RATIONAL DESIGN OF STABLE LYOPHILIZED PROTEIN FORMULATIONS: THEORY AND PRACTICE

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"Give a man a fish and he will eat for a day.
 But teach a man to fish and he will sit around in boat all day drinking beer."
 (Thanks to Eric Bergstrom)

INTRODUCTION

For ease of preparation and cost containment by the manufacturer, and ease of handling by the end user, an aqueous therapeutic protein formulation usually is preferred. However, with many proteins it is not possible -- especially considering the time constraints for product development -- to develop sufficiently stable aqueous formulations. Unacceptable denaturation and aggregation can be induced readily by the numerous stresses to which a protein in aqueous solution is sensitive; e.g., heating, agitation, freezing, pH changes, and exposure to interfaces or denaturants (Arakawa et al., 1993; Cleland et al., 1993; Brange, 2000; Bummer and Koppenol, 2000). Furthermore, even under conditions that thermodynamically greatly favor the native state of proteins, aggregation can arise during months of storage in aqueous solution (e.g., Gu et al., 1991; Arakawa et al., 1993; Chen et al., 1994; Volkin and Middaugh, 1996; Chang et al., 1996a). In addition, several chemical degradation pathways (e.g., hydrolysis and deamidation) are mediated by water. In aqueous formulations, the rates of these and other (e.g., oxidation) chemical degradation reactions can be unacceptably rapid on the time scale of storage (e.g., 18-24 months) for pharmaceutical products (Manning et al., 1989; Cleland et al., 1993; Goolcharran et al., 2000; Bummer and Koppenol, 2000).

In contrast, a properly lyophilized formulation can maintain adequate physical and chemical stability of the protein during shipping and long-term storage, even at ambient temperatures. As will be outlined in this chapter, developing stable lyophilized protein formulations should be a rational, straightforward process, which for most proteins should be a rapid. With liquid formulation development, it may only be possible to obtain adequate protein stability after lengthy studies. Furthermore, sometimes there are conflicting conditions (e.g., pH) needed to slow sufficiently multiple degradation pathways in aqueous solution. Considering these issues plus the fact that formulation scientists now have to deal with numerous proteins and/or variants of a given protein, lyophilization should be considered as a primary mode for product development. Only if a parallel effort to develop an aqueous formulation is successful, will a final lyophilized product not be needed.

Rapid formulation development has important financial ramifications. A drug product has a finite patent life, during which time the company has an exclusive market. Considering that even a moderately successful drug product has annual sales of hundreds of millions of dollars, potentially millions of dollars in sales are lost for each day of delay in bringing a product to market. Unfortunately, there are often delays because the formulations designed during early stage development and clinical trials (e.g., frozen) were not adequate for the final product. With a rational approach to formulation development, pharmaceutical scientists and process engineers can minimize the risk of this problem and the time needed to obtain a successful, final formulation. The success of such efforts depends on frank, open communication between the groups involved. For example, it is critical that the formulation scientists learn from the process engineers the issues for large-scale lyophilization runs, which are usually conducted in units that do not have the capacity to match processing parameters obtained in a small-scale research lyophilizers.

Despite the best efforts of the scientists and engineers, all too often delays in formulation development arise because sufficient resources are not invested in product development.   For example, sometimes purchase of even essential equipment (e.g., a differential scanning calorimeter), which costs a minute fraction of a day's sale of product, is not allowed. To avoid unnecessary delays in product launch, which can have disastrous consequences for the company and for patients, it is essential that companies appreciate that product development is ultimately a key limiting factor in getting a therapeutic to market. Hence, development efforts need to be as well funded as the usually much more visible drug discovery research programs. If a product is not stable, it will not be marketed, no matter how dramatic an impact it can have on human health and the financial status of the company.

MINIMAL CONDITIONS FOR A SUCCESSFUL LYOPHILIZED PROTEIN FORMULATION

Research over the past several years has demonstrated that five criteria define the **minimal** conditions necessary to obtain a successful lyophilized protein formulation (Table 1). The first four criteria can be met with use of the appropriate excipients and lyophilization cycle design. For information on the proper design of lyophilization cycles, the reader is directed to the numerous previous reviews in this area (Franks, 1990; Pikal, 1990; Nail and Gatlin, 1993; Gatlin and Nail, 1994; Carpenter and Chang, 1996; Rey and May, 1999; Cappola, 2000). For the current chapter we will only consider cycle design in terms of the interplay between formulation physical properties (e.g., collapse temperature) and process parameters (see below). The last criterion listed in Table 1 requires insight into the unique physicochemical properties of each therapeutic protein, which will be explained in more detail below. We will explain in turn why each of these criteria is important. Then we will present an explanation of how to design rationally a formulation to meet these criteria.

Inhibition of Lyophilization-Induced Protein Unfolding

The stresses of freezing and drying cause protein unfolding, and the formulation must be designed to inhibit unfolding at each step (Prestrelski et al., 1993a,b; Carpenter et al., 1993; Prestrelski et al., 1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Chen et al., 1999; Bell, 1999; Carrasquillo et al., 2000).   Even if the formulation excipients and/or intrinsic thermodynamic stability of the protein prevent denaturation during freezing, unfolding can arise during subsequent drying (Carpenter et al., 1993; Prestrelski et al., 1993b; Allison et al., 1998; Carrasquillo et al., 2000). Conversely, once a protein unfolds during freezing, it will not regain native structure during dehydration.

For many proteins, unfolding during lyophilization leads to clinically unacceptable, non-native aggregates, even when samples are rehydrated immediately after lyophilization (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a; 1999; Costantino et al., 1998). Aggregates are not necessarily formed during freezing and drying. Rather, during rehydration refolding of structurally perturbed protein molecules competes

with formation of non-native protein aggregates (Prestrelski et al., 1993a). Aggregation can be minimized by including stabilizing excipients (e.g., sucrose or trehalose), which inhibit lyophilization-induced unfolding, in the formulation (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Furthermore, fostering refolding during rehydration (e.g., with surfactants) can reduce aggregation (Chang et al., 1996c; Zhang et al., 1995,1996).

In addition to minimizing protein aggregation during lyophilization/rehydration, maximizing retention of native protein structure in the dried solid is essential for optimizing long-term storage stability (Prestrelski et al., 1995; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Allison et al., 2000; Cleland et al., 2000). Both chemical and physical degradation in the dried solid can be accelerated if protein unfolding is not inhibited during lyophilization. With chemical degradation, a non-native structure may provide an environment conducive to covalent modification of one or more residues. For example, exposure of a methionine, which is normally buried deep in the interior of the native protein, on the surface of an unfolded dried protein may foster oxidation. Increased levels of aggregates noted after storage and rehydration of unfolded proteins could be due to formation of non-native intermolecular contacts within the dried solid, perturbation of refolding during rehydration because of chemical degradation, and/or other undefined processes.

Infrared spectroscopy has been used routinely to compare the secondary structures of a protein in lyophilized formulations to that of the native protein in aqueous solution dried states (Prestrelski et al., 1993a,b; Prestrelski et al., 1995; Dong et al., 1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Carpenter et al., 1998; Chen et al., 1999; Carrasquillo et al., 2000). This method should be considered essential in the development of stable lyophilized formulations, because it allows one to assess rapidly the effectiveness of formulations at inhibiting protein unfolding. Technical details about how to employ infrared spectroscopy to design stable lyophilized protein formulations can be found in the papers cited above.

Storage at Temperatures Below Formulation Glass Transition Temperature

In the dried powder, the protein is a component of an amorphous phase that includes amorphous excipients and water. If this glassy matrix is held below its characteristic glass transition temperature (T_g), the rate of diffusion-controlled reactions, including protein unfolding and aggregation, and chemical degradation, are greatly reduced, relative to rates noted at temperatures > T_g (Roy et al., 1989; Franks, 1990; Franks et al., 1991; Pikal, 1994,1999). T_g can be determined with differential scanning calorimetry (DSC) or other thermal scanning methods (Nail and Gatlin, 1993; Chang and Randall, 1992; Craig and Royall, 1998; Verdonck et al., 1999).

