, 1994). Prestrelski *et al.* (1995) studied the influence of formulation pH

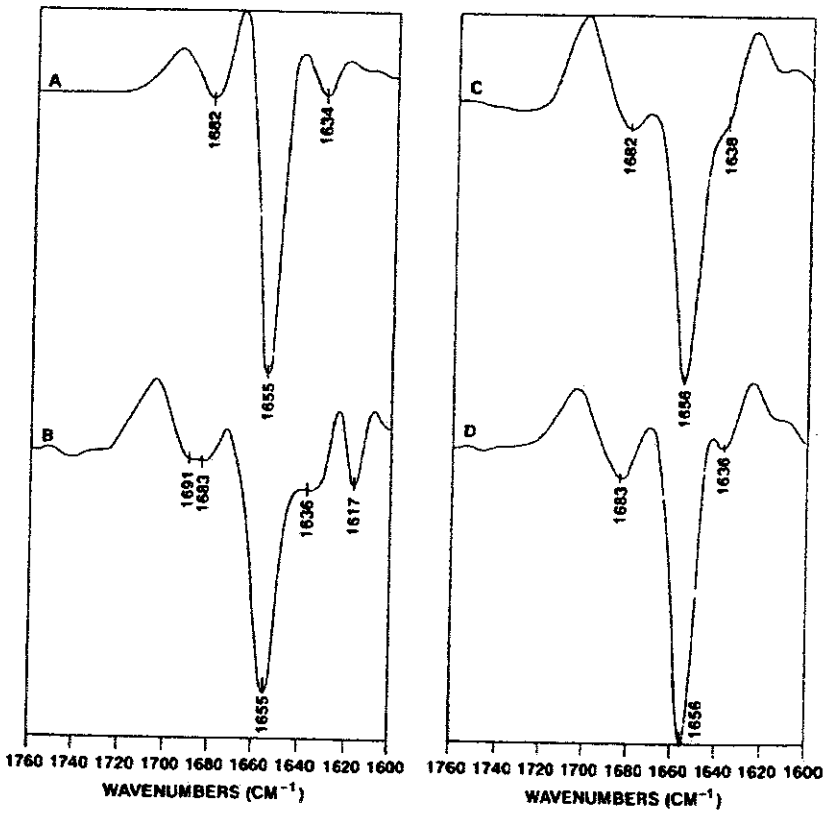

Figure 21. FTIR spectra of freeze-dried interleukin-2: (A) aqueous solution, pH 5; (B) lyophilized, pH 7; (C) lyophilized, pH 6; (D) lyophilized, pH 5. Reproduced with permission from Prestrelski *et al.* (1995). Copyright 1995 Plenum Press.

on lyophilization-induced secondary structural changes and on the solid-state stability of freeze-dried interleukin-2 (IL-2). Drying from a pH 7 solution resulted in significant changes in the spectrum in the amide I region, whereas IL-2 remained native if dried from a pH 5 solution. The spectra are shown in Fig. 21. Note the appearance of the band at 1617 cm^{-1}, which is consistent with a β sheet structure formed by unfolding and aggregation of the protein during lyophilization. Additional unfolding was observed upon incubation at elevated temperature, and a strong correlation was observed between retention of native structure after freeze-drying and stability as a freeze-dried solid.

Remmele *et al.* (1997) described an internal reflection IR technique for *in situ* monitoring of protein structure during freeze-drying. In this study,

the amide I band was used to monitor the effect of sucrose in the stabilization of lysozyme.

4.6. Nuclear Magnetic Resonance

The application of nuclear magnetic resonance to the characterization of frozen solutions and freeze-dried solids has been primarily by use of low-field pulsed NMR, or relaxometry. This NMR technique does not provide chemical shift information, but is used to measure molecular mobility for solids, liquids, and frozen systems through the spin–lattice (T_1) and spin–spin (T_2) relaxation times. The reader is referred to Harris (1986) or other books on theoretical and experimental aspects of NMR spectroscopy for background information.

Izutsu *et al.* (1995, 1996) used low-field pulsed NMR to measure mobility of water and solutes in frozen solutions of various solutes in both water and D_2O, and used the term "softening temperature" to describe the higher temperature transition such as that shown in Fig. 13. Izutsu *et al.* reported that water molecules gain mobility at significantly lower temperatures than solutes, where water molecules become mobile at around the "real" glass transition temperature and solute molecules become mobile at the softening temperature.

Yoshioka *et al.* (1996) measured molecular mobility of BSA and bovine IgG (BGG) as a function of water content by measuring T_2 and correlating this measurement with aggregation susceptibility. Increased aggregation susceptibility appeared to be directly related to T_2. In later work by the same research group (Yoshioka *et al.*, 1997a), the free induction decay (FID) signal of freeze-dried BSA and BGG was analyzed as a function of temperature at different water contents. At low temperature, the FID is composed of a single Lorentzian decay arising from water protons and a single Gaussian decay due to protein protons. As the temperature increases, however, a temperature is reached where the FID is no longer described by these two decays and an additional Laurentian decay is needed. This is illustrated in Fig. 22 for bovine IgG containing 0.212 g H_2O/g protein at 10°C and 90°C. This was interpreted as the appearance of a population of protein protons with higher mobility, and the temperature at which this population appeared was defined as the softening temperature, or $T_{s(T_2)}$. Yoshioka *et al.* proposed that this softening temperature is an important property of protein formulations, particularly because T_g of freeze-dried protein formulations as determined calorimetrically is often uncertain, as discussed above. An abrupt increase in the rate of aggregation of both BSA and BGG was observed above the softening

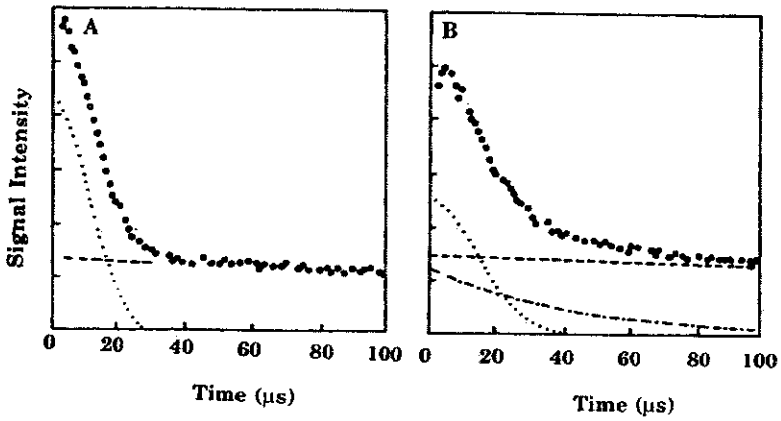

Figure 22. NMR free induction decay signal for freeze-dried bovine IgG containing 0.212 g H_2O/g protein at 10°C (A) and 90°C (B). Reproduced with permission from Yoshioka *et al.* (1997a). Copyright 1997. American Chemical Society and American Pharmaceutical Association.

temperature, although significant aggregation was observed below this temperature, particularly at low moisture levels. The term $T_{s(T2)}$ was later replaced by T_{mc}, or the "mobility changing" temperature (Yoshioka *et al.*, 1997b), and applied to study the stability of BGG in model formulations containing varying molecular weights of dextran and poly(vinyl alcohol) (PVA) (Yoshioka *et al.*, 1998) as an excipient. Both T_{mc} and protein stability increased as the molecular weight of dextran increased, and the temperature dependence of the protein aggregation rate changed at T_{mc}. Above T_{mc}, the temperature dependence of the aggregation rate was related to T_{mc} by the Williams–Landel–Ferry equation, where T_g was replaced by T_{mc}. Formulations containing PVA were less stable than those containing dextran, despite having lower residual water contents. This was attributed to the lower T_{mc} of the PVA formulations.

4.7. Dielectric Analysis

Measurement of the electrical properties of frozen systems by monitoring electrical resistance as a function of temperature has been used as a characterization tool since the early days of freeze-drying. The principle of the technique is that, when an aqueous solution is thoroughly frozen, the mobility of charge-carrying species is restricted. As the frozen system warms and begins to thaw, however, the mobility of charge-carrying species increases markedly and causes a large decrease in electrical resistance. This is a sensitive

method for the detection of eutectic melting. However, the interpretation of electrical resistance data as a function of temperature is uncertain in frozen systems containing amorphous solutes, and widespread use of this technique has been hampered by the frequency dependence of the response and, more generally, by a lack of standardized instrumentation and methodology.

