Rate Constant and Activation Energy for Formation of a Nitrosoascorbic Acid Intermediate Compound

KENJIRO IZUMI, ROBERT G. CASSENS* and MARION L. GREASER

Department of Meat and Animal Science, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

The rate of decomposition of nitrite as a result of its reaction with ascorbic acid was determined from spectrophotometric measurements at various pH values and temperatures. The reaction proceeded initially as second order. The product was nitrosoascorbic acid, and it was formed with an increasing rate constant with decreasing pH. The rate was proportional to the concentration of nitrite from pH 4.2 to 5.38. As the reaction proceeded further at lower pH and higher temperature, it deviated from the second order reaction plot, giving kinetic evidence that the formation of 2,3-dinitrosoascorbic acid occurred more easily at low pH and high temperature. The activation energy for formation of nitrosoascorbic acid decreased with increasing pH from 10.90 Kcal/M at pH 4.35 to 6.46 Kcal/M at pH 5.49. Apparently there are two different reactions in the activation energy; i.e., the reaction of nitrous acid with the undissociated form of ascorbic acid (high activation energy) and the reaction of nitrous acid with once dissociated ascorbic acid (low activation energy). The significance of the reaction of nitrite with ascorbic acid at the pH values encountered in meats cured with nitrite and ascorbate is emphasized in terms of low activation energy and heating which increase the rate constant.

It has long been considered that ascorbic acid reacts with nitrite to form an intermediate compound (*1,2*). We (*5*) have confirmed the existence of an intermediate compound and the potential of ascorbic acid to react with two moles of nitrite to form nitrosoascorbic acid and 2,3-dinitrosoascorbic acid as proposed by Fox et al. (*3*). Since the intermediate compound quickly transferred NO of cytochrome c in an oxidized form at pH values encountered in meat (*5*), formation of the intermediate compound is considered a key step of the nitrosation reaction occurring in cured meat because nitrite essentially does not react with cytochrome c at pH above 5.0 even upon heating (*4,6,7*). The rate of formation of the intermediate compound was apparently dependent upon pH, increasing with decreasing pH, but the extent of the reaction seemed to be larger at higher pH (*5*).

In the present work, the rate constant for decomposition of nitrite by reaction with ascorbic acid was calculated at various pH values and temperatures. The activation energy of nitrite decomposition was estimated from the Arrhenius equation.

MATERIALS AND METHODS

Analytical-grade reagents were used and dissolved in distilled water previously bubbled with nitrogen gas for at least 2 h to prevent oxidation of ascorbic acid by oxygen. To minimize decomposition of nitrite, especially at low pH, sodium nitrite dissolved in distilled water was added to citrate buffer just before initiation of the reaction. The pH of the ascorbic acid solution was adjusted to that of the citrate buffer and any solutions containing ascorbic acid were kept in the dark to prevent photo-oxidation. Solutions of nitrite and ascorbic acid were made up fresh immediately before experimentation.

In the experiment for calculating the initial velocity of nitrite decomposition, the reaction was started by mixing the nitrite solution with the citrate buffer containing ascorbic acid, and the concentration of free nitrite was followed by measurement of absorption of 372 nm using a Gilford 240 spectrophotometer at 22°C (*5*). The reaction medium contained 0.2 M citrate, 50 mM sodium ascorbate, sodium nitrite varying in concentration from 2 to 50 mM, and the pH was 4.20 ± 0.05, 4.78 ± 0.025 or 5.38 ± 0.005. A second order rate constant at time t is expressed as general equation, $kt = \frac{1}{a-b} \ln \frac{(a-x)}{x} - \frac{1}{a-b} \ln \frac{a}{b} (a \neq b)$ where k is a second order rate constant, a and b are the initial concentrations of reactants and x is reaction products at a time t. To make evaluation simple, the equal concentrations of reactants were employed and the equation is $kt = \frac{x}{a(a-x)}$ ($a = b$). The initial velocity of decrease in nitrite was taken from the reaction for the initial 30 s at pH values 4.20 and 4.87. At pH 5.38, where the reaction was small, the initial 5 min was taken to calculate the initial velocity. Data from 6, 4 and 2 mM nitrite at pH 4.87 and 10, 8, 6, 4 and 2 mM nitrite at pH 5.38 were omitted for logarithmic plot of the equation, $v = 50KC^n$, where v is initial velocity, 50 is the concentration of ascorbate (mM), k is a second order rate constant, C is the concentration of nitrite varied and n is the reaction order for nitrite. The pH employed was appropriate for the measurement without rapid kinetic methods. Experiments were repeated twice.

