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Accelerating Effect of Histidylhistidine on the Copper(II)-Catalyzed Oxidation of Ascorbic Acid

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The effect of histidylhistidine on the copper(II)-catalyzed oxidation of ascorbic acid was investigated kinetically by the polarographic technique. At pH 3.8 histidylhistidine had an accelerating effect on the copper(II)-catalyzed oxidation. In contrast, at pH above 5.0 it exerted an inhibitory effect. The visible and CD spectra of the copper(II)-histidylhistidine system obtained at pH 3.8 and 5.9 indicate that different types of complex species are present predominantly at each pH. That is, at the former pH copper(II) is linked by a coordinate bond to the N-groups of the two imidazole rings in histidylhistidine, while at the latter pH two other N-groups take part in further bond formation. It appears that lability of two of the four coordinate bonds in the copper(II)-histidylhistidine complexes with respect to ligand substitution is a prerequisite for catalytic activity.

The effects of histidine, imidazole and acetylhistidine on the copper(II)-catalyzed oxidation of ascorbic acid were compared. The results suggest that the accelerating effect of histidylhistidine arises from the two imidazole rings in the molecule.

Keywords——ascorbic acid; oxidation; histidylhistidine; copper(II)-catalyzed oxidation; accelerating effect of histidylhistidine; copper(II) complexes; imidazole; histidine; acetylhistidine; polarography

The copper(II)-catalyzed oxidation of ascorbic acid has been investigated by many authors in relation to the catalytic action of metal ions occurring in biological systems. One of the most fascinating and far-reaching aspects of this oxidation is its use as a model reaction for the oxidative decomposition of ascorbic acid by copper(II)-containing oxidase enzymes. The catalytic activity of copper(II) is affected by complexing with a variety of ligands.[2]

Various chelating agents, including aminopolycarboxylic acids[3] and flavonoids,[4] are known to inhibit the metal-catalyzed oxidation of ascorbic acid. On the other hand, enhancement of the catalytic activity of metals has been observed only in a few cases.[5] Recently, it has become apparent that polymer ligands such as polyvinylpyridine[6] and polyhistidine[7] cause the catalytic activity of copper(II) ions to increase markedly. Pecht *et al.*[7a] confirmed this by comparing the accelerating effect of histidine dimer with that of polyhistidine.

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3) *a*) I. Onishi and T. Hara, *Bull. Chem. Soc. Japan*, **37**, 1317 (1964); *b*) M.M. Taqui Khan and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 7104 (1967).

4) *a*) W. Heimann and B. Heinrich, *Fetle Seifen Anstrichmittel*, **61**, 1024 (1959); *b*) D. Nomura and Y. Oda, *Hakko Kogaku Zasshi*, **6**, 309 (1962); *c*) K.A. Harper, A.D. Morton, and E.J. Rolfe, *J. Food Technol.*, **4**, 255 (1969); *d*) A.J. Shvikhande and F.J. Francis, *J. Food Sci.*, **39**, 904 (1974); *e*) K. Takamura and M. Ito, *Chem. Pharm. Bull.*, **25**, 3218 (1977).

5) *a*) V.S. Butt and M. Hallaway, *Arch. Biochem. Biophys.*, **92**, 94 (1961); *b*) S. Isaka, *Nature* (London), **179**, 578 (1957).

6) *a*) N.A. Vergerova, Yu. E. Kirsh, and V.A. Kabanov, *Vysokomol. Soedin.*, **A13**, 2509 (1971); *b*) N.A. Vergerova, N.N. Kukoshina, Yu. E. Krish, and V.A. Kabanov, *ibid.*, **A15**, 773 (1973).

7) *a*) I. Pecht and M. Anbar, *Nature* (London), **207**, 1386 (1965); *b*) I. Pecht, A. Levitzki, and M. Anbar, *J. Am. Chem. Soc.*, **89**, 1587 (1967).

However, it was not explained why even low polymers such as histidylhistidine exert an accelerating effect to some extent. It should be useful to study the structure-accelerating effect relationship of simple peptides in order to identify the factors which are responsible for the enhancement of the catalytic activity.

The present work was undertaken to investigate the effect of histidylhistidine on the copper(II)-catalyzed oxidation of ascorbic acid.

