Effect of Freezing on Oxidation of L-Ascorbic Acid

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The rate of oxidation of L-ascorbic acid (AA) in acetate buffer solutions was studied at pH values of 4.6 and 5.5, at various concentrations, and at temperatures ranging from +21° to −23° C. Samples which were initially dilute (0.00087*M* acetate buffer containing 1.7 mg AA per 100 ml of solution) and thereby capable of dissolving substantial quantities of oxygen, and those which had pH values of 5.5, exhibited, upon freezing, either an increased rate of oxidation of AA or a smaller than expected decrease in rate. At higher initial concentrations and a pH value of 4.6 (conditions common to many foods), expected decreases in the rate of oxidation of AA were observed upon freezing. Results from this study provide a reasonable basis for reconciling differences in the effect of freezing on the rate of oxidation of AA in foods as compared to dilute, simple solutions.

In frozen foods, L-ascorbic acid (vitamin C) generally is more stable than in unfrozen foods, and stability is further improved as the temperature of frozen storage is lowered (Davis, 1956; Feister *et al.*, 1950; Guadagni *et al.*, 1957a,b,c; Guerrant *et al.*, 1945; Hucker and Clark, 1961; Ross, 1944). Although the same relationship between stability of L-ascorbic acid (AA) and temperature might be expected in dilute simple soultions, an unexpected departure from this pattern was reported by Grant and Alburn (1965b). They studied the rate of oxidation of AA in frozen (−11° C) and unfrozen (+1° C) solutions containing 10^{-4} *M* AA and 0.02 *M* acetate buffer at pH values of 5.0 and 5.5. At both pH values the rate of oxidation of AA was substantially greater at −11° C than at +1° C, the difference being more pronounced at pH 5.5. The most likely cause for this behavior is freeze concentration, *i.e.*, the increase in concentration of solutes that occurs in the unfrozen phase during freezing (Pincock and Kiovsky, 1966). However, Grant and Alburn (1965a,b) suggested several other factors which also may contribute to this unusual behavior: a catalytic effect exerted by the surface of ice crystals; favorable orientation of reactants in the partially frozen state; and the decrease in dielectric constant or increase in proton mobility that accompanies formation of ice. Although these factors provide a reasonable basis for explaining rates of AA oxidation in dilute simple solutions, they do not explain why freezing should affect the stability of AA in foods so differently from that in dilute simple solutions. Information pertinent to this point is provided here.

MATERIALS AND METHODS

Composition of Samples. Four solutions with different pH values and concentrations, but with the same kind and ratio of solutes (same ratio of AA to buffer), were used to determine the effects of temperature, concentration, and pH on the rate of oxidation of AA. The "standard" sample, referred to as 1x, consisted of 0.02 *M* acetate buffer and 40 mg of AA per 100 ml of solution. This concentration of AA was chosen to represent foods which are good sources of vitamin C. A more dilute sample, referred to as $^1/_{23}$x, consisted of 0.00087 *M* acetate buffer and 1.7 mg of AA per 100 ml of solution ($^1/_{23}$rd the solute concentration of the 1x sample). This concentration of AA was selected because it was close to that employed by Grant and Alburn (1965b) when they observed rate enhancements during freezing. The $^1/_{23}$x sample was studied at pH values of 4.6 (referred to as $^1/_{25}$x–4.6) and 5.5 ($^1/_{23}$x–5.5). A concentrated solution, referred to as 50x–4.6, consisted of 1.0 *M* acetate buffer at pH 4.6 and 2000 mg of AA per 100 ml of solution (50 times the solute concentration of the 1x solution). This sample was included to approximate the concentration calculated to exist in the unfrozen phase of a partially frozen solution at approximately −3° C. Still higher concentrations would have been useful, but they were experimentally difficult to handle in terms of providing an adequate supply of oxygen. The pH values of 4.6 and 5.5 were selected because they are typical of many foods, especially those in which AA is only moderately stable.

