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Salt precipitation during the freeze-concentration of phosphate buffer solutions

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Salt precipitation during the freeze concentration of phosphate solutions was investigated by differential scanning calorimetry (DSC), in view of its practical importance in the cryopreservation or freeze-drying of biological materials. It was found that the fraction of salt precipitated depends on the initial salt concentration; it began to decrease with decreasing concentration at approx. 1 M. Salt precipitation also depends on the cooling rate. In some cases, cooling at approx. 10^3 degree min^{-1} inhibited salt precipitation which had been observed during slow cooling (0.62 degree min^{-1}), without, however, affecting the shape of the ice melting endotherm. In the case of ternary phosphate buffers, the fraction of salt precipitating depends on the salt composition as well as the initial concentration and cooling rate. Near the composition of the ternary eutectic or the composition where two salts are present at the same concentration, salts were prevented from precipitation.

1. Introduction

Freezing and freeze-drying have become popular methods for the preservation and stabilization of biological materials. These methods are subject to some deleterious processes. Salt concentration and pH changes are considered to be two of the contributing factors [1–6]. Thus, salt concentrations and pH changes have been measured during the freezing of phosphate buffer solutions which are frequently used as media for cryopreservation or freeze-drying [1,2]. The extent of the pH change following freezing and the concomitant salt precipitation have been reported, at times, to amount to about 3 pH units, particularly in the case of sodium salt buffers. All such investigations were performed on equilibrium mixtures, with crystal seeding of solid phases.

However, in 'real life' situations, the relevance of equilibrium phase separation must be questioned, because the salts in the buffer solutions are known to supersaturate easily, rather than crystallizing as eutectic mixtures. The mechanism of salt precipitation following ice crystallization, although very important in practice, has scarcely been investigated.

In this study, factors influencing eutectic formation, i.e., salt precipitation during the freezing of phosphate solutions and buffer mixtures, were investigated by DSC.

2. Materials and methods

The phosphates used in this study were of sodium and potassium. The monosodium ($NaH_2PO_4 \cdot 2H_2O$), disodium (Na_2HPO_4) and monopotassium (KH_2PO_4) salts were obtained from Fisons (Loughborough, U.K), the dipotassium salt ($K_2HPO_4 \cdot 3H_2O$) being supplied by BDH Chem-

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icals (Poole, U.K.). These reagents were used without further purification. Distilled water was used throughout the experiments. Phosphate buffers were prepared by mixing two 0.5 M sodium or 1.0 M potassium salt solutions on a volume basis.

About 1 h before the DSC measurement, 5–7 μl of each solution was transferred into an aluminium DSC pan and sealed hermetically. It was then scanned in the DSC. Samples were weighed before and after the measurement to check the weight losses. No weight losses were observed within the accuracy of weighing (< 10 μg).

DSC measurements were carried out with a Perkin Elmer DSC-2 instrument, fitted with Autoscanning Zero and subambient accessories. Samples were cooled, usually at a rate of 0.62 degree min^{-1}, to −53°C; the rate of subsequent heating was 2.5 or 5 degree min^{-1}. For determination of the melting heat of the eutectic, a heating rate of 0.62 degree min^{-1} was used to separate the eutectic endotherm clearly from that due to ice melting. The effect of rapid cooling, by dipping sample pans directly into liquid nitrogen, approx. 10^3 degree min^{-1}, was also investigated.

3. Results

DSC scans for each phosphate solution are shown in figs. 1–4. The NaH_2PO_4 solution showed only one endotherm, independent of the initial concentration. The peaks were broad and the melting temperatures decreased with increasing concentration. The 3.42 M solution, corresponding to the eutectic concentration of the salt, also showed a single broad endothermic peak. As eutectic melting takes place at a fixed temperature, the broad peak cannot be due to eutectic melting, but must correspond to ice melting. The eutectic data of the four phosphate solutions are summarized in table 1.

