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The Copper-catalyzed Autoxidation of Cysteine. The Amount of Hydrogen Peroxide Produced under Various Conditions and the Stoichiometry of the Reaction

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Hydrogen peroxide is shown to be produced as an intermediate from oxygen in the copper-catalyzed autoxidation of cysteine. The amounts of cysteine oxidized and of hydrogen peroxide formed varied depending on the reaction conditions employed. The perpxide upon forming was spontaneously utilized for the oxidation of cysteine. As a result, the ratio of the concentration of hydrogen peroxide formed to cysteine oxidized is variable depending on the conditions. But, in dilute solutions, where the oxidation by the peroxide was slow, a stiochiometric relation of 2:1 was obtained between cysteine consumed and hydrogen peroxide produced. The rate of autoxidation is dependent on the concentration of oxygen dissolved. The double reciprocal plot of the rate against the concentration of oxygen gives a straight line, which indicates a possibility of the Michaelis-Menten type mechanism concerning the reoxidation or oxygenation of Cu(I) species.

Copper ion catalyzes effectively the autoxidation of cysteine (CySH) to cystine(CyS–SCy). Typical features of this autoxidation are that the substrate forms very strong complexes with both Cu(I) and Cu(II) ions and the redox process is assumed to take place within a copper thiolate complex.[1] At high pH and in the presence of excess cysteine, a 2:1(CySH/Cu) complex is formed,[2,3] but this complex is not considered as an intermediate; because it is relatively stable under the conditions.[4] Therefore, a 1:1(CySH/Cu) complex, which has not hitherto been detected by spectroscopic methods, is postulated as a reaction intermediate. During the autoxidation, various amounts of H_2O_2 are formed and subsequently decomposed depending on pH.[2,5] Thus, the primary process of the reaction can be written by Eq. 1, but the stoichiometric relation between cysteine consumed and H_2O_2 formed has not been reported so far.

$$2CySH + O_2 \longrightarrow CyS\text{-}SCy + H_2O_2 \quad (1)$$

Aims of the present work are to verify the stiochiometric relation shown in Eq. 1 and to elucidate the role of oxygen in the reaction. In this paper, we wish to report the following results. 1) The rate of autoxidation depends on the concentration of oxygen. The double reciprocal plot of the rate against the concentration of oxygen gives a straight line, which indicates a possibility that the reoxidation or oxygenation process of Cu(I) species obeys the Michaelis-Menten type mechanism. 2) The stiochiometric relation between cysteine and H_2O_2 was proved to be 2:1. The Cu(I) species may be reoxidized *via* a two-equivalent process.

Experimental

A stock solution of Cu(II) ion was prepared from copper foil of 99.999% purity (Kishida Chemical & Co., Ltd.), which was weighed out and dissolved in a small amount of concd HNO_3. The Cu(II) nitrate solution thus prepared was diluted with deionized, doubly distilled water to a desired concentration. The concentration of the Cu(II) solution was determined by titration with standard 0.01 M EDTA (1M=1 mol dm^{-3}) in ammonium-ammonia buffer at pH 10 with murexide as an indicater[6]. Cysteine, 5,5′-dithiobis(2-nitrobenzoic acid), abbreviated as DTNB, $TiCl_4$ and other reagents were of analytical grade and were used without further purification.

The oxidation was conducted in a 200 ml water-jacketted beacker under pure oxygen or oxygen-nitrogen mixtures (4:1—1:4) at 20 °C. The solution was saturated with oxygen or oxygen-nitrogen mixtures by bubbling for 20 min prior to the kinetic run. The reaction was started by adding the cysteine solution last. Gas bubbling at a rate of 100 ml/min and vigorous agitation by a magnetic stirrer were continued during the reaction. Aliquots were withdrawn at periodic intervals from the reaction mixtures, and applied to the determination of cysteine and H_2O_2. The determination of cysteine and H_2O_2 was carried out spectrophotometrically by using a Hitachi 101 spectrophotometer. Cysteine was determined at 415 nm with DTNB[7], and H_2O_2 at 410 nm with a $TiCl_4$ reagent[8]. The determination of the dissolved oxygen was done before a kinetic run with a Beckman Fieldlab oxygen analyzer, which had been calibrated against air saturated water.

Plots of the decrease of cysteine against time gave a straight line for over 60% reaction. In the kinetic study, the initial rate was calibrated with a graphical method.

