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Copper-catalyzed Oxidation of Ascorbic Acid

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Copper-catalyzed oxidation of ascorbic acid was investigated in various environments by using the conventional Warburg manometric technique. The rate of the oxidation, expressed by the consumption of molecular oxygen, depends on pH and the concentration of ascorbic acid. From the pH dependence of the reaction, it was deduced that the dissociated monoionic ascorbic acid was a molecular species susceptible to the oxidation. Inorganic ligand including buffer anion influenced the oxidation. Nitrate, sulfate and acetate ions, which have a little ability to complex with copper ion, reduce a little the catalytic efficiency. The retardation by acetate ion was ascribed to the formation of copper tetraacetate. Halide, pyrophosphate, thiocyanate and cyanide ions, which complex preferably with cuprous ion thereby change the redox potential between cupric and cuprous ions, retards the oxidation markedly.

Copper-catalyzed oxidation of ascorbic acid has been widely investigated as a model for copper containing oxidase. The reaction pathway for the ascorbate oxidation is composed of two steps of redox reaction. The first, which may be rate-determining, is the redox reaction between copper ion and ascorbic acid. The second is the auto-oxidation of cuprous ion. If ascorbic acid reacts with oxygen forming dehydroascorbic acid and hydrogen peroxide, theoretically one mole of oxygen will be consumed per mole of ascorbic acid.[2] However, the amount of oxygen consumed was reported to vary, depending on the reaction condition, from half to one mole per each ascorbic acid molecule.[3] This variation in the amount of oxygen is explained by intermediate being changed by further oxidation. The catalytic activity is modified markedly by the complexation of copper ion.[4] The chelating agent, such as ethylenediaminetetraacetic acid, 1,10-phenanthroline or 8-hydroxyquinoline, decreases markedly the catalytic efficiency. The activity of copper ion is modified also by electrolyte or other environmental factors,[5] which might affect the redox potential of copper ion. Scaife reported that sodium chloride reduced the catalytic efficiency of copper ion.[6] Some buffer anions were shown to reduce the oxidation rate.[7] However, no detailed information about the effect of electrolyte has hitherto been reported. The present paper dealt manometrically with the catalytic activity of copper ion in various environments.

Experimental

Materials——Unless described otherwise, all compounds were commercially available, reagent grade chemicals, and were used without further purification. The ascorbic acid solution was prepared just prior

1) Location: *Anagawa-4, Chiba.*
2) A. Weissberger and J.E. LuValle, *J. Am. Chem. Soc.*, **66**, 700 (1944).
3) D.B. Hand and E.C. Greisen, *J. Am. Chem. Soc.*, **64**, 358 (1942); R.W. Pertersen and J.H. Walton, *J. Am. Chem. Soc.*, **65**, 1212 (1943).
4) R. Flitman and E. Frieden, *J. Am. Chem. Soc.*, **79**, 5198 (1957); E. Tanner, W. Schuler and R. Meier, *Helv. Chim. Acta*, **42**, 445 (1959); V.S. Butt and M. Hallaway, *Arch. Biochem. Biophys.*, **92**, 94 (1961).
5) I. Pecht and M. Anbar, *Nature*, **207**, 1386 (1965); I. Pecht, A. Levitzki and M. Anbar, *J. Am. Chem. Soc.*, **89**, 1587 (1967).
6) J.F. Scaife, *Can. J. Biochem. Physiol.*, **37**, 1049 (1959).
7) H.G. Steinman and C.R. Dawson, *J. Am. Chem. Soc.*, **64**, 1212 (1942).

to use by dissolving sodium ascorbate in twice distilled water from all glass apparatus. The copper solution was prepared from the stock solution, which was standardized by the conventional complexometric titration.[8] For the investigation of the effect of inorganic ligand, the following chemicals were employed; $NaNO_3$, CH_3COONa, Na_2SO_4, NaCl, KCl, KBr, KI, KSCN and KCN.

