

Phosphate-Catalyzed Decomposition of Nitrite to Nitrate during Freezing and Thawing of Solutions

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A reaction leading to decomposition of nitrite ions to nitrate, catalyzed by phosphate, during freezing–thawing of solutions is reported. Factors such as solution pH, presence of calcium ions, substrate concentration, and temperature and duration of freezing affected the reaction rate. It is postulated that a sharp decrease in the system pH during the freezing process or frozen state, catalyzed by phosphate ions, led to the decomposition of nitrite ions. The results exemplify the potential of major artifacts in research caused by well-accepted laboratory practices such as freezing.

Keywords: Nitrite decomposition; freezing and thawing; NO_3^- formation

INTRODUCTION

Ordinarily, freezing temperatures are believed to minimize, or even stop, the rate of chemical and biological reactions. It is for this reason that most scientists store many of their biological materials and reagents at temperatures below the freezing points. Effects of freezing temperatures on retardation of microbial activity are well-known and a matter of common knowledge. However, little attention is commonly paid to the fact that some reactions may change their pathway or proceed more rapidly in the frozen state despite the decrease in kinetic energy and probably restricted diffusion (Grant and Alburn, 1966). Many researchers have shown that certain biochemical reactions proceed more rapidly in ice than in liquid water (Bruice and Butler, 1964; Pincock and Kiovsky, 1965, 1966; Pincock, 1969; Tong and Pincock, 1969). The commonly given explanation is that the reactants are brought into a favorable vicinity with each other in the ice structure. This would enhance the reaction catalytically or the dielectric properties of ice may facilitate the reaction of the substrates. Other researchers believe that the ice structure around a solute might modify its chemical kinetic properties (Pincock, 1969). An explanation by Grant et al. (1961) suggests that high proton mobility in ice might be a factor. Freezing can accelerate a number of reactions such as hydrolysis, aminolysis, and oxidation (Grant and Alburn, 1966), probably due to higher proton mobility. Takenaka et al. (1992) reported an increase in the reaction rate of NO_2^- oxidation to NO_3^- as the freezing rate was increased.

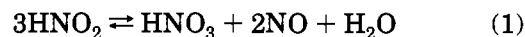
Since NO_2^- is an important intermediate in reactions of many biological systems, such as human, animal, plant, and soil, it was the objective of this research to study how freezing–thawing may cause its decomposition to NO_3^- . Earlier, the unexplained disappearance of large percentages of NO_2^- in some experiments in our laboratory was traced mainly to its decomposition to NO_3^- due to freezing and thawing of solution samples. This research should also serve as an example of how a well-established and accepted scientific practice may generate major artifacts in research.

MATERIALS AND METHODS

All chemicals used were of analytical grade. High-performance liquid chromatography techniques were used for NO_2^- and NO_3^- assay (Thayer and Huffaker, 1980). The freezing at different temperatures was performed in a refrigerated water bath (Model 2095, Forma Scientific) except the -80°C treatment, which was accomplished in a freezer. The freezing was carried out at -20°C for a period of 24 h, unless otherwise specified. The initial pH of the solutions was adjusted using HCl for pH 3 and 4 and NaOH for pH 5 and above. The results are expressed as a percentage of the initial concentration of NO_2^- . Four replicates of 4 mL of 1 mM KNO_2 in test tubes (16×100 mm) were used for each measurement. The standard errors of means are shown as bars in figures (smaller bars are contained within symbols) and as “ \pm ” numbers in tables. Since all experimental conditions and apparatus were very carefully controlled, we speculate that the variability among the replicates was largely due to microphysical factors affecting the patterns of ice crystallization.

RESULTS AND DISCUSSION

The reaction of NO_2^- decomposition to NO_3^- in liquid phase is presented below (Yost and Russell, 1946).



