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Autoxidation of Cysteine catalyzed by Copper in Glycylglycine Buffer

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Autoxidation of cysteine catalyzed by Cu(II) in glycylglycine buffer was examined spectrophotometrically. Upon mixing the Cu(II) and cysteine solutions was produced a transient purple color, due to a Cu(II)-cysteine complex, giving a broad electronic spectrum with $\lambda_{max} = 530$ nm. The purple Cu(II) complex, which is decomposed fleetingly to Cu(I) species, is stabilized relatively as pH increases. The autoxidation of cysteine does not exhibit Michaelis-Menten kinetic behaviors, and appears to be half order in concentration of cysteine over pH 7.3. Increasing concentrations of oxygen in the medium promote the autoxidation. As cysteine is autoxidized, hydrogen peroxide is produced and partially accumulated. The plot of the reciprocal of the rate as a function of the reciprocal of the oxygen concentration gives a straight line. One molar oxygen participates probably in a step of the reoxidation of the Cu(I) species. A pH-rate profile of the autoxidation displays a bell-shaped curve with a maximum at pH 7.4.

In the metal ion-catalyzed autoxidation, a catalyst, M^{n+}, associates first with a substrate, S, to form an intermediate, M^{n+}—S,[2,3] undergoing subsequent decomposition to yield products and an inactive species, $M^{(n-1)+}$, which is soon autoxidized to the active catalyst.[3] The metal ion is thus turned over between the oxidized and reduced states during the reaction. A rate determining step in the autoxidation is generally dissociation of M^{n+}—S, and thereupon the kinetics of overall reaction is treated with the Michaelis-Menten equation.[4] If the substrate possesses an ability to complex strongly with $M^{(n-1)+}$, as well as M^{n+}, the autoxidation of $M^{(n-1)+}$ is interrupted considerably. As a result, the overall reaction is retarded and the kinetics becomes complicate. A typical example of the complicated reactions is the copper-catalyzed autoxidation of cysteine.

In a previous paper, we reported a study of the autoxidation of cysteine by Cu(II) using a conventional Warburg manometric technique.[5] The determination of the rate of oxidation by the method of measuring oxygen uptake has been the subject of various investigations.[6] A manometric study of the cysteine oxidation reveals that an amount of oxygen uptake is not related stoichiometrically with an amount of cysteine decomposed and varies depending upon pH and concentration of the substrate.[5] Then, the rate of oxygen uptake corrected relative to the total amounts of oxygen uptake was used as the rate of the autoxidation.[5] In order to intend to elucidate unequivocally the kinetics and mechanism of the autoxidation, the reaction should be examined by monitoring the substrate or the products. In the present work, decrease of cysteine was followed spectrophotometrically. The present paper deals with a general and overall feature of the copper-catalyzed autoxidation of cysteine.

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Experimental

Materials——A stock solution of Cu(II) ion was prepared from copper foils of 99.999% purity (Kishida Chemicals & Co. Ltd.), which was weighed out accurately and dissolved in a small amount of conc. HNO_3. The $Cu(NO_3)_2$ solution thus prepared was diluted to a desired concentration with purified water, once deionized and twice distilled with an all glass apparatus. If necessary, the solution was standardized against ethylenediaminetetraacetic acid with a conventional complexometric titration method.[7] L-Cysteine hydrochloride monohydrate (Nutritional Biochemicals Co.) and 5,5′-dithiobis(2-nitrobenzoic acid), abbreviated as DTNB, (Wako Pure Chemical Industries, Ltd.) were used without further purification. Cysteine hydrochloride weighed out accurately was dissolved in 0.1 N KNO_3 and adjusted pH with 0.1 N KOH. The cysteine solution was prepared freshly before a kinetic run.

Spectrophotometric Measurement——Electronic spectrum of the intermediate was obtained on a Hitachi RSP-2 rapid scan spectrophotometer using a rapid-mixing and con- tinuous flow technique. The measurement was carried out under nitrogen at 20°.

