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## Freezing-induced pH jump is proved to be an important degradation factor for proteins being frozen

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The freezing of aqueous solutions leads to the separation of ice crystals and the remaining solutes into microscopic veins and pockets; these regions are known as the freeze concentrated solution (FCS). The interactions between the molecules present within this phase are of key importance for the stability or reactivity of compounds in both natural and human-controlled freezing. Pharmaceutical stabilization often involves two steps, namely, freezing and freeze-drying; as each of the procedures can have deleterious effects on active pharmaceutical ingredients (API), we examined them closely.

We investigated the stability of proteins in terms of the enzymatic activity after exposure to repeated freezing and thawing. The most significant losses in enzyme structure and functionality were found in very fast cooling or when the apparent pH had changed markedly. Besides the simple freeze-concentration effect, the two main causes of acidity variation in FCS are as follows: a) preferential crystallization of one buffer component, letting the other one interact with API; b) uneven distribution of ions between the ice and FCS to be later neutralized by the flow of protons. The latter effect is also connected to the Workman-Reynolds freezing potential. The two effects are approximately additive, and we demonstrated that the pH jump induced by the former one can be neutralized by the latter. Moreover, we proved experimentally that when a pH jump was avoided, the fully preserved enzyme activity after reconstitution was sustained. Thus, we proposed a new protein stabilization strategy: adding a neutral salt to the buffer to minimize the pH after freezing. The method was named ionic cryoprotection.

Next, we studied the effects of freeze-drying on the pH in FCS. The apparent pH change after freezing was substantial, but it was found to be much less pronounced after the lyophilization step. This is explainable by additional crystallization of its components. Thus, we consider misleading to assess the pH stress accompanying the lyophilization cycle solely by monitoring the pH of the lyophiles (as practised previously). The unexpected negative dependence between initial pH of the solution and the acidity of the lyophiles further demonstrates the remaining unpredictability accompanying the freezing process.

The pH jump was assessed via the Hammett acidity function by measuring the amount of protonation in sulfonephthalein acid-base indicators. At this stage, sodium and potassium phosphate buffers and several common salts were examined in detail.

In conclusion, we show that a freezing-induced pH change can be harmful to the compounds present in FCS but is easily avoidable if the salts are chosen rationally. The good correlation between the Hammett acidity function in the frozen and lyophilized states and the enzyme recovery activity suggests that the indicator method for the pH assessment exhibits sufficient functionality and can be applied also in other research fields dealing with frozen aqueous solutions.

### Significance statement

The degradation of proteins during freezing often accompanies (pharmaceutical) stabilization steps and occurs in natural conditions too. The substantial apparent pH changes were identified as the most detrimental factors.

Therefore, we focused on the method of cryoprotection by ionic compounds, which preserves the original enzymatic activity.

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