Procedures for Studies Above 0° C. To help supply oxygen in abundance, 10 ml portions of the 1x or $^1/_{23}$x solutions were placed in open 250 ml Erlenmeyer flasks and shaken continuously in a liquid bath at constant temperature. Duplicate samples were removed after 10 min (0 time) and at several subsequent times, and each sample was diluted immediately to 100 ml with 3% HPO_3 and analyzed for AA by titration with a solution of 2,6-dichlorophenolindophenol (Association of Vitamin Chemists, 1966). The 50x samples were treated in essentially the same manner, except the sample size was reduced to 2 ml to help assure an adequate supply of oxygen.

Procedures for Studies of Frozen Samples. Twenty milliliter portions of the 1x or $^1/_{23}$x solutions were placed in closed 60 ml bottles, and each bottle was first rotated in an ice water bath for 30 min to equilibrate the contents with air, then rotated for 1 min in a dry-ice-acetone bath to accomplish freezing, and finally stored for 30 min at the desired reaction temperature to obtain temperature equilibration (0 time). The overcooling and rewarming procedure was employed to help assure solid-liquid equilibrium at the desired subfreezing reaction temperature. Duplicate samples were removed at 0 time and at subsequent intervals, each was thawed by addition of sufficient 3% HPO_3 to bring the volume to 100 ml, then analyzed for AA as described previously. This procedure resulted in rapid thawing (since the frozen matter was present as a thin layer on the inner surface of the bottle) and prompt cessation of the reaction.

The 50x samples were treated in the same manner, except the sample size was reduced to 4 ml for the reason given previously.

Calculation of Reaction Rate Constants. Reaction rate constants for oxidation of AA at a given temperature were obtained by plotting the logarithm of mg-AA-per-100-ml-

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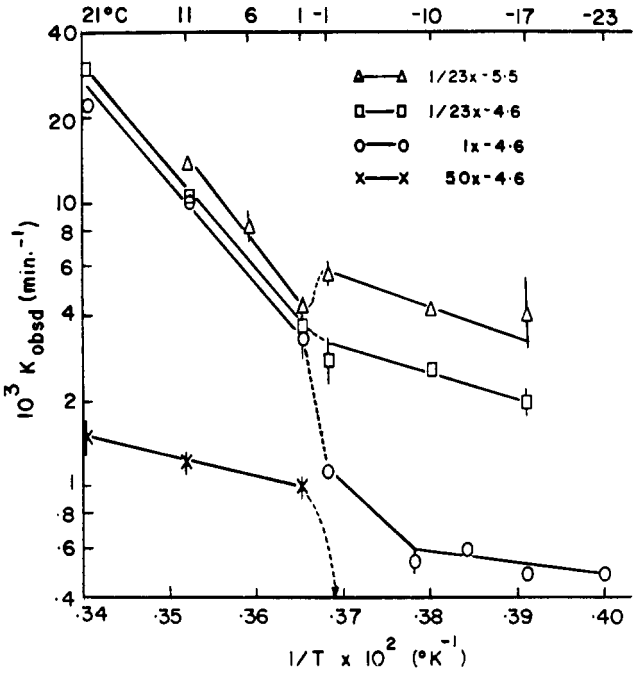

Figure 1. Oxidation of L-ascorbic acid as influenced by pH, concentration, and temperature

Table I. Effect of Freezing on the Reaction Rate Constant for Oxidation of L-Ascorbic Acid

			$k \cdot 10^3$ min^{-1}		
pH	Concentration	Temp. (°C)	Observed (frozen)[a]	Extrapolated (supercooled)[b]	Acceleration value[c]
4.6	50x	−10	0.1	0.7	0.1
4.6	1x	−1	1.1	2.8	0.4
		−8	0.6	1.2	0.5
		−12	0.6	0.7	0.9
		−17	0.5	0.4	1.2
		−23	0.5	0.2	2.5
4.6	1/23x	−1	2.8	2.9	1.0
		−10	2.7	1.0	2.7
		−17	2.0	0.4	5.0
5.5	1/23x	−1	5.8	3.3	1.8
		−10	4.3	1.1	4.2
		−17	4.4	0.4	11.0