Kinetic Procedure——A 100 ml beaker with a water-jacket was used as a reaction vessel. The reaction was carried out at 20° under aerobic or unaerobic conditions. The solution cont aining all the reactants except cysteine was pre-equilibrated in an appropriate circumstance. After 30 min of the equilibration, the reaction was initiated by adding the cysteine solution. Before and during the reaction, the solution was agitated vigorously with a magnetic stirrer and oxygen or oxygen-nitrogen mixture was bubbled at a rate of 100 ml/min. An aliquot was pipetted from the reaction mixture at regular intervals and applied to the determination of the substrate or the product. Determination of cysteine was done, using a Hitachi 101 spectrophotometer, with DTNB at 415 nm ($\varepsilon=13200$)[8] and that of hydrogen peroxide with $TiCl_4$ at 410 nm ($\varepsilon=680$).[9] Plots of the decrease of cysteine against time gave a straight line, from which the rate of the autoxidation of cysteine was calculated, for over 60% reaction. Oxygen dissolved in the medium was deter mined before a kinetic runw ith a Beckman Fieldlab oxygen analyzer, which was calibrated against air-saturated water.

Results and Discussion

When a Cu(II)-glycylglycine solution is added to an excess of cysteine in a neutral or alkaline medium, a transient purple color, which is due to a paramagnetic Cu(II)-cysteine complex, is produced immediately.[10] The electronic spectrum of the purple complex in the visible region, displaying a maximum at 530 nm, possesses characteristic properties differing apparently from the spectrum of the Cu(II) complex of amino acid. Production of a transient purple color has been observed upon mixing the solutions containing Cu(II) ion and thiol compound. The purple color produced in the interaction of Cu(II) ion with penicillamine[11] or with mercaptosuccinic acid[12] is relatively stable, which are a few exceptions. The spectra resulting from the Cu(II)-cysteine interaction for first 100 msec are shown in Fig. 1. The time course of the interaction under nitrogen, monitored by OD_{530}, is shown in Fig. 2. The complex formation, *i.e.*, the ligand substitution, of Cu(II) is generally extremely rapid.[13] In the Cu(II)-cysteine interaction, the formation of the purple complex is also very rapid. A considerable color change was observed during a dead time of the instrument, *i.e.*, 3 msec. The following decrease in OD_{530} is resulted from the decomposition of the Cu(II) complex to colorless Cu(I) species. The purple complex appeared to be stabilized relatively in accordance with an increase of pH.

As regard the oxidation of cysteine by Cu(II)-glycylglycine under nitrogen, the reaction sequence may be that given in eq (1a) and (1b)

$$Cu(II)^{n+} + CySH \rightleftarrows Cu(II)\text{–}SCy^{(n-1)+} + H^+ \quad (1a)$$

$$Cu(II)\text{–}SCy^{(n-1)+} \longrightarrow Cu(I)^{(n-1)+} + \cdot SCy \quad (1b)$$

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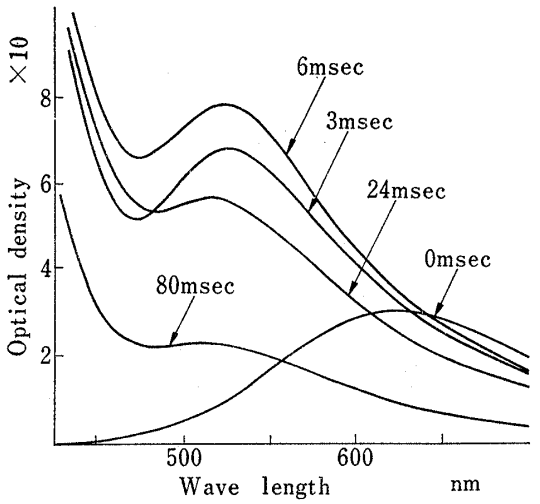

Fig. 1. Electronic Spectra Resulting from the Cu(II)-Cysteine Interaction at pH 7.5 and at 20° under Nitrogen

[Cu(II)]=3.5×10^{-3}M; [glycylglycine]=3.5×10^{-2}M; [cysteine]=3.5×10^{-3}M; ionic strength=0.25 (KNO_3)
The spectrum at 0 msec is due to Cu(II) glycylglycinate.