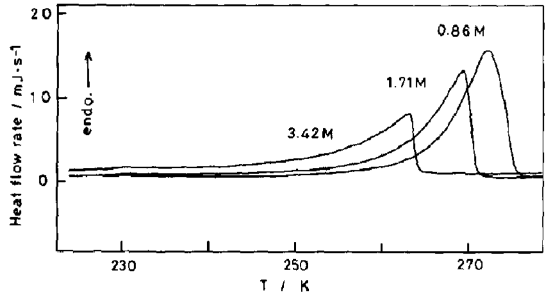

Fig. 1. DSC heating power-time curves of NaH_2PO_4 solutions. Conditions: sample volume, 5 μl; cooling rate, 0.62 degree min^{-1}; heating rate, 5 degree min^{-1}.

The solution of the disodium salt, whose solubility in water is the lowest among the four phosphates (approx. 11 wt% at 25°C, about 0.82 M) [7], also showed only one narrow endothermic peak, near 0°C, during heating. The peak may be due to the overlap of eutectic and ice melting, because the eutectic temperature of this solution (−0.5°C) is very close to the melting temperature of ice, and the eutectic is readily formed. This was confirmed by studying a solution of a concentration (0.5 M) above that of the eutectic. The power-time curve showed a small, broad endothermic peak above 0°C, in addition to the sharp

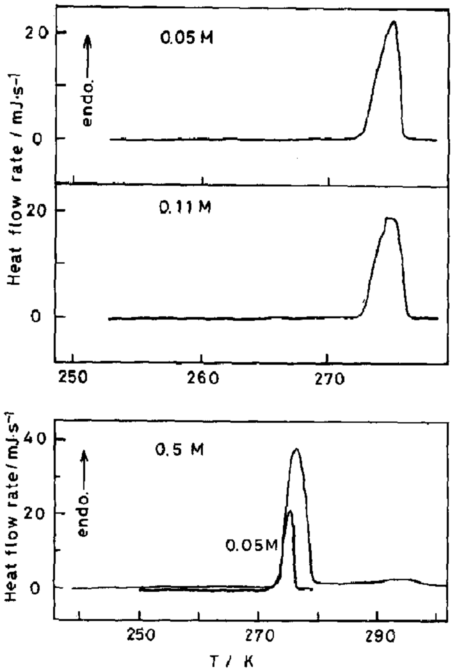

Fig. 2. DSC heating power-time curves of Na_2HPO_4 solutions. Sample volume, 3 μl (for 0.05 and 0.11 M) and 5 μl (for 0.5 M); cooling and heating rates, 2.5 degree min^{-1}.

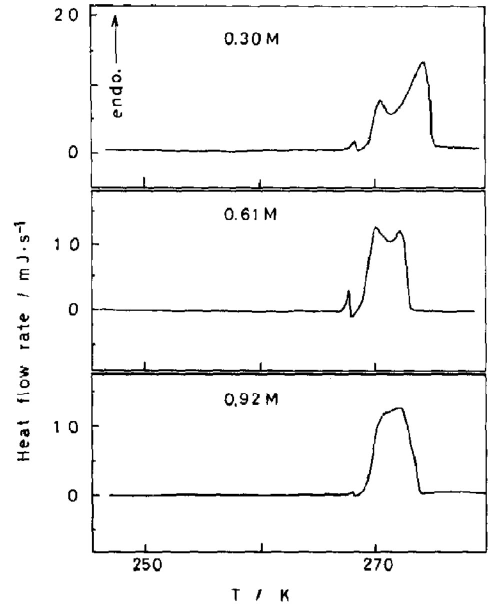

Fig. 3. DSC heating power-time curves of KH_2PO_4 solutions. Sample volume, 3 μl; cooling and heating rates, 2.5 degree min^{-1}.