[a] Means of two k values. [b] These values were obtained by extrapolating the linear above-zero data of the Arrhenius plot (Fig. 1) to the sub-zero temperature under consideration. [c] Acceleration value = observed reaction rate constant ÷ extrapolated reaction rate constant.

of-solution against time, from which $k_{obs} = -2.303$ (slope). Determination of each k_{obs} value involved eight samples (four determinations in duplicate taken during reaction periods ranging from 30 min to 7 hr, depending on temperature). Few plots of log AA concentration *vs.* time were perfectly linear (most had a slight curvature), but lines of best fit were obvious in most instances. Two values for k_{obs} were determined for each condition, and the range in the replicates is indicated by the heights of the symbols in Figure 1.

Although considerable care was exercised to help assure an excess of oxygen within the samples, it is likely that the reaction rates of most samples (especially the 50x samples) were limited somewhat by an inadequate supply of oxygen. The slight curvature present in the plots of log AA concentration *vs.* time support this contention. The reported reaction rate constants, although calculated in accordance with pseudo-first-order kinetics, consequently must be regarded simply as observed values (k_{obs}).

RESULTS AND DISCUSSION

Oxidation of AA was studied at temperatures ranging from +21° C to −23° C, and the maximum reaction period was 7 hr at −23° C. The results are summarized in the Arrhenius plot of Figure 1. At any given temperature above 0° C, observed reaction rate constants of the 1x–4.6 and 1/23x–4.6 samples were of intermediate magnitude and essentially the same, whereas the rate constant of the 1/23x–5.5 sample was somewhat greater, and that of the 50x–4.6 sample was very much smaller than the rate constants of the aforementioned pair of samples. The above-zero reaction rate constants of any given sample type decreased linearly with temperature, as would be expected. The above-zero slopes of the 1x and 1/23x samples were much smaller than the others, again raising the possibility that oxygen was rate limiting in the 50x samples.

When the reaction rate constants of the four samples are compared over the entire temperature range, it is evident that differences among the rate constants are much greater below 0 than above. Furthermore, at each of the various temperatures, the 1/23x–5.5 sample always exhibited the greatest rate constant, followed in order by the 1/23–4.6 sample, the 1x–4.6 sample, and the 50x–4.6 sample. The reaction rate constant of the 50x–4.6 sample at sub-zero temperatures was too small to be accommodated on the figure and too small to be measured accurately. All Arrhenius plots lacked good linearity in the below 0 zone.

When passing from 0° to −1° C (partially frozen), the reaction rate constants of the 50x and 1x samples declined greatly, that of the 1/23x–4.6 sample declined moderately, and that of the 1/23x–5.5 sample increased significantly. A more accurate assessment of the changes in rate constants induced by freezing can be gained by extrapolating the linear above-zero data to appropriate sub-zero temperatures. Then the observed reaction rate constant for a given frozen sample can be compared to a theoretical reaction rate constant for an unfrozen sample (extrapolated value) at the same temperature. The ratio of the two values [observed reaction rate constant (frozen) ÷ extrapolated reaction rate constant (supercooled)] indicates the effect of freezing on the magnitude and direction of change in the rate constant for oxidation of AA. This ratio will be referred to as an "acceleration value," with values greater than 1.0 indicating an increase in the rate constant induced by freezing.

The merit of this approach can be demonstrated by examining the data for sample 1/23x–4.6 in Figure 1. As the 1/23x–4.6 sample is cooled and frozen through the zone −1° to −17° C, there is a tendency for the rate constant to decrease. Based on this observation one could conclude that freezing of this sample inhibits oxidation of AA. However, if the linear above-zero data is extrapolated to the sub-zero zone and the observed rate constant at, for example −10° C (partially frozen sample), is compared to the extrapolated value at −10° C (represents a supercooled sample), it is immediately obvious that ice formation has accelerated rather than inhibited oxidation of AA.