Results and Discussion

The oxidation was conducted over the pH range of 6.5 and 7.8. The rate of cysteine autoxidation depended on the concentration of the dissolved oxygen in the medium. Martell *et al.* reported that the rate of the ascorbate oxidation catalyzed by iron chelates was linearly proportional to the concentration of oxygen, and on the basis of this observation they postulated the formation of the ascorbate–Fe(III)–O_2 ternary complex as an intermediate.[9] But, in the autoxidation of cysteine, the rate *vs.* the concentration of oxygen plot did not give a straight line.

Of much importance is that the double reciprocal plot; *i.e.*, the Lineweaver-Burk plot, of the rate against the oxygen concentration gives a straight line as shown in Fig. 1. A series of reciprocal plots for various cysteine concentrations appear to intercept at one point on the abscissa. Only the slope is affected by the variation in the cysteine concentration. The intercept at the abscissa is $-1/K_{0.5}$, where $K_{0.5}$ represents the half saturation concentration of oxygen for the autoxidation. The intercept at the ordinate is $1/V_{max}$,

where V_{max} represents the maximal velocity. The existence of the linear Lineweaver-Burk plot suggests the kinetic association of the metal catalyst and oxygen during the course of the reaction. Probably, the oxygen dissolved in the medium may associate with Cu(I) to form an adduct, Cu(I)–O_2, through which the electron transfer from Cu(I) to oxygen proceeds. When a small amount of EDTA, less than 10^{-4} M, was added to the medium, the reaction was considerably enhanced.[10] In this case, a Lineweaver-Burk plot also gives a straight line, but a series of the plots appear to be parallel. The $K_{0.5}$ value becomes significantly small in the presence of a small amount of EDTA. Those kinetic parameters are summarized in Table 1.

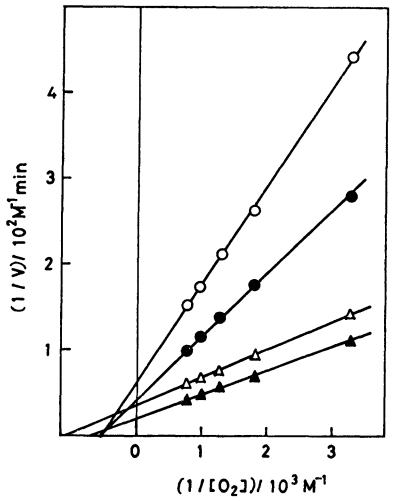
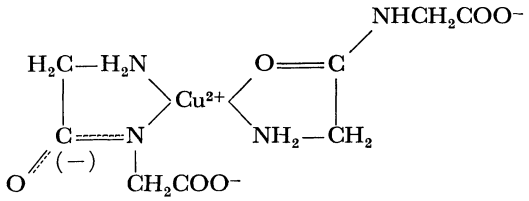


Fig. 1. Double reciprocal plot of the rate against the concentration of the oxygen dissolved in the medium. $[Cu(II)]_0=1.38\times10^{-6}$ M, $[CySH]_0=2.00\times10^{-3}$ M(○) and 4.00×10^{-3} M(●) in the absence of EDTA; $[CySH]_0=2.00\times10^{-3}$ M(△) and 4.00×10^{-3} M(▲) in the presence of EDTA, Buffer: 0.016 M glycylglycine.

The rate of autoxidation was affected by buffers with chelating abilities. Glycylglycine used as a buffer appeared to retard the oxidation, while phosphate did not affect or rather increased the rate of reaction. When the reaction was carried out in buffers containing both glycylglycine and phosphate and the concentration of phosphate was varied, the rate increased significantly with an increase of phosphate ion. This trend is obvious in the pH range of 7—7.5. The examples are shown in Table 2. When the oxidation was conducted in the buffer containing phosphate only, the rate increased approximately 20% as compared with the rate observed in both glycylglycine and phosphate containing buffer. Thus, phosphate is considered to enhance the oxidation.

Since glycylglycine is present excessively; *i.e.*, approximately at 10^4-fold concentration over copper ion, the main species for Cu(II) complex in the medium is $[Cu^{II}(H_{-1}glygly)(glygly)]^-$ ($\lambda_{max}=620$ nm, $\varepsilon=85$ $M^{-1}cm^{-1}$).[11-13] This 2:1 complex competes with cysteine for coordination site on the Cu(II) catalyst. In addition, glycylglycine is present at tenfold concentration over the substrate. Then, the competition



Structure of $[Cu^{II}(H_{-1}glygly)(glygly)]^-$.