General Characterization. Preliminary experiments had shown that NO_2^- was decomposed to NO_3^- upon freezing–thawing, at pH 6 or above, only if the inorganic nutrients required for plant growth (Hoagland and Arnon, 1950) were included in the solution. Of all the nutrients present, only PO_4 (generally included as KH_2PO_4) was effective in NO_2^- decomposition (results not shown). Since no NO_2^- decomposed in the presence of KNO_3 , we inferred that the PO_4 ions from KH_2PO_4 (rather than K^+) catalyzed the reaction during freezing–thawing (Table 1).

Nitrite decomposition during freezing–thawing at pH 6.0 was tested as a function of phosphate salts of four common cations. Of the salts tested, $\text{Ca}(\text{H}_2\text{PO}_4)_2$ and KH_2PO_4 were the most and least effective, respectively (Table 1). However, little NO_2^- decomposed due to the inclusion of $\text{Ca}(\text{NO}_3)_2$. Therefore, Ca^{2+} seemed to only enhance the catalytic activity of PO_4 ions in NO_2^- decomposition (Table 1). Several other inorganic and organic anions were also tested for their effectiveness in NO_2^- decomposition during freezing–thawing. Among those tested, none of the anions, except PO_4 , catalyzed the NO_2^- decomposition (Table 1).

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Table 1. Effect of Various Salts on NO_2^- Decomposition during Freezing-Thawing (Experimental Conditions: 1 mM KNO_2 , pH 6.0, -20°C for 24 h)

salt	% of initial 1 mM NO_2^-		
	NO_2^-	NO_3^- formed	total ($\text{NO}_2^- + \text{NO}_3^-$)
none (KNO_2 only)	99.3 \pm 0.6	ND ^a	99.3 \pm 0.6
KNO_3 , 2 mM	99.2 \pm 0.5	0.50 \pm 0.02 ^b	99.7 \pm 0.6
$\text{Ca}(\text{NO}_3)_2$, 2 mM	99.9 \pm 0.6	1.20 \pm 1.80 ^b	101.2 \pm 2.2
$\text{Ca}(\text{H}_2\text{PO}_4)_2$, 1 mM	41.6 \pm 1.4	57.0 \pm 1.30	98.7 \pm 0.3
$\text{Ca}(\text{H}_2\text{PO}_4)_2$, 2 mM	40.8 \pm 0.0	56.4 \pm 0.00	97.2 \pm 0.0
$\text{Mg}(\text{H}_2\text{PO}_4)_2$, 1 mM	51.0 \pm 5.3	47.6 \pm 4.70	98.6 \pm 0.6
KH_2PO_4 , 2 mM	65.7 \pm 1.2	31.7 \pm 0.50	97.4 \pm 0.6
NaH_2PO_4 , 2 mM	55.3 \pm 4.0	39.7 \pm 4.20	95.0 \pm 3.6
NaI	97.4 \pm 0.9	ND	97.4 \pm 0.9
NaF	93.4 \pm 2.2	ND	93.4 \pm 2.2
NaCl	100.4 \pm 0.5	ND	100.4 \pm 0.5
Na_2SO_4	100.2 \pm 0.8	ND	100.2 \pm 0.8
NaMoO_4O_6	100.8 \pm 0.8	ND	100.8 \pm 0.8
sodium acetate	97.2 \pm 0.4	ND	97.2 \pm 0.4
sodium citrate	97.0 \pm 0.7	ND	97.0 \pm 0.7
sodium tartrate	96.4 \pm 1.1	ND	96.4 \pm 1.1

^a ND none detected. ^b Additional to that was added as NO_3^- .