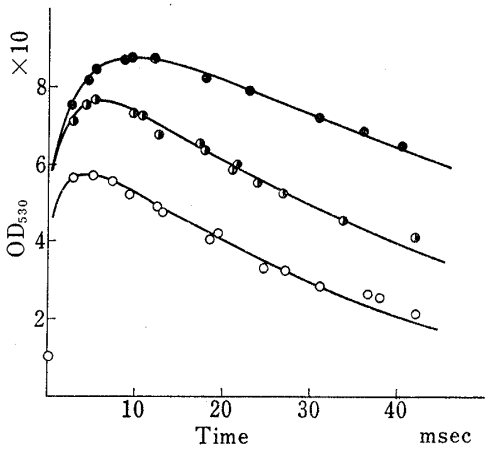

Fig. 2. Time Course of the Cu(II)-Cysteine Interaction at Different pH Values

○: pH 7.0, ◑: pH 7.5, ●: pH 8.0
experimental details as under Fig. 1

where CySH and ·SCy represent cysteine and its free radical respectively. $Cu(II)^{n+}$ and $Cu(I)^{(n-1)+}$ represent Cu(II) and Cu(I) species respectively. Under the experimental condition in Fig. 1 and 2, where glycylglycine used as a buffer is present excessively and the reaction is observed under nitrogen, the Cu(II) species in Eq (1a) corresponds to Cu(II) glycylglycinate and the Cu(I) species in Eq (1b), which is probably thermodynamically stable Cu(I) monocysteinate,[14] does not undergo reoxidation. Then, turnover of copper ion does not occur. The intermediate, the purple complex abbreviated as $Cu(II)\text{-}SCy^{(n-1)+}$, appears fleetingly. Accordingly, the steady state of the reaction could not be observed.

When the reaction is observed under aerobic conditions, the Cu(I) species is reoxidized, copper ion is turned over, and the autoxidation of cysteine is promoted. Generally, divalent transition metal ions prefer complexing with cysteine to complexing with glycylglycine. The regenerated Cu(II) ion may complex preferably with cysteine. Accordingly, under aerobic conditions, $Cu(II)^{n+}$ in the steady state is considered to be mainly Cu(II) monocysteinate. This possibility is indicated in the experiment of the autoxidation in an alkaline medium. Addition of excess cysteine to a solution of Cu(II) ion in 0.1N sodium hydroxide results in an immediate appearance of a yellow color with $\lambda_{max}=330$ nm, which is identified with Cu(II) dicysteinate.[3] In the Cu(II)-cysteine interaction in glycylglycine buffer, production of a transient yellow color with $\lambda_{max}=ca.$ 340 nm was observed. The optical absorptivity of the yellow color is larger, more than fifty times, than that of the purple color. A detailed study of the yellow species is now in progress.

The effect of concentration of cysteine on the rate of the autoxidation apperas entirely complicate. First, Michaelis-Menten kinetics is not observed in the autoxidation. If the reaction obeys Michaelis-Menten kinetic behaviors,[15] the plot of the reciprocal of the rate as a function of the reciprocal of the substrate concentration should give a straight line. However, as shown in Fig. 3, the rate of the autoxidation, when plotted according to the Lineweaver-Burk treatment,[16] does not yield a straight line. Second, as the initial concentration of cysteine increases, the half life of the reaction is lengthened, though the rate is of course increased.

14) W. Stricks and I.M. Kolthoff, *J. Am. Chem. Soc.*, **73**, 1723 (1951).
15) L. Michaelis and M.L. Menten, *Biochem. Z.*, **49**, 333 (1913).
16) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

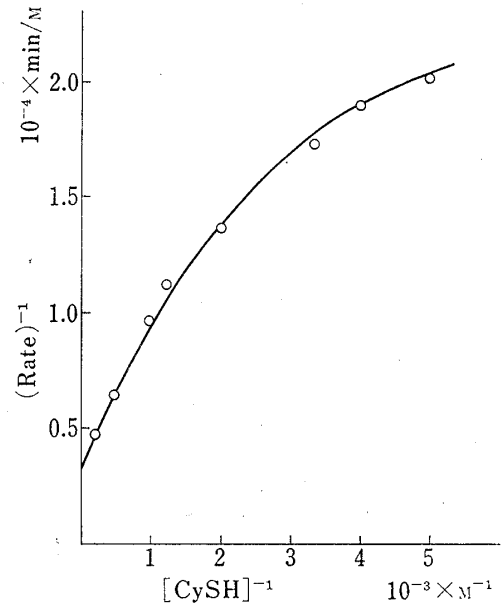

Fig. 3. Plot of the Rate of Oxidation and Concentration of Cysteine at pH 7.4 and at 20° under Oxygen