Manometric Measurement——The uptake of oxygen connected with the ascorbate oxidation was followed by the conventional Warburg manometric technique at 25° using air as the gaseous phase. The main compartment of the reaction vessel contained 2.0 ml of 0.125 (or 0.15) M sodium acetate buffer containing various amounts of inorganic ligands and 2.0 ml of 5.0×10^{-5} M copper nitrate solution. In the side arm, 1.0 ml of 2.50×10^{-2} M sodium ascorbate solution was contained. The total volume of the reaction mixtures was 5.0 ml, and pH was 5.3. The ionic strength was maintained at 0.1 or 1.0 with $NaNO_3$. The substrate was tipped from the side arm after 10 min for the temperature equilibration at 25°. The manometer was closed just before tipping the substrate, and oxygen consumption was read every 1 min thereafter. The reaction vessel was shaken vigorously (120 oscillation per min) throughout the measurement. The rate of oxygen consumption was estimated graphically from the initial step of the oxidation.

Results

The rate of the ascorbate oxidation was measured manometrically in acetate buffer. Since the consumption of molecular oxygen, which is ascribed to the oxidation of ascorbic acid, was increased linearly with time, the rate of the oxidation was expressed as the volume of oxygen consumed per minute. The rate measured at a definite concentration of ascorbic acid was found to increase with pH, from pH 3.8 to 5.5, and reach to a constant level beyond pH 5.5. Then, the rate measured at pH 6.5 in 0.05 M phosphate buffer, which showed a maximum level, was used as a reference. In order to compare the oxidation rates measured under various conditions, the term named the relative activity, which is the ratio of the observed (V_{obsd}) to the maximum (V_{max}) rates, was accepted in this paper. The plot of the relative activity against pH was shown in Fig. 1. Considering the ionization constant of ascorbic acid, $pK_{a1}=4.04$ at 25°,[9] the molecular species of ascorbic acid presented in the reaction mixtures are both the undissociated (AH_2) and the dissociated (AH^-) forms, and the concentration of the latter increases with pH. Since the total concentration of ascorbic acid $[A]_0$ was fixed, the relative concentration of the monoionic acid expressed by $[AH^-]/[A]_0$ also increases with pH. At lower pH value, where the predominant species of ascorbic acid is AH_2, the oxidation becomes slow. Those facts indicate that the reactive species of ascorbic acid for copper-catalyzed oxidation is AH^-, and that some relation is expected to exist between the rate of oxygen consumption and the relative concentration. Provided that AH^- is an only species susceptible to the oxidation and that the catalytic efficiency of copper ion does not vary throughout, the rate would be correlated to the concentration of AH^- and reach to a maximum level when the first acid dissociation of ascorbic acid has been completed. According to those assumptions, the relative activity would be equal to the relative concentration of the monoionic acid and expressed as follows:

$$\text{Relative activity}=V_{obsd}/V_{max}$$
$$=[AH^-]/[A]_0=K_a/(K_a+[H^+]) \quad (1)$$

The curve calculated from Eq. (1), which displays an inflection point at pH 4.04, was shown in Fig. 1. A fairly good coincidence between the calculated and the experimental values indicates the validity of the above assumption.

The rates measured in acetate and phosphate buffers depend on the total concentration of ascorbic acid as shown in Table I. Though the effect of the buffer on the reaction rate appeared to be different, the reciprocal of the rate was related linearly to the reciprocal of the total concentration of ascorbic acid as shown in Fig. 2. Therefore, under a given condition

8) G. Schwarzenbach, "Die Komplexometrische Titration," Ferdinand Enke Verlag, Stutgart, 1955, p. 68.
9) H.M. Taqui Kan and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).