Since water and ice have roughly the same electrical conductivity, the lower proton activity in ice must be compensated by the higher mobility (Kavanau, 1964). The proton mobility in ice is actually 10–100 times greater than that in water (0.19 cm s^{-1} at -10°C). The ice crystal might thus be considered as a "proton semiconductor" (Kavanau, 1964). When an aqueous solution containing a pH-sensitive dye at an initial pH

of 6.0 was frozen, a change in dye color (in ice) was observed, indicating that the pH had dropped to about 4.0 (results not shown). The decrease in pH upon freezing as shown by the dye color was even more pronounced when PO_4 ions were included in the solution. The H^+ activity of solutions, however, decreases with decreasing temperature. Therefore, the decrease in pH upon freezing must be due to an increase in either proton concentration or mobility. The protons that are associated with ionizable groups also become more mobile in relation to their binding sites upon freezing; i.e., proton association becomes weaker (Kavanau, 1964). Therefore, it is possible that more mobile protons were available in samples containing PO_4 ions.

As stated above, the $\text{Ca}(\text{H}_2\text{PO}_4)_2$ matrix was more effective in NO_2^- decomposition upon freezing-thawing than KH_2PO_4 (Figure 1A,B). Since all solutions containing KH_2PO_4 or $\text{Ca}(\text{H}_2\text{PO}_4)_2$ were titrated to the same pH before freezing, they must have had the same H^+ activity in liquid phase. However, upon freezing, the protons in solutions containing $\text{Ca}(\text{H}_2\text{PO}_4)_2$ might be more ionized and mobile than those containing KH_2PO_4 . The crystal structure of NO_2^- salts might also have an influence on proton mobility. It is interesting to note that both ice and $\text{Ca}(\text{NO}_2)_2$ crystals are hexagonal rings, while KNO_2 crystals are granules or rods (Budavari, 1989), which might hinder the flow of protons.

Another interesting phenomenon noted was that usually some of the N could not be accounted for either