Experimental

Reagents and Preparation of Solutions——Acetate buffer solution of pH 3.8 or 5.3 was used as the base electrolyte in the present work. The stock solution (0.1 M) of ascorbic acid was prepared by dissolving 0.1761 g of reagent grade *l*-ascorbic acid (Wako Pure Chemical Industries, Ltd.) in 10 ml of 0.1 M $HClO_4$ because of the stability of ascorbic acid in a strongly acidic medium. The concentration of ascorbic acid in the test solution was adjusted to 1.0×10^{-3} M by pipetting the stock solution into the base electrolyte solution. A stock solution (0.1 M) of copper(II) ions was prepared by dissolving $CuSO_4\cdot5H_2O$ in redistilled water and standardized by titration with EDTA. *l*-Histidylhistidine was synthesized by using N,N′-dicyclohexylcarbodiimide (DCC) as a coupling reagent. Histidine was obtained from Wako Pure Chemical Industries, Ltd., and the other peptides were purchased from Sigma Chemical Company. All other chemicals were of reagent grade and were used without further purification.

Apparatus——Direct current (D.C.) polarograms were recorded on a Yanagimoto pen-recording polarograph, model P8, in the usual manner. All potentials in this paper are relative to a saturated calomel electrode (SCE). Visible spectra were recorded on a Hitachi 200-10 spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J-500C spectropolarimeter.

All the measurements were carried out at 25°.

Kinetic Measurements——It is known that in the absence and presence of copper(II) ions the oxidation of ascorbic acid (reaction (2) in Chart 1) follows first-order kinetics with respect to the concentration of ascorbic acid under conditions of oxygen saturation.[8]

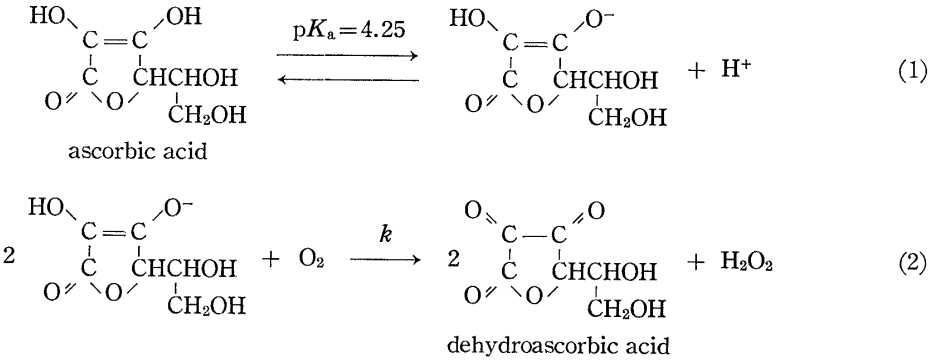


Chart 1

Since the dissociation of ascorbic acid to its monoanion hardly occurs in strongly acidic media, the reaction given in eq. (2) scarcely proceeds below pH 3.0. On the other hand, above pH 7.0 the reaction proceeds so rapidly that the present technique can no longer follow the decay of ascorbic acid concentration. Therefore the kinetic measurements were carried out in the pH range from 3.0 to 7.0.

The initial concentration of ascorbic acid in the test solution was fixed at 1.0×10^{-3} M and was determined polarographically by measuring the anodic limiting current at +0.2 V.[4e] Before each run, pure nitrogen gas was bubbled through the test solution to remove dissolved oxygen. Nitrogen gas was also passed continuously above the test solution to prevent recontamination of the solution by air during the electrochemical measurement.

The rate of oxidation of ascorbic acid in the presence of copper(II) ions with or without ligands was followed by measuring the change in the anodic limiting current of ascorbic acid with time. The oxidation was initiated by adding 0.2 ml of the stock solution of ascorbic acid to the air-saturated reaction mixture (20 ml) with the aid of a pipet within 5 sec. The anodic currents were recorded at +0.2 V at appropriate times to obtain current-time curves. Air was supplied by bubbling it into the solution at a constant flow rate during the reaction, except when the current was being recorded.

8) M.M. Taqui Khan and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).

The anodic limiting current (i_d) directly corresponds to the concentration of ascorbic acid remaining in the reaction mixture, so that the consumption of ascorbic acid during the period of the oxidation can be traced by measurement of the time-dependence of i_d. A plot of log C_0/C *vs* t (C_0 and C are the concentrations of ascorbic acid at time 0 and time t, respectively) was found to be linear, and the first-order rate constant (k) corresponding to reaction (2) was calculated from the slope of the plot.