[Cu(II)]=1.60×10^{-6}M; [glycylglycine]=8.00×10^{-3}M

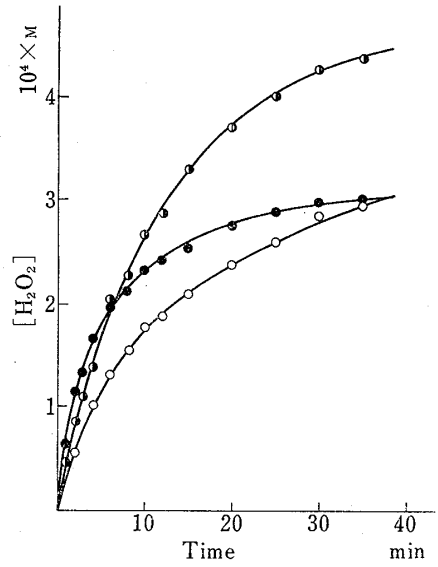

Fig. 4. Time Course of the Production of Hydrogen Peroxide at Different pH Values and at 20° under Oxygen

[Cu(II)]=1.45×10^{-6}M; [glycylglycine]=5.00×10^{-3}M; [cysteine]=2.00×10^{-3}M
○: pH 6.8, ◑: pH 7.0, ●: pH 7.4

TABLE I. Relation between Initial Concentration and Half Life of Cysteine at 20° and at pH 7.4[a)]

10^3 [Cysteine] (M)	Half life (min)	10^3 [Cysteine] (M)	Half life (min)
0.25	2.4	2.00	6.6
0.50	3.5	4.00	9.0
1.00	4.8		

a) [Cu(II)]=1.60×10^{-6}M, [glycylglycine]=8.00×10^{-3}M The reaction was performed under oxygen and at ionic strength 0.1.

An example is shown in Table I. At pH value over 7.3, the half life appears to be linearly proportional to the square root of the cysteine concentration. This indicates that the autoxidation is as a whole one half order in concentration of cysteine. Below pH 7.3, a simple relationship did not exist between the half life and concentration. Thus, increased concentration of cysteine inhibits apparently the autoxidation of cysteine, which has been observed but remains uncertain.[3)]

As cysteine is oxidized, molecular oxygen is consumed and hydrogen peroxide is produced and accumulated. The time course of the peroxide formation at different pH values is presented in Fig. 4. The initial rate of the peroxide formation increases with an increase of pH under the experimental condition below pH 7.5. If cysteine is autoxidized to cystine *via* two electron transfer, one molar hydrogen peroxide is to be produced upon oxidation of two molar cysteine. However, this stoichiometry is not satisfied under any condition of the experiment. The amount of hydrogen peroxide produced and accumulated during the oxidation is less than fifty percent of the theoretical value, and is not correlated with the initial rate of the peroxide formation. As pH increases over 7.2, the amount of the peroxide accumulated decreases gradually. This may be due to the utilization of hydrogen peroxide to oxidize cysteine. In a previous paper, we demonstrated that hydrogen peroxide is utilized to the

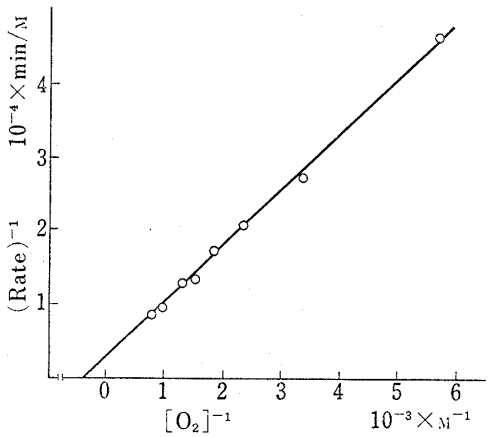

Fig. 5. Plot of the Rate of the Cysteine Oxidation and Concentration of Oxygen Dissolved in the Medium at pH 7.4 and at 20°

[Cu(II)]$=1.60\times10^{-6}$M; [glycylglycine]$=8.00\times10^{-3}$M; [cysteine]$=1.50\times10^{-3}$M