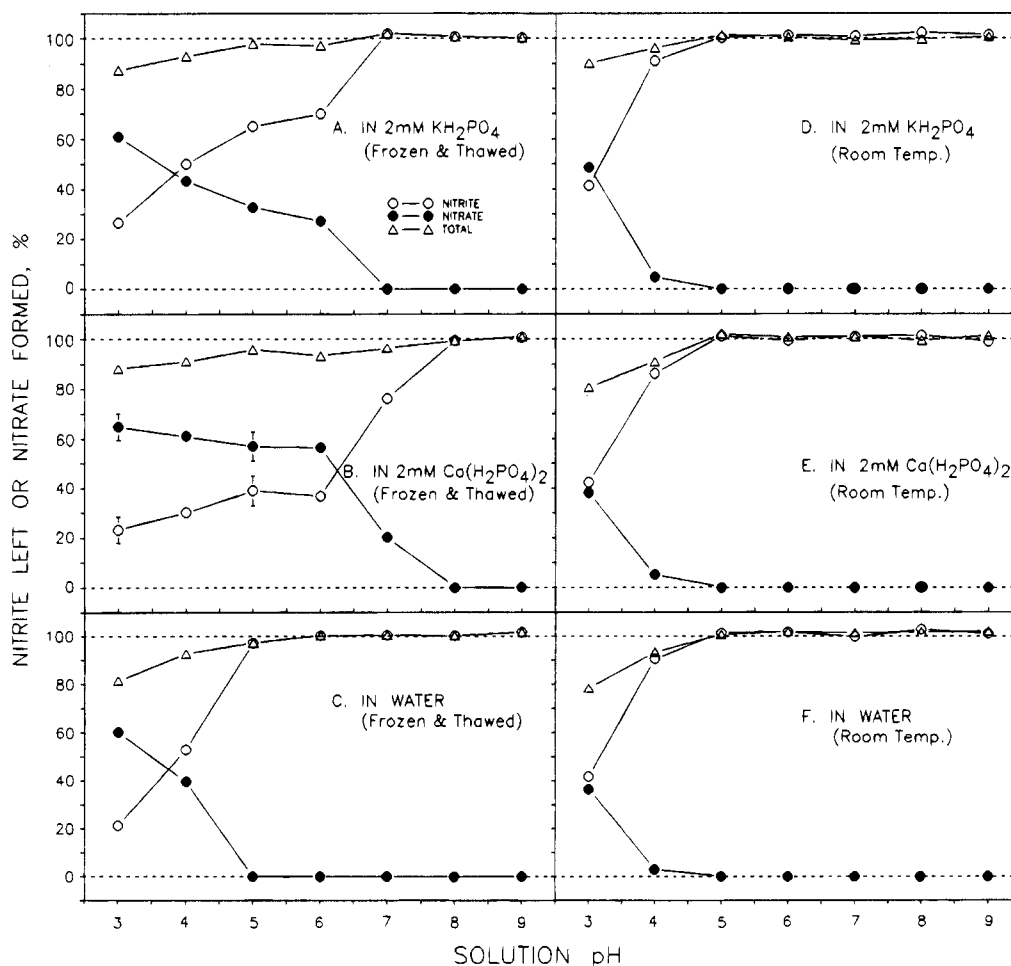


Figure 1. Effect of initial solution pH and calcium or potassium phosphate on the decomposition of nitrite and formation of nitrate ions during freezing-thawing. Solutions at an initial nitrite concentration of 1 mM were frozen at -20°C for 24 h.

as NO₂⁻ or as NO₃⁻. For example, at pH 4.0 with Ca(H₂PO₄)₂, about 30 and 60% of the N was recovered as NO₂⁻ and NO₃⁻, respectively, and about 10% of the N was not accounted for in either form (Figure 1B). Stoichiometrically, 2 mol of nitric oxide gas (NO) would be produced for every 1 mol of NO₃⁻ formed during the NO₂⁻ decomposition reaction (eq 1). If all NO formed during the reaction were lost from the system, the N not accounted for in the NO₂⁻/NO₃⁻ fraction should have been twice the NO₃⁻ formed. Apparently, that was not the case (Figure 1A-C). Therefore, the bulk of the NO produced was probably oxidized back to NO₂⁻ during the process and only the portion of N not recovered in the NO₂⁻/NO₃⁻ fraction was perhaps lost in the gaseous form. However, the conversion of NO₂⁻ to other N compounds cannot be ruled out at this time.

Effect of pH on NO₂⁻ Decomposition. In general, the percentage of NO₂⁻ decomposition to NO₃⁻ decreased with increasing pH of the solutions, and none ever occurred at pH 8.0 or above under any of the conditions tested (Figure 1). At pH 4.0 or below, the decomposition occurred even at room temperature (no freezing) and was not much affected by the PO₄ ions (Figure 1D-F). Nevertheless, as compared to room temperature, freezing-thawing greatly increased the decomposition at all pH values below 8.0 (Figure 1). These observations can be explained by the fact that the H⁺ concentration in solutions increases with decreasing pH and that the proton mobility increases upon freezing of solutions.

In freezing-thawing solutions at pH 3.0, PO₄ ions had little effect on NO₂⁻ decomposition, regardless of the source—Ca(H₂PO₄)₂ or KH₂PO₄. However, at pH 4.0 or above, Ca(H₂PO₄)₂ greatly increased the decomposition relative to no PO₄ ions or even KH₂PO₄ (Figure 1A-C). At pH 5.0 and above, NO₂⁻ decomposition required both freezing-thawing and PO₄ ions (Figure 1) and no decomposition occurred if one or the other was absent. These observations suggest that the NO₂⁻ decomposition primarily occurred due to low pH (eq 1), such as pH 4.0 or below. For the role of freezing, a sharp decrease in the system pH, strongly catalyzed by PO₄ and Ca²⁺, most likely occurred during the freezing-thawing process. Therefore, the actual decomposition of NO₂⁻ may have always happened due to low pH values of 4.0 or less. Since the pK_{a2} of PO₄ ions is 7.2, the pH of solutions at an initial pH of 8.0 or above probably did not drop low enough during freezing-thawing and, hence, no NO₂⁻ decomposition occurred. Therefore, the hypothesis that a decrease in pH during freezing-thawing caused NO₂⁻ decomposition also explains the observation that no NO₂⁻ decomposition occurred at pH 8.0 or above, under any conditions.