Obtaining a formulation T_g in excess of the planned storage temperature (e.g., room temperature) is absolutely essential for optimal protein stability (e.g., Franks et al., 1991; Pikal, 1994; 1999; Carpenter and Chang, 1996; Duddu and Dal Monte, 1997). The T_g of a

given amorphous phase is dependent on the T_g and mass percent of each component, including water (Angell, 1995; Franks et al., 1991; Levine and Slade, 1988, 1992; Pikal, 1994; 1999). Compared to excipients, dried proteins have relatively high T_g's (e.g., > 150ºC; Angell, 1995). Thus, with all other factors being held constant, the formulation T_g varies directly with the mass fraction of protein. However, care must be taken that the mass fraction of protein is not so high that there are not adequate levels of stabilizing excipients to prevent protein unfolding during lyophilization (Cleland et al., 2000; and see below).

Fortunately, sucrose and trehalose, which are the preferred excipients for inhibiting lyophilization-induced protein unfolding (see below), also provide a glassy matrix with acceptably high T_g values. For example, with water contents of 1% the Tg for pure sucrose and trehalose are about 100 and 65ºC, respectively (Crowe et al., 1998).

It has now been documented with several proteins, that simply storing the formulation at temperature below T_g alone does not assure optimal stability. A native protein structure is also required. For example, proteins lyophilized in dextran alone are usually unfolded, but in a glassy matrix with a relatively high T_g (e.g., > 75ºC). Yet they still degrade at relatively rapid rates compared to those for native protein molecules lyophilized with either sucrose or trehalose (Krielgaard et al., 1998a, 1999; Lueckel et al., 1998; Allison et al., 2000; Yoshioka et al., 2000). On pharmaceutical time scales of several months of storage many degradative reactions are not coupled to the glass transition of a formulation. This is because on these times scales there is still significant molecular mobility, even at temperatures well below (e.g., more than 30ºC) the T_g (Hancock et al., 1995; Duddu et al., 1997; Pikal, 1999; Yoshioka et al., 1999).

The Water Content is Relatively Low

Because of its very low T_g (-135º C), water is a potent plasticizer for glasses; increasing water content in the dried formulation will greatly reduce T_g. For example, increasing the water content of pure sucrose from 1 to about 3-4% (g H_2O/100 g dried powder) is sufficient to reduce the T_g to below room temperature (Crowe et al., 1998). It is critical to achieve a sufficiently low water level for a given formulation such that T_g exceeds the planned storage temperature. The lyophilization cycle dictates the initial water content (see reviews listed above). The most important parameter is the temperature for secondary drying, during which the nonfrozen water is desorbed (Pikal et al., 1990).

Water can also be transferred to the product from the vial stoppers during storage (Pikal and Shah, 1992; DeGrazio and Flynn, 1992; Hora and Wolfe, 1999). This effect can be dramatic. For example, let's consider a formulation containing 10 mg of dried protein, 40 mg of sucrose and initial water content of 1% by weight. The total amorphous fraction containing protein and sucrose has 0.50 mg of water. If 1.0 mg of water was transferred from the stopper to the product, the water content of this fraction would increase from 1% to 3.0%. This increase would be sufficient to lower the formulation T_g to below room temperature (Crowe et al., 1998). The risk of transfer of moisture from stoppers can be minimized by drying the stoppers before use, and, if acceptable for a given product, using stoppers coated with a material such as Teflon (see Hora and Wolfe, 1999).

A Strong, Elegant Cake Structure is Obtained

Often the most desired cake has strong, porous structure, without macroscopic collapse or meltback. This structure has a high surface area to volume ratio, which aids in the rapid dissolution of product upon addition of water. A detailed account of how to obtain such a cake structure is beyond the scope of the current chapter, but is available in several previous reviews (see above). For the current purposes it is sufficient to focus on the impact of formulation composition on avoiding collapse or meltback. When a product is frozen, the protein and amorphous excipients (e.g., sucrose) are dispersed between ice crystals and any excipient used as a crystalline bulking agent (e.g., glycine). To obtain an appropriate cake structure during lyophilization, the product temperature during primary drying, when the water in ice is sublimed, must be below the characteristic collapse and eutectic melting temperatures of amorphous and crystalline solutes, respectively. Above the eutectic temperature, the melting of crystalline solutes leads to massive loss of porous structure and macroscopic dissolution of the frozen matrix into a "puddle". Above the collapse temperature, which closely coincides with the glass transition temperature (T_g') of the maximally freeze-concentrated amorphous phase, the amorphous phase cannot support its own weight. The result is also a loss of pore structure and a macroscopic shrinkage or collapse of the cake.

It should be noted that the T_g' thermal event, which can be measured with differential scanning calorimetry, is also referred to as a softening event (T_s), rather than the actual glass transition temperature of the freeze-concentrated amorphous phase. Whatever the exact nature of the thermal event, it can be detected with differential scanning calorimetry as a second order increase in the baseline of the thermogram, which usually occurs just prior to the onset of the endotherm for the melting of ice (e.g., Her and Nail, 1994). The transition can also be measured with electrothermal methods (Her et al., 1994).

Another powerful method, which is essential for rational development of lyophilized formulation, for determining collapse and eutectic melting temperatures is freeze-drying microscopy (e.g., Nail et al., 1994). With this approach, the formulation of interest is directly examined visually for its performance during a simulated freezing, annealing and drying cycle. All of the critical phase changes, including ice formation, solute crystallization, eutectic melting and collapse, can be detected easily, and the temperature of their occurrence can be measured accurately.

Formulation composition dictates collapse temperature. Each pure amorphous excipient has a characteristic T_g' and collapse temperature; the collapse temperature for the formulation is the mass averaged temperatures of all of the components in the amorphous phase. It is important to design a formulation with maximum collapse temperature, because the rate of drying is directly proportional to the sample temperature during lyophilization. To allow for a reasonable drying time the T_g' should not be lower than -40º C. The T_g' values for pure sucrose is -32º C, while that of pure trehalose is -30º C (Skrabanja et al., 1994; Chang and Randall, 1992). In contrast, glucose, which should also be avoided

because it is a reducing sugar, has a T_g' of -43º C. The collapse temperature of pure protein is about -10º C, which means increasing the protein:sugar mass ratio will increase collapse temperature. Finally, collapse temperature will be decreased if salts and excipients are not maximally crystallized. For example, glycine has a Tg' of -45º C, and its contribution to the amorphous phase can reduce collapse temperature to impractically low values (Chang and Carpenter, 1996). This problem can be avoided by using the appropriate annealing (see Carpenter and Chang, 1996) of the frozen product to maximize crystallization.

Meltback can be avoided by using crystalline solutes with relatively high eutectic melting temperatures. In contrast, additives such as calcium chloride have very low eutectic melting temperatures (e.g., -51º C for calcium chloride). If mannitol or glycine is used a crystalline bulking agent, the T_g' of the amorphous excipient phase, which is lower that the eutectic melting temperatures of these excipients, will be the value that dictates the temperature of primary drying.

Collapse can also occur during secondary drying, when unfrozen water is desorbed, if the temperature is increased too rapidly. As water is removed from the amorphous phase the T_g of this phase increases. Thus, product temperature can be raised gradually and collapse can be avoided if product temperature at a given time point does not exceed the T_g. Directions for optimizing secondary drying can be found in previous reviews mentioned above.