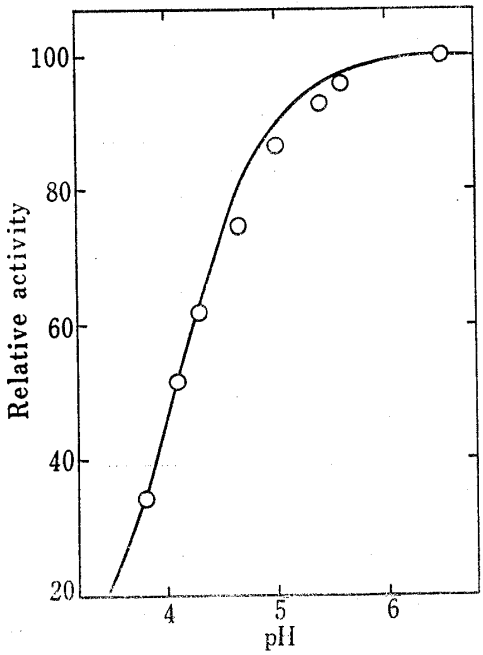

Fig. 1. pH Dependence of the Ascorbate Oxidation at 25°

$[Cu^{2+}]_0=2.00\times10^{-5}$M $[A]_0=6.00\times10^{-3}$M
acetate buffer: 0.1M ○: experimental value
Solid line indicates the calculated rate from Eq. (1).

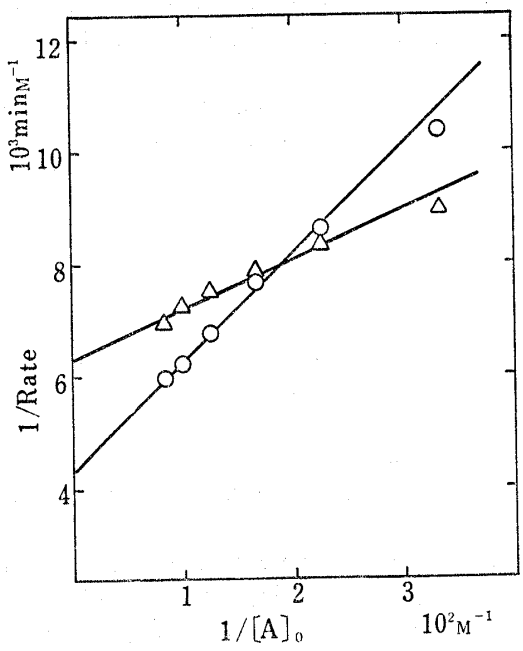

Fig. 2. Concentration Dependence of the Ascorbate Oxidation in Acetate and Phosphate Buffers at 25°

$[Cu^{2+}]_0$: 5.00×10^{-6}M
—○—○—: 0.1M acetate buffer pH 5.3
—△—△—: 0.1M phosphate buffer, pH 6.6

Table I. Effect of Varying Concentrations of Ascorbic Acid on the Ascorbate Oxidation

Concentration 10^{-3}M	Rate of oxygen consumption (μl/min) In acetate buffer pH 5.3	In phosphate buffer pH 6.6
3.0	10.8	12.6
4.5	13.0	13.2
6.0	14.3	14.6
8.0	16.6	14.8
10.0	18.0	15.4
12.0	18.8	16.1

$[Cu^{2+}]_0=5.00\times10^{-6}$M

where the concentrations of all the reactants except ascorbic acid were fixed constant, the relation of the rate to the concentration could be represented as follows;

$$1/\text{Rate}=b/[A]_0+d \qquad (2)$$

where b and d indicate parameters decided by the nature of buffer anion. The term d may correspond to the maximum rate in a certain buffer.

Acetate or phosphate anion, though having a weak complexing ability with copper ion, reduces a little the rate of the oxidation. The buffer anion, which has an ability to form a stable complex, such as pyrophosphate(cumulative stability constant, log β_2,[10] of the complex CuL_2; 8.33 for monohydrogen pyrophosphate),[11] inhibits markedly the oxidation. The effect

10) $\beta_2=[CuL_2]/[Cu^{2+}][L^-]^2$.
11) O.E. Schupp III, P.E. Sturrock and J.I. Watters, *Inorg. Chem.*, 2, 106 (1963).

of buffer was summarized in Table II. Thus, the anion of buffer is expected to affect, probably retard, the oxidation by virtue of its complexing ability.

