of proteins. The interested reader is referred to comprehensive reviews of protein formulation and lyophilization (31–33). This section will cover how freezing itself can have a major influence on success of the formulation and the lyophilization process. Excipient crystallization will be covered in section "Effects on Solute Crystallization."

Proteins are prone to denaturation at both high and low temperatures. Bhatnagar et al. provide a comprehensive review of the subject (34). Although cold denaturation is frequently mistaken for freezing denaturation, it is known to occur in the absence of freeze-concentration. As early as in 1930, it was reported that the rate of ovalbumin denaturation by urea is higher at 0°C than at 23°C (35). Bhatnagar et al. found ice itself to be responsible for loss of lactate dehydrogenase (LDH) activity during freezing (36). They created concentrated solutions to mimic freeze-concentration and found no degradation. Tang et al. discovered that cryopreservatives sucrose, trehalose, and glycerol can protect proteins from cold denaturation, effectively lowering their cold denaturation temperatures (37).

Several have found faster freezing to result in greater protein aggregation. In freeze-thaw studies, Eckhardt et al. found that the formation of insoluble aggregates of recombinant human growth hormone during freezing increased sharply with increased cooling rates (38). The authors stated possible causes to be surface denaturation or recrystallization during thawing of rapidly frozen samples. Skrabanja et al. report on freezing method and formulation effects on monoclonal antibody aggregation formation (39). Rapid freezing by immersion in CO_2/acetone resulted in greater aggregation after freezing than slow freezing by placing vials into a freezer at −20°C. In another study discussed in the same paper they report on recovery of a recombinant protein after lyophilization. The fast freeze yielded marginally higher yield than freezing by either shelf-ramp or a precooled shelf. Chang et al. showed that denaturation of proteins during freezing is closely related to surface denaturation by quantifying both types of denaturation for a range of proteins (40). A strong correlation ($r = 0.99$) was observed between the tendency of a protein to denature by freezing and its tendency to surface denature. Freezing by liquid nitrogen immersion caused more denaturation than shelf-ramp freezing. Small quantities of surfactant provided protection to the proteins against both types of inactivation (six surfactants were tested). A subsequent study from the same research group showed that surfactants stabilize against surface denaturation by competing with stress-induced soluble aggregates for interfaces, inhibiting subsequent transition to insoluble aggregates (41). Jiang and Nail studied catalase, β-galactosidase, and LDH in phosphate buffers, and found that shelf-frozen samples retained more protein activity than those frozen by liquid nitrogen immersion (42). Freezing by placement in a −40°C freezer yielded even better recovery. In all cases, freeze/thaw as well as lyophilization protein activity retention was found to improve with protein concentration. This is possibly an artifact of constant loss of a given mass of protein. As the concentration is increased, the fraction recovered increases; however, the same mass is lost. Jiang and Nail also found that the activity recovery increased with increasing residual moisture, suggesting that the secondary drying process also contributed to loss.

The trend of greater protein losses via faster freezing continued with the finding by Sarciaux et al. that liquid nitrogen quench freezing of an antibody formulation resulted in more and larger insoluble aggregates than shelf-ramp