Steps Taken to Minimize Specific Routes of Protein Chemical Degradation

It is essential that the major routes of chemical, as well as physical, degradation be characterized carefully for each protein, because all of the criteria listed above can be met and a protein might still be damaged during long term storage in the dried solid. For example, methionine oxidation is a common degradation pathway for therapeutic proteins (Manning et al., 1987; Ahern and Manning, 1992; Cleland et al., 1993; Goolcharran et al., 2000). Even in a formulation, which prevents protein unfolding and has a T_g exceeding the storage temperature, methionine oxidation can proceed at an unacceptably rapid rate. If a methionine residue is on the surface of the protein, then maintaining native structure would not prevent this residue's exposure to reactive oxygen species in the dried solid. But why would the glassy matrix not sufficiently retard the mobility of the reactive species to prevent the reaction from occurring? On pharmaceutical time scales (i.e., of many months) relevant motion in a glass is not arrested unless the storage temperature is about 50º C below the T_g (e.g., Hancock et al., 1994; Pikal, 1994; Pikal, 1999). Therefore, the mobility of a relatively small reactive oxygen species and the oxidation of methionine residues are probably not coupled to the glass transition of the formulation. An intriguing alternative, but not mutually exclusive, explanation has been suggested by Steve Prestrelski (personal communication). He proposes that reactive oxygen species accumulating during long-term storage might not be causing damage just in the dried solid. Rather upon rehydration oxygen radicals rapidly react with the protein. This is an important area for future research.

Fortunately, despite the rather complicated and poorly understood theoretical aspects of unacceptable methionine oxidation rates in dried protein formulations, from a

practical viewpoint dealing with the problem is relatively straightforward. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using formulation additives (e.g., free methionine) to compete with protein residues for reactive oxygen species. These approaches should be effective whether the oxidation of residues occurs in the dried solid, during rehydration or at both times.

Rational Design of Stable Lyophilized Protein Formulations

Two critical practical issues need to be considered when choosing excipients for a therapeutic protein formulation. 1) From a financial standpoint, scientists should focus on using excipients and processing approaches that do not need to be licensed from a patent holder. Acceptable protein stability can be achieved readily with using excipients and processing methods that are well known to someone, to use the legal term, "skilled in the art of protein formulation." Clearly, however, the specific applications of such compounds and processes to a given protein drug product and/or a class of proteins often can be patented. Such patents may be critical to the company developing that product.

2) From a regulatory standpoint (and financial perspective, also), the formulation scientist should choose from among excipients that are already used in approved parenteral products. For protein stabilizers, the best choices are the disaccharides, sucrose and trehalose. For bulking agents, the best choices are glycine, mannitol and hydroxyethyl starch. For surfactants, usually the Tweens (20, 40 or 80) are preferred.

Based on these practical concerns and the criteria for a successful lyophilized protein product (Table 1), prototypic rational formulations are presented in Table 2. If the formulations suggested are employed, and the criteria in Table 1 are met, then most likely the product will have acceptable stability.

It is important to emphasize that not all components listed in Table 2 may be needed for a given protein product. For example, it may not be necessary to include a nonionic surfactant to aid in reducing protein aggregation. If there is not clear evidence that a given component is beneficial for a formulation, then that component should not be included in the formulation. Also, some components that are used because of "tradition" and/or for purposes such as final formulation tonicity can cause great difficulties in lyophilization. The most common example is the use of NaCl as a tonicity modifier. During lyophilization, NaCl can greatly reduce the collapse temperature of a formulation, if a fraction of the salt does not crystallize (Her et al., 1995). Crystallization of NaCl during freezing and annealing can be inhibited by other excipients (e.g., bulking agents and stabilizing sugars).   Because of the low collapse temperature, a low temperature cycle must be used, which increases production time and costs. Also, even if formulations with NaCl can be lyophilized successfully in small research lyophilizers, there is great risk that a large fraction of vials will collapse during large scale manufacturing runs. Thus, if at all possible, NaCl should not be used in lyophilized formulations. Alternative tonicity modifiers include mannitol and glycine, which can also serve as crystalline bulking agents.

Every protein and product has unique characteristic, some of which may cause greater difficulty in designing stable formulations. Thus, the suggestions in Table 1 should be considered only as a good starting point in formulation development. For the remainder of this chapter we will discuss the rationale for the choice of each of the formulation components, their mechanisms of action and other practical approaches that can be used to increase protein stability.

Choice of Buffer

In terms of protein stability, the main concern with choice of buffer is the potential for certain buffer salts to precipitate during freezing and cause large changes in pH. For example, crystallization of the dibasic form of sodium results can result in a pH < 4 (van den Burg, 1959; van den Burg and Rose, 1959; Anchordoquy and Carpenter, 1997). Thus, whenever possible, sodium phosphate buffer should be avoided. Although somewhat obvious, it is important to realize that a sodium phosphate system will be present if one starts with potassium phosphate buffer salts and NaCl, as is the case with phosphate buffered saline. Alternative buffers that do not appear to have major pH changes during freezing include Tris and histidine (Anchordoquy and Carpenter, 1996).

A clear example of the detrimental effects of buffer acidification on the stability of a lyophilized protein formulation has been reported by Lam et al. (1996). These researchers documented that a succinate buffer with an initial pH of 5 exhibited a pH drop of 1-2 units during lyophilization. The result was a reduced stability of interferon-γ during storage of the lyophilized, relative to that noted for formulations prepared with glycolate buffer, which does not undergo freezing-induced acidification.

Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein

Often from experience gained during purification, intermediate storage protocols and preformulation studies can provide great insight into specific solution conditions (e.g., pH, ionic strength, buffer type) that confer the greatest stability to a given protein. Before choosing the appropriate "general" stabilizers, which are effective at protecting most proteins, it is absolutely essential that the formulation be optimized for the specific factors that increase the physical and chemical stability of a given protein. For example, simply avoiding extremes in pH can drastically reduce the rate of deamidation (Manning et al., 1987; Goolcharran et al., 2000). Also, specific ligands that increase protein physical stability (e.g., by increasing the free energy of unfolding) should be investigated. The stabilizing effects of heparin and other polyanions on growth factors (e.g., Chen et al., 1994; Volkin and Middaugh, 1996) and calcium on DNase (Chen et al., 1999) provide good examples. As will be described below, increasing the free energy of unfolding directly correlates with increased resistance of proteins to denaturation during freezing. In addition, at least in the case of DNase, there was also dramatic increases in storage stability of the lyophilized protein in formulation containing calcium, which were not due to

increased structural stabilization during lyophilization (Chen et al., 1999). The mechanism for this effect is unknown.

Moreover, sometimes protein unfolding during freeze-drying can be minimized by optimizing initial solution pH (e.g., Prestrelski et al., 1995). However, in other cases it has been found that initial solution pH does not impact the degree of unfolding arising during lyophilization (Costantino et al., 1995; Carrasquillo et al., 2000). For each protein it is necessary to investigate the effect of initial pH (and potential pH changes during freezing) on structural retention during lyophilization. Infrared spectroscopy can be used to monitor secondary structure in the dried solid, and the resulting data can be used to choose the initial pH that results in the most native-like secondary structure in the dried solid.

Trehalose or sucrose to inhibit protein unfolding and provide glassy matrix

In this section we will discuss the rationale for choice of stabilizing excipients. Then we will describe the mechanisms for protection of proteins by additives during freezing and drying. Mechanistic insight is important for a clear understanding of protein stabilization during lyophilization and for guiding critical practical choices such as determining the level of disaccharide needed for optimal protein protection. Finally, we will address this and other practical issues in the use of stabilizing excipients to inhibit protein unfolding during freezing and drying.