Results and Discussion

Catalytic Oxidation of Ascorbic Acid in a System containing Copper(II) and Histidylhistidine

The kinetic measurements of the copper(II)-catalyzed oxidation of ascorbic acid in the presence of histidine and related peptides in amounts equimolar with respect to ascorbic acid were made at pH 3.8, and the rate constants for reaction (2) are compared in Table I. Polyhistidine and histidylhistidine caused the catalytic activity of copper(II) in the oxidation of ascorbic acid to increase. The presence of histidine or dipeptides other than histidylhistidine hardly affected the rate constant obtained with the control solution (*i.e.*, containing no peptide). The accelerating effect of polyhistidine can be attributed to the polymer effect,[7b] but it seems difficult to explain the effect of histidylhistidine on the same basis.

Table I. Rate Constants for the Copper (II)-Catalyzed Oxidation of Ascorbic Acid in the Presence of Some Peptides containing Hisitdine
[ascorbic acid] $= 1.0 \times 10^{-3}$ M,
[Cu^{2+}] = [peptide] $= 1.0 \times 10^{-4}$ M, pH 3.8

Peptide	Rate constant k min^{-1}
None	0.076
Polyhistidine	0.143
Histidylhistidine	0.115
Histidine	0.081
Histidylglycine	0.074
Histidylalanine	0.073
Glycylhistidine	0.072
Alanylhistidine	0.071

Table II. Effect of Histidylhistidine on the Oxidation Rate of Ascorbic Acid at Various pH's
$k_{His-His}$: the rate constant in an equimolar solution of Cu^{2+} and histidylhistidine.
k_0: the rate constant in the absence of histidylhistidine.

pH	3.8	4.2	5.3	5.9
$(k_{His-His}-k_0)/k_0 \times 100\%$	+51	+20	−18	−55

The oxidation rate of ascorbic acid obtained in the presence of copper(II) ions and histidylhistidine depended on pH, as shown in Table II. In this table, the magnitude of the change in the rate constant caused by histidylhistidine is given in the form $(k_{His-His}-k_0)/k_0$, where $k_{His-His}$ and k_0 are the rate constants in the presence and in the absence of histidylhistidine, respectively, because k_0 itself increased with pH. The value of $(k_{His-His}-k_0)/k_0$ decreased with increase in pH. At pH above 5, an inverse effect appeared, that is, histidylhistidine caused inhibition of the copper(II)-catalyzed oxidation.

The ligand effect on the copper(II)-catalyzed oxidation of ascorbic acid has generally been explained on the basis of changes in the nature of the bonding between copper(II) and

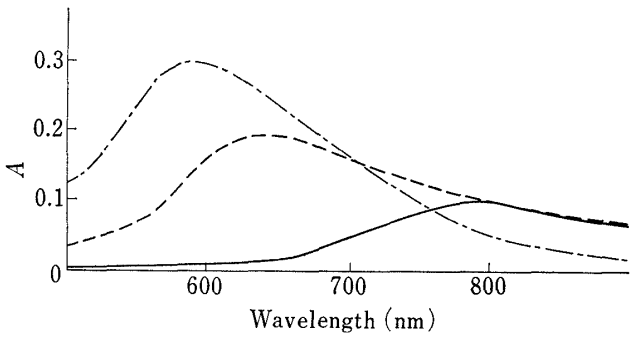


Fig. 1. Visible Spectra of a Solution Containing 5.0×10^{-3} M Copper (II) Ion and 5.0×10^{-3} M Histidylhistidine at pH 5.9 (—·—) and pH 3.8 (----)

The solid line represents the spectrum obtained without histidylistidine at pH 3.8.

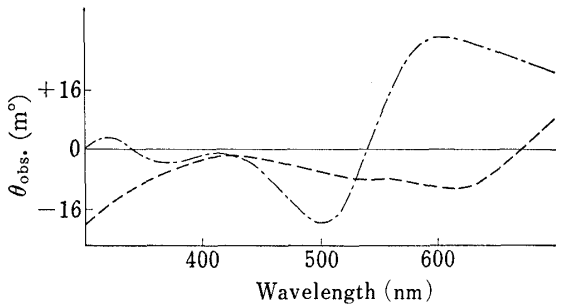


Fig. 2. Circular Dichroism Spectra of a Solution Containing 5.0×10^{-3} M Copper (II) Ion and 5.0×10^{-3} M Histidylhistidine at pH 5.9 (—·—) and pH 3.8 (----)

