TABLE 1 Shifts in pH During Nonequilibrium Freezing with Phosphate Buffer Systems

Concentration (mm)	Initial pH	Frozen pH	Δ pH
Sodium phosphate buffer			
100	7.5	4.1	−3.4
8	7.5	5.1	−2.4
Potassium phosphate buffer			
100	7.0	8.7	+1.7
100	5.5	8.6	+3.1
10	5.5	6.6	+1.1

Source: From Refs. 13 and 14.

close to the equilibrium values. However, lowering the buffer concentration by an order of magnitude considerably reduces the pH shift observed during freezing. It should also be noted that, under some conditions, potassium phosphate buffers also give large pH shifts during freezing. As shown in Table 1, if the initial pH is 5.5, the 100 mM potassium phosphate buffer increases in pH by 3.1 units during freezing. Clearly, if a protein's structural integrity is sensitive to pH shifts, buffer crystallization must be avoided. In our experience, the best solution is to formulate such that the weight ratio of buffer to other solutes is very low (16,17).

It is well-known (18) that protein adsorption to surfaces, such as the air-water interface, may result in a perturbation of the conformation. That is, adsorption is a possible stress. While the surface area of the air-water interface is minimal during a well-designed freezing process, liberation of dissolved air during thawing may generate numerous bubbles, thereby providing a significant surface area for protein adsorption and conformational change. During the freezing process itself, the major interfacial area is the aqueous-ice interface. As the degree of supercooling increases (normally, as the rate of cooling increases), the number of ice crystals increases, thereby increasing the aqueous-ice interfacial area. It is clear that if a protein were to adsorb on the ice crystals and suffer a loss of conformational stability, the formation of ice itself would be a significant stress during freezing. Several observations suggest this speculation has merit. Freezing studies with human growth hormone (hGH) (19) show more insoluble aggregates develop during rapid freezing in a $-80^{\circ}C$ bath than during freezing procedures that cool more slowly. Classically, one expects less aggregation during a fast-cooling process since the residence time in the potentially reactive freeze concentrate is much less than in a slow-cooling process (i.e., the time required to reach the glassy state is much less). However, since the fast-cooling process will generate a greater aqueous-ice interface, which would maximize the fraction of protein adsorbed, the authors concluded that hGH was denatured by adsorption on ice. We have observed that more rapid freezing to lower temperatures results in more air bubbles on thawing, so an alternate interpretation of the hGH data might be made in terms of denaturation at air bubbles.

A recent study of unfolding during freezing provides strong evidence that proteins can indeed unfold as a result of adsorption to the ice surface (20). A summary of the major findings of this study is given in Figure 3 where the solid circles represent the average phosphorescence lifetime of Trp-48 of azurin in a 1 mM potassium phosphate buffer. All these systems contain ice at subzero temperatures and were formed by seeding with ice at $-2^{\circ}C$ followed by rapid equilibration to the temperature of interest. The heavy line gives the