Arrhenius plots of the decomposition of nitrite were obtained in the reaction medium containing 50 mM sodium nitrite, 50

mM sodium ascorbate and 0.2 M citrate at various pH values (4.35, 4.47, 4.61, 4.85, 5.05, 5.31 and 5.49) and temperatures (22, 30 and 39°C). The time-course (0, 1, 2, 3 and 4 min) of nitrite decomposition was analyzed as a second order reaction. In the reaction medium at pH 4.35, a different time-course (0, 0.5, 1.0, 1.5 and 2 min) was used. The correlation coefficient (r^2) from the analysis as the second order reaction was between 0.997 and 1.0. For comparison, Arrhenius plots of the decomposition of nitrite (50 mM) were obtained in 0.2 M citrate only at pH values of 3.15, 3.43 and 3.68 and temperatures of 25 and 41°C, since nitrite decomposition was too slow to make accurate evaluation at high pH values and low temperatures. The rate constant for nitrite decomposition was calculated from a first order reaction plot for 20 h (0, 5, 10, 15 and 20 h), because it was found from preliminary experiments that the decomposition of nitrite in 0.1 M citrate solution was a first order reaction ($r^2 > 0.96$). Arrhenius equation ($k = Ae^{-Ea/RT}$) was plotted against temperature on the logarithmic scale : $\log k = Ea/2.3R \cdot \frac{1}{T} + \log A$ where k is a rate constant, T is absolute temperature, Ea is activation energy, R is the gas constant and A is a constant (factor A). Experiments were repeated at least three times.

RESULTS

The time-course of the decrease in the concentration of free nitrite in a reaction mixture of nitrite (50 mM) and ascorbic acid (50 mM) is shown in Fig. 1. The pH-dependence of the reaction was strong and it proceeded more rapidly at lower pH. The decrease in nitrite concentration for the initial 5 min was analyzed as a second order reaction between nitrite and ascorbic acid (Fig. 2). The plots at pH 4.87 and 5.38 fitted a second order reaction ($r^2 = 1.0$), and the second order rate constants were 5.50×10^{-5} and 6.71×10^{-6} mM^{-1}•sec^{-1} at pH 4.87 and 5.38, respectively. On the other hand, the reaction plot did not obey the second order reaction at pH 4.20, indicating that the reaction product is not a compound formed from only 1 mole of nitrite and 1 mole of ascorbic acid. When the ratio of the concentration of ascorbic acid to nitrite was changed to the condition of low concentration of ascorbic acid and high concentration of nitrite, the reaction time course expressed as the second order reaction became short, resulting in early deviation from the second order kinetics.