Rationale for choice of stabilizing sugar. Among the numerous compounds tested, it appears that the most effective stabilizers of proteins during lyophilization are disaccharides (reviewed in Carpenter et al., 1999). Trehalose and sucrose are the best choices for a stabilizing disaccharide for therapeutic proteins. Both sugars: 1) protect proteins during both freezing and dehydration; 2) are nonreducing; 3) tend to remain amorphous during lyophilization; and 4) have been used in approved parenteral therapeutic products. There are, however, some important differences in the physicochemical properties of these sugars. 1) Trehalose has a higher T_g at a given moisture content than sucrose and, thus, for formulations containing trehalose it may be easier to obtain an appropriate cake structure with an economical lyophilization cycle (Crowe et al., 1998). However, a skilled process engineer should be able to design economical, effective cycles for formulations containing either sugar. In addition, the condition of having a T_g greater than the product storage temperature will hold at higher residual water contents for trehalose. In products with a relatively high protein concentration, the protein could contribute to an increased T_g, which serves to minimize the advantages of trehalose. 2) Trehalose is more resistant than sucrose to acid hydrolysis. Hydrolysis of these disaccharides produces reducing sugars, which must be avoided. Usually this is not a problem, unless very low pH's (ca. < 4) are employed. It should be noted that acid catalyzed hydrolysis of sucrose can occur even in lyophilized solids (Shalaev et al., 2000), suggesting that buffer acidification during freezing (see below) could ultimately result in formation of reducing sugars and resulting damage to proteins in a lyophilized formulation. 3) Sucrose appears to be somewhat more effective at inhibiting unfolding during

lyophilization (Allison et al., 1999). This difference has been most obvious when there is a relatively high protein concentration and a need to employ a relatively high initial concentration of sugar. Evidence to date indicates that less effective stabilization by trehalose is due to the greater propensity of this sugar to phase separate from polymers (Isutzu et al., 1997) and proteins (S.D. Allison, T.W. Randolph, B.S. Chang and J.F. Carpenter, unpublished observation) during freezing and drying. Whether or not this is a problem with a given formulation cannot be predicted. Hence, the relative capacities of sucrose versus trehalose to inhibit lyophilization-induced unfolding of must be examined for each protein. The final choice of sugar for a given protein product should be based on a direct comparison of sucrose and trehalose as stabilizers during the lyophilization cycle and storage in the dried solid.

Reducing sugars such as maltose or lactose should not be used. These compounds may effectively inhibit protein unfolding during the lyophilization cycle, but during storage in the dried solid they can degrade proteins via the Maillard reaction between carbonyls of the sugar and free amino groups on the protein (Hageman, 1992; Li et al., 1996).

Mechanism for freezing protection. Many different compounds, such as sugars, polyols, certain amino acids, methylamines and salting-out salts, nonspecifically stabilize proteins during freezing or freeze-thawing ("cryoprotection"). The same compounds have also been shown in nonfrozen aqueous systems to increase protein thermodynamic stability (e.g., increase resistance to themperature or chaotrope-induced unfolding). Protection of proteins by sugars during freezing and freeze-thawing is due to the same universal thermodynamic mechanism that Timasheff and colleagues have defined for solute-induced protein stabilization in nonfrozen aqueous solution (reviewed in Carpenter and Crowe, 1988; Carpenter and Chang, 1996; Carpenter et al., 1999; Timasheff, 1998).

Usually relatively high concentrations (ca. > 0.3 M) of solute are needed to stabilize proteins because the interactions of the solute with the protein are relatively weak. Stabilizing solutes are excluded preferentially from the surface of the protein.   This statement does not mean that no solute molecules bind to the surface of the protein, but rather that there is a lower concentration of solute in the immediate vicinity of the protein than in the bulk solution. The chemical potential of the protein is increased in the presence of a preferentially excluded solute, which is the basis for the affect of solute of protein thermodynamic stability. Considering a two-state model for protein folding (N $\leftrightarrow$ D), the native state is favored thermodynamically (e.g., under physiological conditions) because it has a lower free energy than the denatured state. The key to increasing the thermodynamic stability of the native state is to increase the free energy barrier between the native and denatured states.   So how can increasing the chemical potential of the native state result in stabilization? The degree of preferential exclusion and concomitant increase in protein chemical potential correlates directly with the protein surface area.   The denatured state has a much greater surface area than the native state. Thus in the presence of a preferentially excluded solute the magnitude for the increase in protein chemical potential will be much greater for the denatured than that for the native state; the native state will be stabilized.

This same mechanism applies to the inhibition of smaller scale structural expansions of the native state, which may be sufficient to promote irreversible protein aggregation, by compounds such as sucrose (e.g., Chang et al., 1996a; Kendrick et al., 1997,1998; Kim et al., 2000). Many proteins are known to form non-native aggregates from species with conformations that are not greatly different from the most compact native state. One example is the molten globule that has perturbed tertiary structure, but native secondary structure. However, even expanded species within that native state ensemble can participate in intermolecular interactions leading to non-native protein aggregates (e.g., Chang et al., 1996a; Kendrick et al., 1997,1998; Kim et al., 2000). Preferentially excluded solutes shift the equilibrium between protein species towards that with the lowest surface area, i.e., the most compact species in the native state ensemble. As a result aggregation is inhibited.

Timasheff's preferential interaction mechanism also explains the influence of solutes on the degree of assembly of multimeric proteins. Preferentially excluded solutes tend to induce polymerization and stabilize oligomers since the formation of contact sites between constituent monomers serves to reduce the surface area of the protein exposed to the solvent. Polymerization reduces the thermodynamically unfavorable effect of preferential solute exclusion. In this case, the assembled protein is much more stable than the constituent monomer because of the direct solute effects, but also because of the increase in oligomer stability gained by intersubunit contacts (e.g., Neet and Timm, 1994). In one example of this effect during lyophilization, maintenance of the native tetramer during freezing has been shown to increase the resistance of lactate dehydrogenase to dissociation and inactivation during subsequent drying (Anchordoquy and Carpenter, 1996; Anchordoquy et al., 2001).

How do we know that the preferential exclusion mechanism is actually operative in the frozen state? It is not possible to measure directly the protein-solute interaction in the frozen state. However, the effects of solutes on protein chemical potential and the resulting protein stabilization can be inferred from a freezing study with hemoglobin, in which the protein was partitioned into separate polyethylene glycol-rich and dextran-rich solution phases (Heller et al., 1996). In a phase separated system, thermodynamic equilibrium requires that chemical potential of each component be equal in both phases. Hence, there should be equivalent effects of the different solute conditions in the two phases on the increase in free energy of protein unfolding. In other words, the impact of preferentially excluded solutes on protein stability is equal in the two phases. As predicted from the preferential exclusion mechanism, the degree of structural protection of hemoglobin during freezing (as observed directly in the frozen state with infrared spectroscopy) was equivalent in both phases (Heller et al., 1996).

Mechanism for inhibition of dehydration-induced unfolding. The interaction of a protein's residues with water are intimately involved with the formation of the native, globular protein structure, and if the protein is dehydrated it will unfold. It has been documented by numerous studies that sucrose and trehalose prevent dehydration-induced unfolding by hydrogen bonding to the dried protein in place of the lost water (e.g., Carpenter and Crowe, 1989; Prestrelski et al., 1993a; Allison et al., 1999; Costantino et al.,

1998; Wolkers et al., 1998; Tzannis and Prestrelski, 1999). This so called "water replacement mechanism" is supported by several different observations.

For example, with infrared spectroscopy, it has been found that the band at 1583 cm^{-1} in the spectrum for lysozyme, which is due to hydrogen bonding of water to carboxylate groups, is not present in the spectrum for the dried protein (Carpenter and Crowe, 1989; Remmele et al., 1997; Allison et al., 1999). When lysozyme is dried in the presence of trehalose or sucrose, the carboxylate band is retained in the dried sample, indicating that the sugar is hydrogen bonding in the place of water. Similar results have been obtained with α-lactalbumin and sucrose (Prestrelski et al., 1993a). The magnitude of the retention of the carboxylate band correlates directly with the level of trehalose or sucrose in the lyophilized formulation, as well as the degree of inhibition of unfolding (Allison et al., 1999). These effects of sugars on proteins in the dried solid are not due to the presence of increased amounts of water in the formulations dried with sugars (Allison et al., 1999; Tzannis and Prestrelski, 1999). The level of water in formulations dried with the sugars is as low as that for the protein lyophilized from just buffer or water, and is so low that the hydration shell of the protein is essentially completely removed (Prestrelski et al., 1993a; Krielgaard et al., 1998a, 1999; Allison et al., 1999).