A more recent advance in electrical thermal analysis is dielectric relaxation spectroscopy (DRS). The reader is referred to Smith *et al.* (1995) for a comprehensive review of the theory and applications of this method. Briefly, DRS measures the rate and extent of polarizability of materials when placed in an oscillating electromagnetic field. At low frequency, the molecular dipoles reorient with the applied field so as to minimize the potential energy of the system. As the frequency is increased, a frequency is reached where the molecular dipoles can no longer reorient fast enough to stay in phase with the applied field. The frequency response of a given material depends on the rotational freedom of the molecule, which is a reflection of its environment and its interaction with neighboring molecules. The method covers a broad frequency range (from about 10^{-5} to about 10^{11} Hz), thus enabling investigation of a wide range of processes. Since multiple frequencies are used, DRS can distinguish between first-order transitions, which show no frequency dependence, and second-order transitions such as the glass transition, which are frequency-dependent (Evans *et al.*, 1995). DRS can be applied to characterization of both frozen systems and dried solids, although there have been few studies reported in the field of freezing and freeze-drying. Morris *et al.* (1994) applied DRS to the prediction of collapse temperatures for binary glass-forming systems, including sucrose, trehalose, an antibiotic, and sorbitol in water. A "take-off" frequency model was used to establish good correlation between the predicted onset of viscous flow as measured by DRS and collapse as directly observed by microscopy.

5. THE DRYING PROCESS

5.1. Annealing in Freeze-Drying

Annealing actually precedes the drying process, and refers to warming the product to a subzero temperature—while avoiding melting the product—and holding for a period of perhaps 2–6 hr in order to improve the freeze-drying characteristics of the formulation. This is generally done in order to facilitate crystallization of a component of the formulation, either the drug substance or an excipient. The rationale for annealing is based on glass transition-associated mobility, where mobility increases dramatically

above the glass transition temperature. Holding the system above the glass transition temperature of the metastable glass may then allow crystallization of components which would otherwise not crystallize. Oguchi *et al.* (1990) used annealing in the temperature range of −13° to −18°C to prepare crystalline inclusion complexes of α-cyclodextrins with a series of benzoic acid derivatives as guest molecules. These investigators pointed out the marked temperature dependence of annealing, where decreasing the annealing temperature to −21°C resulted in no crystallization of the complex even when held for up to 24 hr. Gatlin (1991) studied the kinetics of crystallization of cefazolin sodium during annealing at a range of temperatures above the glass transition. Analysis of the data using the Johnson–Mehl–Avrami equation yielded kinetic data that were consistent with a crystallization mechanism of heterogeneous nucleation of a two-dimensional growth habit.

In addition to promoting crystallization of solutes, annealing can also promote crystallization of unfrozen water in glassy systems. Annealing even a few degrees below the glass transition temperature can thus deplasticize the amorphous phase, resulting in a readily measurable increase in the glass transition temperature.

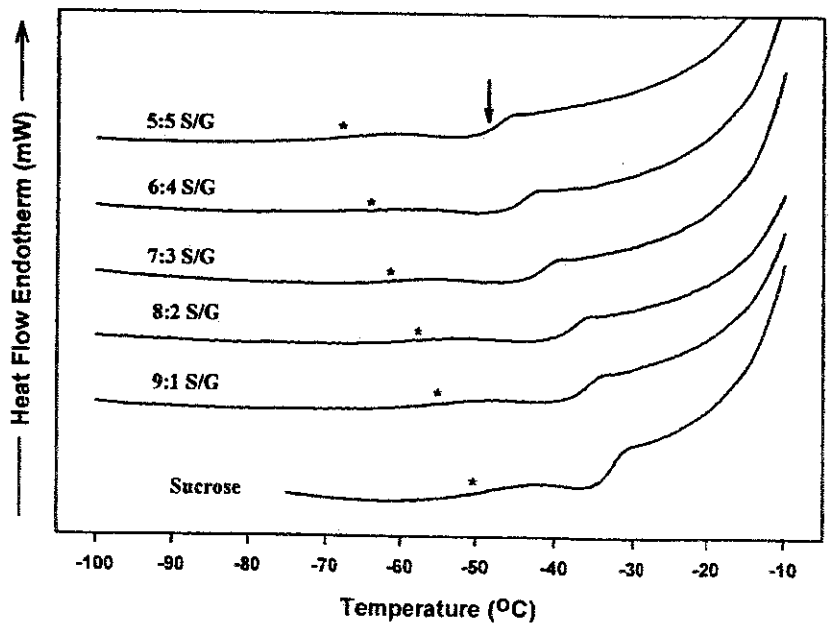

Figure 23. DSC thermograms of sucrose/glycine as a function of ratio of sucrose to glycine. Asterisk indicates lower temperature transition and arrow indicates higher temperature transition.

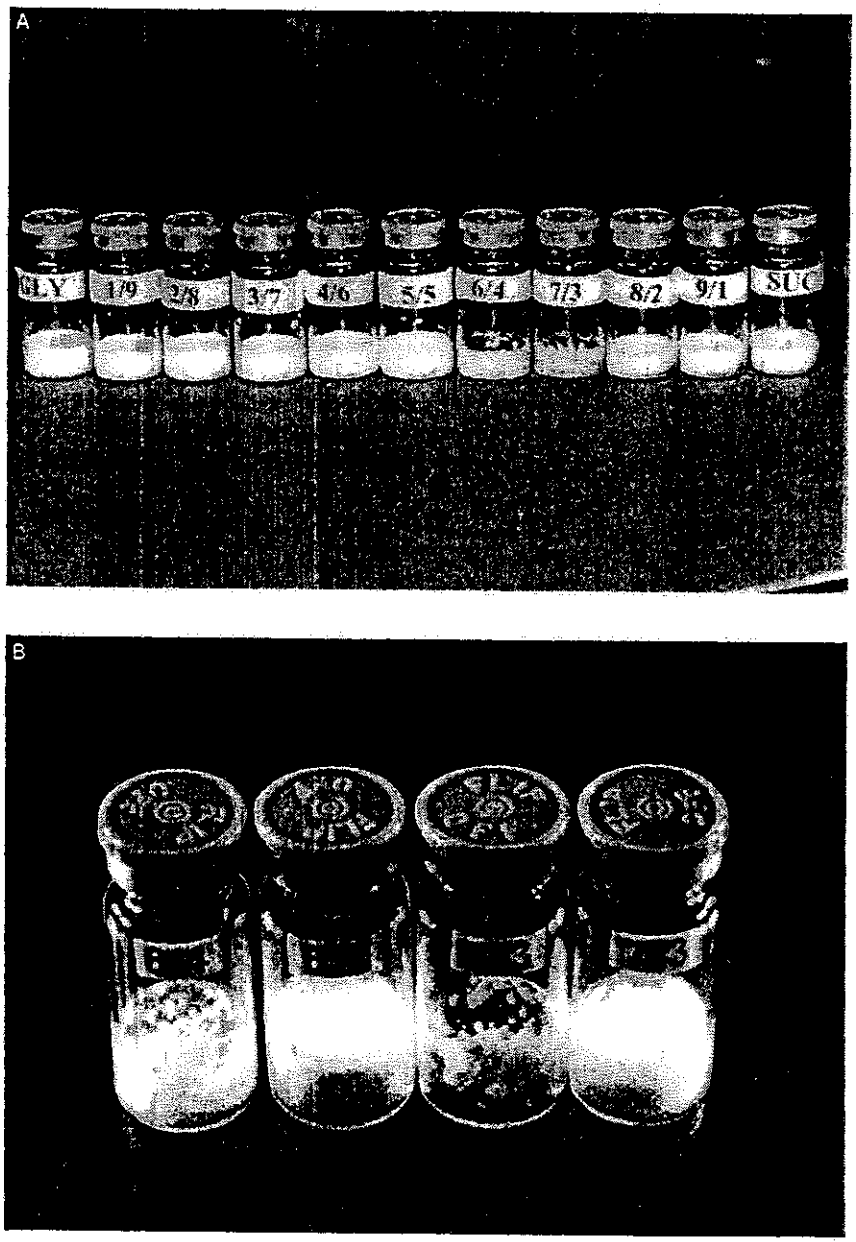

Figure 24. Freeze-dried samples of sucrose glycine at different weight:weight ratios of the two solutes (A) and the effect of annealing on structural collapse during freeze-drying (B) of 7:3 and 6:4 sucrose/glycine systems.