To examine the reaction order of nitrite (n) at a fixed concentration of ascorbic acid (50 mM), the initial velocity of the decrease in nitrite was plotted against the original concentration of nitrite (C) on a logarithmic scale ($v = k50C^n$ ➤ $\log v = n \log C + \log 50k$). When the initial velocity of the decrease in nitrite was plotted against the original concentration of nitrite on a logarithmic scale (Fig. 3), the plots revealed that the reaction was first order for nitrite (n = 1). The second order rate constants (k) were 3.03×10^{-4}, 4.47×10^{-5}, 3.17×10^{-6} mM^{-1}•sec^{-1} at pH 4.20, 4.87 and 5.38, respectively. There was good correspondence with the rate constants calculated from the application plot of the second order reaction at pH 4.87 and 5.38. A small decrease in the rate constant cal-
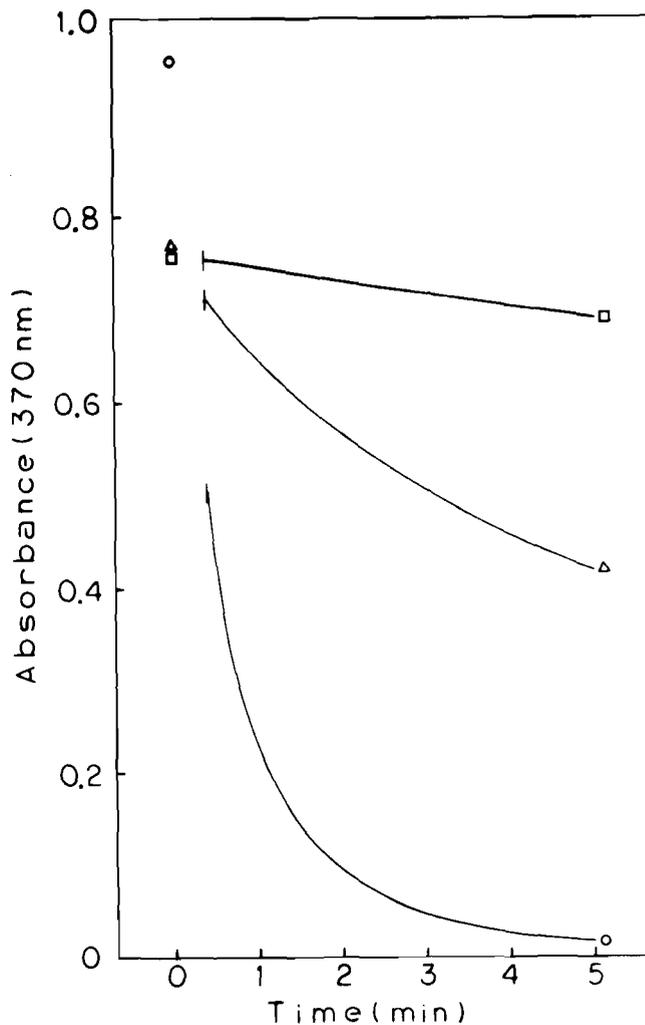

Figure 1. *Time course of decrease in concentration of free nitrite. The reaction medium contained 50 mM sodium nitrite, 50 mM sodium ascorbate and 0.2 M citrate at 22°C. The symbols at the left indicate the original absorbance of 50 mM nitrite solution, and the short vertical lines indicate when the recording on the chart started. pH was 4.20 (○), 4.87 (△) and 5.38 (□).*

culated from the initial velocity is expected by way of calculation. If corrected for nitrous acid (pK 3.36), they were 2.40×10^{-3}, 1.49×10^{-3} mM^{-1}•sec^{-1} and 3.37×10^{-4} mM^{-1}•sec^{-1} at pH 4.20, 4.87 and 5.38, respectively. There remained a strong pH-dependence and the rate constants increased with decreasing pH.