Furthermore, Farhat et al., (1998) used infrared spectroscopy to study dried gelatin-sugar mixtures and the hydration behaviors of the mixtures and concluded that the sugars hydrogen bond to the dried protein. Costantino et al. (1998) studied water sorption of proteins lyophilized with sucrose or trehalose and found that the solid-state interactions between protein and sugar reduced the availability of water binding sites. Tzannis and Prestrelski (1999) found that water sorption behavior of spray-dried protein formulations containing stabilizing levels of sucrose indicated hydrogen bonding between dried protein and the sugar. However, if an excessively high sucrose:protein ratio was employed, due to formation of protein- and sugar-rich phases, hydrogen bonding of sucrose to protein and resulting protein stabilization were reduced. Also, Suzuki et al. (1998; 1999) found that there was a high degree of stabilization of lactate dehydrogenase when sucrose remained amorphous and hydrogen bonded to the dried protein. Crystallization of sucrose abolished hydrogen bonding between the sugar and dried protein, as well as protein stabilization.

Further support for the water replacement mechanisms comes from two studies that compared the relative effectiveness of saccharides of increasing molecular weight for inhibiting lyophilization-induced unfolding. Tanaka and colleagues (1991) found that the capacity to protect catalase during freeze-drying was inversely related to saccharide size. They suggested that as the size of the saccharide increases, steric hindrance interferes with hydrogen bonding between the saccharide and the dried protein. In support of this contention, the carboxylate band is only minimally detectable in the infrared spectrum of lysozyme freeze-dried in the presence of glassy dextran and the protein is unfolded (Allison et al., 1999). With infrared spectroscopy, Prestrelski et al. (1995) found that as the molecular weight of a carbohydrate additive was increased the capacity to inhibit unfolding of interleukin-2 during lyophilization decreased, and the level of protein aggregation after rehydration increased. Also, it was clear that protection of the protein did not correlate directly with the formation of a glass (all samples were found to be amorphous) or with the

glass transition temperature of the sample (the T_g increased as carbohydrate molecular weight increased). Rather, there was a negative correlation between stabilization and molecular weight, which is to be expected if protection during drying is due to the water replacement mechanism.

An alternative mechanism for stabilization of proteins during dehydration states that proteins are simply mechanically immobilized in a glassy, solid matrix during dehydration (e.g., Franks, 1991). The restriction of translational and relaxation processes is thought to inhibit protein unfolding, and spatial separation between protein molecules (i.e., "dilution" of protein molecules within the glassy matrix) is proposed to prevent aggregation. It is clear that protective additives must partition with the protein into the amorphous phase of the dried sample. If the compound crystallizes during lyophilization it does not inhibit protein unfolding (Carpenter et al., 1993; Izutsu et al., 1993; Krielgaard et al., 1999). Also, spatially separating protein molecules can help favor refolding over aggregation during rehydration (Allison et al., 1998, 2000). However, as evidenced by the failure of glassy dextran matrices to prevent unfolding (Prestrelski et al., 1995; Krielgaard et al., 1999; Allison et al., 1998; 1999; 2000), simply forming a glassy solid is not sufficient for inhibiting dehydration-induced unfolding.

Practical considerations. The protein mass in a unit dose (or multidose) vial of therapeutic protein is dictated by the amount to be given to the patient. With a lyophilized product, which can be reconstituted to a volume different from the prelyophilization volume, the initial protein concentration can be varied without changing the total protein mass. Changes in initial protein concentration can affect relative loss of protein due to interaction with vial surfaces (e.g., Page et al., 2000), protein stability during processing, the collapse temperature of the formulation, the T_g of the final dried formulation and the lyophilization process itself. Thus, protein concentration can be an important variable to investigate during formulation development.

Increasing initial protein concentration leads to apparent increased resistance to denaturation during freezing (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). This phenomenon can be demonstrated by determining the percentage protein aggregated after freeze-thawing, which varies inversely with protein concentration. Increasing protein concentration can directly reduce freezing-induced protein unfolding because one component of protein damage during freezing appears to involve protein denaturation during formation of the ice-water interface (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). Assuming that only a finite number of protein molecules can be unfolded per unit area at this interface (Krielgaard et al., 1998b), then increasing the initial protein concentration will lead to a smaller percentage of damaged molecules. Of course, other factors such as direct cold denaturation and freeze concentration of salts may predominate over surface denaturation during freezing. For practical purposes, it is not necessary to sort out the relative contribution of the various stresses to freezing-induced damage. Rather, is important to include realative high protein concentrations during early formulation development in order to increase the

"intrinsic" resistance of the protein to denaturation. The greater the intrinsic stability of the protein, the lower the concentration of excipient that will be needed.

Another advantage of employing relatively high initial protein concentration is that increasing protein:disaccharide mass ratio increases both formulation collapse temperature and T_g of the final dried product. In addition, the formulation volume will be reduced, which will reduce the duration and costs of lyophilization.

Of course, if the ratio of excipient to protein is not sufficiently high, there will not be adequate stabilization of the protein during freezing and dehydration. Freezing protection depends on the initial bulk concentration of the sugar, and if the given protein is freeze-labile sometimes concentrations exceeding 5% (wt/vol) are needed to maximize stabilization. However, often the stability of the protein is sufficient during freezing that the level of sugar needed for optimal protection during lyophilization is dictated by that required for inhibition of unfolding during dehydration. Protection during drying depends on the final mass ratio between the sugar and the protein (Pikal, 1994; Carpenter and Chang, 1996; Cleland et al., 2001). Recently it has been demonstrated that the sugar:protein mass ratio predicts not only degree of native structural retention during lyophilization, but also the long-term storage stability of a lyophilized protein (Cleland et al., 2001). Generally, a weight ratio of sugar to protein of at least 1:1 is required for inhibiting lyophilization-induced unfolding, with optimal stability being reached at around 3-5:1. In practice, with the protein concentration held constant, a range of sugar concentrations can be tested during formulation screening to discern the optimal concentration needed for retention of native protein in the dried solid. In general, the optimal sugar concentration for stabilizing the protein during lyophilization will also provide storage stability, if the final dried powder has a T_g well above the storage temperature. Finally, it should be stressed that the minimal effective level of sugar should be employed, because of the effect of changing protein:excipient ratio on glass transition temperatures. Also, if excess levels of sugar are employed there is an increased risk of sugar crystallization if a product should happen to be held at temperatures near or above the formulation T_g (e.g., Krielgaard et al., 1999b).

Bulking agent (e.g., mannitol, glycine or hydroxyethyl starch)

If the product has a relatively low mass of protein per vial, often it will necessary to have a bulking agent in the formulation to prevent the protein from being lost from the vial during drying and to form the product cake. Mannitol and glycine are examples of bulking agents, which can also serve as tonicity modifiers, that usually crystallize to a substantial degree during lyophilization (Pikal, 1994; Carpenter and Chang, 1996). A disaccharide protein stabilizer will need to be used in combination with these bulking agents. One drawback of mannitol and glycine is that often an annealing step is required to assure maximum crystallization. If a fraction of the bulking agent remains amorphous during lyophilization, there may be problems with obtaining a suitable cake structure, as well as a risk of excipient crystallization during subsequent storage in the dried solid (e.g., Carpenter and Chang, 1996; Carpenter et al., 1997). An alternative, amophous bulking agent is the polymer hydroxyethyl starch, which is used clinically as a plasma volume expander. Its

main advantages in lyophilization are that it has a high collapse temperature (ca. -10º C), it forms strong cakes and it has a relatively high Tg for a given moisture content (e.g., > 200º C at 2% residual moisture). However, as is the case with dextran, the large polymer hydroxyethyl starch does not inhibit protein unfolding during dehydration, and may actually foster additional protein unfolding due to phase separation from the protein during freezing and drying. Again, stabilizing disaccharides must be used with hydroxyethyl starch to inhibit lyophilization-induced protein unfolding.

