some of the damage noted during freezing (63–65). Also, the protein, which partitions into the non-ice phase, is exposed to extremely high solute concentrations as the sample is frozen. If solutes that are destabilizing to the protein are present, then this concentrating effect can contribute to protein denaturation. Finally, as noted earlier, there can be dramatic pH changes during freezing. For example, the dibasic form of sodium phosphate crystallizes in frozen solution, which results in a system that contains essentially solely the monobasic salt and has a very low pH (66,67). Other components in a formulation may inhibit crystallization of dibasic sodium phosphate (25,39). However, such inhibition is not predictable and must be investigated for each formulation with methods such as calorimetry (39) or direct pH measurements on the frozen systems (25). To minimize problems associated with pH changes, whenever possible, sodium phosphate buffer should be avoided. Also, 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.

Fortunately, to prevent freezing-induced damage to proteins, it is usually not necessary to discern which stresses are responsible for the damage or to target selectively each of these specific stresses. Rather, the most efficient approach is to design a formulation that provides the greatest overall resistance of the protein to denaturing forces. And as noted earlier, the first step in any stabilization process is to choose the specific conditions (pH or ligand) that maximize the stability of the given protein. These specific conditions, or adding a cryoprotectant solute such as sucrose, will protect the protein during freezing, whether the ultimate cause of denaturation is low temperature, high solute concentration, or some combination of stresses. This is because ultimately the stabilization of the protein derives from increasing the free energy barrier between the native and denatured states, which increases resistance to damage by any stress.

A wide variety of compounds have been found to provide nonspecific cryoprotection to proteins. These include sugars, amino acids, polyols, salting-out salts, methylamines, alcohols, other proteins, and synthetic polymers (59,61,68–70). During the initial screening of compounds as cryoprotectants, it is important that a relatively wide range of concentrations be tested for each compound. The range to be tested will be dictated by other formulation concerns (e.g., total excipient mass and tonicity of final rehydrated product) and the effectiveness of the cryoprotectant. Compared to sugars, polymers such as PEG and PVP and other proteins (e.g., albumins) are much more potent cryoprotectants (70). Especially for proteins that must be formulated at low concentration, polymers can be useful as protectants and to minimize loss of active protein on the walls of the vial. Also, if high excipient mass is a concern, polymers are good candidates for cryoprotectants because they are effective at relatively low concentrations (i.e., less than 1% wt/vol). For some proteins, sufficient freezing protection can be obtained by using a disaccharide (e.g., sucrose), which has the added benefit of also protecting the protein during subsequent drying. However, often much higher concentrations (e.g., more than 30% wt/vol) of such low molecular weight solutes are needed to confer adequate protection during freezing.

Finally, at least one aspect of protein stability during freezing is qualitatively unique from that noted in aqueous solution. That is, with numerous proteins it has been found that increasing the initial protein concentration increases protein stability during freeze-thawing. This is usually not the case in unfrozen aqueous solution, except in the special instance in which increasing the