The decomposition of nitrite in 0.1 or 0.2 M citrate without ascorbic acid was expressed as a first order reaction, and Arrhenius variables are given in Table 1. The activation energy estimated was about 10.2 Kcal/M and seemed to be independent of pH. In addition to the values in Table 1, the first order rate constants of nitrite decomposition in citrate solution at various pH values were as follows: 1.16×10^{-5} sec^{-1} at pH 2.95, 0.38×10^{-5} sec^{-1} at pH 3.75 and 0.12×10^{-5} sec^{-1} at pH 4.30 in 0.1 M citrate at 23°C, and 0.23×10^{-5} sec^{-1} at pH 4.04 and 0.89×10^{-6} sec^{-1} at pH 4.48 in 0.2 M citrate at 25°C.
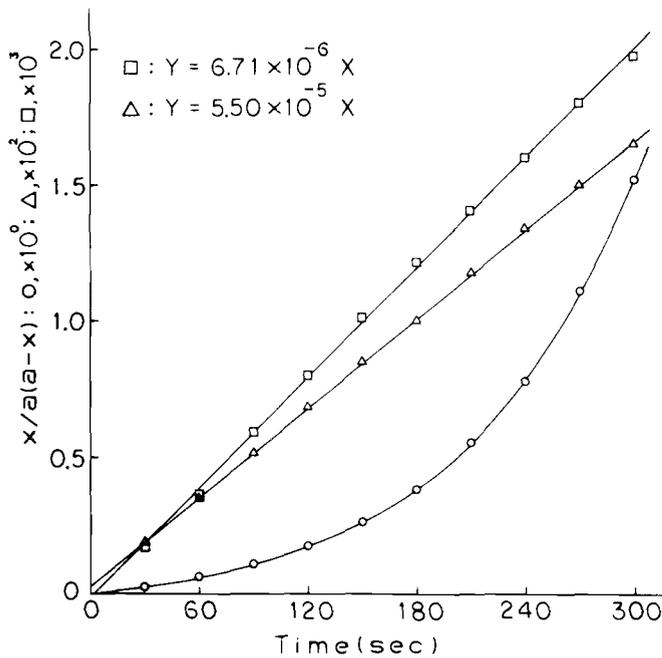
Figure 2. *Analysis of time course of decrease in nitrite as a second order reaction. Data were obtained from Fig. 1. Symbols indicate pH and are 4.00 (○), 4.87 (△) and 5.38 (□).*
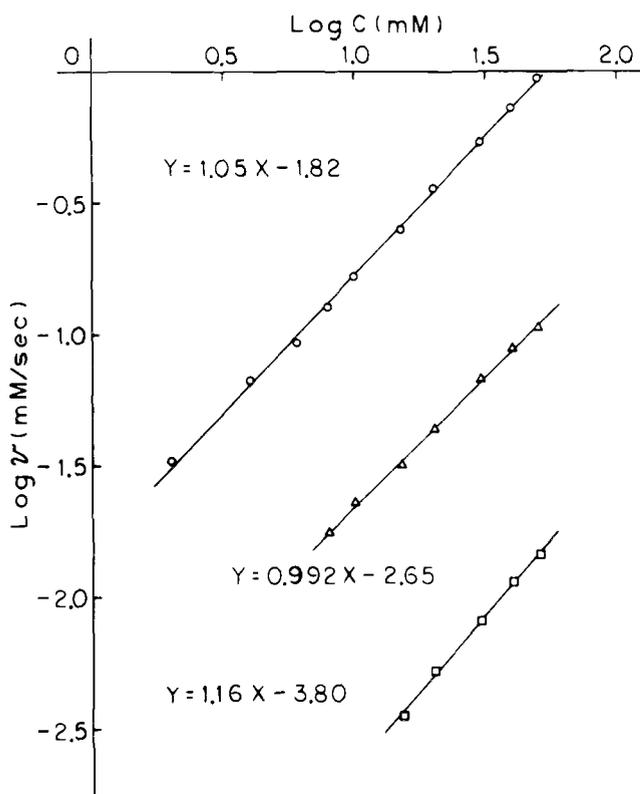

Figure 3. *Logarithmic plots of initial velocity of decrease in nitrite against original concentration of nitrite. The reaction medium contained 50 mM sodium ascorbate, various concentrations of sodium nitrite and 0.2 M citrate at 22°C (○, pH 4.20; △, pH 4.87; □, pH 5.38).*

There was no difference in the values of the rate constant between ionic strengths of 0.1 M and 0.2 M citrate.

In the presence of ascorbic acid, the rate constant and Arrhenius plots of nitrite decomposition are shown in Table 1 and Fig. 4. It was found that the rate constant of nitrite decomposition by reaction with ascorbic acid $(4.372 \times 10^{-4} \text{ mM}^{-1} \text{ sec}^{-1}$ at pH 4.47 and 39°C) was more than 100 times greater than that of nitrite decomposition without ascorbic acid $(1.800 \times 10^{-6} \text{ sec}^{-1}$ at pH 4.48 and 41°C) at an almost equal pH and temperature. The activation energy of nitrite decomposition in the presence of ascorbic acid decreased with increasing pH from 10.90 to 6.46 Kcal/M at pH 4.35 to 5.49. Since the activation energy increased with decreasing pH, making the rate constant short, the rate constant of the reaction of nitrite and ascorbic acid became greater at lower pH in terms of factor A, but smaller with respect to the activation energy.

