Metal Ion and Metal Chelate Catalyzed Oxidation of Ascorbic Acid by Molecular Oxygen. I. Cupric and Ferric Ion Catalyzed Oxidation[1,2]

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Abstract: The kinetics of the uncatalyzed and the Cu(II) and Fe(III) ion catalyzed oxidation of ascorbic acid are measured at 25 and 0.4°. The rate of the uncatalyzed oxidation of ascorbic acid was found to be proportional to oxygen concentration at 20% and higher concentration of molecular oxygen. In the pH range investigated (2–5.5), only the monoionic species of ascorbic acid was found to be reactive toward molecular oxygen. For the spontaneous oxidation, a mechanism is proposed whereby molecular oxygen takes part directly in the oxidation of the ascorbate anion. The rates of the ferric and cupric ion catalyzed oxidations were found to be first order with respect to the concentration of molecular oxygen. For the catalyzed oxidation, a reaction path involving molecular oxygen bound to the metal–ascorbate complex is proposed. The catalytic activity of cupric ion was found to be more than that of ferric ion toward the oxidation of the ascorbate anion. In the oxidation of the neutral species, however, ferric ion is a better catalyst than cupric ion. The difference in the catalytic activity of the two metal ions toward the monoionic and neutral species of ascorbic acid is explained on the basis of the difference in their relative tendencies to form complexes with the two species of ascorbic acid. The mechanistic implications of the catalyzed and uncatalyzed reactions are discussed.

Because of its biochemical significance most of the studies of the oxidation of ascorbic acid have been carried out in or near the physiological pH range. A complicating factor in much of the previous work on Cu(II) catalysis was the use of buffers, which interact to different extents with Cu(II) ions to form complexes. Because of the intricate and generally unknown equilibria occurring in these systems, most of the early workers reported results from which no fruitful reaction mechanism could be derived.

From the chemical point of view, the oxidation of ascorbic acid has been the subject matter of many investigations. A study of the Cu(II) ion catalyzed oxidation of ascorbic acid was carried out by Barron, De Meio, and Klemperer,[4] Weissberger, Luvalle, and Thomas,[5] Weissberger and Luvalle,[6] and more recently by Nord[7] and by Grinstead.[8]

No studies of the separate catalytic effects of metal ion and metal chelate compounds on the oxidation of ascorbic acid by molecular oxygen have been reported previously. The present investigation was undertaken to determine the kinetics and oxidation of ascorbic acid catalyzed by cupric and ferric ions. The study of oxidation reactions catalyzed by other metal ions, and by metal chelates, will be described in subsequent communications.

Experimental Section

Reagents. The l-ascorbic acid used in this investigation was Kodak White Label grade, and was used without further purification. Solutions of Cu(II) and Fe(III) nitrates were prepared from Fisher analytical grade materials. The Cu(II) solution was standardized both by an iodometric procedure with standard sodium thiosulfate and also by titration in ammoniacal solution with standard disodium salt of EDTA according to the procedure described by Schwarzenbach.[9] The Fe(III) solution was standardized by an oxidation–reduction titration with potassium permanganate and also by EDTA titration[9] with Tiron as an indicator. The results of the two methods agree within the experimental error.

Potentiometric Measurements. The dissociation constants of l-ascorbic acid were determined by potentiometric titration in a medium of 0.100 *M* ionic strength. A Beckman Model G pH meter fitted with extension glass and calomel electrodes was used. The pH meter was calibrated in terms of hydrogen ion concentration with acetic acid buffer as well as with standard HCl and KOH. The data given by Harned and Owen[10] were used to correct for the hydrogen ion concentration. The solutions were prepared in air-free distilled water, and an atmosphere of purified nitrogen was maintained in the titration cell to avoid any disturbing effects resulting from oxidation.

Kinetic Measurements. Duirng each run the pH value was maintained constant by a Beckman Model K automatic titrator fitted with extension glass and calomel electrodes. It was calibrated with acetic acid buffer and by titration of standard HCl and KOH solutions. The ionic strength of the experimental solution was maintained at approximately 0.1000 *M* with KNO_3. After adjusting the pH to the desired value, a stream of oxygen was passed through the cell in such a way as to ensure very intimate contact between gas phase and solution. The oxygen was 99% pure, and was passed through an ascarite tube to remove carbon dioxide. It was presaturated with water vapor by streaming through a wash bottle maintained at the same temperature and electrolyte concentration as the reacting solution. As the rate of reaction is low compared to the rate of dissolution of oxygen, the reacting solution was considered to be saturated with oxygen at all times. The rate of oxidation was measured by the amount of dehydroascorbic acid produced during the course of oxidation.

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(4) E. S. G. Barron, R. De Meio, and F. W. Klemperer, *J. Biol. Chem.*, 112, 625 (1936).

(5) A. Weissberger, J. E. Luvalle, and D. S. Thomas, Jr., *J. Am. Chem. Soc.*, 65, 1934 (1943).

(6) A. Weissberger and J. E. Luvalle, *ibid.*, 66, 700 (1944).

(7) H. Nord, *Acta Chem. Scand.*, 9, 442 (1955).

(8) R. Grinstead, *J. Am. Chem. Soc.*, 82, 3464 (1956).

(9) G. Schwarzenbach, "Complexometric Titrations," Interscience Publishers, Inc., New York, N. Y., 1958, pp 77 and 82.

(10) H. S. Harned and B. B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed, Reinhold Publishing Corp., New York, N. Y., 1958, pp 638 and 752.

Analysis of Dehydroascorbic Acid. The analytical procedure employed for the estimation of dehydroascorbic acid was that established by Roe and co-workers.[11] According to this method, a 1.00-ml sample of the experimental solution was pipetted into a 5-ml solution of 2,4-dinitrophenylhydrazine–thiourea ragent in a 25-ml volumetric flask. Because of a high concentration of thiourea and a very low pH (<1), the oxidation reaction stopped immediately. At the low pH of the solution, dehydro-1-ascorbic acid underwent spontaneous hydrolysis to diketo-1-gulonic acid, which reacted with 2,4-dinitrophenylhydrazine to give the bis(2,4-dinitrophenylhydrazone). The reaction was completed in 3 hr by placing the flask in a bath at 37°. The reddish brown 2,4-dinitrophenylhydrazone was dissolved slowly in 85% H_2SO_4, while the solutions were cooled by immersing the flasks in an ice bath. This gave a highly stable reddish brown solution which has absorption maxima at 500–550 mμ and 300–380 mμ. The intensity of the color was measured spectrophotometrically at 525 mμ, and the amount of dehydroascorbic acid was estimated with the aid of a calibration curve determined by Roe's method.[11] The results were highly reproducible (within 1%) and were unaffected by the presence of metal ions.

Results

Dissociation Constants. The pK values of ascorbic acid were calculated from the titration curves at 25 and 0.4°. The dissociation constants used in the calculations are given in Table I.

Table I. Dissociation Constants of Ascorbic Acid[a]

Temp, °C	K_1	K_2
25	9.16×10^{-5}	4.57×10^{-12}
0.4	3.24×10^{-5}	1.91×10^{-13}

[a] $\mu = 0.10\ M\ KNO_3$.