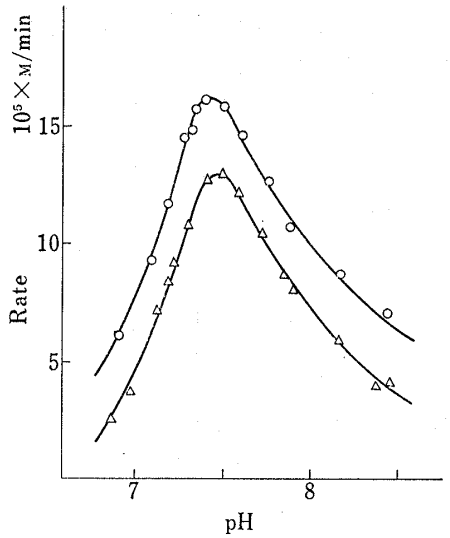

Fig. 6. pH-Rate Profile of the Oxidation of Cysteine at 20° under Oxygen

[Cu(II)]$=1.60\times10^{-6}$M;
○: [cysteine]$=2.00\times10^{-3}$M; [glycylglycine]$=8.00\times10^{-3}$M
△: [cysteine]$=8.00\times10^{-4}$M; [glycylglycine]$=4.00\times10^{-3}$M

noncatalytic oxidation of cysteine and the rate is elevated with an increase of pH.[17] In an alkaline medium, on the contrary, hydrogen peroxide accumulated during the autoxidation is not used to oxidize the excess cysteine and suddenly destroyed when the oxidation has been finished.[18]

The rate of the autoxidation increases with increasing amounts of oxygen dissolved in the medium. Oxygen may play a role in oxidizing $Cu(I)^{(n-1)+}$ to $Cu(II)^{n+}$. The reoxidation of the Cu(I) species by molecular oxygen is a much faster reaction than the reduction of the Cu(II) species by cysteine. Therefore, whenever the steady state is satisfied, concentration of the active catalyst, $Cu(II)^{n+}$, is kept relatively constant. At a fixed concentration of cysteine, a Lineweaver-Burk plot of the rate of the autoxidation against the concentration of oxygen gives a straight line as shown in Fig. 5. It indicates that one molar oxygen participates in the autoxidation of cysteine, probably in a step of the reoxidation of the Cu(I) species. In the reoxidation of the Cu(I) species, molecular oxygen associates with Cu(I) ion or Cu(I) monocysteinate to form an active intermediate, $Cu(I)\text{-}O_2^{(n-1)+}$, undergoing subsequently rapid decomposition to generate the active catalyst, which is expressed by Eq (2a) and (2b)

$$Cu(I)^{(n-1)+} + O_2 \rightleftarrows Cu(I)\text{–}O_2^{(n-1)+} \quad (2a)$$

$$Cu(I)\text{–}O_2^{(n-1)+} \longrightarrow Cu(II)^{n+} + \cdot O_2^- \quad (2b)$$

The superoxide radical anion, $\cdot O_2^-$, is highly reactive and would oxidize cysteine or the Cu(I) species, or would be dismuted to oxygen and hydrogen peroxide, which is a detectable species among the intermediates in the reduction of oxygen.

An intercept at $(\text{rate})^{-1}=0$ of the Lineweaver-Burk plot indicates the reciprocal of the half saturation concentration of oxygen for the autoxidation of cysteine. The half saturation concentration, which is used as an index indicating the extent of dissociation of the intermediate, $Cu(I)\text{-}O_2^{(n-1)+}$, was estimated approximately 2×10^{-3}M at pH 7.4 and at 20°. In the copper-catalyzed autoxidation of ascorbic acid, it was reported to be 2×10^{-4}M.[19] Since

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19) E. Frieden, O. Osaki, and H. Kobayashi, *J. Gen. Physiol.*, **49**, 313 (1965).

the thiol group of cysteine stabilizes preferably Cu(I) ion rather than Cu(II) ion, the reoxidation of the Cu(I) species is interrupted considerably in the presence of an excess amount of cysteine. As a result, a higher value of the half saturation concentration of oxygen was observed in the autoxidation of cysteine.

The rate of the cysteine autoxidation varies depending upon pH, and the pH-rate profile gives a bell-shaped curve with a maximum at pH 7.4 as shown in Fig. 6. The value of pH_{max} was shifted to acidic side as concentration of cysteine increased. Below pH 7.3, a plot of the logarithm of the rate against pH gives a straight line with a slope of approximately unity. In order to explain the bell-shaped profile, at least two rate-controlling steps should be considered. One contributes to the acceleration and another to the retardation. The formation and decomposition of Cu(II)-$SCy^{(n-1)+}$ proceed depending on pH. The reoxidation of Cu(I)-$^{(n-1)+}$, which is estimated from the half saturation concentration of oxygen, might also depend on pH. The pH dependence of those reactions are being investigated.