DISCUSSION

It has been established that the stoichiometry of the reaction of nitrite with ascorbic acid is such that ascorbic acid reacted with more than one mole of nitrite (5). For example, when 2.5 mM ascorbic acid reacted with 4 mM nitrite (in a reaction mixture of 2.5 mM sodium ascorbate, 5 mM sodium nitrite and 0.1 M citrate, pH 3.45) the reaction reached a plateau by 10 min. The reaction of nitrite with ascorbic acid could occur in two steps;

$$A + NOX \xrightarrow{k_1} A\text{-NO} + NOX \xrightarrow{k_2} A(NO)_2,$$

where A is ascorbic acid, NOX is nitrous acid, A-NO is nitrosoascorbic acid, $A(NO)_2$ is 2,3-dinitrosoascorbic acid, and k_1 and k_2 are rate constants. Both steps did not seem to be in equilibrium to an appreciable extent, because the intermediate compound decomposed monomerically, resulting in regeneration of nitrite, but ascorbic acid was oxidized (5). Moreover, the intermediate compound was semistable so that regeneration of nitrite due to decomposition of the intermediate compound was small compared to formation of the intermediate compound. The decomposition of nitrite in citrate without ascorbic acid was essentially negligible (about one-one hundredth the rate of that with ascorbic acid). Therefore, the rate of decrease of nitrite measured in the presence of ascorbic acid is not significantly affected by regeneration of nitrite from the intermediate compound and the decomposition of nitrite alone.

Since neither the decrease in concentration of ascorbic acid nor formation of the reaction product was measured, the decrease in concentration of nitrite measured is considered to be possibly responsible for both the steps of k_1[A] [NOX] and $k_1 k_2$[A] $[NOX]^2$, if k_2 is much larger than k_1. However, the fact that the reaction condition established high concentration of ascorbic acid (50 mM) and low concentration of nitrous acid (6.32 mM at pH 4.20, 1.50 mM at pH 4.87 and 0.47 mM at pH 5.38 in the presence of 50 mM nitrite) favors the step of k_1 [A] [NOX] as being responsible for nitrite decrease. In addition, the data in Fig. 3 (where the initial concentra-

TABLE 1. *The values of the variables of Arrhenius equation of nitrite decomposition at various pH values. The reaction medium contained 0.2 M citrate, 50 mM sodium nitrite in the presence or absence of 50 mM sodium ascorbate.*

pH	Temp[a]	k[b]	Ea[c]	A[d]
In the absence of ascorbic acid				
3.15	25	1.043×10^{-5}	10.24	3.4×10^2
	41	2.516		
3.43	25	0.653×10^{-5}	10.29	2.3×10^2
	41	1.583		
3.68	25	0.416×10^{-5}	10.18	1.3×10^2
	41	1.000		
In the presence of ascorbic acid				
	22	3.133		
4.35	20	5.171×10^{-4}	10.90	3.8×10^4
	39	8.643		
	22	1.674		
4.47	30	2.691×10^{-4}	10.27	7.0×10^3
	39	4.372		
	22	0.802		
4.61	30	1.210×10^{-4}	8.97	3.9×10^2
	39	1.850		
	22	3.718		
4.85	30	5.262×10^{-5}	7.34	1.0×10^1
	39	7.361		
	22	1.701		
5.05	30	2.399×10^{-5}	7.48	6.0×10^0
	39	3.414		
	22	0.626		
5.31	30	0.858×10^{-5}	7.12	1.2×10^0
	39	1.217		
	22	3.341		
5.49	30	4.416×10^{-6}	6.46	2.1×10^{-1}
	39	6.300		

[a]Temperature, °C.

[b]First order rate constant (sec^{-1}) in the absence of ascorbic acid and second order rate constant $(mM^{-1} \cdot sec^{-1})$ in the presence of ascorbic acid.