Spontaneous Oxidation. The spontaneous oxidation of ascorbic acid was carried out at 25 and 0.4° in the pH range 2–5.5. In the presence of a large excess of oxygen (saturation) the spontaneous oxidation was first order with respect to the concentration of the ascorbate anion at a particular pH. First-order rate constants were obtained from plots of log $[a/(a - x)]$ *vs.* time and multiplying the slopes by 2.303, where a is the initial concentration of ascorbic acid and x is the concentration of the product (dehydroascorbic acid) at time t. The rate under pseudo-first-order conditions may be expressed by the equation

$$-dT_A/dt = k_1A_1 + k_2A_2 + k_3A_3 \qquad (1)$$

where T_A is the total analytical concentration of A species, A_1, A_2, and A_3 are the concentrations of neutral, monoionic, and diionic forms of ascorbic acid, respectively, and k_1, k_2, and k_3 are the corresponding specific rate constants. In the pH range below 6, the undissociated and the monoionic forms of ascorbic acid are the main species present in solution; the concentration of the diionic species may be neglected. The specific rate constants k_1 and k_2 were calculated in the pH range 2–6. The total spontaneous rate of oxidation observed was found to vary linearly with the concentration of the monoionic species of ascorbic acid, the contribution of the neutral form being zero, within experimental error. The rate profile for the spontaneous oxidation at 25° is shown in Figure 1. A similar profile was obtained at 0.4°. The specific rate constant for the spontaneous oxidation at 25 and 0.4° differs from the calculated rate by less than 2% below pH 5. The specific rate constants, k_2, calculated from the pH dependence of the rate at 25 and 0.4° are 5.87×10^{-4} sec^{-1} and 1.06×10^{-4} sec^{-1}, respectively.

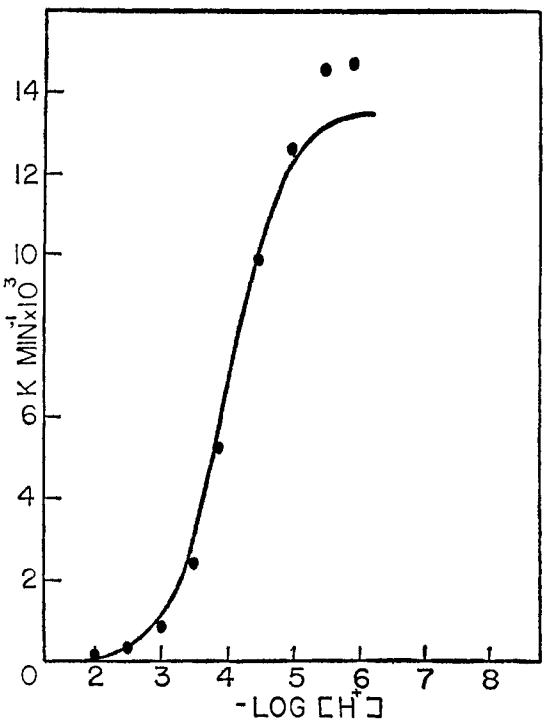


Figure 1. Rate profile for the spontaneous oxidation of ascorbic acid at 25°: ●, measured rate constants; the solid curve indicates the calculated rates.

Oxygen Dependence of Spontaneous Oxidation. Mixtures of oxygen and nitrogen varying in composition from 80% oxygen to 5% oxygen, as well as pure oxygen, were employed for the present studies. The mixtures were purchased from Matheson Co., and were carefully analyzed for oxygen content. The gas mixture was passed rapidly through the experimental solution with a fritted glass diffusing tube at a very rapid rate (700 cc/min). Since the rate of dissolution of oxygen is much faster than any possible reaction, the amount of oxygen present at a given time may be considered constant and dependent only on its partial pressure. The rate of oxidation was followed by measuring the amount of dehydroascorbic acid as described in the experimental part. It was observed that down to a partial pressure of 0.40 atm of oxygen, the rate of oxidation was directly proportional to the partial pressure, but below 0.40 atm linearity was not observed. At these low pressures a very long time was required for the reaction to proceed to any appreciable extent (2 days in the case of 5% O_2 mixture). The nonlinearity may be attributed to side reactions of the same type as suggested by Weissberger, *et al.*[5] The significance of the results below a partial pressure of 0.40 is not clear at present. However, in the range 0.40–1-atm O_2 pressure where linearity is observed, the rate law may be given by the expression

$$-dT_A/dt = k_1[HA^-][O_2] \qquad (2)$$

The pseudo-first-order rate constants for the various

(11) J. H. Roe, "Methods of Biochemical Analysis," Vol. I., Interscience Publishers, Inc., New York, N. Y., pp 115–139.

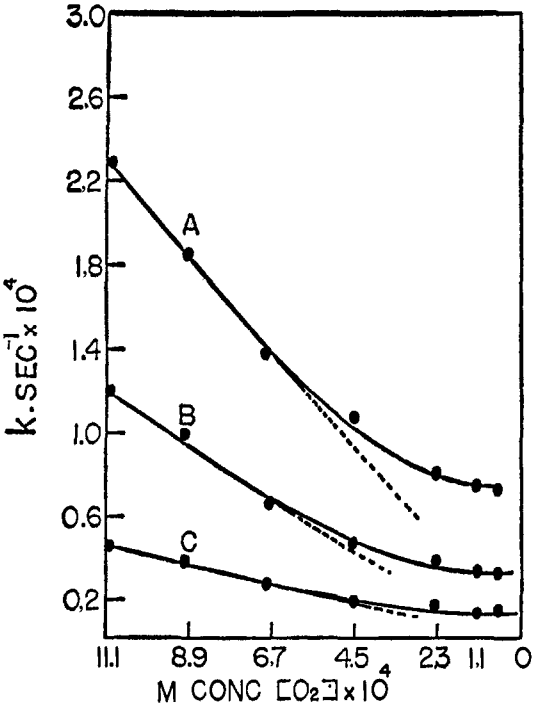


Figure 2. Dependence of the rate constant k for the spontaneous oxidation of ascorbic acid on oxygen concentration; temperature, 25°; μ = 0.100 M KNO_3, at − log [H^+] values of: A, 3.85; B, 3.45; and C, 3.00.

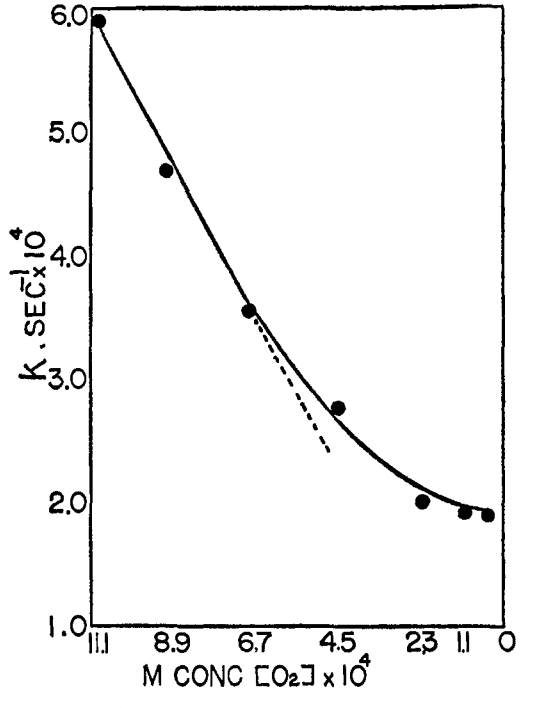


Figure 3. Dependence of the rate constant k for the oxidation of the monoionic species of ascorbic acid on oxygen concentration; temperature, 25°; μ = 0.10 M KNO_3.