Sarciaux *et al.* (1999) studied the effect of annealing on aggregation of bovine IgG after freeze-drying, and observed decreased aggregation following annealing along with a significant reduction in the specific surface area of the freeze-dried solid. This observation is consistent with a mechanism involving denaturation at the ice–freeze-concentrate interface, which becomes irreversible after freeze-drying.

An important example of annealing with respect to protein formulations is the sucrose/glycine system. Figure 23 shows how the thermogram of the frozen system changes with composition, where the total solids concentration is 10% (w/v), and the ratios of the two solutes are indicated in the figure. Addition of glycine to a sucrose solution at relative concentrations too low for glycine to crystallize causes a decrease in the apparent glass transition of the frozen system. Figure 24A shows the appearance of freeze-dried solids as a function of the composition of this system, where samples were frozen at −45°C and freeze-dried at a shelf temperature of −30°C. Under these drying conditions, the samples containing 7:3 and 6:4 weight ratios of sucrose-to-glycine exhibit collapse. As the relative concentration of glycine increases, glycine is able to crystallize. Glycine crystallization allows formation of a freeze-dried solid which retains its macroscopic structure during freeze-drying and results in a pharaceutically acceptable cake. If the frozen solutions with sucrose-to-glycine ratios of 7:3 and 6:4 are annealed at −20°C for 3 hr prior to drying, glycine crystallizes from these systems. Freeze-drying of the annealed samples results in a pharmaceutically acceptable cake, shown in Fig. 24B.

5.2. Collapse during Freeze-Drying

If for no other reason than pharmaceutical acceptability, drying conditions must be developed which avoid collapse during freeze-drying. However, a more fundamental question must be asked: Does collapse during freeze-drying cause a marked decrease in recovery of activity after freeze-drying? Furthermore, does collapse during freeze-drying result in a product which is less stable as a freeze-dried solid? The answers to these questions have important product quality implications, particularly in the widely observed case of partial or localized collapse in a few vials within a batch of product. Total collapse can be easily observed visually, and these vials can be culled from the batch during inspection. However, areas of collapse may be present within the freeze-dried solid which are not apparent during inspection. Since measurement of protein activity after freeze-drying is based on statistical sampling, a small percentage of partially collapsed vials could easily go undetected. If collapsed regions within the lyophilized

Addendum 332a

Searles, Carpenter, and Randolph (2001a, 2001b) reported that the primary determinant of primary drying rate is the ice nucleation temperature, where a higher degree of supercooling results in smaller ice crystals, a higher resistance to mass transfer, and a slower drying rate. However, ice nucleation is not directly controllable in normal pharmaceutical freeze-drying. Annealing at temperatures above the T_g' of the formulation was found to increase ice crystal size. This not only reduces the heterogeneity in drying rate caused by variation in ice nucleation, but also results in increased primary drying rate by as much as a factor of 3.5. Annealing below T_g' did not result in increased drying rates.

Table V
Recovery of Activity of Model Proteins after Freeze-Drying under Conditions of Retention of Structure and Collapse of Cake

Protein	Structure retention	Collapse
Catalase	92	88
β-Galactosidase	95	85
Lactate dehydrogenase	95	50

solid were associated with significantly lower recovery of activity or significantly decreased stability, the consequences could be subpotent vials, which is far more significant than pharmaceutical elegance.

There has been little published literature on the effect of collapse during freeze-drying on stability of proteins against damage during freeze-drying, although there is widespread acceptance of the idea that collapse can cause a sharp drop in recovery of activity. Jiang and Nail (1998) measured the profile of activity loss during freeze-drying of the model proteins catalase, β-galactosidase, and lactate dehydrogenase under conservative drying conditions resulting in complete retention of microstructure versus aggressive conditions which resulted in collapse during primary drying. Results are shown in Table V. The only protein for which a large decrease in recovery of activity was observed is LDH. This may be due at least in part to the sensitivity of LDH to freeze–thaw damage, since some melting of frozen solution during primary drying could not be ruled out. These results may also be partially confounded with the effect of residual moisture in the freeze-dried material. It was found in this study that the most important determinant of activity recovery after freeze-drying was the amount of residual moisture in the lyophilized solid, and collapsed material, having a lower specific surface area, tends to have a high residual moisture level.

5.3. Heat and Mass Transfer in Primary Drying

The heat of sublimation of ice must be supplied by transfer of heat through a series of resistances from the freeze-dryer shelf to the ice at the sublimation front. Of course, this must be done without melting the frozen material in contact with the bottom of the container. The rate of the solid-to-vapor phase change is, relative to the time course of freeze-drying, instantaneous. Following the phase change, water vapor must be removed by flow through the porous matrix of partially dried product. A knowledge of both heat and mass transfer

is important for the development of optimum processing conditions. In particular, it is important to understand the dominant, or controlling, resistance in both of these transfer operations. There is a small, but important body of literature on heat and mass transfer in freeze-drying of pharmaceuticals, most notably by Pikal and co-workers (Pikal, 1985; Pikal *et al.*, 1983, 1984), and the reader interested in a detailed analysis is referred to these papers. The discussion below is intended to summarize this work and to provide broad guidelines pertaining to freeze-dry cycle development.

There are three mechanisms by which heat can be transferred:

- *Conduction*, the flow of heat by molecular motion between one differential volume element of a material and the next.
- *Convection*, exhange of heat caused by bulk flow of a fluid.
- *Radiation*, flow of heat by thermal excitation which gives rise to electromagnetic radiation.

Because freeze-drying takes place in an evacuated system, heat transfer by convection is probably not a significant mechanism. This has been demonstrated by measuring the rate of heat loss from a heated filament as a function of orientation of the filament at pressures representative of freeze-drying (Dushman and Lafferty, 1962). Because of the modest temperatures used in freeze-drying, it has been shown that thermal radiation probably does not account for more than about 5% of the total heat flow (Nail, 1980), where the total heat flow is given by

$$Q = \Delta H_s \cdot m \tag{3}$$

where Q is the heat flow, which is linked to the rate of sublimation m by the heat of sublimation of ice ΔH_s. However, thermal radiation can be significant in some situations, particularly in scaleup from laboratory to production-scale equipment.

The dominant mechanism of heat transfer in freeze-drying is conduction. Analysis of this heat transfer process (Nail, 1980) has demonstrated that the controlling resistance to heat transfer by conduction is the poor thermal contact between the surface of the freeze-dryer shelf and the product. This contact resistance is particularly important when freeze-drying from vials. The rate of heat transfer is thus determined by the thermal conductivity of the vapor phase, which is a function of the pressure in the chamber. Under conditions of free molecular flow of the vapor phase (mean free path is larger than the characteristic dimension of the system), the thermal conductivity of the vapor increases linearly with pressure. As the system pressure increases to above about 0.1 mm Hg, the vapor flow regime becomes transitional, and thermal conductivity increases more slowly with increased pressure (at still higher pressure, where the flow regime is viscous,

thermal conductivity of a gas is independent of pressure). Analysis of heat transfer leads to the conclusion that, contrary to earlier belief, very low chamber pressure during freeze-drying slows the rate of drying by inhibiting heat transfer.

Selection of an optimum system pressure involves a trade-off between heat transfer and mass transfer considerations. The pressure in the chamber, which consists almost entirely of water vapor during primary drying (Nail and Johnson, 1992), must be lower than the vapor pressure of ice in the product in order for drying to take place. However, a pressure which is too low unnecessarily retards the process. The optimum chamber pressure depends on the desired product temperature during primary drying. A reasonable guideline for selection of an optimum chamber pressure during primary drying is that the chamber pressure should be no more than about one-half and not less than about one-fourth of the vapor pressure of ice at the desired product temperature. For example, assume that the target product temperature during primary drying is −20°C. From Table I, the vapor pressure of ice at this temperature is 0.76 mm Hg. An optimum pressure for primary drying would be in the range of 0.25–0.35 mm Hg (250–350 μm Hg). If the product temperature must not exceed, for example, −40°C during primary drying, then the viewpoint that "the lower the pressure, the better" is appropriate, because the optimum system pressure would be less than 0.05 mm Hg.