ligands.[7a] Therefore the kinetic properties of the catalytic oxidation of ascorbic acid in the system involving copper(II) ion and histidylhistidine are expected to be affected by differences in the complex species formed by copper(II) and histidylhistidine at different pH's. The existence of such species was confirmed by direct comparison of the visible and CD spectra of an equimolar solution of copper(II) ions and histidylhistidine obtained at pH 3.8 with those obtained at 5.9. The data are shown in Figs. 1 and 2. The solution exhibited an absorption maximum at 645 nm at pH 3.8, while the maximum shifted to 595 nm and became larger at pH 5.9 (Fig. 1). The spectral change with pH was even more marked in the CD spectra. As shown in Fig. 2, two negative and positive bands appeared distinctly around 500 nm and 605 nm at pH 5.9, whereas the broad band lay to the negative side at pH 3.8. Those data suggest different modes of complex formation between copper(II) and histidylhistidine depending upon pH.

The continuous variation plot was applied to the copper(II)-histidylhistidine system at pH 3.8 to determine the composition of the complex formed, using the absorbances at 640 nm (Fig. 3). The results indicate the presence of only a 1:1 complex. Since the dissociation of a proton in the α-amino group of histidylhistidine ($pK_a=7.92$[9]) scarcely occurs at pH 3.8 in comparison with that of the imidazole rings ($pK_a=5.36$ and 6.70[9]), the structure illustrated in Chart 2A seems reasonable for the 1:1 complex of copper(II)-histidylhistidine at this pH. This structure is supported by the data on the potentiometric titration of Cu^{2+} and histidylhistidine with base reported by Martell *et al.*,[9] *i.e.*, the amount of base added per mole of histidylhistidine present was 3 moles at pH around 4. On the other hand, they proposed the structure illustrated in Chart 2B for the predominant complex at pH above 5.6, in which copper(II) is linked by coordinate bonds to three or four N-groups of histidylhistidine.[9] It

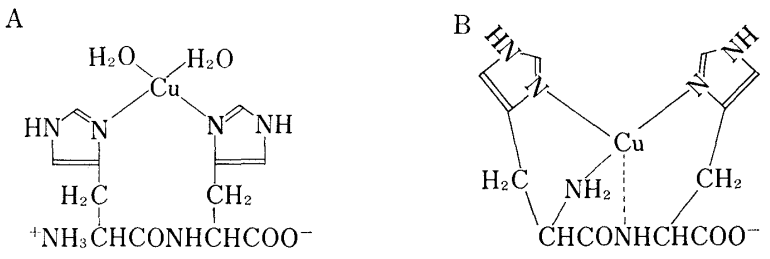


Chart 2

9) M.A. Doran, S. Chaberek, and A.E. Martell, *J. Am. Chem. Soc.*, **86**, 2129 (1964).

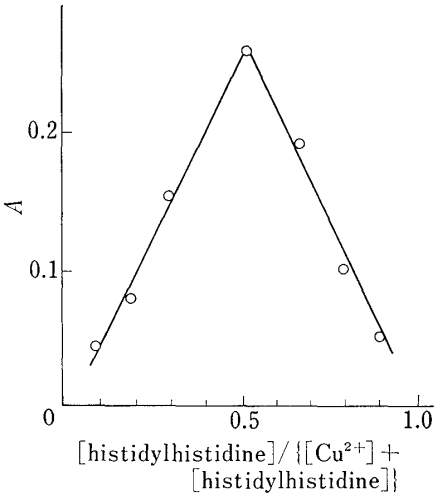


Fig. 3. Continuous Variation Plot for the Copper(II)-Histidylhistidine System at pH 3.8

$[Cu^{2+}]+[\text{histidylhistidine}]=1.0\times10^{-2}$ M
Absorbance was measured at 640 nm.

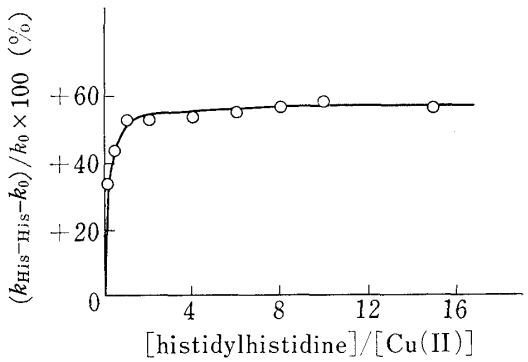


Fig. 4. Dependence of $(k_{\text{His-His}}-k_0)/k_0$ on the Concentration of Histidylhistidine at a Fixed Concentration of Copper (II) Ion and at pH 3.8

can be considered that the accelerating effect is attributable to the complex species shown in Chart 2A, and that the inhibition is attributable to that shown in Chart 2B.