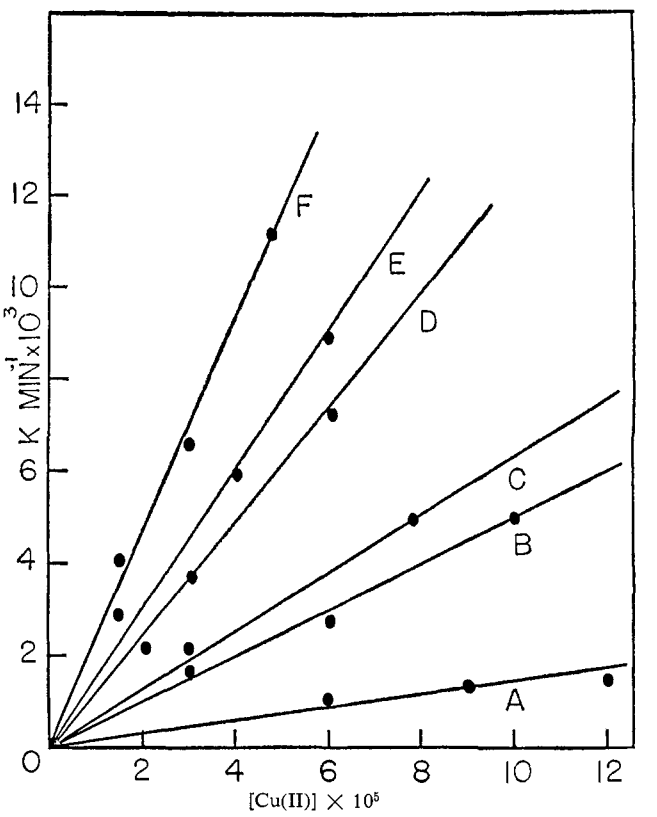


Figure 4. Catalytic effect for the oxidation of ascorbic acid in the presence of Cu(II) ion at 25°, at − log [H^+] values of: A, 1.50; B, 2.00; C, 2.25; D, 2.50; E, 2.85; and F, 3.45. μ = 0.10 M KNO_3; k = difference between the first-order rate constants in the presence and in the absence of the metal ion.

Figure 3. The second-order rate constant for the oxygen dependence is calculated from the slope of the straight line in Figure 3, and the result is indicated at the bottom of Table II.

Table II. Variation of Rate Constants (sec^{-1}) with Partial Pressure of Oxygen[a]

Partial pressure of O_2, atm	Pseudo-first-order rate constants, at −log [H^+] values indicated, × 10^4 [b]			Specific rate constant, k_2, of ascorbate anion × 10^4
	3.85	3.45	3.00	
1.00	2.31	1.21	0.49	5.87
0.81	1.84	1.01	0.39	4.68
0.62	1.39	0.64	0.28	3.53
0.40	1.08	0.48	0.20	2.75
0.19	0.79	0.40	0.18	2.01
0.10	0.76	0.38	0.16	1.93
0.05	0.75	0.36	0.16	1.91

[a] Temperature, 25°; −log [H^+] = 3.85, 3.45, and 3.00. [b] Second-order rate constant = 5.68 × 10^{-1} M^{-1} sec^{-1} for 0.40–1.00 partial pressure O_2.

pressures of oxygen are given in Table II. The results are presented graphically in Figure 2. Specific rate constants, k_2, were calculated for the monoionic species of ascorbic acid from the data in Table II and eq 1. The specific-first-order rate constants for the monionic species are presented in the last column of Table II, and plotted as a function of oxygen concentration in

Copper(II) Ion Catalyzed Oxidation. The experimental results at 25 and 0.4° indicate a first-order rate of oxidation with respect to the total concentration of unreacted ascorbic acid. In the pH range 1.5–3.5, the rate varied linearly with the concentration of Cu(II) ions. Figure 4 indicates the variation of rate at 25° with the concentration of Cu(II) ions at constant pH.

Table III. Specific Rate Constants (M^{-1} sec^{-1}) of Cu(II) Ion Catalyzed Oxidation of Ascorbic Acid[a]

− Log [H+]	25°	0.4°
1.50	9.7×10^1	
2.00	33.8×10^1	1.40×10^1
2.25	42.8×10^1	1.90×10^1
2.50	8.5×10^2	2.70×10^1
2.85	10.4×10^2	6.3×10^1
3.45	16.2×10^2	28.8×10^1
3.85	...	7.8×10^2

[a] $\mu = 0.10$ KNO_3; $P_{O_2} = 1$ atm.

Similar results were obtained at 0.4°. The specific rate constants given in Table III were calculated from the slopes of the straight lines in Figure 4, and from similar data obtained at 0.4°. The data for a particular straight line were obtained by measuring the rate over a wide range of catalyst concentration.

Fe(III) Ion Catalyzed Oxidation. As in the case of the Cu(II) ion catalyzed oxidation, the experimental results indicated a first-order rate of oxidation with respect to the total concentration of unreacted ascorbic acid. In the pH range 1.5–3.85, a linear variation of rate with the concentration of Fe(III) ions was observed, indicating the truly catalytic behavior of Fe(III) ions. Extensive hydrolysis of Fe(III) ions, and a very rapid rate of reaction, restricted the study of the catalytic oxidation to pH values below 3.85.

Ferric ion undergoes extensive hydrolysis even at low pH values. The concentration of ferric ion was corrected for hydrolysis with the aid of the hydrolysis constants given by Hedstrom.[12] The hydrolysis equilibria are represented by the following equations.

$$Fe^{3+} \rightleftharpoons Fe(OH^{2+}) + H^+$$

$$K_1^M = [Fe(OH)^{2+}][H^+]/[Fe^{3+}] \quad (3)$$

$$Fe(OH^{2+}) \rightleftharpoons Fe(OH)_2^+ + H^+$$

$$K_2^M = [Fe(OH)_2^+][H^+]/[Fe(OH)^{2+}] \quad (4)$$

If $[Fe_T^{3+}]$ represents the total concentration of metal species, then the concentration of the unhydrolyzed ferric ion, Fe^{3+}, may be calculated with the help of the equation

$$[Fe^{3+}] = [Fe_T^{3+}](1 + K_1^M/[H^+] + K_1^M K_2^M/[H^+]^2)^{-1} \quad (5)$$

The hydrolysis data at 0.4° were calculated with the help of the thermodynamic data given in the Stability Constant Tables.[13]

The variation of rate at 25° with the concentration of unhydrolyzed ferric ion at a particular pH is indicated in Figure 5. Similar results were obtained at 0.4°. The specific rate constants given in Table IV were calculated from the slopes of the straight lines in Figure 5, and from those obtained at 0.4°. As in the case of the Cu(II) ion catalyzed oxidation, the data for a particular straight line were obtained by measuring the rate over a wide range of catalyst concentration.

Specific Rate Constants of Metal Ion Catalyzed Oxidation. In both the cupric and ferric ion catalyzed oxidation reactions, the rate showed an inverse dependence on the hydrogen ion concentration. The rate law describing the oxidation may be expressed in the form

$$-dT_A/dt = k'T_A[M^{n+}] \quad (6)$$

$$-dT_A/dt = k_1'[H_2A][M^{n+}] + k_2'[HA^-][M^{n+}] \quad (7)$$

where k_1' and k_2' are the rates for the catalytic effects of the metal ion on the neutral and monoionic forms of ascorbic acid, respectively, on the assumption that the two species of ascorbic acid react independently with the metal ion. Equation 7 may be rewritten as