When freeze-drying from vials, the vial itelf is the main reason for the poor thermal contact between the shelf and the product. Seemingly insignificant variations in vial geometry can have a measurable impact on heat transfer and, consequently, drying rate. It is often useful to measure the vial heat transfer coefficient. These data can be valuable in vial selection for new products and evaluation of the impact of a change in vial supplier, vial size, or vial type (molded vs. tubing glass) with respect to impact on freeze-drying characteristics. A simple procedure for measuring the vial heat transfer coefficient is as follows (Pikal, 1985). First, the ~~number of~~ vials to be measured are filled to ~~a depth of perhaps 2 cm~~ with water, marked for identification, and weighed. Thermocouples are carefully placed in several vials such that the thermocouple is touching the bottom center of the vial (see Fig. 1) and at least one other thermocouple is placed on the surface of the shelf. Vials are then transferred to the freeze-dryer and frozen. Freeze-drying is carried out at the desired shelf temperature and at constant chamber pressure. After a period of time during which at least half of the ice is sublimed, the process is stopped, the drying time is noted, and the vials are weighed. The mass loss per unit time is calculated, and the total rate of heat transfer is calculated from Eq. (3). The vial heat transfer coefficient is then calculated from

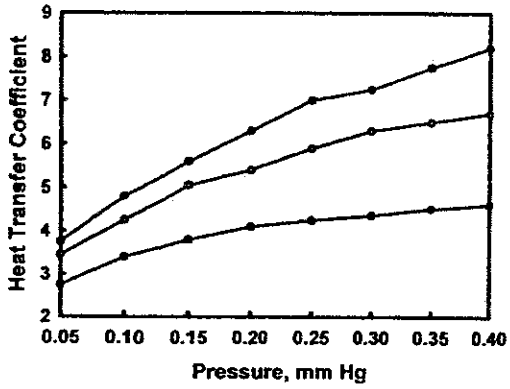

Figure 25. Representative vial heat transfer coefficient data illustrating dependence on type of vial and chamber pressure. Reproduced with permission from Pikal (1985). Copyright 1985.

$$Q = A_v K_v (T_s - T_b) \tag{4}$$

where A_v is the cross-sectional area of the vial based on the outside vial diameter, T_s is the shelf surface temperature, and T_b is the temperature measured at the bottom center of the vial. Since the vial heat transfer coefficient is a function of pressure, K_v should be calculated at several pressures in the range of 0.05 to perhaps 0.4 mm Hg. Representative vial heat transfer coefficient data are shown in Fig. 25, which illustrates that the vial heat transfer coefficient is strongly dependent on both the pressure and the type of vial.

Analysis of mass transfer follows the same general approach as analysis of heat transfer, where

$$\text{Sublimation rate} = \text{Pressure difference/Resistance}$$

and the total resistance to mass transfer consists of three components: (1) the dried product layer, which increases in thickness as drying proceeds, (2) the partially inserted stopper, and (3) the resistance of the freeze-dryer itself to flow of vapor from the drying chamber to the condenser. Mass transfer in freeze-drying has been rigorously analyzed by Pikal *et al.* (1983; Pikal, 1985), and the limiting resistance has been identified as the dried product layer, which usually accounts for more than 90% of the total resistance to mass transfer. Measurement of dried product resistance is more involved than measurement of heat transfer coefficients. The most precise method is the microbalance technique reported by Pikal, which requires specialized equipment. The "vial" method, also reported by Pikal, also requires special equipment in order to measure the pressure in the headspace of the vial. Pikal accomplished this by a sidearm connected to a pressure gauge. A more recent

method, reported by Milton *et al.* (1997) and discussed below, uses analysis of the transient pressure response when the valve between the chamber and condenser is briefly closed during primary drying. Although this method requires only an external condenser design with a valve that closes quickly (about 0.5 sec), further study is needed in order to verify the accuracy of this method for resistance of the product to water vapor transport.

Given that the product resistance is the controlling resistance to mass transfer and that this resistance increases with product thickness, the depth of fill should be minimized as much as practical. In general, it is good practice to avoid filling a vial more than about one-third full. This also helps to prevent vial breakage during freezing, as discussed above for mannitol-based formulations. There is a trade-off here, however, because minimizing the depth of fill also means that less product can be placed in the freeze-dryer.

5.4. Secondary Drying

Secondary drying is removal of water which did not freeze. This is a particularly important aspect of drying of protein formulations because, unlike traditional, low molecular weight drugs, proteins can be subject to loss of activity by low overdrying. For example, Hsu *et al.* (1992) studied the effect of residual moisture in an excipient-free freeze-dried formulation of tissue-type plasminogen activator (tPA) on both turbidity upon reconstitution (insoluble aggregates) and stability of the biological activity upon storage. This was related to the water vapor adsorption isotherm of the protein. Samples that were dried to a residual water level below monolayer coverage of the solid exhibited greater turbidity upon reconstitution than samples containing a higher residual moisture level, and the turbidity of the driest material increased during storage at 50°C more rapidly than samples containing a higher residual moisture content. However, samples that were dried to a residual moisture level corresponding to monolayer or multilayer coverage showed more rapid loss of biological activity upon storage. The results suggest that dryer is not always better, and that the optimum moisture level must be chosen based on both physical stability and chemical stability considerations.

As with heat and mass transfer during primary drying, it is useful to know the rate-limiting process during the secondary drying phase of freeze-drying. The possibilities are (1) diffusion in the glassy matrix to the surface of the solid, (2) evaporation at the solid surface, and (3) water vapor transport through the porous bed of solids. Pikal *et al.* (1990) studied the kinetics of secondary drying as a function of temperature and pressure, although protein formulations were

not used for these studies. Mannitol was used as a model crystalline solid along with two amorphous materials, moxalactam disodium and polyvinylpyrrolidone. It was reported that residual moisture decreases rapidly during the first few hours of secondary drying, then slows considerably, and reaches a plateau level which exceeds the equilibrium moisture content of the solid calculated from the desorption isotherm and the partial pressure of water vapor in the chamber. This plateau level is very temperature-sensitive, and higher secondary drying temperatures cause a significant reduction in the plateau level. The secondary drying rate increases with specific surface area of the dried solids, and the rate of secondary drying was observed to be independent of the chamber pressure during secondary drying. The rate of secondary drying was found not to be dependent on the depth of the dried product layer. This, along with the independence of secondary drying rate on pressure, supports the conclusion that the rate-limiting step is not water vapor transport through the porous bed of solids. The rate-limiting step must therefore be either diffusion of water through the solid or evaporation at the solid surface.

Spieles *et al.* (1995) studied secondary drying using aqueous solutions of hydroxyethyl starch, which were concentrated to the point where ice formation was prevented during freezing. In this case, the entire drying process consists of secondary drying. Drying kinetics was studied by measuring weight loss at constant temperature in the range of 254–293 K and pressure in the range of 5–44 Pa until equilibrium was reached after 5–8 days. The main focus of this study was the relationship between water activity and residual moisture content, which was described by a Freundlich relationship

$$W = q(a_w)^r \tag{5}$$

where q and r are empirical constants. The relationship between water content and water activity was independent of temperature over the range studied. This finding is not consistent with Pikal's data, although the experimental conditions and, in particular, the time scale of the drying experiments were quite different.

When small changes in residual moisture have a significant impact on a critical product quality attribute such as stability, the distribution of residual moisture levels between vials and within a lyophilized cake may be as important as the average residual moisture level. Vial-to-vial variability should be determined by extensive sampling during process validation, ideally by "mapping" residual moisture level as a function of position in the freeze-dryer. "Edge" effects can be significant, where vials at the edge of a shelf dry faster and have higher residual moisture levels than the bulk of the lot because of lateral heat transfer from the sides, the door, and the back of

lower

the chamber. Pikal and Shah (1997) studied the intravial distribution of moisture. Theoretical analysis predicted that residual moisture near the top of the lyophilized cake is lower than at the bottom, particularly for large fill depths. Experimental data demonstrated that the top is drier than the bottom of the cake, but that the "outer" section of the cake—that nearest the vial wall—is consistently the driest.

Better definition of the role of residual moisture in the stability of freeze-dried proteins and better control of the secondary drying process are subjects of significant practical importance where more research is needed.