Table II. Effect of Buffer on the Ascorbate Oxidation

Concentration of buffer (M)	Rate of oxygen consumption (μl/min) Acetate pH 5.35	Phosphate pH 6.50	Pyrophosphate pH 6.85
0.04	30.9	31.4	2.85
0.08	30.4	30.9	1.11
0.12	29.9	29.7	0.56
0.16	27.8	27.0	0.42
0.20	27.4	24.8	0.16

$[Cu^{2+}]_0=1.00\times10^{-4}$M $[A]_0=6.00\times10^{-3}$M

All the anions influenced more or less the rate of the oxidation. Nitrate and sulfate ions associating very weakly with copper ion showed little effect over the concentration range below 0.1 M. At higher concentration, the rate was reduced a little probably due to the complex formation with copper ion. The retardation curve, a plot of the relative activity against the logarithm of the total concentration of anion, was shown in Fig. 3. All the halogen ions, as well as thiocyanate and cyanide ions, retard the oxidation. The concentration of anion required for the 25 and 50 percent retardation was presented in Table III. The retardation effect of those anions appears to parallel with the stability constant of the cuprous complex as shown in column 4 of Table III. As to the retardation by chloride, Mapson assumed that chloride retarded the reaction by decreasing the rate of cuprous ion oxidation, probably by forming the more stable cuprous

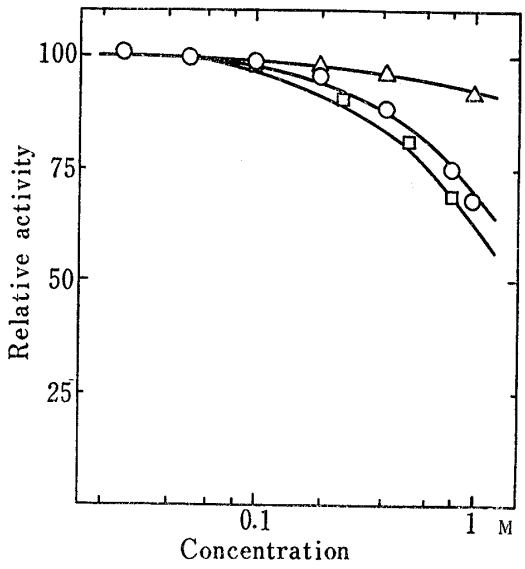

Fig. 3. Retardation Curve of the Oxidation by Nitrate, Acetate and Sulfate Ions

$[Cu^{2+}]_0=2.00\times10^{-5}$M $[A]_0=5.00\times10^{-3}$M
acetate buffer: 0.05M, pH 5.3 (Ionic strength 1.0)
—△—△—: nitrate —○—○—: acetate
—□—□—: sulfate

Table III. Retardation of the Ascorbate Oxidation by Halide, Thiocyanate and Cyanide Ions

Anion	Concentration of 25% retardation (M)	50% retardation (M)	Stability constant of cuprous complex[a] $\log\beta_1$ (M^{-1})	$\log\beta_2$ (M^{-2})	Redox potential (v)
Cl^-	1.4×10^{-2}	2.4×10^{-2}	5.5	5.7	0.54
Br^-	8.8×10^{-3}	1.5×10^{-2}	5.92		0.64
I^-	4.8×10^{-5}	6.2×10^{-5}	9.03		0.86
SCN^-	1.2×10^{-5}	1.8×10^{-5}		9.90	
CN^-	1.6×10^{-5}	1.7×10^{-5}	21.7	26.3	1.12

$[Cu^{2+}]_0=2.00\times10^{-5}$M

a) from "Stability Constants," ed. by L.G. Sillen and A.E. Martell, Special Publication No. 17, The Chemical Society, London (1964).