In contrast, NO₂⁻ oxidation to NO₃⁻ at pH 4.5 due to freezing was negligible in the absence of dissolved oxygen (Takenaka et al., 1992). They believed that dissolved oxygen must be reacting with NO₂⁻ to form NO₃⁻. However, in our system, at pH 4.0 and in the presence of Ca(H₂PO₄)₂, lack of dissolved oxygen did not affect the percentage of NO₂⁻ decomposed. Nevertheless, the NO₃⁻ formed and hence the recovery of N in the NO₂⁻/NO₃⁻ fraction was about 60% lower (results not shown). This suggests a large loss of N via NO, which, in the presence of oxygen, was most likely oxidized back to NO₂⁻ to again participate in the reaction (eq 1).

Effect of Phosphate Salt Concentration on NO₂⁻ Decomposition. In general, NO₂⁻ decomposition dur-

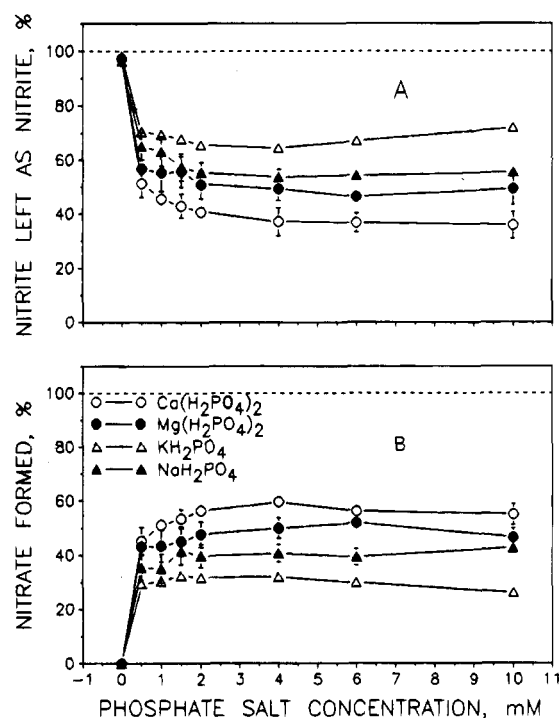


Figure 2. Effect of sodium, potassium, magnesium, or calcium phosphate at different concentrations on the decomposition of nitrite and formation of nitrate ions during freezing-thawing. Solutions at an initial nitrite concentration of 1 mM and pH 6.0 were frozen at -20°C for 24 h.

ing freezing-thawing (as measured by NO₂⁻ remaining or NO₃⁻ formed) increased with increasing PO₄ salt concentration up to 1 mM (Figure 2). In some cases, a minor increase was seen at 1.5 mM also. At all concentrations tested (from 0.5 to 10 mM), Ca(H₂PO₄)₂ and KH₂PO₄ were, respectively, the most and the least effective in NO₂⁻ decomposition, while Mg(H₂PO₄)₂ and NaH₂PO₄ had an intermediate effect (Figure 2).

Comparison of different PO₄ salts at equimolar concentrations may not be valid due to differences in cation valency. At 1 mM Ca(H₂PO₄)₂ or Mg(H₂PO₄)₂, H₂PO₄ will be present at 2 mM, which is twice as much of that in 1 mM KH₂PO₄ or NaH₂PO₄. However, the effect of KH₂PO₄ or NaH₂PO₄ was always lower than that of Ca(H₂PO₄)₂ or Mg(H₂PO₄)₂, even at the highest concentration of 10 mM (Figure 2). This, once again, shows that the cation accompanying H₂PO₄ also had a significant role in NO₂⁻ decomposition during freezing-thawing.