Nonionic Surfactant to Inhibit Aggregation.

Surfactants are often included in protein formulations to minimize interfacial denaturation and aggregation, e.g., at vial surfaces, due to bubble entrainment during filling or because of agitation (Chang et al., 1996c; Krielgaard et al., 1998b; Page et al., 2000). For a lyophilized product, a surfactant can be beneficial to minimize protein aggregation during vial filling, freezing and rehydration. Generally, a surfactant will not inhibit protein unfolding during dehydration (Krielgaard et al., 1998a). The mechanisms by which surfactants inhibit protein aggregation will be described in a separate chapter in this volume. For the current discussion it is sufficient to stress that a surfactant should not be included in a lyophilized product, unless there is direct evidence that increases recovery of native protein in the rehydrated sample. Surfactants can decrease the free energy of unfolding of some proteins, which may cause the compound to actually foster aggregation. Also, they have been shown to inhibit the assembly of small soluble aggregates into higher order soluble oligomers and insoluble aggregates (e.g., Krielgaard et al., 1998b). This effect can manifest itself during lyophilization and rehydration. As a result, the presence of a surfactant can cause an undesirable increase in the level of soluble aggregates.

REFERENCES

Allison, S.D., Dong, A., and Carpenter, J.F., 1996. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying and rehydration. *Biophys. J.* 71:2022.

Allison, S.D., Randolph, T.W., Davis, A., Middleton, K., and Carpenter, J.F., 1998. Effects of drying methods and additives on structure and function of actin: Mechanisms of dehydration-induced damage and its inhibition. *Arch. Biochem. Biophys.* 358:171.

Allison, S.D., Randolph, T.W., Chang, B.S., and Carpenter, J.F., 1999. Hydrogen bondingbetween sugar and protein is responsible for inhibiting dehydration-induced protein unfolding. *Arch. Biochem. Biophys.* 365:289.

Allison, S.D., Manning, M.C., Randolph, T.W., Middleton, K., Davis, A., and Carpenter, J.F., 2000. Optimization of storage stability of lyophilized actin using combinations of disaccharides and dextran. *J. Pharm. Sci.* 89:199.

Anchordoguy, T.J. and Carpenter, J.F., 1996. Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. *Arch. Biochem. Biophys* 332:231.

Anchordoquy, T.J., Izutsu, K.I., Randolph, T.W., and Carpenter, 2001**.** Maintenance of quaternary structure in the frozen state stabilizes lactate dehydrogenase during freeze-drying. *Arch. Biochem. Biophys.* (In Press).

Angell, C.A., 1995. Formation of glasses from liquids and biopolymers. *Science* 267:1924.

Arakawa, T., Prestrelski, S., Kinney, W., and Carpenter, J.F., 1993. Factors affecting short-term and long-term stabilities of proteins. *Adv. Drug Delivery Rev*. 10:1.

Bell, J.A., 1999. X-ray crystal structures of a severely desiccated protein. *Prot. Sci.* 8:2033.

Bummer, P.M., and Koppenol, S., 2000. Chemical and physical considerations in protein and peptide stability, in: *Protein Formulation and Delivery*, E.J. McNally, ed., Marcel Dekker, New York.

Brange, J., 2000. Physical stability of proteins, in: *Pharmaceutical Formulation Development of Peptides and Proteins*, S. Frokjaer and L. Hovgaard, eds., Taylor and Francis, London.

Cappola, M.L., 2000. Freeze-drying concepts: The basics, in: *Protein Formulation and Delivery*, E.J. McNally, ed., Marcel Dekker, New York.

Carpenter, J.F., and Chang, B.S., 1996. Lyophilization of protein pharmaceuticals, in: *Biotechnology and Biopharmaceutical Manufacturing, Processing, and Preservation*, K.E. Avis and V.L. Wu, eds., Interpharm Press, Buffalo Grove, IL.

Carpenter, J.F., K. Izutsu and T.W. Randolph, 1999. Freezing- and drying-induced structural changes in proteins and their inhibition by stabilizing additives. *Pharmaceutical Freeze-Drying*, L. Rey (Ed.)

Carpenter, J.F., Prestrelski, S., and Arakawa, T., 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins by stress-specific stabilization: I. Enzyme activity and calorimetric studies. *Arch. Biochem. Biophys.* 303:456.

Carpenter, J.F., Prestrelski, S.J., and Dong, A., 1998. Application of infrared spectroscopy to development of stable lyophilized protein formulations. *Europ. J. Pharm. Biopharm.* 45:231.

Carpenter, J.F. and Crowe, J.H., 1988. The mechanism of cryoprotection of proteins by solutes. *Cryobiology* 25:244.

Chang, B.S., and Randall, C.S., 1992. Use of subambient thermal analysis to optimize protein lyophilization. *Cryobiology* 29:632.

Chang, B.S., and Fisher, N.L., 1995. Development of an efficient single-step freeze-drying cycle for protein formulations. *Pharm. Res.* 12:831.

Chang, B.S., Beauvais, R.M., Arakawa, T., Narhi, L.O., Dong, A., Aparisio, D.I., and

Carpenter, J.F., 1996a. Formation of an active dimer during storage of interleukin-1receptor antagonist in aqueous solution. *Biophys. J.* 71:3399.

Chang, B.S., Beauvais, R.M., and Carpenter, J.F., 1996b. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: Glass transition and protein conformation. *Arch. Biochem. Biophys.* 331:249.

Chang, B.S., Kendrick, B.S., and Carpenter, J.F., 1996c. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J. Pharm. Sci.* 85:1325.

Chang, B.S., Kendrick, B.S., Carpenter, J.F. 1996c. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J. Pharm. Sci.* 85:1325.

Chen, B-L., Arakawa, T., Hsu, L., Narhi, L., Tressel, T.J., and Chen, S.L., 1994. Strategies to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development. *J. Pharm. Sci.* 83:1657.

Chen, B., Costantino, H.R., Liu, J., Hsu, C.C., Shire, S.J., 1999. Influence of calcium ionson the structure and stability of recombinant human deoxyribonuclease I in the aqueous and lyophilized states. *J. Pharm. Sci.* 88:477.

Cleland, J.L., Powell, M.F., and Shire, S.J., 1993. The development of stable protein formulations – A close look at protein aggregation, deamidation and oxidation. *Crit. Rev. Ther. Drug* 11:60.

Cleland, J.L., Lam, X., Kendrick, B.S., Yang, J., Yang, T-Z., Overcashier, D., Brooks,D., Hsu, C., and Carpenter, J.F., 2000. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *J. Pharm. Sci.* (In press).

Carrasquillo, K.G., Sanchez, C., Griebenow, K., 2000. Relationship between conformational stability and lyophilization-induced structural changes in chymotrypsin. *Biotechnol. Appl. Biochem.* 31:41.

Costantino, H.R., Curley, J.G., Wu, S., and Hsu, C.C., 1997. Water sorption behavior of lyophilized protein-sugar systems and implications for solid-state interactions. *Int. J. Pharm.* 166:211.

Costantino, H.R., Griebenow, K., Mishra, P., Langer, R., and Klibanov A.M., 1995. Fourier-transform infrared spectroscopic investigation of protein stability in the lyophilized form. *Biochim. Biophys. Acta* 1253:69.