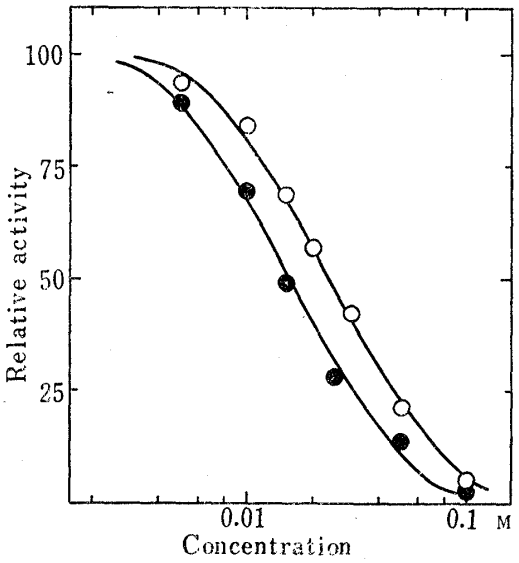

Fig. 4. Retardation Curve of the Oxidation by Chloride and Bromide Ions

—○—○—: chloride —●—●—: bromide
experimental details as under Fig. 3

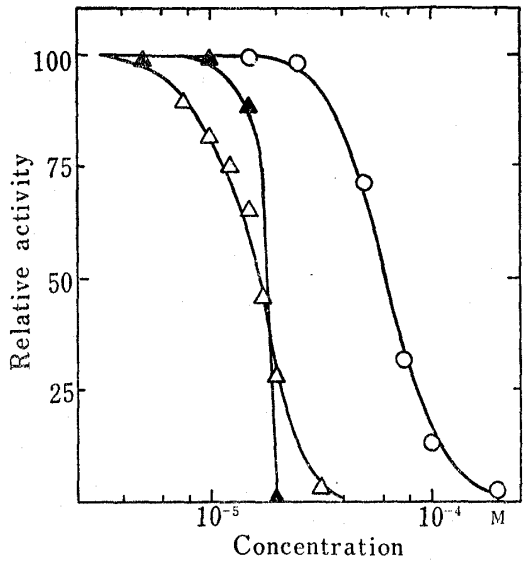

Fig. 5. Retardation Curve of the Oxidation by Iodide, Thiocyanate and Cyanide Ions

—○—○—: iodide —△—△—: thiocyanate
—▲—▲—: cyanide
experimental details as under Fig. 3

complex.[12] The retardation curves displayed symmetric sigmoid shapes in all the halogen ions as presented in Fig. 4 and 5. The relative activity in this case was expressed empirically by Eq. (3);

$$\text{Relative activity} = 1/(1+K[L^-]^n) \qquad (3)$$

where K and n indicate constants. In copper chloride and bromide, $[L^-]_0$ can be used instead of $[L^-]$, because the ligand is present excessively as compared with copper ion. From the slope of the retardation curve, n can be estimated. In copper chloride and bromide, a reliable value for n was 2. K values were calculated as 1.7×10^3 and 4.5×10^3, respectively, for chloride and bromide ions. Owing to the strong complexing ability, iodide ion inhibits intensely the oxidation, and $[L^-]$ is lower than $[L^-]_0$. In addition, the copper complex in the reaction mixtures is not probably a single species, the evaluation of $[L^-]$ seems very difficult. Accordingly, K and n values were not calculated from the retardation curve.