$$-dT_A/dt = k'_1[H_2A][M^{n+}] + k_2'[H_2A]K_1[M^{n+}]/[H^+] \quad (8)$$

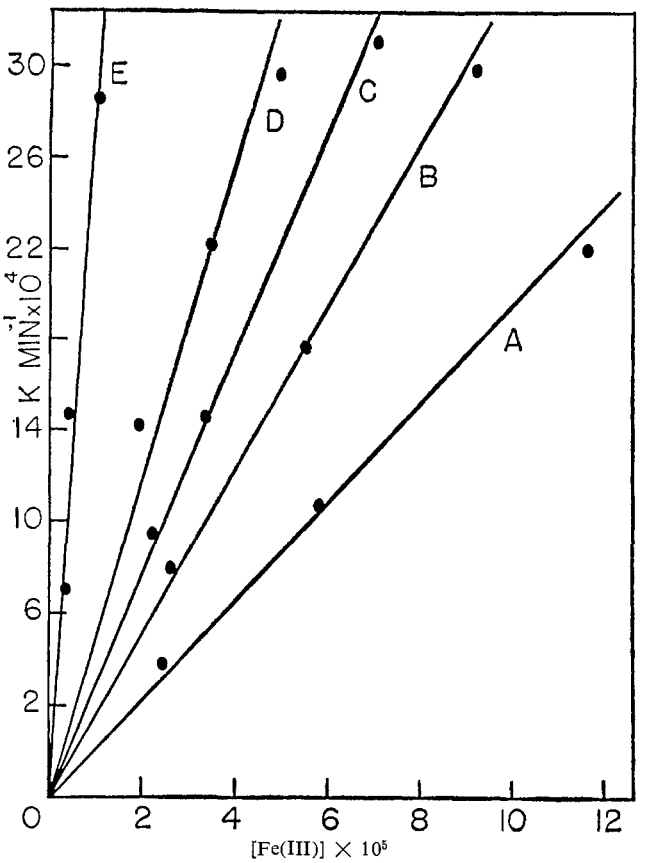


Figure 5. Catalytic effect for the oxidation of ascorbic acid in the presence of Fe(III) ion at 25°, at − log [H+] values of: A, 1.50; B, 2.00; C, 2.42; D, 2.94; and E, 3.44. $\mu = 0.10$ M KNO_3; k = difference between the first-order rate constants in the presence and in the absence of the metal ion.

Table IV. Specific Rate Constants (M^{-1} sec^{-1}) of Fe(III) Ion Catalyzed Oxidation of Ascorbic Acid[a]

− Log [H+]	25°	0.4°
1.50	12.4×10^1	...
2.00	22.1×10^1	9.2×10^1
2.42	29.7×10^1	...
2.50	30.5×10^1	13.5×10^1
2.94	43.9×10^1	14.9×10^1
3.00	...	15.8×10^1
3.44	19.3×10^2	17.7×10^1
3.50	20.9×10^2	18.2×10^1

[a] $\mu = 0.10$ KNO_3; $P_{O_2} = 1$ atm.

(12) B. O. A. Hedstrom, *Arkiv Kemi*, 6, 1 (1953).
(13) L. G. Sillen and A. E. Martell, "Stability Constants," Special Publication No. 17, The Chemical Society, London, 1964.

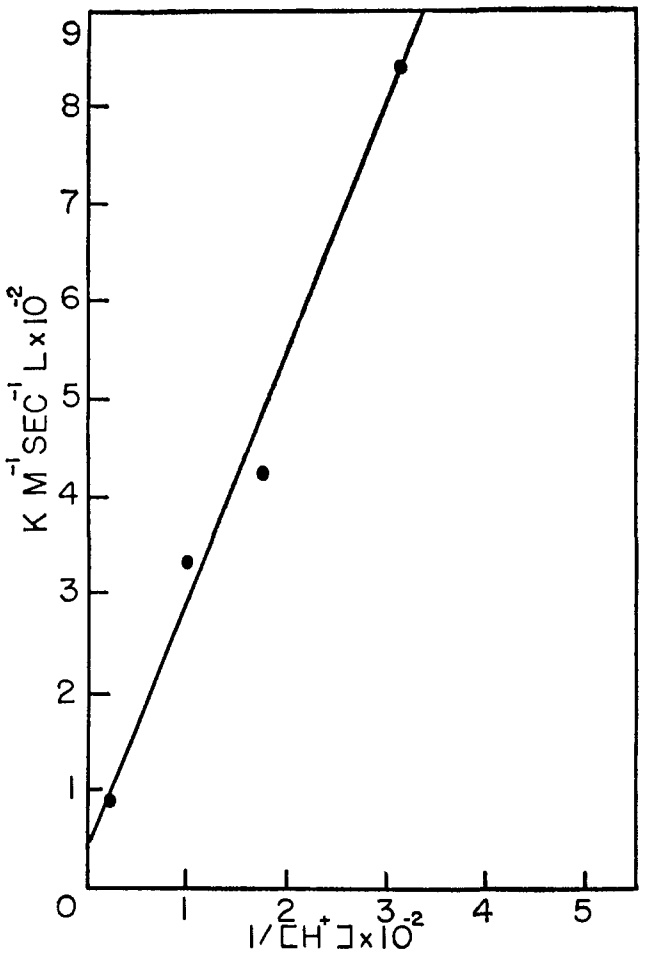


Figure 6. Dependence of the specific rate constants k on the hydrogen ion concentration for cupric ion catalyzed oxidation of ascorbic acid at 25°; μ = 0.10 M KNO_3.

where

$$[HA^-] = [H_2A]K_1/[H^+] \quad (9)$$

(K_1 stands for the first dissociation constant of ascorbic acid)

$$-dT_A/dt = [H_2A][M^{n+}](k_1' + k_2'K_1/[H^+]) \quad (10)$$

and

$$k' = k_{obsd} = \{k_1' + k_2'(K_1/[H^+])\}\{[H^+]/([H^+] + K_1)\} \quad (11)$$

In strongly acid solution where H_2A is not appreciably ionized, K_1 is small compared to $[H^+]$ and (11) simplifies to

$$k_{obsd} = k' + k_2'K_1/[H^+] \quad (12)$$

A plot of the specific rate constant against the reciprocal of the hydrogen ion concentration gives a straight line the intercept of which is k_1' and the slope, k_2'/K_1. Figures 6 and 7 represent such plots for the cupric and ferric ion catalyzed oxidations, respectively. Similar plots were obtained at 0.4°. The values of k_1' and k_2' are listed in Table V. In case of both cupric and ferric ion catalyzed oxidation, $k_1' \ll k_2'$. The ratio of the rates k_2'/k_1' for cupric ion is much higher than for the ferric ion, the values at 25° being 848 and 40 (M^{-1} sec^{-1}), respectively. However, k_1' is much higher for ferric than for cupric ion, which can be readily seen from the intercepts of Figures 7 and 6, respectively.

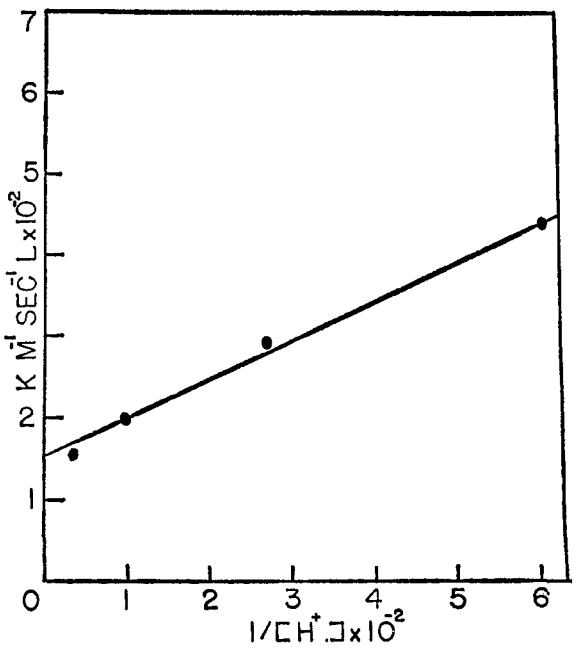


Figure 7. Dependence of the specific rate constants k on the hydrogen ion concentration for ferric ion catalyzed oxidation of ascorbic acid at 25°; μ = 0.10 M KNO_3.

