Because of the many cases where equilibrium is not achieved during freezing and freeze-drying, it is important to understand what happens under these conditions. It has been shown that under non-equilibrium freezing conditions (e.g., relatively fast cooling rates and small sample size), monosodium phosphate did not typically crystallize, whereas disodium phosphate readily crystallize [33]. It has been more recently shown that conditions that influence crystallization kinetics such as cooling rate, sample size, and initial buffer concentration also play a role in pH changes. For example, it was shown that an initial buffer with a pH 7.4 drops to pH 5.2 for 8 mM buffer as compared to pH 4 for 50 and 100 mM buffers [20].

The pronounced pH shifts in frozen solutions may be minimized by inhibiting the selective crystallization of a buffer component. Studies have shown that the crystallization of sodium phosphate buffer can be inhibited in the frozen state by the incorporation of glycine at a low ratio [52]. However, if the molar ratio of glycine to sodium phosphate is too high, this can lead to the crystallization of glycine. Furthermore, it was shown that at low glycine concentrations ($\leq$50 mM), pH shift in 10 and 100 mM sodium phosphate buffer can be prevented [37]. These findings show the importance in the selection of the buffer and excipient components for a freeze-dried protein formulation.

While it is well understood that pH shifts occur in frozen aqueous phosphate buffer solutions due to disodium phosphate buffer crystallization, there is recent evidence that shows a pH shift upon cooling to −25 °C in the absence of solute crystallization. For example, a pH shift from 6.2 to 4.5 was observed in a buffer system containing sucrose with no evidence of buffer salt crystallization [7]. We speculate that this could be due to preferential inclusion of the basic component (disodium phosphate) by the ice crystals, thus leaving more acidic freeze-concentrated solution in a continuous freeze-concentrated phase, in which pH is measured by low-temperature pH electrode. It should be noted, that a similar explanation of microheterogeneity was proposed in the original study, although specific reasons why heterogeneity would result in acidic shift were not elaborated [7].

Carboxylic Acid Buffers

The risk of pH shifts in frozen solutions for phosphate buffers has played a role in the investigation of other buffer types. Carboxylic buffers are of interest because of its broad buffering capacity ranging from 3 to 7. The crystallization propensity of carboxylic buffers has been studied. Several studies with sodium and potassium citrates have shown that these buffers do not crystallize and have minimal pH change during freezing. Examples include DSC freeze/thaw experiments [44] and a low-temperature pH probe study [27]. In this latter study, it was shown that the pH upon freezing of a citrate buffer increased slightly from 6 to 6.4 [27]. Similar findings were observed with a pH indicator study, which showed no pH change for citric acid/sodium citrate pH 5.5 during freezing [34]. Lastly, a concentration study from 0.1 to 0.6 molal showed no significant change in pH for citrate buffers at pH 6 and 4.4 [5].