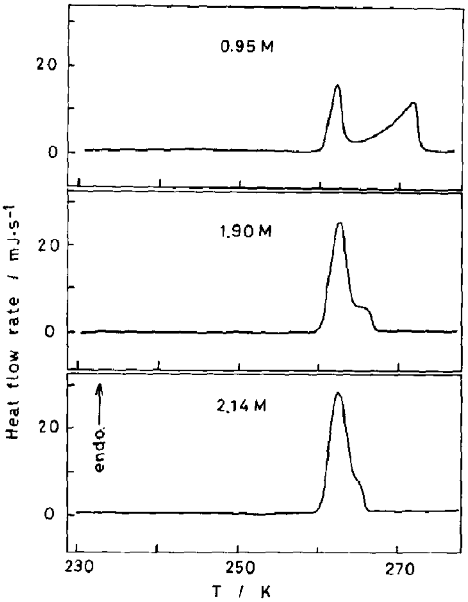

Fig. 4. DSC heating power-time curves of K_2HPO_4 solutions. Sample volume, 3 μl; cooling rate, 2.5 degree min^{-1}; heating rate, 5 degree min^{-1}.

peak characteristic of more dilute solutions. The broad peak corresponds to the heat of solution of the salt, and the sharp one is, in this case, due to eutectic melting, as ice melting cannot be expected at this concentration.

In the case of KH_2PO_4 solutions, the endotherm due to eutectic melting was observed at about −3° C, in addition to the ice melting peak. However, another small endotherm was also usually observed just below that of eutectic melting.

With the K_2HPO_4 solution, which has the lowest eutectic temperature of the four phosphate solutions, eutectic melting was observed at about −14° C, separated clearly from the ice melting endotherm.

The heat of eutectic melting per g salt was calculated and is plotted in fig. 5. As it was difficult to deconvolute the superimposed endotherms due to eutectic and ice melting in the case of the Na_2HPO_4 solution, and eutectic formation

Table 1

Eutectic data of phosphate solutions (taken from ref. 2)

(1) Monosodium or monopotassium salt; (2) disodium or dipotassium salt.

	T_e (°C)	C_e(1) (M)	C_e(2) (M)	Crystal type	$C_e(1)/C_e(2)$
NaH_2PO_4	−9.7	3.42	–	$NaH_2PO_4 \cdot 2H_2O$	–
Na_2HPO_4	−0.5	–	0.11	$Na_2HPO_4 \cdot 12H_2O$	–
NaH_2PO_4-Na_2HPO_4	−9.9	3.42	0.06	$NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$	57 (0.72) [a]
KH_2PO_4	−2.7	0.92	–	KH_2PO_4	–
K_2HPO_4	−13.7	–	2.85	$K_2HPO_4 \cdot 6H_2O$	–
KH_2PO_4-K_2HPO_4	−16.7	1.30	2.70	KH_2PO_4, $K_2HPO_4 \cdot 6H_2O$	0.48 (0.72) [a]

[a] Corresponds to the pH 7 buffer ratio.

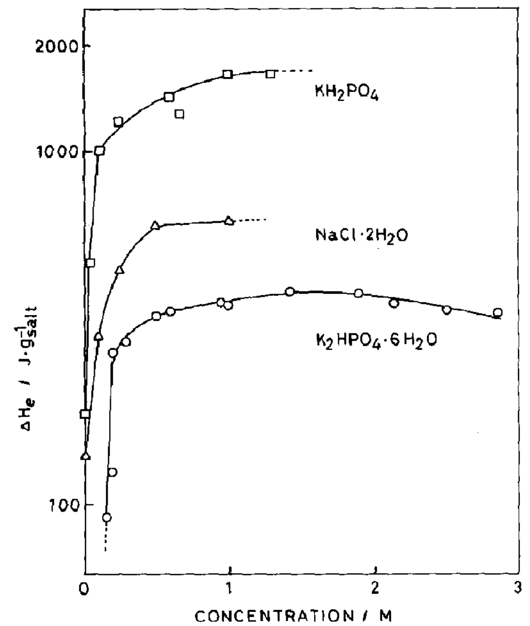

Fig. 5. Dependence of the heat of eutectic melting on the concentration. The ordinate is plotted logarithmically.

was not observed at all with NaH_2PO_4, the values for the latter salts have been omitted from fig. 5. Instead, the values for NaCl solutions are included for comparison. It is clear that the fraction of salt which precipitates to form eutectic during the freezing of each solution decreases with a decrease in the concentration to below approx. 1 M.