Effect of Freezing Temperature and Duration on NO₂⁻ Decomposition. Freezing temperatures from -1 to -20°C were tested on solutions containing 1 mM KNO₂ and 2 mM Ca(H₂PO₄)₂, at pH 6.0, kept frozen for 24 h. The NO₂⁻ decomposition (NO₂⁻ disappeared or NO₃⁻ formed) increased rapidly as the temperature was decreased from -1 to -2°C and continued to increase with decreasing temperature, at progressively lower rates, until about -12.5°C (Figure 3). No further increase in decomposition activity was seen with temperatures lower than -12.5°C (Figure 3). A system can be considered to be completely frozen solid only below its eutectic point. Below the freezing point and above the eutectic point, a solid is believed to be in equilibrium with a liquid phase (Pincock and Kiovsky, 1966). The immediate direct result of freezing is a gradual concentration of solutes, which will increase the ionic strength and cause changes in pH. Therefore, at -1°C , a liquid phase concentrated in salts must have

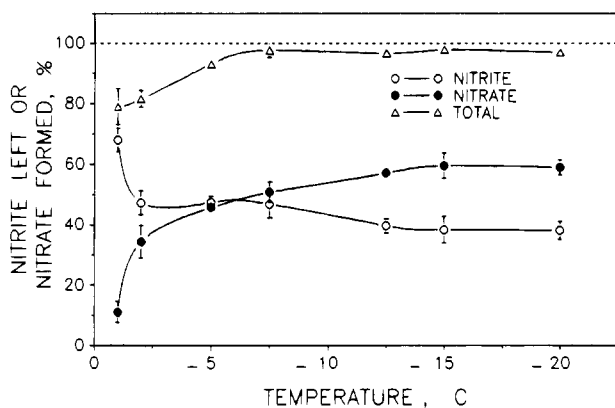


Figure 3. Effect of freezing temperature on the decomposition of nitrite and formation of nitrate ions during freezing-thawing. Solutions at an initial nitrite concentration of 1 mM and pH 6.0 contained 2 mM calcium phosphate and were frozen for 24 h.

existed. The pH of this liquid probably became low (dye experiments mentioned above), which promoted NO_2^- decomposition similar to that in solutions at pH 3 or 4 and at room temperature (Figure 1). However, since the system may not have been fully frozen, the relatively high proton mobility (which may be needed for NO_3^- formation) was perhaps not achieved. Consequently, a lower percentage of NO_3^- was formed (Figure 3).

When solutions containing 1 mM KNO_2 and 2 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$ at pH 6.0 were frozen in a -80°C deep freezer for 24 h, NO_2^- decomposition was only about 65% of those frozen at -20°C (results not shown). The decomposition was even less when solutions were frozen at -197°C using liquid nitrogen. Takenaka et al. (1992) reported the appearance of the same NO_3^- concentration at freezing temperatures of -21 and -45°C ; temperatures lower than -45°C were not tested. According to Kavanau (1964), studies show that the proton configuration in ice is highly mobile, varies with the thermal fluctuations, and becomes constant at about -183°C . Luyet (1966) suggested that under increased cooling velocity (fast freeze at -80°C , for example) during crystallization, some of the elements of hexagonal symmetry are lost and irregular arrangements of ice crystals are obtained, which also might influence the proton mobility.

Although the NO_2^- decomposition activity at -1°C was the lowest, the percent of N not recovered in the $\text{NO}_2^-/\text{NO}_3^-$ fraction was the highest (20%) among all the temperatures tested. At -1°C , about 70 and 10% of the original NO_2^- was recovered as NO_2^- and NO_3^- , respectively, after freezing-thawing. In other words, of the 30% NO_2^- decomposed at -1°C , only about 10% (about 33% of decomposed) was recovered as NO_3^- and a rather large part (67% of decomposed) was not accounted for in the $\text{NO}_2^-/\text{NO}_3^-$ fraction (Figure 3). As the temperatures were decreased further, the percentage of N recovered in the $\text{NO}_2^-/\text{NO}_3^-$ fraction increased rapidly. At -7.5°C almost all of the original NO_2^- was accounted for in $\text{NO}_2^-/\text{NO}_3^-$ fraction after freezing-thawing. This suggests that at relatively lower temperatures the bulk of the NO formed may be readily oxidized back to NO_2 ions, while at higher temperatures more NO may be lost in gaseous form.

