process to assure recovery of a native protein in the dried solid and after rehydration (8,9,33–36).

Although most carbohydrates used for protein formulations remain amorphous in frozen solutions and during drying (e.g., sucrose and trehalose), some exhibit eutectic phase separation from frozen solutions (34–39). For example, mannitol readily crystallizes during freeze-drying, but the degree of crystallization can be manipulated by altering processing conditions and formulation components (34–39). In the concentration range where it remains mostly amorphous, mannitol has been shown to protect enzymes during freeze-drying in a concentration-dependent manner (35,36). A relatively high mass ratio of protein to mannitol will serve to inhibit mannitol crystallization, whereas with excess mannitol, crystallization and loss of stabilization arise. Similarly, substantial stabilization has been achieved with solutes (including buffer salts) that alone crystallize but in combination interfere with each other's crystallization. For example, Izutsu et al. (35) found that with a sufficiently high ratio of potassium phosphate to mannitol, mannitol remained amorphous and protected LDH during freeze-drying. However, when there was excess mannitol, its crystallization obviated protein protection. Similarly, Pikal et al. (40) found that appropriate ratio of mannitol and glycine resulted in a sufficiently large amorphous fraction to protect freeze-dried human growth hormone.

Although it is well established that an amorphous excipient is needed to protect proteins during dehydration, the nature of the protective interaction of amorphous solutes with the protein in the dried solid has been a subject of controversy in the literature. There are at least two nonexclusive mechanisms proposed. Before describing these mechanisms, we wish to emphasize that neither mechanism alone is sufficient to fully explain stabilization during lyophilization. Both mechanisms focus only on the effect of stabilizers during the terminal stress of dehydration and essentially ignore the freezing step. As documented below, no matter what the nature of the interaction of the additive with the dried protein, the most important factor is that the additive(s) prevent unfolding during *both* freezing and dehydration.

Proponents of one mechanism state that proteins are simply mechanically immobilized in the glassy, solid matrix during dehydration (41). The restriction of translational and relaxation processes is thought to prevent protein unfolding, and spatial separation between protein molecules (i.e., "dilution" of protein molecules within the glassy matrix) is proposed to prevent aggregation. Although it is clear that protective additives must partition with the protein into the amorphous phase of the dried sample, simply forming a glassy solid does not assure protein stabilization. First, if all that were needed to prevent damage to a protein is the formation of a glass, then the protein by itself should be stable. Clearly, this is not the case because proteins themselves should form an amorphous phase in the dried solid (42); however, most unprotected proteins are denatured during lyophilization (8–14). In some cases adding another protein (e.g., BSA), which should simply add to the mass of the final protein glass, confers protection (25).

One might further qualify the mechanism by proposing that the requisite mechanical restriction to unfolding and aggregation can only be achieved if another amorphous compound is present to provide immobilization and spatial separation of the protein drug molecules. However, then the question becomes what amount of additive is sufficient to provide the desired physical properties of the dried solid, which are not achieved with the protein alone? This question