The dependence of the heating curves on the cooling history is shown in fig. 6a–d. The heating traces of the NaH_2PO_4 solution were almost identical, independent of the cooling rate. In the case of Na_2HPO_4, the temperature at which the melting started was lower for the rapidly cooled solution by about 1.5 degree, although the heat of melting remained constant. The heating curves of the KH_2PO_4 solution following slow and rapid cooling were almost identical, except for a small peak at about −5°C which was observed only after slow cooling. In the case of the K_2HPO_4 solution, the endotherm due to eutectic melting was observed during heating following slow cool-

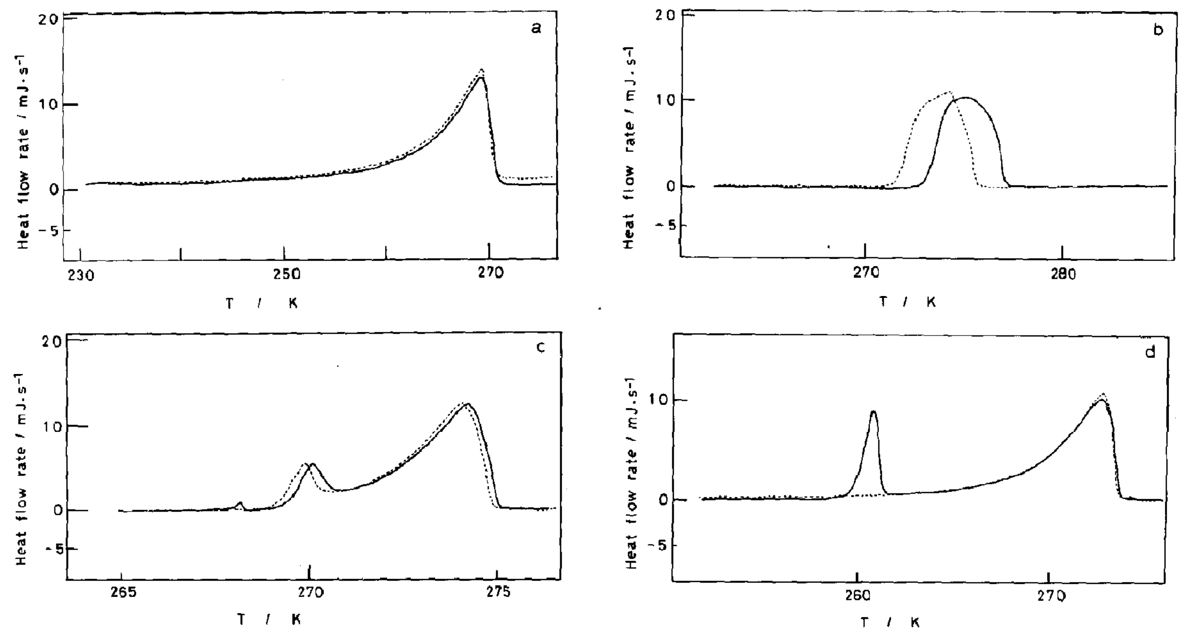

Fig. 6. Dependence of DSC heating power-time curves on the rate of previous cooling; (———) slow cooling (0.62 degree min^{-1}); (- - - - -) cooling at approx. 10^3 degree min^{-1}; (a) 1.71 M NaH_2PO_4; (b) 0.2 M Na_2HPO_4; (c) 0.2 M KH_2PO_4; (d) 0.5 M K_2HPO_4.

ing, but not following rapid cooling. However, the shape of the endotherm due to ice melting was almost independent of the eutectic formation.