Oxygen Dependence of the Rate of Metal Ion Catalyzed Oxidation. The dependence of the rates of the reaction on the partial pressures of oxygen was determined both for cupric and ferric ion catalyzed oxidation at pH values of 3.10, 3.45, and 3.85. The results are given in Tables VI and VII. The experimental procedure was exactly the same as described for the uncatalyzed oxidation. In every case the specific rates were found to be proportional to the partial pressure of oxygen. The results are presented graphically in Figures 8 and 9 for cupric and ferric ion catalyzed oxidations, respectively. The specific rates for the oxygen dependence of the oxidation of ascorbic acid are calcu-

Table V. Specific Rates (M^{-1} sec^{-1}) for the Metal Ion Catalyzed Oxidation of the Ionic Species of Ascorbic Acid[a]

Metal ion	25°		0.4°	
	k_1	k_2	k_1	k_2
Cu^{2+}	3.1×10^1	2.5×10^4	0.60×10^1	2.2×10^3
Fe^{3+}	1.6×10^2	6.4×10^3	8.9×10^1	8.3×10^2

[a] μ = 0.100 KNO_3; P_{O_2} = 1.0 atm.

Table VI. Specific Rate Constants (M^{-1} sec^{-1}) for the Cupric Ion Catalyzed Oxidation of Ascorbic Acid under Different Partial Pressures of Oxygen[a]

Partial pressure of oxygen, atm	−Log [H⁺] 3.00	3.45	3.85
1.00	11×10^2	16×10^2	8.2×10^3
0.81	8.6×10^2	13×10^2	7.0×10^3
0.62	6.0×10^2	9.5×10^2	4.8×10^3
0.40	4.6×10^2	6.5×10^2	3.9×10^3
0.19	1.8×10^2	3.2×10^2	2.0×10^3

[a] μ = 0.100 M KNO_3; temperature, 25°.

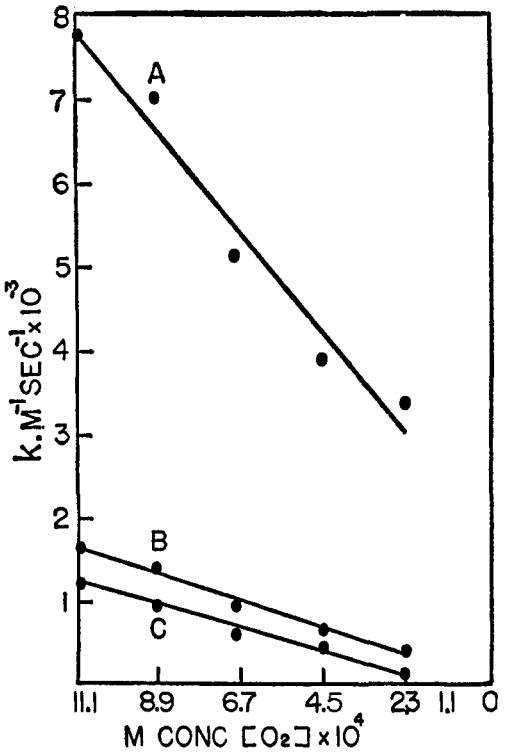


Figure 8. Dependence of the specific ate constants k on oxygen concentration for Cu(II) ion catalyzed oxidation of ascorbic acid at 25°; μ = 0.10 M KNO_3; − log [H^+] values of: A, 3.85; B, 3.45; and C, 3.00.

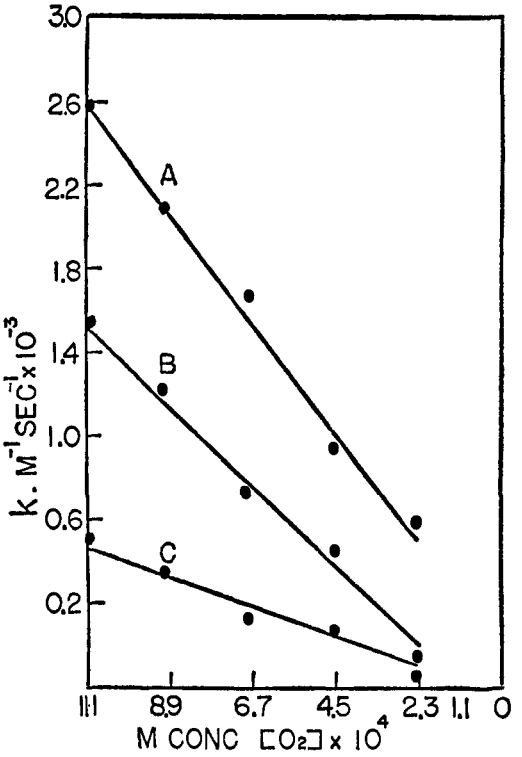


Figure 9. Dependence of the specific rate constants k on oxygen concentration for Fe(III) ion catalyzed oxidation of ascorbic acid at 25°; μ = 0.10 M (KNO_3); − log [H^+] values of: A, 3.85; B, 3.45; and C, 3.00.

lated from the slopes of the straight lines in Figures 8 and 9, and are given in the second column of Table VIII. The specific rates given in Table VIII are calculated from the pH dependence of the rates presented in column 2 with the relationship given by (12), and are presented in columns 3 and 4, respectively.

Table VII. Specific Rate Constants (M^{-1} sec^{-1}) for the Ferric Ion Catalyzed Oxidation of Ascorbic Acid at Different Partial Pressures of Oxygen[a]

Partial of oxygen, atm	−Log [H^+] 3.00	3.45	3.85
1.00	5.6×10^2	1.5×10^3	2.6×10^3
0.81	3.6×10^2	1.2×10^3	2.1×10^3
0.62	1.6×10^2	7.6×10^2	1.6×10^3
0.40	1.0×10^2	4.6×10^2	9.6×10^2
0.19	7.5×10^1	2.1×10^2	6.0×10^2

[a] μ = 0.100 KNO_3; temperature, 25°.

Table VIII. Third-Order Rate Constants (M^{-2} sec^{-1}) of the Oxygen and Metal Ion Dependent Oxidation of Ascorbic Acid, H_2A, and of the Monoprotonated Species of Ascorbic Acid, H_2A^- [a]

Metal ion	Ascorbic acid, −log [H^+] 3.00	3.45	3.85	H_2A	HA^-
Cu^{2+}	11×10^5	15×10^5	4×10^6	3.8×10^5	6.0×10^7
Fe^{3+}	7×10^5	9×10^5	20×10^6	4.0×10^5	2.4×10^7

[a] μ = 0.10 KNO_3; temperature, 25°; − log [H^+] = 3.00, 3.45, and 3.85.

Discussion

The study of the oxygen dependence of the rate of oxidation of ascorbic acid, in the pH range 2–5.5, indicated that the rate is dependent on the concentration of the monoionic species, HA^-, and O_2 concentration, and followed the rate law given by eq 2. A mechanism which is consistent with these kinetic studies and which conforms to the rate law is given in Chart I.

Weissberger, *et al.*,[5] have measured the rate of oxidation of ascorbic acid in the presence of an excess of oxygen, and also as a function of the concentration of molecular oxygen. When the concentration of molecular oxygen was less than 1 atm, the rate of oxidation of ascorbate anion was reported to be independent of the concentration of molecular oxygen. Based on their observation of the kinetics of the oxidation of ascorbic acid as a function of oxygen concentration, they concluded that the rate-determining step in the oxidation of ascorbate anion is semiquinone formation by the interaction of ascorbate anion with dehydroascorbic acid.

