LDH during both treatments by inhibiting dissociation in the frozen state. Dissociation was not induced by freezing itself but rather during an extended residency time in the frozen state (e.g., due to thawing with slow warming or during the early stages of primary drying) at a relatively high subzero temperature (e.g., −20°C). The main factor causing dissociation appeared to be a decrease in the pH of the sodium phosphate buffer system from 7.5 at room temperature to 4.5 in the frozen solution at −20°C. Dissociation did not occur when buffers that did not acidify were employed (e.g., Tris and potassium phosphate). PVP and BSA protected the enzyme during lyophilization, at least in part, by inhibiting the reduction in pH in the frozen state. These experiments provide direct evidence that stabilization during freezing is essential for inhibiting protein damage during lyophilization. Further examples are presented below.

EVIDENCE FOR FREEZING-INDUCED UNFOLDING DURING LYOPHILIZATION

If a protein is not adequately protected during freezing, the protein will be unfolded in the final dried solid, no matter how effective the stabilization during the dehydration step (9,36). There is considerable evidence documenting freezing sensitivity of proteins. First, many proteins are irreversibly denatured by freeze-thawing (59). Since this damage is due primarily to freezing, similar damage should also arise during the freezing step of lyophilization. Second, the capacity of an additive to protect during freeze-drying is often directly related to its initial bulk concentration and not to the final mass ratio of additive to protein (60). For example, the data in Figure 7 show that the recovery of PFK activity after lyophilization and rehydration increases as the prefreeze concentration of trehalose increases, even though the same mass ratio of trehalose to protein was used for all the samples. As will be explained later, freezing protection by sugars is governed by initial concentration of the additive, whereas drying protection is related primarily to the mass ratio between the additive and protein.

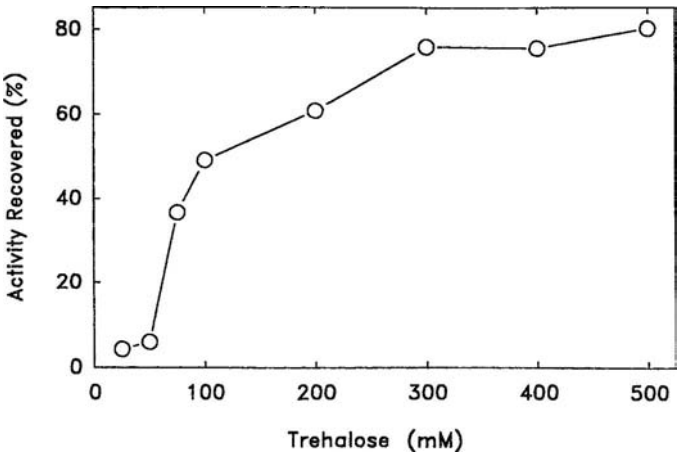

FIGURE 7 The effect of varying concentration of trehalose, while maintaining a constant sugar/protein mass ratio, on recovery of phosphofructokinase (PFK) activity after freeze-drying and rehydration. The protein concentration was adjusted concomitantly with the sugar concentration to maintain a constant sugar/protein mass ratio of 945. *Source*: Data taken from Ref. 59.