The DSC heating curves of the phosphate buffer solutions are shown in figs. 7 and 8. In the case of the sodium buffer, 0.5 M solutions were used, compared to 1 M solutions for the potassium buffer, as the solubility of Na_2HPO_4 is low compared to the other three phosphates used. The sodium buffer rich in disodium salt showed a large endotherm near 0 °C and a small endotherm extending over a broad temperature range above 0 °C. The endotherm may correspond to the heat of solution of the salt, as shown in fig. 2. The large endotherm may be due to eutectic melting of ice and disodium salt. With the ternary buffer, the composition of which is approx. 0.3 M NaH_2PO_4 and 0.2 M NaH_2HPO_4, a small endotherm near −12 °C was also observed during heating. However, no melting of a ternary eutectic could be observed for any buffer composition.

The potassium buffer, on the other hand, clearly showed the endotherms due to the melting of two types of binary eutectics; the melting of the eutectic of ice and monopotassium salt was observed with the buffer rich in monopotassium salt and that of ice and dipotassium salt was observed with the buffer rich in dipotassium salt. With the buffer rich in dipotassium salt, moreover, an en-

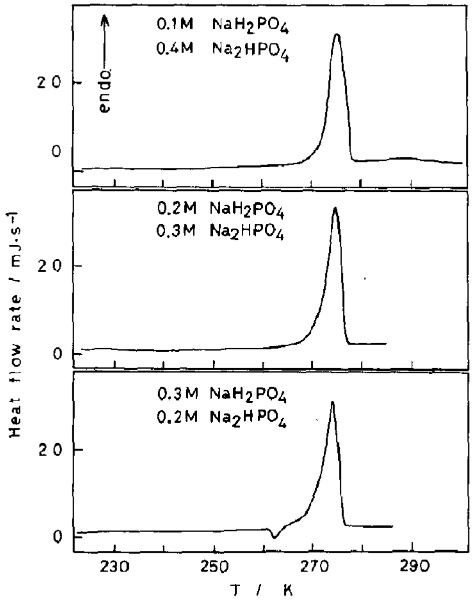

Fig. 7. DSC heating power-time curves of the sodium phosphate buffers. Sample volume, 5 μl; cooling rate, 0.62 degree min^{-1}; heating rate, 5 degree min^{-1}.

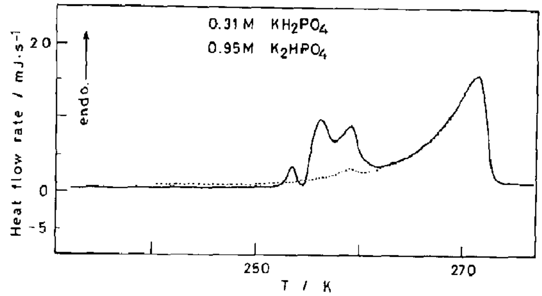

Fig. 8. DSC heating power-time heating curves of the potassium phosphate buffers. Sample volume, 5 μl; cooling rate, 0.62 degree min^{-1} (———) and 5 degree min^{-1} (------); heating rate, 5 degree min^{-1} (both curves). Starting from the low-temperature side, the sequentially observed endotherms for the solid curve are due to meltings of the ternary eutectic, the binary eutectic of ice and dipotassium salt and ice, except for the smallest endotherm observed at the lowest temperature.