Cyanide and thiocyanate ions showed complicated behaviors against the oxygen consumption, probably because of the instability of the complex against the oxidation and/or the reduction. The retardation curves of both ions displayed unsymmetric patterns as shown in Fig. 5. Cyanide ion inhibits the oxygen consumption completely at the concentration of 2×10^{-5}M ($[CN^-]/[Cu^{2+}]_0=1$), which indicates that the copper cyanide complex having probably a high stability constant is a catalytically inactive species. Below 2×10^{-5}M, cyanide ion showed a little or no inhibition against the oxidation. In the concentration range below 2×10^{-5}M, both free copper ion, which is a catalytically active species, and the cyanide complex should be presented in the reaction mixtures. If the cyanide complex would be stable chemically against the oxidation, the oxygen consumption might be related to the concentration of free copper ion; *i.e.*, the inhibition might be related linearly to the concentration of cyanide ion. However, it was found that the retardation was observed only in the initial step, and that the oxygen consumption was measured after the retardation period and raised up to an approximately constant level irrespective of the initial concentration of cyanide ion. The length of the retardation period; *i.e.*, the latent period for the oxygen consumption, was in

12) L.W. Mapson, *Biochem. J.*, 39, 228 (1945).

accord with the amount of cyanide ion. The reaction profile for the retardation by cyanide ion was presented in Fig. 6. Those findings suggest that the cyanide complex might be unstable unexpectedly against the copper-catalyzed oxidation and would be decomposed to a catalytically active species. Thiocyanate ion retarded the oxidation below 2×10^{-5}M, probably by forming the stable cuprous complex. In this concentration range, the retardation curve showed a symmetric sigmoid pattern and the relative activity appeared to obey Eq. (3). Beyond this concentration, however, the oxygen consumption was reduced rapidly with the increasing amounts of thiocyanate ion, and the retardation curve appeared to be close to that of cyanide ion.

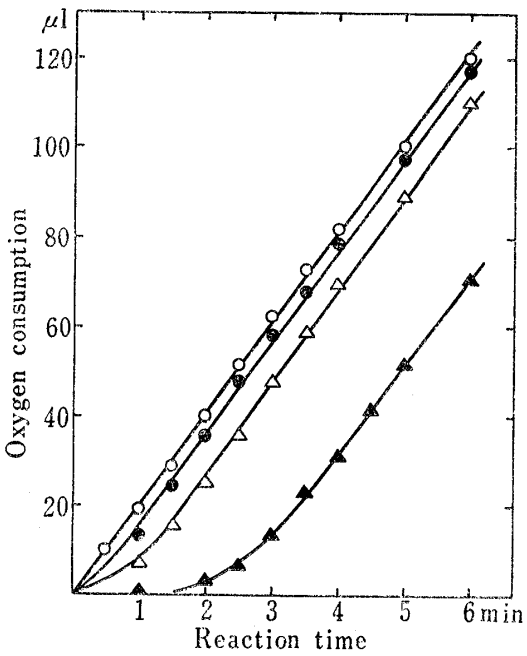

Fig. 6. Reaction Profile for the Retardation by Cyanide Ion

$[Cu^{2+}]_0$: 2.00×10^{-5}M
[CN$^-$]:
—○—○—: 0M —●—●—: 0.5×10^{-5}M
—△—△—: 1.0×10^{-5}M —▲—▲—: 1.5×10^{-5}M

Discussion

It has been demonstrated that the rate of the copper-catalyzed oxidation of ascorbic acid depends on the concentration of the monoionic ascorbic acid which increases with pH. Although the concentration of the monoionic acid is a main factor controlling the oxidation rate, the oxidizability of ascorbic acid itself, which is determined by the redox potential (dehydroascorbic acid/ascorbic acid), increases with pH.[13] However, under the condition even when ascorbic acid is completely dissociated to the monoionic acid, the measured rate increases gradually with pH. A question why the rate profile in Fig. 1 is not consistent completely between the calculated and the measured rates might be explained from the pH dependence of the redox potential of ascorbic acid.