In the mechanism proposed in the present paper, the rate-determining step involves a one-electron oxidation of ascorbate anion, HA^-, by molecular oxygen. The proposed mechanism is based on the study of the oxygen dependence of the rate of oxidation of ascorbic acid. The rates were found to be directly proportional to the concentration of molecular oxygen in the concentration range 50–100% of molecular oxygen. In the presence of an excess of oxygen and at least in the initial phases of the reaction, the oxidation of ascorbate anion by molecular oxygen, step k, is relatively more important, since dehydroascorbic acid is not present in appreciable concentration during the initial phase of the reaction. As the concentration of molecular oxy-

gen becomes much less than that of the reaction products, the mechanism of the oxidation of ascorbate anion becomes complicated, and as suggested by Weissberger, Luvalle, and Thomas,[5] dehydroascorbic acid may also take part in the oxidation of ascorbate anion as a side reaction. This does not explain, however, the nonlinearity of the rate of oxidation of ascorbic acid on oxygen concentration when the concentration of the latter is less than 50% (Figure 2), since the rate constants are determined primarily from data taken during the initial phases of reaction runs. Thus there seems to be another oxidation path that becomes important at low oxygen concentration. One such path could be direct oxidation of the substrate by $HO_2\cdot$.

Metal Ion Catalyzed Oxidation. Any mechanism proposed for the metal ion catalyzed oxidation of ascorbic acid must explain or be in accord with the following: (1) the dependence of the specific rate constant on hydrogen ion concentration, (2) the dependence of the specific rate constant on oxygen concentration, (3) higher reactivity of metal ion toward the ascorbate anion than the deionized acid, (4) the difference in reactivities of cupric and ferric ions toward the oxidation of ascorbate anion, and (5) the difference in the reactivities of Cu(II) and Fe(III) ions toward the oxidation of the neutral form of ascorbic acid. In the rate studies with pure oxygen, a first-order dependence was observed in every case. If dehydroascorbic acid is directly involved in the rate-determining step to any appreciable extent, a first-order rate would not have been obtained under the conditions employed, and a correction for the competitive reaction (oxidation of ascorbate anion by dehydroascorbic acid) would have been necessary. At least for one half-life of the reaction in the presence of excess oxygen, the oxidation of ascorbate anion by molecular oxygen is much slower than any other reaction, and no complications arise in the interpretation of the first-order kinetics.

Examples of a one-step electron transfer to oxygen of the type proposed in the rate-determining step have been reported. Halpern and Smith[14] proposed a one-step electron transfer for the oxidation of U(IV) by molecular oxygen. Kaden and Fallab[15] and Fallab[16] have also proposed an initial one-step reduction of oxygen in the reaction of ferrous complexes with molecular oxygen. Further support of an initial one-electron transfer to oxygen comes from the kinetic evidence of Dahn, *et al.*,[17] in the oxidation of ascorbic acid by HNO_2. Dahn, *et al.*,[17] have interpreted their results on the basis of the formation of an ascorbic acid free radical. The presence of free radical in the oxidation of ascorbic acid is recently confirmed by the esr measurements at pH 5.7 by Lagercrantz.[18] Thus a two-electron transfer to oxygen in a single step may be ruled out on the basis of esr evidence.

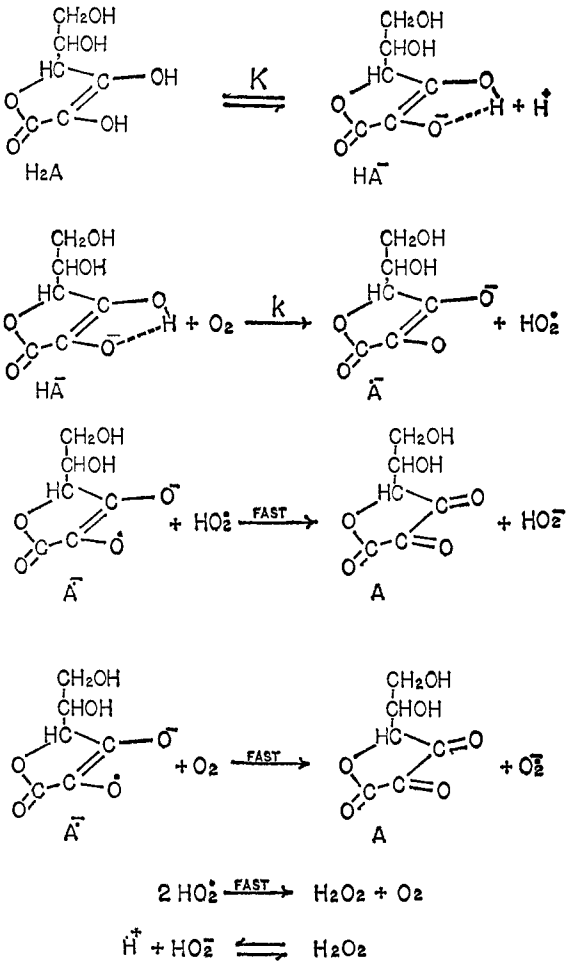
The accumulation of hydrogen peroxide during the course of oxidation was found to have no effect on the rate-determining step. These observations are consistent with the work of Grinstead,[8] who obtained the same rate constant in the oxidation of ascorbic acid by the ferric complex of EDTA with different concentrations of hydrogen peroxide.

In the mechanism suggested by Weissberger and Luvalle,[6] the rate-determining step in the cupric ion catalyzed oxidation of ascorbate anion involves the initial formation of a metal complex between copper ion and ascorbate anion, followed by a rate-determining electron transfer to the metal ion. The semiquinone (HA^-) and Cu(I) are both oxidized by molecular oxygen in subsequent fast steps. This is in accord with factors 1, 3, 4, and 5 but not 2.

Two possible mechanisms may be considered for metal ion catalyzed oxidation of ascorbic acid.

In a mechanism indicated as pathway 1 (Chart I), a metal–ascorbate complex MHA is suggested in a preequilibrium step. This is followed by a rate-determining electron transfer within the complex from ascorbate anion to metal ion. The reduced complex then dissociates in a fast step to the lower valence metal ion and semiquinone, A^-. Reoxidation of the lower valence metal ion takes place in a subsequent fast step as suggested by Dekker and Dickinson.[19] In such a case the rate of the reaction should be independent of oxygen concentration. This fact is contradictory to the present investigation of the metal ion catalyzed oxidation where a direct dependence of rate on partial pressure of oxygen is observed. The same dependence has also

Chart I



(14) J. Halpern and J. G. Smith, *Can. J. Chem.*, 34, 1419 (1956).
(15) T. Kaden and S. Fallab, "Advances in the Chemistry of Coordination, Compounds," S. Kirschner, Ed., The MacMillan Co., New York, N. Y., 1961, pp 654–663.
(16) S. Fallab, *Chimia* (Aarau), 16, 189 (1962).
(17) H. Dahn, L. Loewe, and C. A. Bunton, *Helv. Chim. Acta*, 43, 320 (1960).
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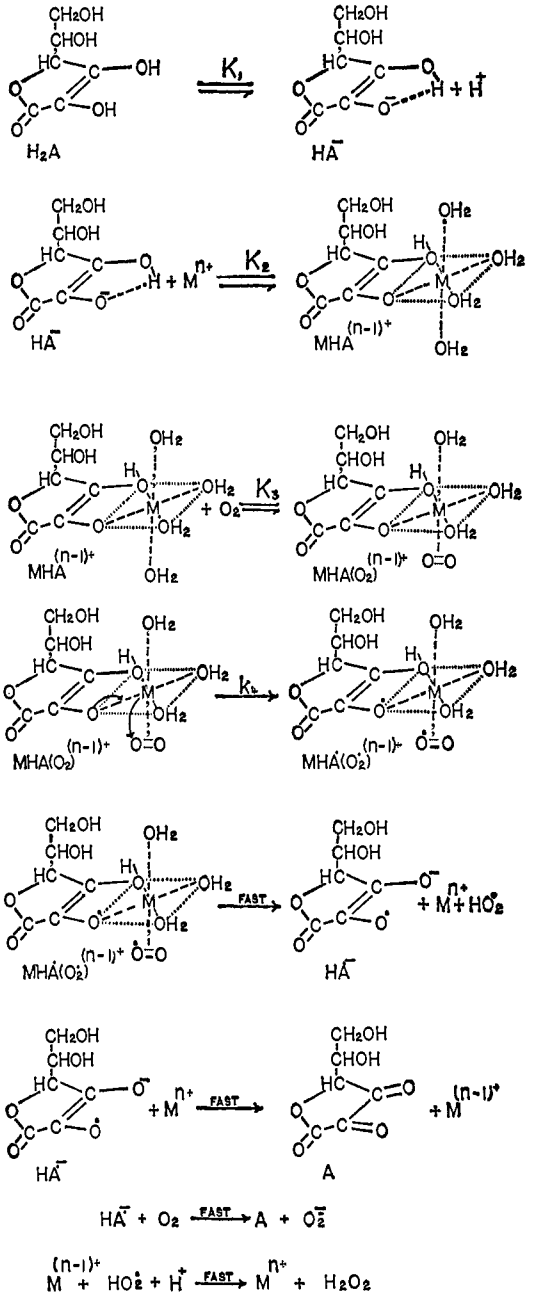
(19) A. O. Dekker and R. G. Dickinson, *J. Am. Chem. Soc.*, 62, 2165 (1940).