Table 1. Kinetic parameters for the autoxidation of cysteine

[Cysteine] / 10^{-3} M	With EDTA		Without EDTA	
	$K_{0.5}$ / 10^{-3} M	V_m / 10^{-4} M min^{-1}	$K_{0.5}$ / 10^{-3} M	V_m / 10^{-4} M min^{-1}
2.00	1.05	3.12	2.13	1.79
4.00	1.43	5.00	2.13	2.70

$[Cu(II)]_0=1.38\times10^{-6}$ M, $T=20$ °C, pH 7.5.

Table 2. Effect of phosphate ion on the enhancement of the rate of cysteine autoxidation

Buffer		Rate/10^{-5} M min^{-1}			
		pH 7.10		pH 7.35	
[Glygly.] / 10^{-2} M	[Phosphate] / 10^{-2} M	Partial pressure of O_2			
		100%	40%	100%	40%
1.6	0	6.58	3.80	6.78	3.74
1.6	0.1	6.68	3.92	6.74	3.58
1.6	0.25	7.70	3.95	8.98	4.90
1.6	0.5	7.88	4.06	12.4	6.52
0	0.5			14.7	7.60
1.6	1.0	8.48	4.10	14.7	7.70
0	1.0			16.9	8.78

$[Cu(II)]_0=1.48\times10^{-6}$ M, $[CySH]_0=2.00\times10^{-3}$ M.

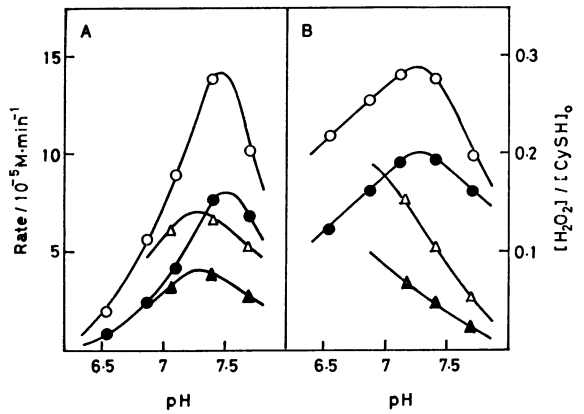


Fig. 2. Effects of buffers on the pH-rate and pH-$[H_2O_2]/[CySH]_0$ Plots.
$[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M, buffer: 0.016 M glycylglycine(△; 100% O_2, ▲; 40% O_2), 0.016 M glycylglycine-0.01 M phosphate(○; 100% O_2, ●; 40% O_2).

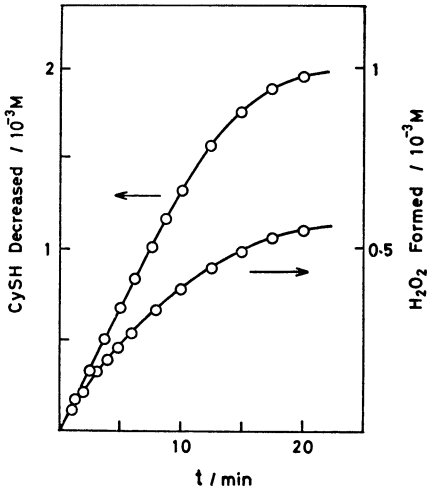


Fig. 3. Time course of the cysteine consumption and the formation of H_2O_2 at pH 7.4.
$[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M, buffer: 0.016 M glycylglycine-0.01 M phosphate, partial pressure of O_2: 100% at 760 mmHg.

of cysteine with glycylglycine; *i.e.*, the ligand substitution from glycylglycine to cysteine in the Cu(II) ion, may be much more retarted. When an equivalent amount of phosphate to glycylglycine is added to the medium, the absorption spectrum of $[Cu^{II}(H_{-1}glygly)(glygly)]^-$ undergoes red shift (λ_{max}=640 nm, ε=83 M^{-1} cm^{-1} at pH 7.1), which suggests that the chelation between Cu(II) and glycylglycine is loosed and is likely dissociated. Under these conditions, the association of cysteine with Cu(II) becomes facilitated and the oxidation is stimulated.