Shown in Table I are various acceleration values derived from observed and extrapolated data of Figure 1. The only samples exhibiting reductions in their rate constants (acceleration values of 1.0 or less) during freezing are the 50x samples, the 1x–4.6 samples at subfreezing temperatures of −12° C or higher, and the 1/23x–4.6 sample at −1° C. The remaining samples exhibit acceleration values ranging

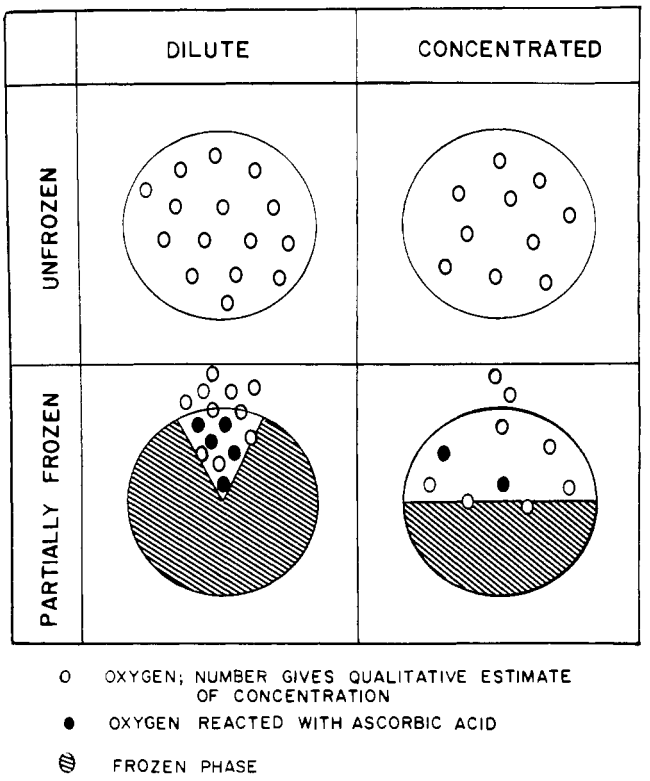

Figure 2. Schematic illustration of the concentration and disposition of oxygen in dilute and concentrated solutions during freezing

from 1.2 to 11.0. It is also evident that at any given subfreezing temperature a large acceleration value is associated with the higher of the two pH values and/or an initially dilute sample, and that within a given type of sample, the acceleration value increases as the temperature is lowered over the range studied. For example, at $-17°$ C, it is evident that: the high pH sample ($^1/_{23}$x–5.5) exhibits an acceleration value approximately twice that of the low pH sample ($^1/_{23}$x–4.6); and the initially dilute sample ($^1/_{23}$x–4.6) exhibits an acceleration value approximately four times that of the initially concentrated sample (1x–4.6).

An alternative way to compare the effects of initial pH and concentration on the rate constant for oxidation of AA during freezing is to calculate half-lives from the relationship $t_{1/2} = 0.693/k$. When considering samples at $+1°$ and $-1°$ C, it is found that the times required to oxidize half of the total amount of ascorbic acid at $+1°$ C (unfrozen) are 3.5, 3.2, and 2.6 hr, respectively, in the 1x–4.6, $^1/_{23}$x–4.6, and $^1/_{23}$x–5.5 samples; whereas, in these same samples at $-1°$ C (frozen) the times required are, respectively, 10.5 hr (three times longer), 4.1 hr (slightly longer), and 2.0 hr (slightly less). This again shows that a high initial pH and a low initial concentration (sample $^1/_{23}$x–5.5) favors an increase in reaction rate during freezing.

Effect of Initial Concentration on the Rate of Oxidation of L-Ascorbic Acid at Temperatures above 0° C. The solubility of oxygen in an aqueous solution decreases with increasing solute concentration. If the solubility of oxygen in these test solutions is similar to the solubility of oxygen in sodium chloride solutions of corresponding concentrations, then the 1x and $^1/_{23}$x samples at 0° C would contain essentially the same amount of oxygen as pure water at 0° C, whereas the 50x samples would contain 0.55 as much oxygen as the other samples (Linke, 1965). The differences in oxygen content probably had a substantial influence on the results observed at above-zero temperatures.