[c]Activation energy (Kcal/M).

[d]Arrhenius factor.

tion of nitrite was decreased for measuring initial velocity of nitrite decrease) strongly support the idea that the rate constant measured was k_1 and the product was nitrosoascorbic acid. k_2 remained unknown. It is, however, roughly estimated that k_2 is similar to k_1 at pH 4.20, as follows; when k_1 at pH 4.20 was taken as 3.03×10^{-4} $mM^{-1} \cdot sec^{-1}$ (Fig. 3), formation of A-NO for the initial 30 s is calculated as 22.725 mM. The decomposition of nitrite for the initial 30 s was 28.171 mM, so that formation of A(NO)$_2$ is 2.723 mM and the concentration of free ascorbic acid is 24.552 mM at 30 s after reaction. These values were employed for the next 30 s to calculate k_2 using the equation $v = 30 k_1 [A] [NOX] + 30 k_2 [A-NO] [NOX]$, where v is the decomposition of nitrite for 30 s, k_1 is 3.03×10^{-4} $mM^{-1} \cdot sec^{-1}$, [A] is the con-

centration of free ascorbic acid, [NOX] is the concentration of free nitrite, k_2 is the rate constant and [A-NO] is the concentration of nitrosoascorbic acid. This process was applied for each 30 s from 30 to 180 s. k_2 obtained was 3.365×10^{-4} (30 to 60 s), 2.621×10^{-4} (60 to 90 s), 2.635×10^{-4} (90-120 s), 2.348×10^{-4} (120 to 150 s) and 2.278×10^{-4} (150 to 180 s) $mM^{-1} \cdot sec^{-1}$. After 180 s, the concentration of free nitrite was too small (less than 2.5 mM) to estimate k_2. The rate constant k_2 estimated in an appropriate value explains that, as the reaction proceeded at pH 4.20, the participation of k_2 in the decrease in nitrite concentration significantly occurred, resulting in the deviation of the reaction from the second order reaction plot. Such was true at 30 and 39°C at pH 4.35 in the late phase of the reaction after 3 min.
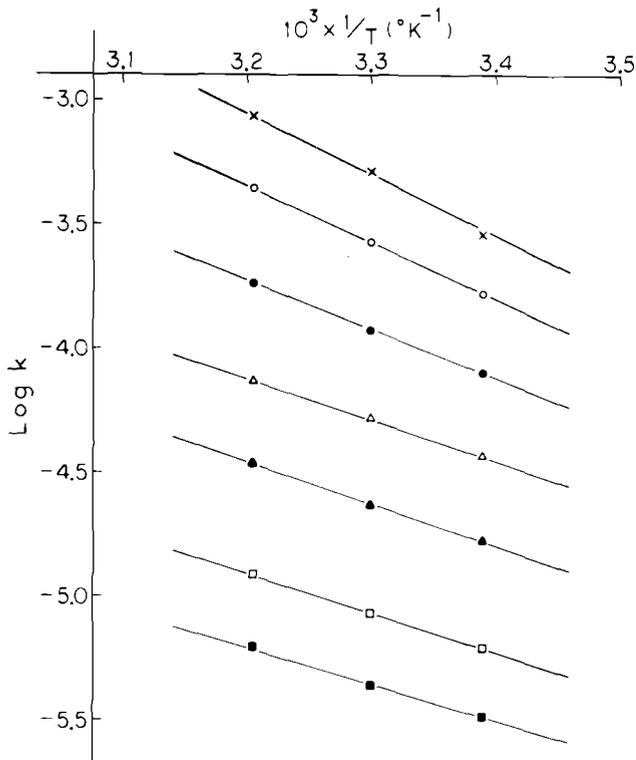
Figure 4. *Arrhenius plots of decomposition of nitrite in the presence of ascorbic acid. The reaction medium contained 50 mM sodium nitrite, 50 mM sodium ascorbate and 0.2 M citrate at temperature 22, 30 and 39°C (X, pH 4.35; ◯, pH 4.47; ●, pH 4.61; △, ph 4.85; ▲, pH 5.05; □, pH 5.31; ■, pH 5.49).*