been reported by Barron, *et al.*,[4] at pH 7.05 and also by Weissberger and co-workers[5] at pH 7.04 and 5.85. If the reoxidation of the metal ion is slow enough to be rate determining, the reaction would probably not show the observed hydrogen ion dependence. Thus pathway 1 is incapable of accounting for all the features of the metal ion catalyzed oxidation of ascorbic acid.

Chart II



In a possible pathway 2 (Chart II), a metal–ascorbate complex is first formed as indicated above. This is followed by an attack of oxygen on the metal–ascorbate complex with the formation of an oxygen complex in a second preequilibrium step as indicated above. In the rate-determining step k_4, an electron is transferred through the metal ion to oxygen. The rate of the metal ion catalyzed oxidation of ascorbic acid by pathway 2 may be expressed by the equation

$$-\mathrm{d[MHA(O_2)]}/\mathrm{d}t = k_4[\mathrm{MHA(O_2)}] \quad (13)$$

Since $MHA(O_2)$ is in equilibrium with MHA and HA^-, rate eq 13 may be written as

$$-\mathrm{d[MHA(O_2)]}/\mathrm{d}t = k_4K_2K_3[\mathrm{HA^-}][\mathrm{M^{n+}}][\mathrm{O_2}] \quad (14)$$

Equation 13 shows a simple dependence of the rate of oxidation of ascorbic acid on oxygen concentration. The validity of this relationship may be seen in the dependence of the rate of Cu(II) and Fe(III) ion catalyzed oxidation of ascorbic acid on the partial pressure of oxygen (Figures 8 and 9).

Equilibrium Evidence for MHA. Ascorbic acid was tritated very rapidly with cupric ion at 0.4°. The "*a*" value (the neutralization value) was preset by adding the calculated quantity of base to ascorbic acid solution and the metal solution was then very rapidly added within 3 sec. The pH was noted immediately and the drop in the value of pH was followed as a function of time for 3 sec up to 25 sec in each case. The value of $-\log [H^+]$ was then extrapolated to zero time. The value of $\log K_{CuHA}$ calculated by this technique was 1.57 ± 0.03. The value of ferric ion could not be obtained by a similar procedure due to much faster oxidation. However, it may be concluded that the complex formed with the ferric ion is analogous to that formed by the Cu(II) ion.

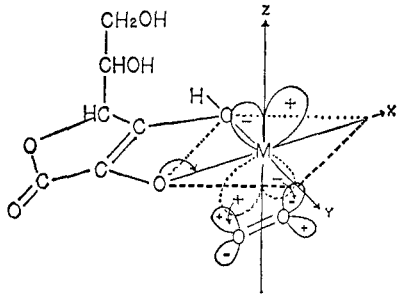
Kinetic Evidence for MHA. The inverse dependence of the specific rates for the metal ion catalyzed oxidation of the monoionic species on hydrogen ion concentration rules out any possibility of a complex of the type MA and suggests that one proton is still on the metal–ascorbate complex.

The formation of a complex with oxygen to give $MHA(O_2)$ is analogous to oxidation mechanisms reported in the literature. For the oxidation of ferrous ion by molecular oxygen, George[20] suggested a rate-determining step which involves the transfer of an electron through a ferrous complex of the type $Fe(O_2)^{2+}$. The studies of Kaden and Fallab[15] and Fallab[16] for the oxidation of ferrous chelates by molecular oxygen further support the proposed electron transfer through the metal ion. Beck and Gorog[21] have found the cobalt(II)–glycylglycine oxygen carrier to be very effective as a catalyst in the oxidation of ascorbic acid in the pH range 7–9.5.

In the case of the metal–ascorbate complex, electron transfer from ascorbate anion to oxygen may be visualized as taking place in the following manner. An electron is first transferred from a 2p orbital of the ascorbate oxygen to a t_{2g} nonbonding or an e_g antibonding metal orbital. This process is followed by transfer of the electron to an antibonding π_y*2p or π_z*2p orbital of the oxygen molecule, according to Scheme I. It may be readily seen from Scheme I that, in order to have maximum overlap between the metal t_{2g} orbital, oxygen should be in a plane parallel to that of the metal ion (the *xy* plane), and perpendicular to the axial bond of the metal ion. The bonding of metal ion to oxygen would be of the same type that occurs in the π bonding of ethylene to metal ions, with overlap between the π orbitals of oxygen and a hybrid axial bond of the metal ion (pd or d^2sp^3). Such a bonding scheme is now well known in cobalt(II) oxygen carriers, where it has

(20) P. George, *J. Chem. Soc.*, 4349 (1954).
(21) M. T. Beck and S. Gorog, *Acta Chim. Acad. Sci. Hung.*, **29**, 401 (1961).

Scheme I. Proposed Metal–Ascorbate–Oxygen Complex



been shown on the basis of molecular orbital treatment that oxygen is perpendicular to the Co–Co axis with bonding[22] similar to that in olefinic complexes. X-Ray work of Brosset and Vannerberg[23] supports the proposed structure. Recently, X-ray structures by Ibers and LaPlaca[24] confirm an olefinic type of metal–oxygen bond and indicate further that the bonded oxygen is not of the peroxo type.

It should be noted that the precise structure of the metal–ascorbate chelate compound illustrated in Scheme I is not known with certainty. A tautomeric form in which the proton is attached to the other coordinated oxygen is also quite probable. There is also a possibility that the metal ion is coordinated to the oxygen atom attached to carbon atoms 5 and 6, although such a structure would be rendered less stable because of the low basicity of the lactone carbonyl group.

It is possible that the greater rate for cupric ion may be due to a higher equilibrium constant, K_3, for the formation of the oxygen complex. On the basis of the information presently available, however, there seems to be no way of estimating the affinity of oxygen for aquo Cu(II) and Fe(III) ions or their ascorbate complexes in aqueous solution.

It may be emphasized that the electron-transfer process from the ascorbate anion to metal ion as suggested in the rate-determining step involves the reducibility of the metal ion without an actual valence change. Unless the metal ion has a stable lower valence form, it will not be expected to participate in the electron-transfer scheme and thus will be inactive as catalysts. This possibility has been experimentally verified by testing the catalytic activity of the members of the first transition series, VO^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}. Except for vanadyl ion, all the ions investigated were found to be inactive as catalysts for the oxidation of ascorbic acid.

Based on the zero dependence of the rate, k_1 (in eq 14), on hydrogen ion concentration, a mechanism analogous to pathway 2 (Chart II) may be suggested for the neutral form of ascorbic acid.