The dependence of the catalytic activity on the concentration of histidylhistidine at a fixed copper(II) ion concentration and at pH 3.8 is shown in Fig. 4. The $(k_{\text{His-His}}-k_0)/k_0$ value increases with increase in the concentration ratio of histidylhistidine to copper(II) up to about unity and then tends to a limiting value. The results suggest that the enhancement of the catalytic activity for the oxidation of ascorbic acid under the present conditions is closely associated with the presence of the copper(II)-histidylhistidine complex.

The mechanism of the catalytic oxidation of ascorbic acid by the copper(II)-histidylhistidine complex seems to be essentially the same as that in the case catalyzed by simple copper(II) ions proposed by Taqui Khan and Martell.[8] This is because in the present experiment too, the rate of oxidation was dependent on the initial amount of oxygen, and hydrogen peroxide was detected as a reaction product. The following reaction scheme can be set down:

$$HA^- + CuL^{2+} \overset{K_1}{\rightleftharpoons} HA\,CuL^+ \quad (3)$$

$$HA\,CuL^+ + O_2 \overset{K_2}{\rightleftharpoons} HA\,CuL(O_2)^+ \quad (4)$$

$$HA\,CuL(O_2)^+ \overset{k}{\longrightarrow} HA\,CuL(\dot{O}_2)^+ \quad (5)$$

$$HA\,CuL(\dot{O}_2)^+ \overset{\text{fast}}{\longrightarrow} A + CuL^+ + H\dot{O}_2 \quad (6)$$

$$CuL^+ + H\dot{O}_2 + H^+ \overset{\text{fast}}{\longrightarrow} CuL^{2+} + H_2O_2 \quad (7)$$

where HA^- represents the ascorbate anion, and L is histidylhistidine.

Mixed ligand complexes of copper(II) involving ascorbate anion are formed in the pre-equilibrium steps, as shown in eq. (3) and eq. (4). These steps are followed by a rate-determining electron transfer step within the mixed ligands complex (eq. (5)).[3b,8] Accordingly, it appears quite likely that lability of two of the four coordinating bonds in the copper(II)-histidylhistidine complex for ligand replacement is a prerequisite for the catalytic activity.

Table III. Effects of Imidazole, Histidine and Acetylhistidine on the Copper (II)-Catalyzed Oxidation of Ascorbic Acid
$[Cu^{2+}]=1.0\times10^{-4}$ M, [ascorbic acid] $=1.0\times10^{-3}$ M, pH 5.3

$[X]/[Cu^{2+}]^{a)}$	$(k_x-k_0)/k_0$ ($\times100\%$)		
	Im	His	Ac-his
1	+7	+4	+2
2	+17	−13	+39
4	+30		+41
10	+50	−53	+45
40	−14		
100	−20		

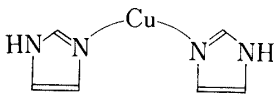
a) X=Im (imidazole), His (histidine), Ac-his (acetylhistidine).

Thus, one would expect the complex shown in Chart 2A to be much more favorable for the catalytic oxidation of ascorbic acid than that in Chart 2B.

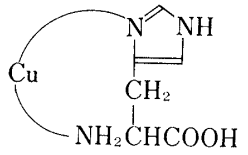
Structural Features of Histidylhistidine contributing to the Accelerating Effect

It is of interest to identify the major structural feature of histidylhistidine responsible for the accelerating effect. Thus, the effects of the constituent groups of histidylhistidine, such as histidine and imidazole, were examined. The results are shown in Table III. The rate constants were measured in the presence of imidazole and histidine at various concentration ratios to copper(II) ions in the test solution at pH 5.3. In the case of imidazole, an accelerating effect became clear in the presence of more than two equivalents of imidazole relative to copper(II) ions, and attained a maximum at about ten equivalents. However, upon addition of a large excess of imidazole (about 40 equivalents) the value of $(k_{\text{imidazole}}-k_0)/k_0$ became negative, that is, the oxidation rate decreased below that obtained without any additive.