pH not only affects the existing forms of nitrite, but also those of ascorbic acid (pk_1 4.17 and pk_2 11.57). There is no information about how much dinitrogen trioxide is formed with a change in pH of the system used. As shown in Fig. 3, the rate of the reaction of nitrite with ascorbic acid was proportional to the concentration of nitrite, but not the square of the concentration of nitrite as expected from the reaction scheme proposed by Fox and Thomson (2). Therefore, there is no particular reason to assume that the reactive species of nitrite is dinitrogen trioxide. Rather it may be considered to be nitrous acid in reaction with ascorbic acid even at pH 4.2 to 5.38.

As for ascorbic acid, the undissociated and singly dissociated forms are 24.15 and 25.85 mM at pH 4.20, 8.35 and 41.65 mM at pH 4.87, and 2.90 and 47.10 mM at pH 5.38, respectively. The doubly dissociated form does not exist at these pH values. Ascorbic acid decomposed more nitrite at high pH that low pH regardless of the low rate, if the reaction reached a plateau in early phase within 10 min (5). This probably indicates that the reaction of nitrous acid with once dissociated ascorbic acid (AH⁻) occurs more easily than that of nitrous acid with undissociated ascorbic acid (AH₂); namely, the former reaction has lower activation energy than that of the latter. In this context, the decrease in the activation energy with increasing pH can be explained. However, the rate constant is also a function of factor A, which greatly increased with decreasing pH (Table 1), so that the pH-de-

pendence of the rate constant was brought about by the factor A which concealed the effect of the increasing activation energy on the decrease in the rate constant. Among the conditions affecting factor A, nitrous acid content is of primary importance. The fact that the rate constant corrected for nitrous acid was also pH-dependent may indicate that the formation of H_2^+ONO plays a significant role in the reaction of nitrite with ascorbic acid at pH above 4.0.

With respect to the reaction of nitrite with ascorbic acid in cured meat at pH 5.5 to 6.5, the contribution of this reaction to decomposition of nitrite during the curing process is expected to be quite small. In fact, the level of nitrite added to bacon did not decrease so much (about 12%) for 24 h until application of heat (8). The rate of the reaction of nitrite with ascorbic acid was dependent upon temperature. A rate constant of about 10^{-5} mM^{-1}•sec^{-1} for nitrite decomposition is expected from pH dependence of the rate constants at pH values encountered in meat cured with nitrite and ascorbate during heat processing at 80°C. It is clear that the reaction of nitrite with ascorbic acid leads to a powerful nitrosating intermediate compound which plays a significant role during heat processing of meat (especially bacon with high pH) as indicated by the increase in the rate and extent of the reaction of nitrite with cytochrome c in the presence of ascorbic acid upon heating (6).

In conclusion, decomposition of nitrite by reaction with ascorbic acid proceeded as a second order reaction in the initial phase. The reactants were ascorbic acid and nitrous acid, and it was thought that the product was nitrosoascorbic acid. The rate constant increased with decreasing pH. The activation energy of nitrite decomposition (formation of nitrosoascorbic acid) decreased with increasing pH from 10.9 Kcal/M at pH 4.35 to 6.46 Kcal/M at pH 5.49. When the reaction proceeded at lower pH values (4.20 and 4.35) and higher temperatures (30 and 39°C) it deviated from the second order reaction plot, indicating formation of 2,3-dinitrosoascorbic acid. A term of the Arrhenius equation increased with decreasing pH and was the primary factor determining the rate constants dependent upon pH. The probability of two different reactions between the undissociated form and the once dissociated form of ascorbic acid with nitrous acid in terms of the activation energy was present. A significant role of the reaction of nitrite with ascorbic acid on nitrite decomposition followed by formation of a powerful nitrosating intermediate compound was emphasized by meat cured with nitrite and ascorbate during heat processing. In contrast, decomposition of nitrite in citrate without ascorbic acid took place with an increasing first order rate constant with decreasing pH. The activation energy of nitrite decomposition was about 10.2 Kcal/M and seemed to be independent of pH value.

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