The percent of NO_2^- decomposed at pH 6.0 and -20°C increased linearly and rapidly as the time elapsed increased from 2.5 to 10 min (Figure 4). After 2.5 and 10 min, 10 and 30% of the NO_2^- decomposed, respec-

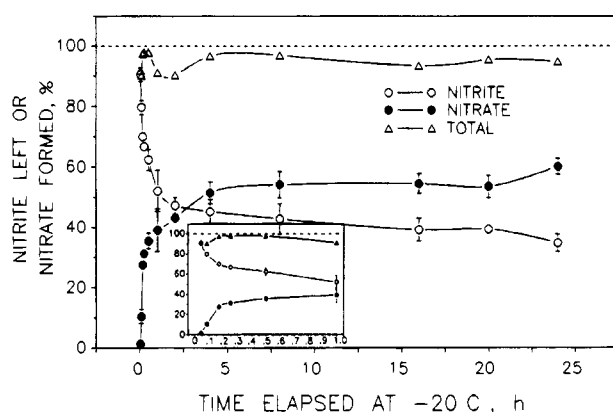


Figure 4. Effect of freezing duration on the decomposition of nitrite and formation of nitrate ions during freezing-thawing. The solutions at an initial nitrite concentration of 1 mM and pH 6.0 contained 2 mM calcium phosphate and were frozen at -20°C .

Table 2. Effect of Initial NO_2^- and NO_3^- Concentration on NO_2^- Decomposition during Freezing-Thawing (F-T) [Experimental Conditions: pH 6.0, -20°C for 24 h, Matrix of 2 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$]

initial concn, mM		concn after F-T, mM		% of initial NO_2^-	
NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
0.5	0.0	0.158 ± 0.003	0.352 ± 0.002	31.3 ± 0.3	68.2 ± 1.0
1.0	0.0	0.489 ± 0.715	0.523 ± 0.007	49.1 ± 0.7	51.7 ± 1.0
1.5	0.0	0.871 ± 0.010	0.624 ± 0.007	58.2 ± 0.7	40.9 ± 0.4
2.0	0.0	1.302 ± 0.009	0.682 ± 0.006	65.4 ± 0.5	33.4 ± 0.3
1.0	0.5	0.516 ± 0.008	1.004 ± 0.008	51.8 ± 0.8	48.7 ± 1.1
1.0	1.0	0.556 ± 0.013	1.458 ± 0.127	56.0 ± 1.3	44.2 ± 1.9
1.0	1.5	0.582 ± 0.023	1.899 ± 0.196	58.9 ± 2.4	40.3 ± 1.8
1.0	2.0	0.513 ± 0.007	2.393 ± 0.003	59.7 ± 0.6	41.7 ± 0.6

tively, and about 1 and 30% of the original NO_2^- was recovered as NO_3^- (Figure 4, inset). In other words, 10 and 100% of the NO_2^- decomposed after 2.5 and 10 min, respectively, was recovered as NO_3^- . After 10 min, the effect started to level off and essentially no further increase in NO_2^- decomposition was noticed after 4 h, when almost 55% of the NO_2^- decomposed (Figure 4).

The effect of shorter durations (e.g., 2.5 min) at -20°C seems to be similar to that observed at relatively higher temperatures with longer durations, e.g., -1 or -2°C for 24 h (Figure 3), in terms of lower NO_2^- decomposition and poor recovery of decomposed NO_2^- as NO_3^- . The reasons in both cases were probably the same as discussed above.