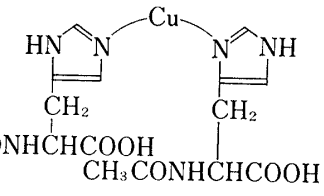
In the copper(II)-imidazole system, complex species with compositions from 1:1 to 1:4 are expected to form consecutively as the pH or imidazole concentration increases. Edsall *et al.*[10] reported that the average coordination number of imidazole molecules per one copper(II) is 2.19 in the presence of about twelve equivalents of imidazole relative to copper(II) ions at pH 5.13. Accordingly, with ten equivalents of imidazole at pH 5.3 (conditions giving the maximum catalytic effect), the 1:2 species of copper(II)-imidazole complex (Chart 3A) would be predominant. This view is also supported by the results of calculation using the formation constants[11] given in Chart 3. The results in Table III thus indicate that the existence of the

A 

k_{f1} 4.20[11)]
k_{f2} 3.42
k_{f3} 2.88
k_{f4} 2.05

B 

k_{f1} 10.56[12)]
k_{f2} 8.26

C 

k_{f1} 4.35[13)]
k_{f2} 3.40
k_{f3} 2.55

Chart 3

10) J.T. Edsall, G. Felsenfeld, D.G. Goodman, and F.R.N. Gurd, *J. Am. Chem. Soc.*, **76**, 3054 (1954).
11) W.L. Koltun, *J. Am. Chem. Soc.*, **80**, 4188 (1958).

1:2 complex is essential for the accelerating effect. Further increase in the imidazole concentration alters the predominant complex species in the system to the 1:3 or 1:4 complex. The data in Table III suggest that such complexes have an inhibitory effect.

Histidine showed only a slight accelerating effect at lower concentration but inhibition became clear at higher concentrations. In the former case, the 1:1 complex species (Chart 3 B) may be predominant,[9] in which copper(II) keeps two orbitals available for further coordination of ascorbic acid. However, even in this case hardly any accelerating effect was observed. In the latter case, an increase in the concentration of the 1:2 complex will reduce the catalytic activity of copper(II) for the oxidation of ascorbic acid.

Overall, it seems quite likely that the first requisite for enhancement of the catalytic activity is the presence of two imidazole groups bonded to copper(II), keeping further coordination positions available for ascorbic acid. In order to confirm this, the effect of acetylhistidine was examined for comparison. The results are shown in the last column of Table III. Acetylhistidine has one imidazole ring and behaves as a monodentate ligand in the lower pH region because of blocking by the acetyl group attached to the side chain amino group. Since the formation constants of copper(II)-acetylhistidine complexes[13] are approximately equal to those of copper(II)-imidazole complexes, the addition of acetylhistidine should cause an accelerating effect similar to that of imidazole. As expected, the enhancement of the catalytic activity of copper(II) was pronounced in the concentration range from two to ten equivalents of acetylhistidine relative to copper(II) ions, at which the 1:2 type of copper(II)-acetylhistidine complex (Chart 3C) should be predominant.

In conclusion, the present results suggest that the accelerating effect of histidylhistidine arises from the two imidazole rings in the molecule.

Based on the assumption that the oxidation of ascorbic acid proceeds through the reaction pathway given in eqs. (3)—(7), it can readily be seen that the values of both K_1 and k are characteristic of the added ligand (L). Thus it can be presumed that the coordination of imidazole groups causes copper(II) to exhibit a higher affinity for the ascorbate anion compared with hydrated copper(II) and also leads to stabilization of the lower valent copper in the transition state. Such effects of the imidazole groups may be explained in terms of electron back donation from the metal ion to the π orbitals of the heterocyclic aromatic ligands containing N atoms.

Ligand effects similar to the above have actually been found for some complexes. For example, copper-bipyridyl complex forms stable ternary complexes with oxygen-containing ligands such as oxalic acid or pyrocatechols.[14] The divalent state of iron is stabilized through complexation with bipyridyl and phenanthroline compared to its trivalent state.[15] The contribution of bipyridyl or phenanthroline to such effects was interpreted on the basis of electron back donation.[14,15] The imidazole effect observed in the present experiments may therefore be explicable similarly.

12) R. Leberman and B.R. Rabin, *Trans. Faraday Soc.*, **55**, 1660 (1959).
13) R.B. Martin and J.T. Edsal, *J. Am. Chem. Soc.*, **82**, 495 (1960).
14) R. Griesser and H. Sigel, *Inorg. Chem.*, **9**, 1238 (1970); H. Sigel, P.R. Huber, R. Griesser, and B. Prijs, *Inorg. Chem.*, **12**, 1193 (1973).
15) D.P. Graddn, ed., "An Introduction to Coordination Chemistry," Pergamon Press, London, 1961, p. 67.