The second step of the oxidation is the formation of the labile copper ascorbate complex. If another ligand is added into the reaction mixtures, the formation of the ascorbate complex may be hindered according to the complexing ability of the ligand. For instance, ethylenediaminetetraacetic acid inhibits completely the formation of the ascorbate complex, though it is an extreme case, and thereby the oxidation is interrupted. Acetate or sulfate ion, though having a weak complexing ability, also retards slightly the oxidation. When a monodentate ligand is presented in the reaction mixtures, this ligand associates with copper ion and forms the complexes, CuL^+, CuL_2, CuL_3^- and CuL_4^{2-}. Some of those complexes have to possess the catalytic activity and others are inactive. The apparent catalytic activity of this complex system may be related to the concentration of the catalytically active complexes. Since the total concentration of copper ion is fixed, the activity is related to the relative concentration, namely the distribution, of the active complex. In order to clarify which complex is active, the distribution of each complex, functional to the concentration of free ligand, is calculated. The distribution of a complex $CuL_p^{(2-p)+}$ is given by Eq. (4).

$$[CuL_p^{(2-p)+}]/[Cu^{2+}]_0=\beta_p[L^-]^p/\sum_{i=0}^{n}\beta_i[L^-]^i \qquad (4)$$

In Eq. (4), β_p represents the cumulative stability constant of $CuL_p^{(2-p)+}$ Provided that the catalytic activities of all the active complex, $Cu,^{2+}$ CuL^+,···and $CuL_m^{(2-m)+}$ are equal, the relative activity can be calculated from Eq. (5).

13) E.G. Ball, *J. Biol. Chem.*, **118**, 219 (1937).

$$\text{Relative activity} = \sum_{j=0}^{m} [CuL_j^{(2-j)+}]/[Cu^{2+}]_0$$

$$= \sum_{j=0}^{m} \beta_j [L^-]^j / \sum_{i=0}^{n} \beta_i [L^-]^i \qquad (5)$$

In copper acetate system, the integral distribution curves of the composite complexes, *i.e.*, Cu^{2+}, $Cu(CH_3COO)^+$, $Cu(CH_3COO)_2$, $Cu(CH_3COO)_3^-$ and $Cu(CH_3COO)_4^{2-}$, were presented in Fig. 7. Because of the low stability constant of those complexes,[14] the total concentration of ligand is close to the free ligand concentration. Therefore, the term $[L^-]$ in Eq. (5) can be replaced by $[L^-]_0$. From the curves it was found that the retardation by acetate ion was ascribed to the formation of copper tetraacetate. Thus, the complexing ability of anion is an important factor affecting the oxidation rate.

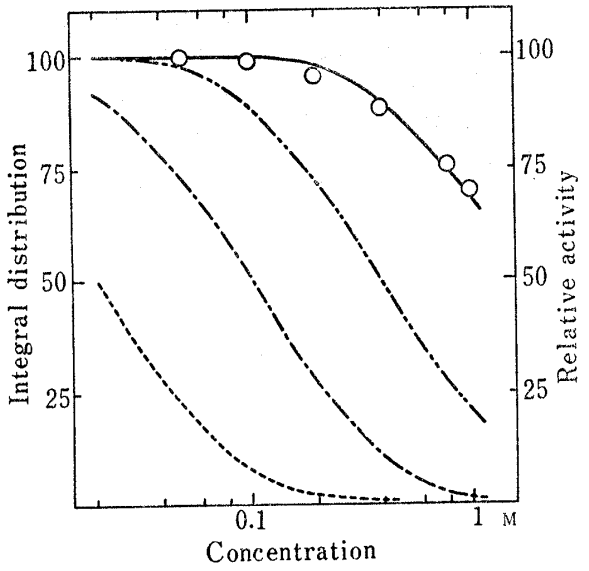

Fig. 7. Integral Distribution Curve of the Composite Copper Acetate Complexes and the Relative Catalytic Activity