The rate at which oxygen was absorbed in the agitated samples could have been influenced somewhat by viscosity differences among the samples. The differences, however, would be very small, since the viscosity of a 50x (1 *M*) sodium acetate solution (1.42 centipoise at 15° C) is only 1.3 times tha tof a $^1/_{23}$x (0.00087 *M*) solution (Landolt-Bornstein, 1969).

Factors Influencing the Rate of Oxidation of L-Ascorbic Acid at Subfreezing Temperatures. When samples are brought to solid-liquid equilibrium at a given subfreezing temperature, a greater amount of ice is formed and a greater increase in solute concentration occurs in initially-dilute samples than in initially-concentrated samples of the same kind and ratio of solutes. This situation arises because ice crystals exhibit a strong tendency to form in a pure state, and once solid-liquid equilibrium is achieved at a given subfreezing temperature, the unfrozen phase of any aqueous sample will have, by definition, a freezing point equal to the equilibration temperature, and provided the initial samples differed only in water content, their unfrozen phases will have essentially identical compositions (% basis) but not necessarily identical volumes (Mazur, 1966). This situation might lead one to conclude that samples differing only in initial water content (disregarding oxygen) should exhibit identical reaction rate constants for oxidation of AA when they are equilibrated at any given subfreezing temperature. This conclusion would appear valid except that samples with different initial water contents almost certainly would have different initial oxygen contents, *i.e.*, the initial oxygen content of a 50x sample, as mentioned previously, surely would be much less than that of more dilute samples. In addition, it should be noted that oxygen would be almost totally rejected from the ice crystals (Scholander *et al.*, 1953).

Consider now the amount of oxygen that must be dissolved in, suspended in, or sparged through the unfrozen phase of a sample during arrival at a given subfreezing temperature. Initially-dilute samples with large initial oxygen contents would form large quantities of oxygen-free ice during freezing, and would transmit a large quantity of oxygen to or through a small volume of unfrozen phase, thereby providing conditions for rapid oxidation of AA. On the other hand, the initially-concentrated 50x sample, with a small initial oxygen content, would form a small quantity of ice during freezing and would transmit a small quantity of oxygen to or through a relatively large volume of unfrozen phase, thereby providing conditions favoring a less than maximum rate of AA oxidation. This hypothesis is depicted schematically in Figure 2, and companion semi-quantitative calculations for the $^1/_{23}$x and 50x samples are presented in Table II. Since the initial difference in calculated oxygen content of the two samples (oxygen concentration in the $^1/_{23}$x sample is *ca.* twice that in the 50x sample) is increased greatly by freezing (at $-6°$ C, the oxygen concentration in the unfrozen phase of the $^1/_{23}$x sample is *ca.* 2300 times that in the unfrozen phase of the 50x sample), this occurrence would appear to be instrumental in the results obtained in Figure 1.

The calculations in Table II are regarded as semi-quantitative, since there is no proof that the assumptions of a pure ice phase and solid-liquid equilibrium were satisfied perfectly. It was determined, however, that freezing rate was not extremely critical, since the same results were obtained when samples were either quiescently immersed or shell frozen in dry ice-acetone. In any event, minor violations of these assumptions, which is all that would be expected with the

Table II. Calculated Changes in Oxygen Concentration of Initially Dilute and Initially Concentrated Solutions During Freezing

	Initially Dilute Sample ($^1/_{23}$x)				Initially Concentrated Sample (50x)			
	Volume (ml)		Concentration in unfrozen phase (amount/liter)		Volume (ml)		Concentration in unfrozen phase (amount/liter)	
Temperature	unfrozen phase	frozen phase	AA-buffer	oxygen	unfrozen phase	frozen phase	AA-buffer	oxygen
Unfrozen 0° C	1000	0	$^1/_{23}$x[a]	Y[b]	1000	0	50x[a]	0.55Y[b]
Partially frozen −3° C	0.87	999.13[c]	50x	1150Y[d]	1000	0	50x[e]	0.55Y
Partially frozen −6° C	0.44	999.56[f]	100x[g]	2300Y[h]	500	500[f]	100x	1.10Y[h]