Costantino, H.R., Carrasquillo, K.G., Cordero, R.A., Mumenthaler, M., Hsu, C.C., and Griebenow, K., 1998. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. *J. Pharm. Sci.* 87:1412.

Craig, D.Q., Royall, P.G., 1988. The use of modulated temperature DSC for the study of pharmaceutical systems: potential uses and limitations. *Pharm Res.* 15:1152.

DeGrazio, F., Flynn, K., 1992. Lyophilization closures for protein based drugs. J. Parenter. Sci. Technol. 46:54.

Dong, A., Prestrelski, S.J., Allison, S.D., and Carpenter, J.F., 1995. Infrared spectroscopicstudies of lyophilization- and temperature-induced protein aggregation. *J. Pharm. Sci.* 84:415.

Duddu, S.P., and Dal Monte, P.R., 1997. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. *Pharm. Res.* 14:591.

Duddu, S.P., Zhang ,G., Dal Monte P.R., 1997. The relationship between protein aggregation and molecular mobility below the glass transition temperature of lyophilized formulations containing a monoclonal antibody. *Pharm Res.* 14:596.

Farhat, I.A., Orset, S., Moreau, P., Blanchard, J.M.V., 1998. FTIR study of hydrationphenomena in protein-sugar systems. *J. Coll. Interface Sci.* 207:200.

Franks, F., 1900. Freeze-drying: From empiricism to predictability. *Cryoletters* 11:93.

Franks, F., 1991. Material science and the production of shelf stable biologicals. *BioPharm* 4(9):38.

Gatlin, L.A., and Nail, S.L., 1994. Protein purification process engineering. Freeze-drying: A practical overview. *Bioprocess Technol.* 18:317.

Goolcharran, C., Khossravi, M., and Borchardt, R.T., 2000. Chemical pathways of protein and peptide degradation, in: *Pharmaceutical FormulationDevelopment of Peptides and Proteins*, S. Frokjaer and L. Hovgaard, eds., Taylor and Francis, London.

Griebenow, K., and Klibanov, A.M., 1995. Lyophilization-induced reversible changes inthe secondary structure of proteins. *Proc. Natl. Acad. Sci. USA* 92:10969.

Gu, L.D., Erds, E.A., Chiang, H.-S., Calderwood, T., Tsai, K., Visor, G.C., Duffy, J., Hsu,W.C., and Foster, L.C., 1991. Stability of interleukin-1 beta (IL-1 beta) in

aqueoussolution: analytical methods, kinetics, products, and solution formulation implications. *Pharm. Res.* 8:485.

Hageman, M., 1992. Water sorption and solid state stability of proteins. in: *Stability of Protein Pharmaceuticals. Part A. Chemical and Physical Pathways of Protein Degradation*, T. Ahern and M.C. Manning, eds., Plenum Press, New York.

Heller, M.C., Carpenter, J.F., and Randolph, T.W., 1996. Effects of phase separating systems on lyophilized hemoglobin. *J. Pharm. Sci.* 85:1358.

Hancock, B.C., Shamblin, S.L., and Zografi, G., 1995. Molecular mobility of amorphous pharmaceutical solids below their glass transition temperature. *Pharm. Res.* 12:799.

Her, L.M., Jefferis, R.P., Gatlin, L.A., Braxton, B., and Nail, S.L., 1994. Measurement of glass transition temperatures in freeze concentrated solutions of non-electrolytes by electrical thermal analysis. *Pharm. Res.* 1994 11:1023.

Her, L.M., and Nail, S.L., 1994. Measurement of glass transition temperatures of freeze-concentrated solutes by differential scanning calorimetry. *Pharm. Res.* 11:54.Pharm Res 1995 May; 12(5):768-72

Her, L.M., Deras, M., Nail, S.L., 1995. Electrolyte-induced changes in glass transition temperatures of freeze-concentrated solutes. *Pharm. Res.* 12:768.

Hora, M.S., and S.N. Wolfe, 1999. Critical steps in the preparation of elastomeric closures for biopharmaceutical freeze-dried products, in: *Freeze-Drying/Lyophilization of Pharmaceuticals and Biological Products*, L. Rey and J.C. May, eds., Marcell Dekker, New York.

Iztusu, K., Yoshioka, S., Kojima, S., Randolph, T.W., and Carpenter, J.F., 1996. Effects of sugars and polymers on crystallization of poly(ethylene glycol) in frozen solutions: Phase separation between incompatible polymers. *Pharm. Res.* 13:1393.

Isutzu, K., Yoshioka, S., and Teroa, T., 1991. The effects of additives of the stability of freeze-dried β-galactosidase stored at elevated temperatures. *Int. J. Pharm.* 71:137.

Izutsu, K., Yoshioka, S., and Teroa, T., 1993. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. *Pharm. Res.* 10:1232.

Kendrick, B.S., Chang, B.S., Arakawa, T., Peterson, B., Randolph, T.W., Manning, M.C., and Carpenter, J.F., 1997. Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: Role in restricted conformational mobility and compaction of native state. *Proc. Nat. Acad. Sci.* 94:11917.

Kendrick, B.S., Carpenter, J.F., Cleland, J.L., and Randolph, T.W., 1998. A transient expansion of the native state precedes aggregation of recombinant human interferon-gamma. *Proc. Nat. Acad. Sci.* 95:14142.

Kim, Y., Wall, J.S., Meyer, J., Murphy, C., Randolph, T.W., Manning, M.C., Solomon, A., and Carpenter, J.F., 2000. Thermodynamic modulation of light chain amyloid fibril formation. *J. Biol. Chem.* 275:1570.

Kreilgaard, L., Frokjaer, S., Flink, J.M., Randolph, T.W., and Carpenter, J.F., 1998a. Effects of additives on the stability of recombinant human Factor XIII during freeze-drying and storage in the dried solid *Arch. Biochem. Biophys.* 360:121.

Kreilgaard, L., Jones, L., Randolph, T.W., Frokjaer, S., Flink, J., Manning, M.C., and Carpenter, J.F., 1998b. Effects of Tween 20 on agitation- and freeze-thawing-induced aggregation of recombinant Factor XIII. *J. Pharm. Sci.* 87:1597.

Kreilgaard, L., Frokjaer, S., Flink, J.M., Randolph, T.W., and Carpenter, J.F., 1999. Effects of additives on the stability of *Humicola lanuginosa* lipase during freeze-drying and storage in the dried solid. *J. Pharm. Sci.* 88:281.

Lam, X.M., Costantino, H.R., Overcashier, D.E., Nguyen, T.H., and Hsu, C.C., 1996. Replacing succinate with glycolate buffer improves the stability of lyophilized interferon-gamma. *Int. J. Pharm.* 142:85.

Levine, H., and Slade, L., 1988. Thermomechanical properties of small-carbohydrate-water glasses and "rubbers": Kinetically metastable systems at subzero temperatures. *J. Chem. Soc. Faraday Trans.* 1(84): 2619.

Levine, H., and Slade, L., 1992. Glass transitions in foods, in: *Physical Chemistry of Foods*, H.S. Shartxberg and R.W. Hartel, eds., Marcel Dekker, NewYork.

Li, S., Patapoff, T.W., Overcashier, D., Hsu, C., Nguyen, T.H., and Borchardt, R.T., 1996. Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state. *J. Pharm. Sci.* 85:873.

Lueckel, B., Helk, B., Bodmer, D., and Leuenberger, H., 1998. Effects of formulation and process variables on the aggregation of freeze-dried interleukin-6 (IL-6) after lyophilization and on storage. Pharm. Dev. Technol. 3:337.

Nail, S.L., and Gatlin, L.A., 1993. Freeze-drying: Principles and practice, in: *Pharmaceutical Dosage Forms: Parenteral Medications*, K.E. Avis, H.A. Lieberman and L. Lachman, eds., Marcel Dekker, New York.