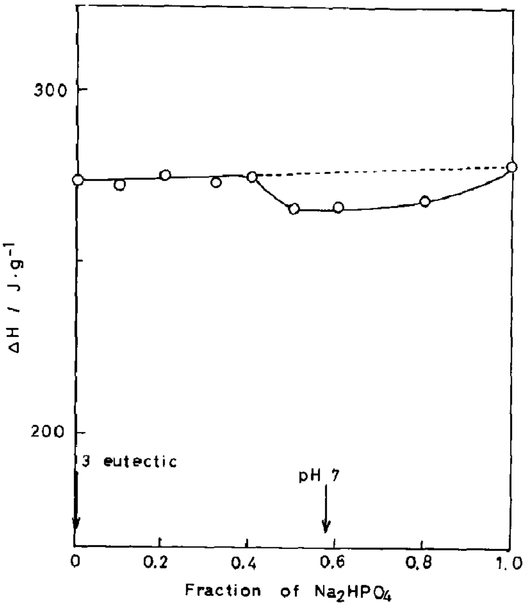

Fig. 9. Heat of melting of 0.5 M sodium phosphate buffers. Broken line: refers values when both mono- and disodium salt solutions contribute to ΔH additively. Arrows indicate the compositions of the ternary eutectic and the pH 7 buffer, respectively.

dotherm due to the melting of the ternary eutectic was also observed for a limited buffer composition range, as shown in fig. 8. The endotherms shown in fig. 8 correspond to ice, binary eutectic of ice and dipotassium salt and ternary eutectic. The nature of the small endotherm appearing in the lowest temperature region remained unclear. The appearance of the eutectic melting depended on the rate of previous cooling: after rapid cooling, eutectic meltings of binary and ternary mixtures were rarely observed.

The total melting enthalpy per g solution *ΔH*, due to both ice and eutectic melting, was calculated and is plotted in figs. 9 and 10. The broken lines in these figures refer to the values when both the monosalt and disalt solutions contribute additively to *ΔH*. No major changes in *ΔH* for the sodium phosphate buffer could be observed. Near the composition where the buffer contains mono- and disodium salts at the same concentration, however, the values were slightly lower than the ideal values (broken line). The tendency was more pronounced for the potassium salt buffer, although the total concentration used was higher than that of the sodium buffer. *ΔH* decreased with an increase in the fraction of dipotassium salt, to the composition where the fraction is unity, i.e., pure dipotassium phosphate solution, when neither binary eutectic of ice and dipotassium salt nor ternary eutectic was formed. As the binary eutectic usually was formed and the ternary eutectic was sometimes formed near the composition rich in the dipotassium salt, *ΔH* showed minima around the composition of the ternary eutectic.

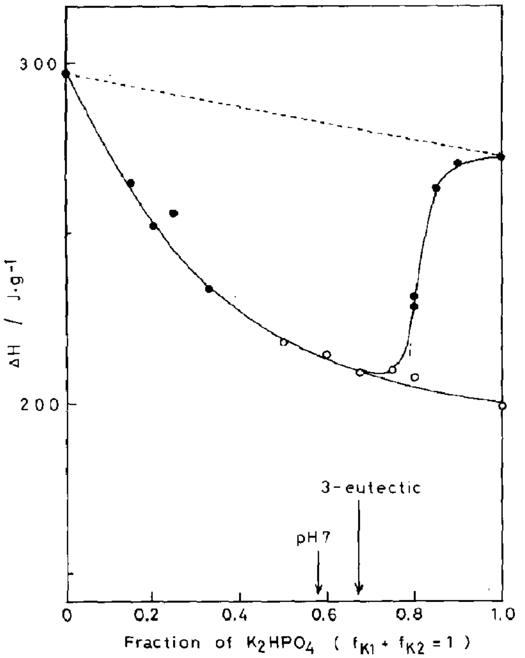

Fig. 10. Heat of melting of 1 M potassium phosphate buffers. Broken line: refers to values when both mono- and dipotassium salt solutions contribute to *ΔH* additively. Arrows indicate the compositions of the ternary eutectic and the pH 7 buffer, respectively.