The rate of autoxidation and the amounts of H_2O_2 formed depended on pH. The pH-rate profile gave bell-shaped curves in both buffer systems as shown in Fig. 2A. But, the profile is different in detail. The maximal rates appeared at pH 7.25 in glycylglycine and at pH 7.45—7.5 in glycylglycine–phosphate buffers. The most considerable differences appeared in the relation between the amounts of H_2O_2 formed and pH. Typical examples are shown in Fig. 2B. The amounts of H_2O_2 formed in glycylglycine buffer, which was determined after cysteine had been completely consumed, decreased monotonously with pH, while in phosphate containing buffer the pH profile also gave a bell-shaped curve. In the latter case, a maximal value for the ratio of the concentration of H_2O_2 formed to the initial concentration of cysteine; *i.e.*, $[H_2O_2]/[CySH]_0$, is approximately 0.3 under pure oxygen. As mentioned above, Cu(II) in glycylglycine buffer possesses a relatively low catalytic ability. In the presence of excess cysteine and at high pH, the 2:1(CySH/Cu) complex is formed increasingly and stabilized with an increase of pH.[14] As a result, the oxidation of cysteine by copper in glycylglycine buffer is reduced at high pH, while the rate of oxidation of cysteine by H_2O_2, which is also catalyzed by copper, is shown to increase with pH.[15] Then, the formation and subsequent decomposition of H_2O_2 may be concerted and thereby the amounts of detectable H_2O_2 is apparently reduced.

Various amounts of H_2O_2 were formed concomitantly with the decrease of cysteine. Under the condition at $[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M and the partial pressure of oxygen in the gas phase=20—100% at 760 mmHg (1 mmHg≈133.322 Pa), a certain stoichiometric relation between the concentrations of cysteine consumed and H_2O_2 formed did not exist. A reason why the stoichiometry does not observed may be due to the consumption of H_2O_2 during the reaction. When cysteine has been almost consumed, the concentration of H_2O_2 remained is kept at constant, which indicates that H_2O_2 is utilized; *i.e.*, decomposed, in the presence of the substrate but not decomposed in the absence of the substrate. The time course for the cysteine consumption and H_2O_2 formation at pH 7.4 is shown in Fig. 3.

H_2O_2 is assumed to be a primary product from oxygen in the autoxidation process. If H_2O_2 formed were inert to the substrate, it would be accumulated quantitatively relative to the amounts of cysteine consumed. But, H_2O_2 upon forming does react inevitably and rapidly with cysteine. If the reaction proceeds at a very slow rate, the stoichiometric relation might be estimated; because the extent of H_2O_2 decomposition may be negligible. In an attempt to estimate the stoichiometry of the cysteine autoxidation, the reaction was carried out at various concentrations of cysteine from 5×10^{-4} M to 4×10^{-3} M, where the concentration of the copper catalyst and oxygen were kept at constant. The plot of the initial concentration of cysteine against $[H_2O_2]/[CySH]_0$ is shown in Fig. 4. Expectedly, as the initial concentration of cysteine reduced, the rate decreased and the ratio increased. The value of $[H_2O_2]/[CySH]_0$ was extraporated to 0.5 at the infinitively dilute concentration of cysteine. This fact indicates that the first-step in the autoxidation process involves naturally a two-equivalent oxidoreduction, in which oxygen is reduced to H_2O_2 directly, or two one-equivalent reduction, in which oxygen is reduced *via* superoxide as an intermediate. But. the

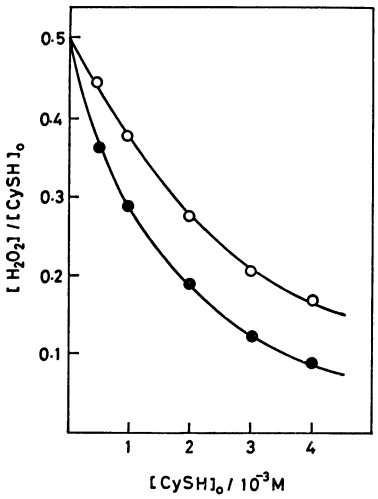


Fig. 4. Relation between the initial concentration of cysteine and $[H_2O_2]/[CySH]_0$; estimation of the stoichiometric relation of the cysteine autoxidation. $[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, partial pressure of O_2: 100%(○) and 40%(●).

superoxide, O_2^-, could not be detected. Accordingly, it is proposed that the autoxidation of cysteine catalyzed by copper involves a two-equivalent process as shown by Eq. 1.

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