5.5. Process Monitoring

Optimization of freeze-drying conditions requires control of shelf temperature and chamber pressure such that the product temperature is just below the maximum allowable temperature during primary drying. Measurement of product temperature is therefore necessary for cycle development and scale-up. In order to obtain the most meaningful data from product temperature measurement, it is important to pay attention both to use of an appropriate temperature-measuring device and placement of the sensor within individual vials. The best place to measure product temperature is in the center of a vial, with the sensor touching the bottom of the vial. The reason for this placement is that, whereas the sublimation front is planar at the beginning of primary drying, it becomes concave (as viewed from the bottom of the vial) as the sublimation front recedes, and the curvature increases with time. This effect can be easily demonstrated by observing freeze-drying of ice, where the last ice to sublime is at the bottom center of the vial. Thermocouples are better for measurement of temperature at a precise location than resistance-type temperature detectors (RTDs), because the measuring element for a RTD is larger, and the temperature measured is an average temperature over the surface of the measuring element. Thin-gauge thermocouples are recommended not only because they facilitate precise location of the thermocouple, but also because the light weight and better flexibility of the lead minimizes problems with the knocking down of vials by the thermocouple wire. A device similar to that shown in Fig. 1 is useful for precise placement of thermocouples, because the thermocouple is held in place by a cap which fits over the top of the vial, and the wire is flexible enough to be located accurately, yet rigid enough to stay in position during the drying cycle.

Although these measures can minimize inaccurate process data arising from sensor placement, there are other sources of uncertainty in product

temperature measurement which are not a function of placement. The temperature sensor facilitates nucleation of ice, so that monitored vials generally undergo less supercooling during freezing than nonmonitored vials. This results in a slower effective freezing rate for monitored vials, a larger average ice crystal size, larger pores in the dried solid, less resistance to mass transfer during primary drying, and faster drying. Monitored vials are generally considered to dry about 10% faster than the nonmonitored vials. For this reason, soak times are used when product temperature data are used to sequence a cycle automatically from freezing to freeze-drying.

The trend in the pharmaceutical industry is toward larger production freeze-dryers, and scaleup to large equipment brings about more problems with product temperature measurement, where an important issue is location of monitored vials within the batch. If vials are placed in metal trays, it is best to monitor vials in the center of the tray, because warpage (with resulting poor thermal contact with the shelf) of a tray tends to be maximum at the center, and vials in the center receive less lateral heat transfer from the sides of the trays. Both of these factors contribute to vials in the center of a tray drying slower than vials near the perimeter. Likewise, the dryer has its own "edge effect" due to heat transfer from the side walls and the door of the chamber. This means that the most appropriate trays to monitor are in the center of a given shelf. However, easy access to the center of a shelf can be problematic, particularly for large freeze-dryers. Since placement of temperature probes is largely a manual process requiring close proximity of an operator to open vials of product, placement of product temperature probes compromises sterility assurance to some extent.

For these reasons, there is a need for technology which enables monitoring of product temperature without placing temperature probes in individual vials or product. Although there is currently no way to do this directly, Milton *et al.* (1997) explored measurement of product temperature by a manometric method. In this technique, which requires an external condenser design, the isolation valve between the chamber and the condenser is closed for between 10 and 20 sec during primary drying, and the transient response of the chamber pressure is monitored. The data are fitted to an equation based on a theoretical model of the transient pressure response, which takes into account (1) continued sublimation of ice while the valve is closed, which stops when the chamber pressure reaches the vapor pressure of ice at the temperature of the sublimation front, (2) continued heating of the product during the measurement, and (3) equilibration of temperature across the frozen layer during the measurement. Least squares fitting of the data to the model results in estimates of the value of three variables: (1) product temperature at the sublimation front, (2) the resistance of the dried product

layer to transport of water vapor, and (3) the vial heat transfer coefficient. Although further refinement of this method is needed in order to explain the effects of batch size and edge effects on accuracy of temperature and product resistance data, it does represent a step toward developing an approach to measuring product temperature while avoiding the problems associated with monitoring the temperature of individual vials.

Another reason for monitoring individual vials of product is to detect the end of drying. The end of drying (see Fig. 2) is approached as the product temperature approaches the shelf temperature during secondary drying. Again, this information is necessary for cycle development, even though the nonrepresentative nature of monitored vials must be recognized and compensated for by the use of "soak times" at the end of the process. When it is desired to eliminate product temperature probes for routine monitoring of production lots, it is helpful to have another way to detect the end point of drying, preferably a method that is based on monitoring of the composition of the vapor phase in the chamber. There have been a few published reports of such methods for detection of drying end point. Nail and Johnson (1992) described a comparative pressure measurement technique, where a capacitance manometer pressure gauge (which measures total pressure, independent of vapor composition) is used to control the pressure, and a thermal conductivity-type pressure gauge (which is preferentially sensitive to water vapor) is used to monitor pressure. At the end of drying, the apparent pressure as determined by the thermal conductivity gauge decreases in response to the decreased partial pressure of water vapor in the chamber. Roy and Pikal (1989) described use of an electronic hygrometer to measure the partial pressure of water vapor in the chamber and demonstrated that this is a sensitive method for detecting the end of drying. A limitation of this type of instrumentation is that, at this writing, it cannot be steam-sterilized. A "pressure rise" technique can be used, similar to the manometric temperature measurement method described above, except that the valve is closed during secondary drying. When drying ceases, the pressure rise due to continued evolution of water vapor from the product ceases, and the pressure rise is only due to leaks in the system. This can be an effective method, provided that the "background" leak rate is low and approximately constant. Use of a residual gas analyzer (RGA) for monitoring the lyophilization process has been described by Connelly and Welch (1993) and Nail and Johnson (1992). The RGA is a small, quadrupole mass spectrometer with an upper limit of mass-to-charge ratio m/e of perhaps 200. Monitoring of the composition of the vapor in the freeze-dry chamber provides a sensitive method for endpoint detection as the partial pressure of water vapor decreases. Connelly and Welch (1993) present data on partial pressure of water vapor versus time for freeze-drying of bovine serum albumin showing a

gradual decrease in partial pressure of water vapor during secondary drying. Nail and Johnson (1992), using a crystalline freeze-dried solid (mannitol), demonstrated a sharp drop in partial pressure of water vapor at the end of the process. As discussed above, this would be expected, because there is virtually no secondary drying when the solute crystallizes during freezing. In addition to end point detection, the ability to monitor vapor phase composition during the process has other applications, such as (1) monitoring oxygen partial pressure in the chamber during stoppering of protein formulations which are sensitive to oxygen in the headspace of the vial, (2) leak detection, either from an external leak or from an internal leak of refrigerant, and (3) monitoring evolution of solvents from the material being freeze-dried, either residual solvents from the purification process or organic solvents, such as ethanol or *t*-butanol, used in the formulation.

6. STABILITY OF FREEZE-DRIED FORMULATIONS

Stability assessment and shelf-life prediction is usually a major focus of a pharmaceutical scientist's attention in the development any freeze-dried dosage form, and this is especially true of freeze-dried protein formulations. Ideally, the product would demonstrate at least a 2-year shelf life during storage at room temperature. However, approximately 90% of all freeze-dried protein pharmaceuticals sold in the United States require refrigerated storage. This can be a major logistical problem, particularly in under-developed countries, where a suitable "cold chain" cannot be maintained. In addition, the predictive value of accelerated stability testing for freeze-dried products is questionable, and the Food and Drug Administration (FDA) does not recognize accelerated stability data in support of the requested expiration dating; that is, the expiration date must be based on actual data at the intended storage temperature. For these reasons, there is a need for further development of the application of pharmaceutical materials science to assist in designing more stable freeze-dried formulations as well as development of the scientific basis for meaningful interpretation of accelerated stability data.

6.1. Molecular Motion in Amorphous Systems and Relevance of the Glass Transition Temperature to Stability

Freeze-dried protein formulations are at least partially amorphous. Whereas a crystalline fraction is usually desireable in maintaining

the mechanical integrity of the solid, it is the amorphous phase which contains the protein and predominates in determining both physical and chemical stability. The concepts of a glass transition and the temperature dependence of molecular motion in the region of the glass transition are central to understanding the behavior of amorphous materials. Given that molecular motion slows dramatically below T_g, there is a body of opinion which holds that all molecular motion relevant to storage stability ceases a few degrees below T_g (Franks, 1990; Hemminga *et al.*, 1993) and that storage below T_g is necessary and sufficient for stability. This point of view, sometimes known as the *vitrification hypothesis*, is an oversimplification, however, and a better understanding of the relationship between the temperature dependence of the molecular mobility and stability of pharmaceutical systems is a subject for which much more research is needed. This has been the subject of several excellent reviews, particularly by Hancock and Zografi (1997) and Angell (1995). The reader is referred to these reviews for a more in-depth treatment.