[a] This value is the starting concentration used experimentally. $^1/_{23}$x consisted of 0.017 g AA made up to 1 l. with 0.00087 *M* acetate buffer; 50x consisted of 20 g of AA made up to 1 l. with 1.0 *M* acetate buffer. [b] Oxygen concentration is based on aqueous solutions of sodium chloride (Linke, 1965). Y = *ca.* 0.05 ml of oxygen (S.T.P.) per ml of solution at 0° C. [c] Amount of ice which must be formed in order to concentrate the fluid phase from $^1/_{23}$x to 50x in terms of AA and buffer (concentration factor of 1150). A pure ice phase is assumed. [d] As a result of freezing to −3° C, Y amount of oxygen, which was initially dissolved in 1000 ml of unfrozen solution, is concentrated (dissolved, suspended, or sparged through) 1150-fold in the 0.87 ml of unfrozen phase. On a liter basis of unfrozen phase, this results in an oxygen concentration of 1150Y. [e] The calculated freezing point of a 50x AA-buffer solution is approximately −3° C. [f] Computed in a fashion similar to that explained in footnote (c). [g] Calculated freezing point of a 100x AA-buffer solution is approximately −6° C. [h] Explanation is smiliar to that given in footnote (d).

types of solutions used here (Tiller, 1967), would not damage the essence of the hypothesis depicted in Figure 2 and Table II since the magnitude of the calculated differences in oxygen concentration is large.

Initial pH is another factor which obviously influences the reaction rate constant in the subfreezing range. This effect is apparent from the fact that the $^1/_{23}$x–5.5 samples exhibited substantially greater reaction rate constants than the $^1/_{23}$x–4.6 samples in the subfreezing range. This is expected, since it is well known that the rate of ascorbic acid oxidation declines greatly as the pH is reduced below 4.6.

Changes in pH during freezing also should be considered (van den Berg, 1961, 1964; van den Berg and Rose, 1959). For example, van den Berg and Rose (1959) studied well-buffered phosphate solutions and found that the pH of many samples declined as much as one unit during passage from 0 to −10° C. There is, however, no evidence suggesting that the solutions studied here underwent declines in pH during freezing. In fact, the pH values of the acetate buffers used in this study were measured over the concentration range of 0.00087 *M* ($^1/_{23}$x) to 4.0 *M* (200x), and the variation at room temperature was only ±0.1 units from the mean. This indicates that freeze concentration is not likely to produce a significant change in pH over the temperature range used in this study provided all of the buffer constituents remain in solution. Formation of a sodium acetate-ice eutectic could promote a decrease in pH during freezing, but this eutectic can form only at temperatures below −18° C, and even then it is unlikely to do so because aqueous solutions at this temperature exhibit a strong tendency to supersaturate, especially when frozen rapidly, as was done in this study (van den Berg and Rose, 1959; Green, 1908). It therefore must be assumed that the initial pH values remained essentially unchanged during freezing.

The influence of a change in buffer type was not determined in this study. However, in a somewhat similar study of AA-buffer solutions where the concentration effect was achieved by clathrate formation rather than by freezing, a change of buffer type from acetate to disodium phosphate-citrate to potassium phosphate-citrate had no significant effect on the results (Thompson and Fennema, 1971).

If the above conclusions are accepted, it is possible to account at least qualitatively for the different effects of freezing on the rate of AA oxidation in foods, as compared to the simple systems studied by Grant and Alburn (1965b). During the freezing of dilute, simple solutions, Grant and Alburn (1965b) observed greater rates of AA oxidation in the subfreezing range than at 1° C, and their data yields acceleration values in excess of 1. This result apparently can be attributed to the fact that their unfrozen samples were a relatively favorable medium for dissolved oxygen (dilute) and possessed relatively high initial pH values. On the other hand, the normal pattern during freezing of foods is for the rate of oxidation of AA to decline well below values at 0° C. This result appears normal, considering that foods are a relatively poor medium for dissolved oxygen (fairly high solute concentration) and many have initial pH values of less than 5.5.

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