4. Discussion

The freeze-thaw behaviour of phosphate solutions differs depending on the type of phosphate. NaH_2PO_4 did not precipitate at all under the experimental conditions. Therefore, the salt concentrates during freezing, becomes supersaturated and turns into an amorphous solid. Even a solution saturated with the salt at ambient temperatures did not show any indication of precipitation in our experiments.

As the solubility of Na_2HPO_4 in water is low and the eutectic temperature ($-0.5\,^\circ$C) is close to the melting temperature of pure ice, it was not possible to observe any eutectic melting separate from that of ice. However, it can be concluded that the salt is easy to precipitate, forming a eutectic, because the heat of solution of the salt was observed above 0 °C, indicating the precipitation of the salt from the solution during cooling. The ease of precipitation of the salt was confirmed by the observation of a freeze-dried sample by scanning electron microscopy (N. Murase et al., manuscript in preparation).

KH_2PO_4 was found to precipitate readily, forming a eutectic. However, the origin of the small endotherm remained unclear.

The eutectic temperature of K_2HPO_4 is the lowest among the four phosphates. This suggests that the salt is not easy to precipitate, since the rates of diffusion of ions as well as water molecules are reduced at low temperatures. Nevertheless, eutectic formation readily occurred. However, when smaller samples were used, eutectic formation was often inhibited. In that case, a high degree of undercooling may have depressed the nucleation temperature to below −53° C which was the operational low temperature limit of the DSC.

The results demonstrate that salt precipitation depends on the initial salt concentration. The salt fraction which precipitates forming eutectic, decreases with a decrease in the initial concentration to below approx. 1 M, in every case. Although the fraction of salt precipitated cannot be calculated without the knowledge of the heat of eutectic formation, it can be estimated by assuming that the maximum ΔH in fig. 5 corresponds to complete precipitation. At 0.1 M, approx. 70% of KH_2PO_4 precipitates. On the other hand, no K_2HPO_4 precipitates spontaneously.

When a dilute solution is cooled, ice is the first component to separate [8–10]. The salt distribution in the freeze concentrate may be determined by the original concentration because, initially, ice crystallization is fast and will determine the morphology of the frozen mixture. The salt-concentrated domains in the frozen sample of lower initial concentration may then be smaller and more finely dispersed than those existing in mixtures of higher initial concentration. The smaller salt-concentrated domains may then become subject to substantial undercooling and supersaturation, eventually into an amorphous state. This accounts for the observation that the fraction of salt which precipitates depends on the initial concentration.

Whether salt precipitates at a eutectic point from the freeze-concentrated domains may depend on the cooling rate. The melting heat of a eutectic depends scarcely on the cooling rate in the cases of Na_2HPO_4 and KH_2PO_4, as shown in fig. 6b and c, which indicates that the rates of nucleation and crystal growth of these salts are high compared to that of cooling. However, some cooling-rate-dependent change in the shape of the domains was observed in a scanning electron microscopic study of freeze-dried preparations (N. Murase et al., manuscript in preparation). The temperature at which the Na_2HPO_4 starts to melt was lower after rapid cooling than after slow cooling. It might possibly have been caused by the smaller crystal size which is obtained by rapid cooling. In the case of K_2HPO_4, the endotherm due to eutectic melting disappeared after the rapid cooling, which indicates that the rate of crystal formation of this salt is low compared to that of cooling. It is interesting, however, that the shape of the endotherm due to ice melting remained almost independent of the cooling rate and that eutectic formation is independent of the previous ice formation.