The temperature dependence of molecular motion in amorphous systems is described by the empirical Vogel–Tammann–Fulcher (VTF) equation,

$$\tau = \tau_0 \exp(B/T - T_0) \tag{6}$$

where τ is the molecular relaxation time, T is the temperature, τ_0 and B are constants, and T_0 is the temperature at which the configurational entropy of the system reaches zero. T_0 is also known as the Kauzman temperature, and is believed to be roughly 50°C below the experimentally measured glass transition temperature, depending on the nature of the system (see discussion below). Note that, when T_0 is zero, the VTF equation reduces to the Arrhenius equation, where B is analogous to the activation energy. The Williams–Landel–Ferry equation, originally developed to describe the temperature dependence of viscosity of solutions above the glass transition temperature, is another special case of the VTF equation:

$$\eta = \eta_g \exp[C_1(T - T_g)/(C_2 + (T - T_g))] \tag{7}$$

where η_g is the mean viscosity at T_g and C_1 and C_2 are constants equal to B/$(T_g - T_0)$ and $(T_g - T_0)$, respectively. The constants have been found to be universal for a variety of polymer systems (−17.4 and 51.6 for C_1 and C_2, respectively). Roy *et al.* (1991) tested the applicability of the WLF equation to the prediction of the temperature dependence of the stability of a freeze-dried monoclonal antibody–vinca conjugate above the T_g of three different formulations, all of which were below 40°C. Three degradation processes were monitored: aggregation, hydrolysis of the hydrazone linkage, and decomposition of the vinca moiety. Stability data at 25°C and 40°C were

plotted as ln R/R_g versus $(T - T_g)$ for each of the processes. All were observed to be linear, which indicates that $C_2 > (T - T_g)$. The slope of the line, C_1/C_2, was constant for all of the degradation reactions.

Glasses have been classified as "strong" or "fragile," depending on the temperature dependence of the activation energy for molecular motion in the region of T_g (Angell, 1995; Hancock and Zografi, 1997). Strong glasses exhibit an Arrhenius temperature dependence of molecular mobility and have small changes in heat capacity at T_g and broad glass transition regions (when they can be detected at all). Proteins are good examples of strong glass-formers. Fragile glasses, on the other hand, undergo much larger changes in heat capacity at T_g and have narrower T_g ranges. The molecular mobility around T_g of a fragile glass is much more temperature-dependent than that described by the Arrhenius equation: The molecular mobility may change by an order of magnitude with a 10 K change in temperature, as opposed to roughly a factor of two over the same temperature range for a mobility which is described by the Arrhenius relationship. The values of B and T_0 in the VTF equation are related to glass fragility. Fragile glasses are characterized by low values of B (<10), and $(T_g - T_0)$ is generally less than 50. Strong glasses, on the other hand, have high values of B (> 100), and $(T_g - T_0)$ is usually greater than 50. Another method for estimating the fragility of a glassy system simply uses the ratio of the melting temperature to the glass transition temperature (T_m/T_g, in K); strong glasses have ratios greater than 1.5 (Hancock and Zografi, 1997).

Hancock *et al.* (1998) described a method for determining fragility of some amorphous pharmaceutical materials based on measuring the width of the glass transition as measured by DSC. Fragility m has been defined as

$$m = \Delta H/(2.3RT_g) \tag{8}$$

where ΔH is the activation energy for structural relaxation and R is the gas constant. Further,

$$\Delta H = CR/(1/T_g^{\text{onset}} - 1/T_g^{\text{offset}}) \tag{9}$$

where C is a constant. Hancock and co-workers examined 11 pharmaceutical materials by this method, finding values of m in the range of 36–60, which corresponds to intermediate fragility. However, values of m were found to vary considerably with the experimental conditions used, particularly on whether measurements were made during heating or cooling. It also appears that the relationship between the width of the glass transition region and fragility is dependent upon the material being examined, which makes measurement of the width of the glass transition as a means of measuring fragility somewhat suspect.

When a glassy solid is allowed to "age" below its glass transition temperature, its normal molecular motions combined with the thermodynamic driving force toward a lower enthalpy state causes it to "relax," or assume a more ordered structure. This exothermic process is termed *enthalpy relaxation.* When the glass is reheated through the glass transition region, energy must be added to the system in order to reestablish the degree of disorder present in the viscous liquid state above the glass transition. This process is called *enthalpy recovery.* The rate at which the material relaxes is a measure of the molecular mobility below the glass transition, and the limited published data support the idea that this relaxation takes place at about the same time and temperature conditions for both polymeric species and low molecular weight glass-formers (Hancock *et al.*, 1995). This mobility is expressed quantitatively as the relaxation time constant, which is the average time required for a motion of a particular type. The time constant is calculated from enthalpy recovery data as follows. First, the maximum enthalpy recovery at a given temperature ΔH_∞ is calculated using the equation

$$\Delta H_\infty = (T_g - T)\Delta C_p \tag{10}$$

where T_g is the glass transition temperature, T is the experimental temperature, and ΔC_p is the change in heat capacity at T_g. The degree to which a material relaxes at any time and temperature is then given as

$$\phi_t = 1 - \Delta H_t/\Delta H_\infty \tag{11}$$

where ΔH_t is the measured enthalpy recovery at a given time and temperature of annealing. The structural relaxation time is then calculated, typically by using the Williams–Watts equation, where the ϕ_t data are fit to a stretched exponential function,

$$\phi_t = \exp(-t/\tau)^\beta \tag{12}$$

where τ is the relaxation time constant and β is a relaxation time distribution parameter, with a value between 0 and 1. Values of β and τ are calculated by nonlinear least squares regression. Values of β close to unity are representative of strong glasses, and the glass becomes more fragile as the value of β decreases. This approach was used by Duddu *et al.* (1997) to study sucrose and trehalose formulations of a monoclonal antibody in order to determine whether physical stability (aggregate formation in this case) is related in a predictable way to molecular mobility below the glass transition temperature. Values of β calculated from enthalpy recovery data were 0.38 and 0.44 for sucrose and trehalose formulations, respectively, and these values were confirmed by dielectric relaxation studies. Based on these data, the sucrose formulation was more fragile than the trehalose formulation.

Although the sucrose formulation had the lower glass transition temperature (59°C and 81°C for the sucrose and trehalose formulations, respectively), the greater temperature dependence of the mobility of the sucrose formulation results in the relaxation time constant τ of the sucrose formulation being less than that of the trehalose formulation below about 12°C. It was not possible to test the significance of this observation with respect to rate of aggregation due to the very slow rate of aggregation at low temperatures. Although much more research is needed, these results suggest that both the glass transition temperature and fragility data are needed in order to correctly interpret accelerated stability data.

The data for the nonenzymatic browning reaction between xylose and lysine in a carboxymethylcellulose/lactose matrix as a function of $(T - T_g)$, shown in Fig. 26, are consistent with the idea of molecular motion relevant to storage stability ceasing below T_g. There is as yet insufficient published literature on actual rates of degradation reactions in protein formulations as a function of $(T - T_g)$ to allow broad generalizations, particularly at temperatures below T_g. Duddu and Dal Monte (1997) investigated the role of the glass transition temperature in the stability of a monoclonal antibody in either sucrose or trehalose. The sucrose formulation ($T_g = 59$°C) was observed to undergo aggregation during storage of the freeze-dried solid at 60°C, whereas the trehalose formulation ($T_g = 80$°C) was stable. Increasing the protein/sucrose ratio increased the T_g of both formulations. For example, increasing the protein concentration from 5 to 50 mg at a constant sucrose content of 62.5 mg resulted in an increase in T_g from 59°C to 89°C.

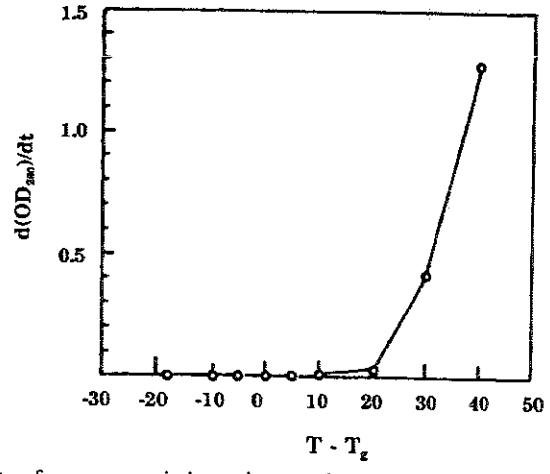

Figure 26. Rate of nonenzymatic browning reaction in a system of lactose:carboxymethylcellulose:xylose:lysine as a function of $(T - T_g)$. Reproduced with permission from Karel *et al.* (1993). Copyright 1993.