As in the case of the oxidation of the monoionic species, the rate of the metal ion catalyzed oxidation of the neutral species may be expressed by the equation

$$-\mathrm{d}[MH_2A(O_2)]/\mathrm{d}t = k_3'[MH_2A(O_2)] = k_3'K_1'K_2'[H_2A][M^{n+}][O_2] \quad (15)$$

where

$$K_1' = [MH_2A^{n+}]/[M^{n+}][H_2A]$$

$$K_2' = [MH_2A(O_2)^{n+}]/[MH_2A^{n+}][O_2]$$

Summarizing the mechanisms suggested for the metal ion catalyzed oxidation of monoionic and neutral species of ascorbic acid and comparing with the mechanism for cupric ion catalyzed oxidation of ascorbic acid by Weissberger and Luvalle,[6] the following points merit consideration.

The formation of the proposed complex between ascorbate anion and the metal ion in a preequilibrium step explains the hydrogen ion dependence of the reaction and also the higher reactivity of metal ion toward the ascorbate anion than the neutral acid. The difference in the reactivities of cupric and ferric ions toward the oxidation of ascorbate anion or neutral form of ascorbic acid may be explained to be due to the difference in the formation constants of the metal ion with the two species of ascorbic acid. The oxygen dependence of the rates of catalyzed and uncatalyzed oxidation of ascorbic acid may be only explained by the mechanisms proposed in this paper, whereby oxygen takes part in the rate-determining step through formation of a metal–oxygen–ligand complex.

The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ corresponding to the rate constants k_1' and k_2' (rate eq 12) are listed under E_1 and E_2 in Table IX, and were calculated from the temperature dependence of k_1' and k_2', respectively. One may see a significant change in the entropy of activation if one compares the spontaneous oxidation with the metal ion catalyzed reaction. The entropy of activation increases in the order $\Delta S^{\ddagger}_{(\text{spontaneous})} < \Delta S^{\ddagger}_{E_1} < \Delta S^{\ddagger}_{E_2}$, where $\Delta S^{\ddagger}_{E_1}$ and $\Delta S^{\ddagger}_{E_2}$ are the entropies of activation corresponding to the oxidation of neutral and monoionic forms of ascorbic acid catalyzed by metal ion.

Table IX. Activation Parameters of Spontaneous and Metal Ion Catalyzed Oxidation of Ascorbic Acid

Metal ion	E_2[a,b] $-\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$	E_1[a,b] $\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$
None[c]	+10.7	−37	+22	...	...	...
Cu^{2+}	+15.5	+14	+11.3	+9.7	−19	+15.4
Fe^{3+}	+13.0	+2	+12.4	+3.3	−37	+14.4

[a] E_2 and E_1 are the activation parameters corresponding to the oxidation of monoionic and neutral forms of ascorbic acid, respectively. [b] $\Delta H^{\ddagger}$, kcal/mole; $\Delta S^{\ddagger}$, cal/deg mole; $\Delta G^{\ddagger}$, kcal/mole. [c] Spontaneous oxidation.

The factor responsible for increase in value of entropy of activation for the monoionic form compared to that of the neutral form may be due to the influence of the metal ion in bringing the oxidant and substrate together through coordination. The entropy increase in the formation of the activated complex HA^- would be greater than for the neutral form of the substrate H_2A since there would be greater displacement of solvated water molecules on combination of HA^- with the metal ion. This effect would be characterized by higher equilibrium concentration of the intermediate MHA and $MHA(O_2)$. The rate constant K_1 for oxidation of the neutral form also seems to correlate with the concentration of products formed in the prequilibrium

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(23) C. Brosset and N. G. Vannerberg, *Nature*, 190, 174 (1961).
(24) J. A. Ibers and S. J. LaPlaca, "Proceedings of the Eighth International Conference on Coordination Chemistry," V. Gutman, Ed., Springer-Verlag, Vienna, 1964, pp 95–97.

steps. Ferric ion is more reactive than cupric ion toward the oxidation of the neutral form H_2A. This may be correlated with a much higher probability of formation of a ferric complex of the type FeH_2A^{3+} compared to CuH_2A^{2+}, because of the higher charge of the ferric ion. One would expect a higher entropy increase and higher enthalpy decrease on the formation of the ferric complex.

The enthalpies of activation of the ferric ion are lower than for cupric ion. This seems to correlate with the expected greater binding energy of ferric over that of cupric ion. The lower enthalpies for the neutral form compared to the monoionic species may be due to the difference in the relative stabilities of the metal complexes corresponding to the two species. The neutral form, as compared to the monoionic form, is expected to give a less rigid complex which is easily deformed on electron transfer as a result of the change in hydration energy. This would result in a lowering of $\Delta H^{\pm}$.

Modification of the Radiolytic Oxidation of Ribonuclease Induced by Bound Copper

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Abstract: The specific effect of cupric ions on the radiolytic oxidative inactivation of enolase and ribonuclease has been investigated. It has been shown that cupric ions bound to the enzymes enhance the radiolytic inactivation induced by OH radicals. The destruction of the amino acid residues was followed in parallel to enzymatic inactivation in the absence and in the presence of Cu^{2+}. It is shown that complexing of Cu^{2+} by RNAase does not alter the *total* yield of radiolytic destruction of amino acid residues, but induces a significant change in the radiolytic yield of individual amino acid residues sensitive to oxidation. A significant increase in the yield of radiolyzed histidyl residues on RNAase in the presence of Cu(II) was observed, accompanied by a corresponding decrease in damaged S–S bonds. A quantitative evaluation of the effect of OH radicals on RNAase in the presence and absence of copper suggests an intramolecular charge transfer in the protein molecule following its reaction with OH radicals. The radiobiological implications of the copper-induced "sensitization" of vital sites in a bipolymer are discussed.

Cupric ions have been shown to enhance the radiolytic decomposition of diamines and amino acids.[1,2] The effect of cupric ions on the radiolytic inactivation of α-amylase and catalase has been subsequently investigated and it was found that binding of Cu(II) to both enzymes resulted in an enhanced radiolytic inactivation of the enzymes by γ irradiation.[3] This enhanced sensitization to ionizing radiation was shown to be due to the action of oxidizing radicals. Other metal ions investigated did not exhibit a comparable effect, thus confirming the suggestions on the possible specific role of copper in radiobiological damage.[4,5] In order to investigate more thoroughly the role of copper in radiation damage to proteins, the Cu(II)–enolase and the Cu(II)–ribonuclease complexes were investigated. In both cases it is known that Cu(II) inhibits enzyme activity.[6,7] For enolase it has been shown that copper ions displace other metal ions essential for the enzymatic activity,[8] suggesting that Cu(II) is bound to a site essential for enolase activity. In the case of RNAase there is accumulating evidence that the Cu(II) interacts with the histidine residues[9–11] which are involved in the catalytic function.[12] Thus, if Cu(II) "sensitizes" its ligands to ionizing radiation,[2] one should expect a preferential radiolytic destruction of the active site in the presence of copper. In the present work we have investigated, in addition to the copper-induced enhancement of radiolytic inactivation of enolase and RNAase, also the effect of bound copper on the radiolytic destruction of individual amino acid residues in the RNAase molecule.

Experimental Section

Reagents and Materials. Crystalline rabbit muscle enolase supplied by Boheringer and Sons, Germany, was used. In order to remove low molecular weight impurities and metal ions the enzyme was dialyzed at 0–4° against 0.02 *M* sodium phosphate buffer, pH 7.0, containing 0.01 *M* EDTA, for 20 hr, and then against phosphate buffer alone (20 hr) to remove the EDTA. This process was shown not to affect the enzymatic activity.

Pure ribonuclease A, lyophilized, phosphate free, obtained from Worthington (lot no. 6508 and 6022), was used without further treatment.

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