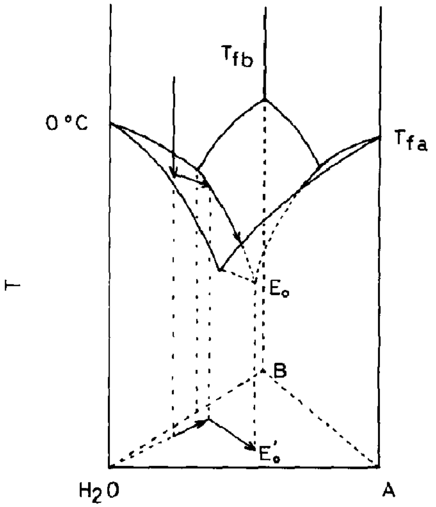

Fig. 11. Schematic phase diagram of a three-component system. (A, B) Components other than water; (T_{fa}, T_{fb}) freezing points of components A and B, respectively; (E_0) the ternary eutectic point.

A schematic phase diagram of a three-component system, e.g., the ternary phosphate buffer, is shown in fig. 11. When the dilute buffer solution is cooled, ice first precipitates and concentrates the solution without change in the ratio of the two phosphate salts. When the composition of the residual solution reaches that of one of the two binary eutectics, one salt begins to separate out, forming a eutectic with ice. Further cooling causes the composition of the solution to follow the binary eutectic through to the ternary eutectic point, when the residual liquid solidifies. The process shown in fig. 11 corresponds to the ideal behaviour, according to the equilibrium phase diagram.

The freezing and/or melting behaviour of phosphate buffer mixtures can be rationalized, to some extent, from the known behaviour of the binary solutions mixed in the buffer. The endotherm close to 0 °C, observed with the sodium phosphate buffer and shown in fig. 7, can be considered as being due to melting of the binary eutectic of ice and Na_2HPO_4, since the initial salt concentration is higher than that of the eutectic and this salt readily precipitates. The binary eutectic of ice and the monosodium salt was not observed to precipitate at all from buffers of any composition, neither was the formation of the ternary eutectic observed. This is predictable, as the ratio of monosodium salt to disodium salt in the eutectic is large, as shown in table 1, and the monosodium salt is difficult to precipitate. With the sodium salt buffer, a small exotherm was observed during heating within a limited range of composition and concentration. This is of interest from the viewpoint of solution structure [11,12], but the reason for such an exotherm remains unclear. In the case of the potassium phosphate buffer, two types of binary eutectic were observed which could be predicted from the freezing behaviour of the binary potassium salt solutions. The ternary eutectic was also formed occasionally.

The difference between the broken and continuous lines in figs. 9 and 10 indicates that the freezing behaviour of the buffer cannot be predicted completely from the knowledge of the behaviour of each binary phosphate solution. The difference between the broken and continuous lines corresponds to the amount of unfrozen, amorphous eutectic mixture. Thus, the amount of salt in the supersaturated, amorphous state is largest close to the composition of the ternary eutectic. Presumably, the simultaneous precipitation of three crystal types from the solution is a complicated and slow process. Moreover, the buffer of this composition can exist as a liquid down to the lowest temperature, where the diffusion rates of water and ions are reduced. For the sodium buffer, the greatest difference between the two lines was observed near the composition where mono- and disodium salts are present at the same concentration. The observed difference may be due to residual amorphous disodium salt, since monosodium salt does not precipitate at all over the whole composition range. Salt precipitation of one salt in a mixture may be prevented most efficiently around this composition by the other salt.

If a pH 7 sodium phosphate buffer is cooled, then, after freezing begins, the composition of the buffer moves towards the binary eutectic of ice and Na_2HPO_4, as shown in fig. 9 and table 1. As this salt is easy to precipitate, the composition may actually change until that of the ternary eutectic is reached. During the process, the pH of the buffer is expected to change dramatically toward acidic values, since the salt compositions of the pH 7 buffer and of the ternary eutectic differ substantially [2,3]. In the case of the potassium salts, the composition of the pH 7 buffer is close to that of the ternary eutectic, as shown in fig. 10 and table 1. Precipitation of KH_2PO_4, following the binary eutectic formation, does not readily take place, and the pH change is likely to be small, toward alkaline values, provided that precipitation takes place at all.

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