Increasing the protein/sugar ratio also significantly improved the stability of the formulation. It should be remembered, however, that increasing the concentration of protein, in general, improves stability, and this observation may not be totally explained by changing the glass transition temperature. Duddu and Weller (1996) measured the stability of a freeze-dried aspirin formulation in the region of T_g and pointed out the significant error introduced by measuring the degradation rate above T_g and extrapolating to temperatures below T_g. For a system with a T_g of about 36°C, extrapolation of 40°C, 45°C, and 50°C data yielded a predicted rate constant of 0.009 day^{-1} at 22°C, whereas the observed value was 0.003 day^{-1}.

In studying the relationship between T_g and stability, it is important to recognize that water can serve not only as a plasticizer of the amorphous phase, but also as a reactant. Bell and Hageman (1994) used a freeze-dried preparation of aspartame in different molecular weights of polyvinylpyrrolidone (PVP) in order to address the question of which is more important, the glass transition temperature or residual water activity. The solid-state degradation of aspartame via rearrangement to a diketopiperazine was measured at 25°C after equilibrating the model formulations containing different molecular weights of PVP at different relative humidities. It was found that reaction rates at constant water activity, but different T_g values, were not significantly different. However, rates at similar $(T - T_g)$ values, but different water activities, were significantly different. Therefore, in this system, water activity is more important than $(T - T_g)$. This same approach has been applied in the authors' laboratory, where the model proteins LDH and β-galactosidase were freeze-dried in matrices consisting of different molecular weights of maltodextrin. The freeze-dried cakes were equilibrated for 2 days in desiccators containing saturated salt solutions at a range of relative humidities, and recovery of enzyme activity was measured at 40°C. The results are shown for β-galactosidase and LDH in Fig. 27 and Fig. 28, respectively, where the rate constant (first order for β-galactosidase, second order for LDH) is plotted against $(T - T_g)$ for different water activities. For β-galactosidase, the contrast of the rate versus $(T - T_g)$ plot to Fig. 26 is striking. The relatively high rates of loss of activity recovery at large values of $(T - T_g)$ are probably attributable to overdrying. There is no sharp increase in degradation rate above T_g, although it was not feasible to examine large values of $(T - T_g)$ and still maintain moderate accelerated stability temperatures for this system.

For LDH, the rate constant versus $(T - T_g)$ plot more closely resembles that of Fig. 26, but significant differences remain. First, the rate of loss of activity is easily measurable far below T_g. Second, in looking at the rate versus $(T - T_g)$ pattern for different water activities, it can be seen that, similar to the data reported by Bell and Hageman (1994), there is not a

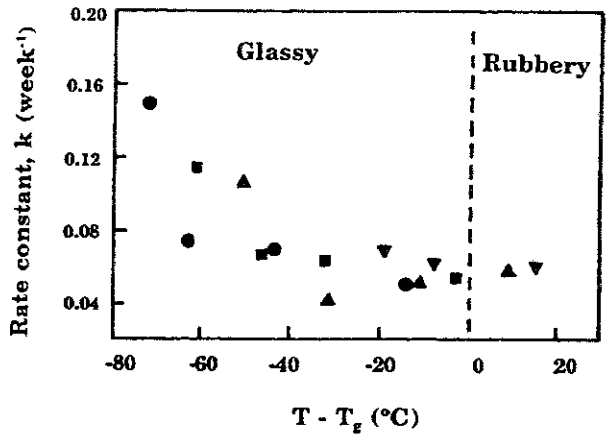

Figure 27. Solid-state stability of freeze-dried β-galactosidase versus $(T-T_g)$. (●) Solids equilibrated at 11% RH; (■) solids equilibrated at 23% RH; (▲) solids equilibrated at 33% RH; (▼) solids equilibrated at 43% RH.

significant difference in rate constant at 40°C for the 11% RH group, despite a difference in $(T-T_g)$ of more than 50°C. This same pattern holds for the samples at higher water activity, despite some scatter in the data. The data above T_g are very limited for the reason mentioned above, but only maltose exhibits a sharp increase in rate constant above T_g. Based on width of the glass transition region, maltose appears to be the most fragile of the excipients studied, and would be expected to show the sharpest increase above T_g, assuming that the concept of strong and fragile glasses

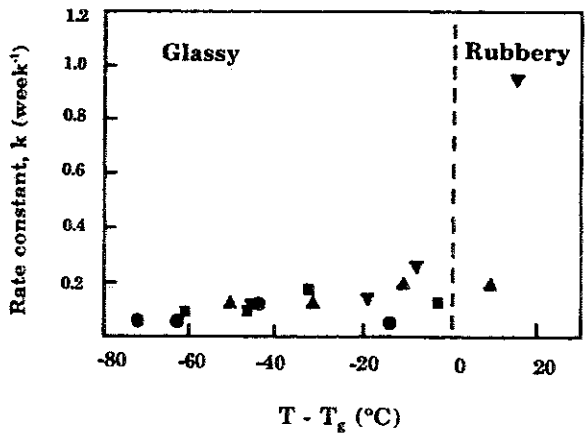

Figure 28. Solid-state stability of freeze-dried lactate dehydrogenase versus $(T-T_g)$. (●) Solids equilibrated at 11% RH; (■) solids equilibrated at 23% RH; (▲) solids equilibrated at 33% RH; (▼) solids equilibrated at 43% RH.

applies quantitatively to stability assessment of freeze-dried protein formulations. The maltodextrin system seems to offer a convenient model system for further investigation. Nonetheless, data from both the β-galactosidase and the LDH model systems support the conclusion that water activity plays a more important role in the stability than glass transition-associated mobility.

6.2. Cake Shrinkage and Crystallization of Excipients during Storage

The glass transition temperature is certainly relevant to physical stability of the lyophilized solid, particularly during accelerated stability testing, because glass transition-associated mobility can cause shrinkage of the cake as the storage temperature approaches the glass transition temperature. This is particularly evident for predominantly amorphous solids, as illustrated in Fig. 29 for a series of sugars having a range of glass transition temperatures. If the freeze-dried solid contains a substantial crystalline fraction, this cake shrinkage may not be observed, because the collapsed amorphous phase is supported by the crystalline component, and little if any cake collapse is observed.

Crystallization of stabilizing excipients, usually sugars and polyols, has been shown to result in decreased stabilization of model proteins. Izutsu *et al.* (1993) used β-galactosidase in mannitol, inositol, and glucose to study the effects of excipient crystallinity on the short-term stability of the model protein. As the relative amount of mannitol and inositol in the formulation increases, the stabilizer crystallizes more readily, with a corresponding decrease in recovery of protein activity after freeze-drying. Annealing during the process was also shown to promote crystallization of the excipient and to decrease recovery of protein activity. This work was extended to the enzymes lactate dehydrogenase and L-asparaginase in a later study by the same authors (Izutsu *et al.*, 1994).