Effect of Initial NO_2^- or NO_3^- Concentration on NO_2^- Decomposition. As the initial NO_2^- concentration in the solution was increased from 0.5 to 2.0 mM, the percent of NO_2^- that decomposed to NO_3^- due to a freeze-thaw cycle decreased; i.e., the percent of NO_2^- remaining increased (Table 2). This suggests that a reactant (PO_4 and/or Ca ions) may have become limiting at higher initial NO_2^- concentrations. However, at 1 mM NO_2^- , no further increase in its decomposition was observed due to an increase in $\text{Ca}(\text{H}_2\text{PO}_4)_2$ concentration beyond 1.5 mM (Figure 2A). In this experiment, $\text{Ca}(\text{H}_2\text{PO}_4)_2$ was used at 2.0 mM and therefore could not have been limiting. The initial concentration of NO_3^- also had little effect on the NO_2^- decomposition (Table 2). The reasons for the relatively higher percentage of decomposition at lower NO_2^- concentration are not clear at this time.

Influence of Repeated Freeze-Thaw Cycles on NO_2^- Decomposition. Each freeze-thaw cycle caused further decomposition of NO_2^- such that no NO_2^- was

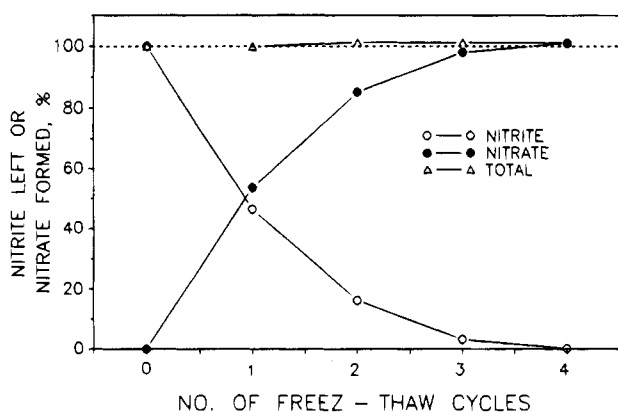


Figure 5. Effect of repeated freeze-thaw cycles on the decomposition of nitrite and formation of nitrate ions. Solutions at an initial nitrite concentration of 1 mM and pH 6.0 contained 2 mM calcium phosphate and were frozen at -20°C for 24 h in each cycle.

detected in a 1 mM solution after four cycles (Figure 5). A progressively larger percentage of NO₂⁻ was decomposed with each freeze-thaw cycle (start of cycle = 100). As noted earlier, a greater percentage of NO₂⁻ decomposed as its initial concentration in solution was decreased (Table 2). Hence, the progressively larger percentage of NO₂⁻ decomposition with each freeze-thaw cycle was most likely due to the progressively lower concentration of NO₂⁻ at the start of each cycle.

In conclusion, a significant percentage of NO₂⁻ decomposes, mainly to NO₃⁻, during freezing-thawing of solutions. The decomposition is strongly effected by several factors, e.g., solution pH, NO₂⁻ concentration, presence of Ca and PO₄ ions, and freezing temperature and duration. The decomposition of NO₂⁻ at low pH (4.0 or below) could occur at room temperature. Therefore, we believe that freezing-thawing affects the decomposition by lowering the solution pH at some point. Regardless of freezing-thawing, little NO₂⁻ decomposes at pH 5.0 or above, unless PO₄ ions are present. Hence, PO₄ ions probably catalyze the decrease in pH during freezing-thawing, which in turn causes the NO₂⁻ decomposition. Calcium ions seem to enhance the catalytic activity of PO₄ ions in decreasing the pH. No NO₂⁻ decomposition was observed at pH 8.0 or above, regardless of other factors. Some NO₂⁻ was not recovered either as NO₂⁻ or as NO₃⁻. We think this represents a loss of N from the system as NO gas.

Finally, we recommend that before freezing is employed as a means of preserving experimental and

research materials, a thorough investigation on its effect on the research parameters should be conducted.

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