----------: $[Cu^{2+}]/[Cu^{2+}]_0$
— — — —: $([Cu^{2+}]+[CuL^+])/[Cu^{2+}]_0$
— · — · —: $([Cu^{2+}]+[CuL^+]+[CuL_2])/[Cu^{2+}]_0$
————: $([Cu^{2+}]+[CuL^+]+[CuL_2]+[CuL_3^-])/[Cu^{2+}]_0$
β_1: 47 β_2: 450 β_3: 1170 β_4: 760
○: measured rate
experimental details as under Fig. 3

The copper ascorbate complex, being unstable, may be dissociated to cuprous ion and the ascorbate free radical, which might be changed spontaneously to dehydroascorbic acid. This reaction involving probably one electron transfer has been considered as the rate-determining step. Cuprous ion generated in this step, being very unstable in the aerobic condition, is oxidized spontaneously to cupric ion. Thus, copper ion is turned over very rapidly between cupric and cuprous states during the reaction as if there exists an equilibrium depending on the redox potential of the copper complex.

The retardation by halide, thiocyanate and cyanide ions is ascribed to the stabilization of the cuprous complex, by which the reoxidation of cuprous ion is hindered. In order to explain the reoxidation of the cuprous complex, it would be conventional to postulate the formation of perhydroxy radical $\cdot O_2H$ or superoxide ion $\cdot O_2^-$ as the result of one electron transfer from cuprous ion to oxygen. However, considering the redox potential ($E^\circ = -0.32$ V for $O_2/\cdot O_2H$ at $[H^+]=1$, and $E^{\circ\prime} = -0.45$ V for $O_2/\cdot O_2^-$ at pH 7),[15] the formation of perhydroxy radical or superoxide ion may be extremely unlikely, though it could not be exclude experimentally. If those peroxides were produced, the electron transfer between those peroxides and cuprous ion or the ascorbate radical would be convenient because of their high reactivities ($E^\circ = 1.68$ V for $\cdot O_2H/H_2O_2$ at $[H^+]=1$, and $E^{\circ\prime} = 0.98$ V for $\cdot O_2^-/H_2O_2$ at pH 7).[15] A thermodynamically feasible oxidation of cuprous ion is the following reaction, which involves two electron transfer.

$$2Cu^+ + O_2 + 2H^+ \rightleftarrows 2Cu^{2+} + H_2O_2$$

$E^0 = 0.68$ V for O_2/H_2O_2 at $[H^+]=1$

$E^{0\prime} = 0.27$ V for O_2/H_2O_2 at pH 7

14) S. Fronaeus, "Komplex system hos Kopper," (Diss.) Lund (1948).
15) P. George, "Oxidase and Related Systems," ed. by T.E. King, H.S. Mason and M. Morrison, John Wiley and Sons, Inc., New York, 1965, p. 3.

Concerning this reaction mechanism, Frieden postulated that a binuclear copper compex in which two copper ions are bridged by one molecular oxygen would be formed as a reaction intermediate.[16]

Another factor controlling the oxidation of cuprous halide may be the formation of a binuclear complex. In the presence of both cupric and cuprous ions, it has been shown that halide ion bridges between cupric and cuprous ions to form a binuclear complex Cu^{2+}-X-Cu^{+}, which has the similar skeleton to the oxygen bridged complex.[17] Since both cupric and cuprous ions should be presented in the reaction mixtures, the binuclear complex may be formed in the course of the oxidation. For the formation of the binuclear complex, halide ion would compete with oxygen molecule. If the stability constant of the halogen bridged complex, which would be related to K in Eq. (3), is higher than that of the oxygen bridged complex, the reoxidation of cuprous ion would be retarded.

16) E. Frieden, S. Osaki and H. Kobayashi, *J. Gen. Physiol.*, **49**, 213 (1965).
17) S.B. Watkins and H.G. Denham, *J. Chem. Soc.*, **115**, 1269 (1919); H. McConnell and N. Davidson, *J. Am. Chem. Soc.*, **72**, 3164 (1950).