has not been answered or addressed in the literature. However, it is expected that, in general, the capacity of an additive to protect a protein specifically during dehydration should depend on the final additive protein mass ratio. Increasing this ratio will favor spatial separation and immobilization of the protein within the glassy matrix. Also, the mass ratios between all compounds in the dried solid affect the influence of the compounds on each other's crystallization (e.g., with glycine and mannitol).

Several studies have shown that formation of a glassy phase by an additive, even when it is used in large excess relative to the protein, is not a sufficient condition for acute stabilization of proteins during lyophilization. For example, formulations of 100 mg/mL interleukin-l receptor antagonist, prepared with sucrose concentrations ranging from 0% to 10% (wt/vol), formed a glass during lyophilization and all had glass transition temperatures of 66 ± 2°C (13). Yet only in formulations containing $\geq 5\%$ sucrose was lyophilization-induced unfolding prevented. Tanaka et al. (43) have found that the capacity of carbohydrates to protect freeze-dried catalase decreased with increased carbohydrate molecular weight. Dextrans were the largest and least effective of all of the carbohydrates tested, and the larger the dextran molecule the less it stabilized catalase. Although they did not determine whether their dried samples were amorphous, it is well known that as the molecular weight of the carbohydrate is increased, the glassy state is formed more readily (44–46). In addition, more recent studies have shown (T. Randolph, M. Zhang, S. Prestrelski, T. Arakawa, and J. Carpenter, unpublished data) that PFK was not protected, and LDH was inactivated further, by dextran during freeze-drying and rehydration. Differential scanning calorimetry documented that the dried samples were amorphous. The potential mechanistic bases for these observations will be described below. For now, it is important to stress the conclusion that it is necessary for stabilizing additives to remain amorphous to protect proteins during lyophilization, but glass formation alone appears not to be sufficient for stabilization of proteins against the severe stress of dehydration.

There are several studies supporting the other mechanism, which is often referred to as the water replacement hypothesis. According to this hypothesis, sugars protect labile proteins during drying by hydrogen bonding to polar and charged groups as water is removed, thus preventing drying-induced denaturation of the protein. For example, in early studies, using solid-state Fourier transform infrared (FTIR) spectroscopy, it was 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 (33). When lysozyme is dried in the presence of trehalose or lactose, 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 (8). More recently, it has been documented that the carboxylate band can be titrated back by freeze-drying lysozyme in the presence of increasing concentrations of either trehalose or sucrose (S. Allison and J. Carpenter, unpublished observations). This effect correlates directly with an increased inhibition of protein unfolding in the presence of increasing amounts of sugar.

Three other recent studies on enzyme preservation provide further support for the water replacement mechanism. Tanaka et al. (43) have found that the capacity of a saccharide to protect catalase during freeze-drying is inversely related to the size of the saccharide molecule. They suggest that as the size of