Nail, S.L., Her, L.M., Proffitt, C.P., and Nail L.L., 1994. An improved microscope stage for direct observation of freezing and freeze drying. *Pharm. Res.* 11:1098.

Neet K.E., and Timm D.E., 1994. Conformational stability of dimeric proteins:quantitative studies by equilibrium denaturation. *Prot. Sci.* 3:2167.

Page C, Dawson P, Woollacott D, Thorpe R, Mire-Sluis A Development of a lyophilization formulation that preserves the biological activity of the platelet-inducing cytokine interleukin-11 at low concentrations. J Pharm Pharmacol 2000 Jan; 52(1):19-26

Pikal, M.J., 1990. Freeze-drying of proteins. Part I: Process design. *BioPharm* 3(8):18.

Pikal, M.J., 1994. Freeze-drying of proteins, in: *Formulation and Delivery of Proteins and Peptides*, J.L. Cleland and R. Langer, eds., Symposium Series 567, American Chemical Society, Washington, DC.

Pikal, M.J., 1999. Mechanisms of protein stabilization during freeze-drying and storage: The relative importance of thermodynamic stabilization and glassy state relaxation dynamics, in: *Freeze-Drying/Lyophilization of Pharmaceuticals and Biological Products*, L. Rey and J.C. May, eds., Marcell Dekker, New York.

Pikal, M.J., and Shah, S., 1992. Moisture transfer from stopper to product and resulting stability implications. *Dev. Biol. Standard.* 74:165.

Pikal, M.J., Shah, S., Roy, M.L., and Putman, R., 1990. The secondary drying stage of freeze-drying: Drying kinetics as a function of temperature and chamber pressure. *Int. J. Pharm.* 60:203.

Prestrelski, S.J., Tedeschi, N., Arakawa, T., and Carpenter, J.F., 1993a. Dehydration-induced conformational changes in proteins and their inhibition by stabilizers. *Biophys. J.* 65: 661.

Prestrelski, S.J., Arakawa, T., and Carpenter, J.F., 1993b. Separation of freezing- and drying-induced denaturation of lyophilized proteins by stress-specific stabilization: II. Structural studies using infrared spectroscopy. *Arch. Biochem. Biophys.* 303:465.

Prestrelski, S.J., Pikal, K.A., and Arakawa, T., 1995. Optimization of lyophilization conditions for recombinant interleukin-2 by dried state conformational analysis using Fourier transform infrared spectroscopy. *Pharm. Res.* 12:1250.

Remmele, R.L. Jr., Stushnoff, C., and Carpenter, J.F., 1997. Real-time in situ monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. *Pharm. Res.* 14:1548.

Rey, L., and May, J.C., 1999. *Freeze-drying/Lyophilization of Pharmaceuticals and Biological Products*, Marcel Dekker, New York.

Roy, M.L., Pikal, M.J., Rickard, E.C., and Maloney, A.M., 1992. The effects of formulation and moisture on the stability of a freeze-dried monoclonal antibody-vinca conjugate: A test of the WLF glass transition theory. *Dev. Biol. Standard.*74:323.

Shalaev, E.Y., Lu, Q., Shalaeva, M., Zografi, G., 2000. Acid-catalyzed inversion of sucrosein the amorphous state at very low levels of residual water. *Pharm. Res.* 17:366.

Skrabanja, A.T.P., de Meere, A.L.J., de Ruiter Rien, and van der Oetelaar, P.J.M., 1994. Lyophilization of biotechnology products. *PDA J. Pharm. Sci. Technol.* 48:311.

Strambini, G.B., and Gabellieri, E., 1996. Proteins in frozen solutions: Evidence of ice-induced partial unfolding. *Biophys. J.* 70:971-976.

Suzuki, T., Imamura, K., Fujimoto, H., and Okazaki, M., 1998. Relationship betweenthermal stabilizing effect of sucrose on LDH and sucrose-LDH hydrogen bond. *J. Chem. Eng. Japan* 31:565.

Suzuki, T., Imamura, K., Fujimoto, H. and Okazaki, M., 1999. Role of sucrose-LDH hydrogen bond for thermal stabilizing effect of sucrose on freeze-dried LDH. *Drying Technol.* 17:1429.

Tanaka, T., Takeda, T., and Miyajama, R., 1991. Cryoprotective effect of saccharides on denaturation of catalase during freeze-drying. *Chem. Pharm. Bull.* 39:1091.

Timasheff, S.N., 1998. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. *Adv. Prot. Chem.* 51:355.

Tzannis, S.T., and Prestrelski, S.J., 1999. Moisture effects on protein-excipient interactions in spray-dried powders. Nature of destabilizing effects of sucrose. *J. Pharm. Sci.* 88:360.

van den Berg, L., 1959. The effect of addition of sodium and potassium chloride to the reciprocal system: KH_2-PO_4-Na_2HPO_4-H_2O on pH and composition during freezing, *Arch. Biochem. Biophys.* 84:305-315.

van den Berg, L., and Rose, D., 1959. The effect of freezing on the pH and composition of sodium and potassium solutions: The reciprocal system KH_2-PO_4-Na_2HPO_4-H_2O, *Arch. Biochem. Biophys.* 81:319.

Verdonck, E., Schaap, K., and Thomas, L.C., 1999. A discussion of the principles and applications of Modulated Temperature DSC (MTDSC). *Int. J. Pharm.* 192:3.

Volkin, D.B., and Middaugh, C.R., 1996. The characterization, stabilization, and formulation of acidic fibroblast growth factor. *Pharm. Biotechnol.* 9:181.

Wolkers, W.F., van Kilsdonk, M.G., Hoekstra, F.A., 1998. Dehydration-induced conformational changes of poly-L-lysine as influenced by drying rate and carbohydrates. *Biochim Biophys Acta* 1425:127.

Yoshioka, S., Aso, Y., Kojima, S., Tanimoto, T., 2000. Effect of polymer excipients on the enzyme activity of lyophilized bilirubin oxidase and beta-galactosidase formulations. Chem. Pharm. Bull. 48:283.

Yoshioka, S., Aso, Y., Kojima, S., 1999. The effect of excipients on the molecular mobility of lyophilized formulations, as measured by glass transition temperature and NMR relaxation-based critical mobility temperature. *Pharm Res.* 16:135.

Zhang, M.Z., Wen, J., Arakawa, T., and Prestrelski S.J., 1995. A new strategy for enhancing the stability of lyophilized protein: The effect of reconstitution medium on keratinocyte growth factor. *Pharm. Res.* 12:1447.

Zhang, M.Z., Pikal, K., Nguyen, T., Arakawa, T., and Prestrelski, S.J., 1996. The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukin-2 and ribonuclease A. *Pharm. Res.* 13:643.

Table 1. Minimal Criteria for a Successful Lyophilized Protein Formulation

1. Protein unfolding during freezing and drying is inhibited
2. The glass transition temperature of the product exceeds the planned storage temperature (e.g., Tg > 40$^{\text{o}}$C)
3. The water content is relatively low (e.g., ca. 1% by mass)
4. An elegant cake structure is obtained (i.e., collapse and meltback are avoided)
5. Steps taken to minimize specific routes of protein chemical degradation (e.g., seal product vials under nitrogen to reduce rate of methionine oxidation)

Table 2. Example of Rational Lyophilized Protein Formulation

1. Buffer that does not acidify during freezing (e.g., Tris, histidine, citrate)
2. Specific ligands/pH that optimizes thermodynamic stability of protein
3. Trehalose or sucrose to inhibit protein unfolding and provide glassy matrix
4. Bulking agent (e.g., mannitol, glycine or hydroxyethyl starch)
5. Nonionic surfactant to reduce protein aggregation