freezing (43). As discussed in detail above, Hsu et al. showed a clear correlation between surface area and cooling rate by using various cooling methods and vial sizes. They also found that poorer *stability* of their protein correlated inversely with the specific surface area, providing a linkage between the freezing method and rate and product stability through time after lyophilization (44). Stability was even affected by the choice of vial and volume of fill through this linkage to the cooling rate for freezing—smaller fills in smaller vials resulted in greater product specific surface areas. No freeze/thaw protein loss was found. The authors stated that the phenomenon of lower stability for higher surface area samples might arise from possible lack of protection by the arginine excipient for those protein molecules at the surface of the amorphous phase. Citing preliminary results, Patapoff and Overcashier also found that annealing significantly reduced the rate of protein aggregation during storage stability studies (10).

Nema and Avis found contradictory evidence for protein denaturation in that fast freezing by liquid nitrogen resulted (88% recovery) in better retention of LDH activity than shelf-ramp freezing (68%) (45). The authors did not identify a specific mode of action responsible. The protein was formulated neat with no buffers or cryopreservatives. They did test a number of cryoprotectants with shelf-ramp freezing and found three formulations, which afforded protection: 1% w/v bovine serum albumin, 1 M sucrose, and 0.05% w/v Brij 30. It is possible that during shelf-ramp freezing degradative reactions are taking place in the partially freeze-concentrated product phase during the slow solidification after ice nucleation. For this reason one would expect that a postfreezing annealing step would adversely affect any products for which shelf-ramp freezing yields are poorer than those for liquid nitrogen immersion. The finding by Nema that product yields across fast freezing are better is not the only such case—some live viruses respond in the same manner.

Sarciaux et al. found that lyophilization resulted in insoluble aggregates of their bovine IgG formulations, but the damage was not observed after freeze/thaw (43). Freezing by shelf-ramp resulted in less postlyo aggregation than liquid nitrogen immersion freezing, and annealing reduced the damage further. The authors correlated the extent of aggregation with the surface area, and found that aggregation progressed through the secondary drying step of lyophilization. Annealing reduced the percentage of aggregate in the final product from 33% to 12% (46). The reduction was attributed to the lower surface area of the annealed samples. However, a new theory posits that these benefits may have been due to stress relaxation: the publication by Webb et al. reports that liquid nitrogen immersion and lyophilization resulted in greater yields of human interferon-γ than spray-freeze-drying (47). As discussed above, spray-freezing yielded several times greater specific surface area than the immersion method. However, like Sarciaux et al., the authors did not find any freeze-thaw damage caused by spray-freeze-drying, rather the damage was found to occur during the terminal stages of drying. The authors speculated that, instead of surface denaturation, which was ruled out by the ultrahigh cooling rates for spray-freezing, residual stress retained within the solid matrix was responsible for the protein damage. Additional evidence to support their finding is provided by the fact that annealing relieves this stress and reduces the protein loss. Unfortunately, Sonner et al. do not comment on the effects of annealing upon protein recovery (48). However, they did find that while spray-freezing did not