Sarciaux and Hageman (1997) examined the effect of relative concentration of recombinant bovine somatotropin (rbSt) and sucrose on the tendency of sucrose to crystallize. Sucrose crystallization was measured by MTDSC (see discussion above), using the temperature of the crystallization exotherm for sucrose. Addition of up to 20% rbSt to sucrose did not measurably change the T_g of sucrose; however, the crystallization temperature of sucrose increased from 110°C for sucrose alone to 140°C when 20% sucrose was added. Sucrose crystallization could be monitored gravimetrically by use of a controlled atmosphere microbalance at room temperature and elevated relative humidity. Induction time for sucrose

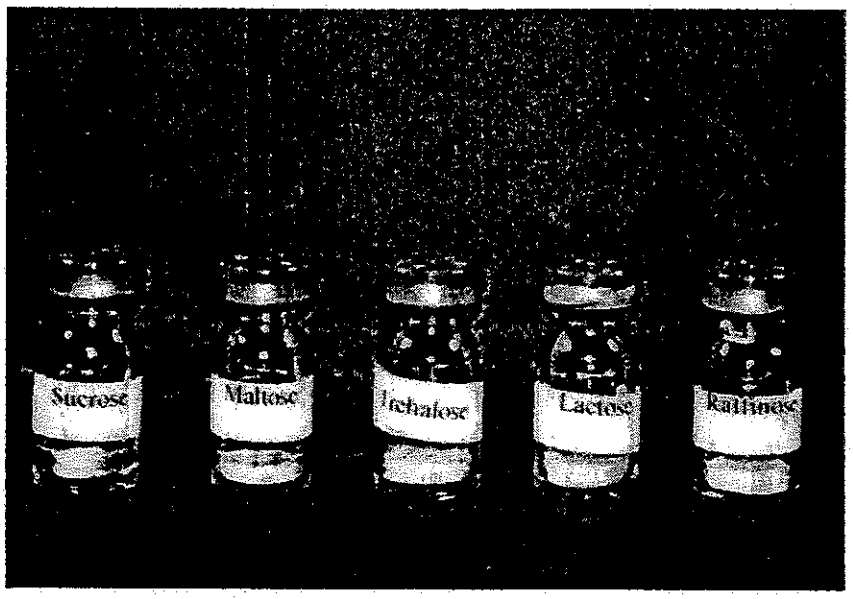

Figure 29. Freeze-dried disaccharides, showing collapse during storage at 50°C for 24 hr. As storage temperature increases, the solids collapse in order of increasing glass transition temperature.

crystallization at room temperature and 45% RH increased almost 10-fold with addition of 10% rbSt to sucrose. The authors conclude that using high protein concentrations results in a more robust formulation with respect to physical stability.

No reports have been published on the effect of excipient crystallization on long-term stability of a protein formulation. Herman *et al.* (1994) examined the long-term stability of a steroid, methylprednisolone sodium succinate, in the presence of either mannitol or lactose. The mannitol formulation, initially amorphous by X-ray powder diffraction, was shown to crystallize during storage at 40°C after a few weeks, and the steroid hydrolyzed at a relatively rapid rate as a freeze-dried solid. When mannitol was replaced with lactose, the formulation remained amorphous over the duration of the study and the drug was stable. This effect was attributed in part to redistribution of water in the freeze-dried solid as a result of mannitol crystallization. The increased water activity in the microenvironment of the drug is a likely cause of accelerated reactions in the solid state.

It is good practice to include periodic examination of the physical state of freeze-dried protein formulations by X-ray powder diffraction during

stability studies. Designing formulations with high T_g, although no assurance against chemical instability, is a rational method for minimizing physical instability such as collapse or crystallization of a critical component of the formulation.

6.3. Phase Separation during Freezing: Protein Stability Implications

As discussed in the section on freezing behavior, liquid–liquid phase separation can take place when enthalpically unfavorable interactions between solutes outweigh the favorable entropy of a homogeneous solution. Examples are polymer–polymer phase separation and polymer–phosphate buffer phase separation. This phase separation can also occur between different proteins and between proteins and polysaccharides. Heller *et al.* (1996) used recombinant human hemoglobin as a model protein and combinations of various concentrations of PEG and dextran to study the effects of phase separation on the secondary structure of hemoglobin after both freeze-thawing and freeze-drying. A solution of 7% PEG and 7% dextran separates into PEG-rich and dextran-rich phases at 0°C, and the concentration of hemoglobin in the dextran-rich phase is approximately three times that in the PEG-rich phase. The α-helical content, as measured by FTIR at 1656 cm^{-1}, was used to estimate the effects of freezing and freeze-drying on both phases. Both polymers have about the same protective effect during freezing, but these effects differ significantly during drying, with the PEG-rich phase showing the greater protective effect.

In an extension of this work, Heller *et al.* (1997) used 4% PEG and 4% dextran in a phosphate-buffered saline solution containing either NaCl or KCl. This combination of polymer concentrations is below the two-phase region at 0°C, and the concentrations of salt used were shown to have little effect on the phase diagram at room temperature. However, freeze-concentration would be expected to force this system into the two-phase region. Evidence of phase separation was observed by SEM for the formulation containing sodium chloride, and this phase separation was enhanced by annealing at −7°C. Loss of secondary structure was shown to correlate with the extent of phase separation during freezing. This effect was particularly striking for annealed versus unannealed samples in the sodium chloride-containing system. Some protection of native structure was observed for unannealed samples; however, the amount of α-helical structure remaining after freeze-drying decreases with annealing time up to about 18 hr. The decrease in α-helix content with annealing does not occur

for the system containing potassium chloride, however. The authors attribute this observation to the formation of a stable glass in the potassium chloride system, which kinetically prevents phase separation. Regardless of the mechanism, preventing phase separation prevents loss of the native structure of hemoglobin.

7. SUMMARY

Given the increasing importance of reducing development time for new pharmaceutical products, formulation and process development scientists must continually look for ways to "work smarter, not harder." Within the product development arena, this means reducing the amount of trial and error empiricism in arriving at a formulation and identification of processing conditions which will result in a quality final dosage form. Characterization of the freezing behavior of the intended formulation is necessary for developing processing conditions which will result in the shortest drying time while maintaining all critical quality attibutes of the freeze-dried product. Analysis of frozen systems was discussed in detail, particularly with respect to the glass transition as the physical event underlying collapse during freeze-drying, eutectic mixture formation, and crystallization events upon warming of frozen systems. Experiments to determine how freezing and freeze-drying behavior is affected by changes in the composition of the formulation are often useful in establishing the "robustness" of a formulation. It is not uncommon for seemingly subtle changes in composition of the formulation, such as a change in formulation pH, buffer salt, drug concentration, or an additional excipient, to result in striking differences in freezing and freeze-drying behavior.

With regard to selecting a formulation, it is wise to keep the formulation as simple as possible. If a buffer is needed, a minimum concentration should be used. The same principle applies to added salts: If used at all, the concentration should be kept to a minimum. For many proteins a combination of an amorphous excipient, such as a disaccharide, and a crystallizing excipient, such as glycine, will result in a suitable combination of chemical stability and physical stability of the freeze-dried solid.

Concepts of heat and mass transfer are valuable in rational design of processing conditions. Heat transfer by conduction—the dominant mechanism of heat transfer in freeze-drying—is inefficient at the pressures used in freeze-drying. Steps should be taken to improve the thermal contact between the product and the shelf of the freeze dryer, such as eliminating metal trays from the drying process. Quantitation of the heat

transfer coefficient for the geometry used is a useful way of assessing the impact of changes in the system such as elimination of product trays and changes in the vial. Because heat transfer by conduction through the vapor increases with increasing pressure, the commonly held point of view that "the lower the pressure, the better" is not true with respect to process efficiency. The optimum pressure for a given product is a function of the temperature at which freeze-drying is carried out, and lower pressures are needed at low product temperatures. The controlling resistance to mass transfer is almost always the resistance of the partially dried solids above the submination interface. This resistance can be minimized by avoiding fill volumes of more than about half the volume of the container. The development scientist should also recognize that very high concentrations of solute may not be appropriate for optimum freeze-drying, particularly if the resistance of the dried product layer increases sharply with concentration.

Although the last 10 years has seen the publication of a significant body of literature of great value in allowing development scientists and engineers to "work smarter," there is still much work needed in both the science and the technology of freeze-drying. Scientific development is needed for improving analytical methodology for characterization of frozen systems and freeze-dried solids. A better understanding of the relationship between molecular mobility and reactivity is needed to allow accurate prediction of product stability at the intended storage temperature based on accelerated stability at higher temperatures. This requires that the temperature dependence of glass transition-associated mobility, particularly at temperatures below the glass transition, be studied in greater depth. The relevance of the concept of strong and fragile glasses to frozen systems and freeze-dried solids has only begun to be explored. The list of pharmaceutically acceptable protective solutes is very short, and more imagination—and work—is needed in order to develop pharmaceutically acceptable alternative stabilizers.

There is a need for technology development in process monitoring, particularly in developing a way to measure the status of the product during freezing and freeze-drying without placing temperature measurement probes in individual vials of product. The current practice of placing thermocouples in vials is uncertain with respect to reliability of the data, inconsistent with elimination of personnel in close proximity to open vials of product in an aseptic environment, and incompatible with technology for automatic material handling in freeze-drying. In addition, a method for controlling the degree of supercooling during freezing would allow better control of freezing rate and would, in many cases, result in more consistent product quality.

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