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Participation of Cu (II) Ion in the Oxidation of Cysteine with Hydrogen Peroxide

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The oxidation of cysteine with hydrogen peroxide was performed in a nitrogen atmosphere and in the presence and absence of Cu (II) ion. The reaction was followed spectrophotometrically by measuring the decreases of cysteine and hydrogen peroxide. One molar hydrogen peroxide could oxidize exactly two molar cysteine and this stoichiometric relation was satisfied under any condition examined. As pH was raised, the oxidation was advanced monotonously. Though addition of Cu (II) to oxygen-free cysteine solution containing hydrogen peroxide enhanced the oxidation significantly, cysteine was oxidized at a fairly rapid speed even in the absence of Cu (II) ion. From the observation on the anaerobic oxidation with hydrogen peroxide, as well as on the autoxidation, a probable mechanism for the copper ion-catalyzed autoxidation of cysteine was deduced.

When molecular oxygen is the oxidant, it is reduced to water, hydrogen peroxide being an intermediate. In the metal ion-catalyzed autoxidation of cysteine, production of hydrogen peroxide was demonstrated with the luminol chemiluminescence reaction,[2] and with the spectrophotometric method.[3] Addition of the peroxide to metal ion-containing cysteine solution increases the rate of oxidation.[4] Those facts appears to indicate that the peroxide generated as an intermediate in the metal ion-catalyzed autoxidation is utilized further to oxidize cysteine in the presence of the metal ion. In order to confirm this prediction, the oxidation of cysteine with hydrogen peroxide in the presence and absence of Cu (II) ion was attempted.

Experimental

Materials——Unless otherwise stated, all the chemicals were of analytical grade and were used without further purification. The Cu (II) solution was prepared from copper foil of 99.999% purity (Kishida Chemicals & Co., Ltd.), which was weighed out accurately and dissolved in a minimum amount of conc. HNO_3. The $Cu(NO_3)_2$ solution thus prepared was diluted to a desired concentration with doubly distilled water. The H_2O_2 solution was prepared from the 30% solution (Mitsubishi-Edogawa Kagaku, Ltd.) and was standardized regularly against 0.1 N $KMnO_4$. The cysteine solution was made freshly just before use from commercially available L-cysteine hydrochloride monohydrate (Nutritional Biochemicals Corporation).

Instruments——The spectrophotometric measurement was done with a Hitachi 101 spectrophotometer. The measurement of pH was made using a Toa-Dempa HM-5A pH meter and the glass electrode was calibrated against the phosphate and phthalate buffers of JIS standard.

Kinetic Procedure——The reaction was carried out in a N_2 atmosphere and a 100 ml double walled beaker was used as a reaction vessel. The solution containing an appropriate amount of Cu (II) and 1.6×10^{-2} M glycylglycine was thermostatted and deoxygenated by bubbling N_2 gas, which was once passed through 0.1N KNO_3. After the temperature equilibration for 30 min, a definite amount of H_2O_2 was added into the solution and subsequently the cysteine solution was added quickly, by which the reaction was started. The intial concentration of cysteine was 2.00×10^{-3}M. An aliquot was pipetted at a certain interval from the reaciion mixture and applied to assay of cysteine or H_2O_2. During the kinetic run, N_2 gas was bubbled into the solu-

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[2] P. Holtz, G. Triem, *Z. Physiol. Chem.*, **248**, 1 (1937); O. Schales, *Ber.*, **71B**, 447 (1937); D. Cavallini, C. DeMarco, S. Dupre, *Arch. Biochem. Biophys.*, **124**, 18 (1968).

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[4] N.W. Pirie, *Biochem. J.*, **25**, 1565 (1931).

tion at a rate of 100 ml/min, and the solution was agitated vigorously with a magnetic stirrer. In the experiment of autoxidation, H_2O_2 was removed from the reaction mixture and the reaction was carried out in an O_2 atmosphere. Determinations of cysteine and H_2O_2 were performed spectrophotometrically using 5,5′-dithiobis-(2-nitrobenzoic acid)[5] and $TiCl_4$,[6] respectively. The pH value of the reaction mixture was checked after the reaction.

Result and Discussion

In the copper ion-catalyzed autoxidation of cysteine, hydrogen peroxide was produced from molecular oxygen. If hydrogen peroxide is the final product from oxygen, one mole of hydrogen peroxide would be produced upon oxidation of two molar cysteine. However, this stoichiometric relation was never satisfied under any condition examined. The molar ratio of hydrogen peroxide generated to cysteine oxidized was varied even in the course of oxidation, which indicated that the peroxide generated was consumed, according to the situation, to oxidize the substrate. The possibility that the peroxide is decomposed catalatically is denied because the level of the peroxide is continued constant for one hour after the substrate has been completely oxidized. An example at pH 7.4 is presented in Table I. In order to confirm the participation of the peroxide in the reaction, the oxidation of cysteine was attempted using hydrogen peroxide as an oxidant. The measurement was done in an anaerobic environment by bubbling N_2 gas to avoid the participation of molecular oxygen in the oxidation. In the oxidation with various concentrations of hydrogen peroxide, the time course of oxidation is shown in Fig. 1. The initial rate of oxidation was very fast and appeared to be proportional to the concentration of the peroxide. The stoichiometry of the reaction is shown in Fig. 2, where the concentrations of cysteine oxidized, $[CySH]_{ox}$, and of hydrogen peroxide remained, $[H_2O_2]$, are plotted against the initial concentration of the peroxide, $[H_2O_2]_0$. In this experiment, the total concentrations of Cu (II) and cysteine were fixed constant. The determination was performed after either cysteine or hydrogen peroxide had been exhausted in the medium. It appears in Fig. 2 that two molar cysteine is oxidized quantitatively with one molar hydrogen peroxide and that the peroxide is not decomposed after the reaction. This stoichiometric relation, attributed to that the equilibrium for the oxidation-reduction couple of (cysteine–hydrogen peroxide)/(cystine–water) is inclined perfectly to the oxidized state under the experimental condition, is satisfied throughout the course of oxidation.

TABLE I. Relation of Cysteine Oxidation with Hydrogen Peroxide Formation in the Copper-catalyzed Autoxidation System at pH 7.4

Time (min)	$[CySH]_{ox}$ (10^{-4}M)	$[H_2O_2]$ (10^{-4}M)	$[H_2O_2]/[CySH]_{ox}$
3	3.51	1.32	0.38
6	6.62	1.96	0.30
9	9.34	2.24	0.24
12	11.87	2.45	0.21
15	14.10	2.55	0.18
30	20.00	3.00	0.15
60	20.00	3.04	0.15
90	20.00	3.03	0.15

$[CySH]_0=2.00\times10^{-3}$M, $[Cu(II)]_0=1.45\times10^{-6}$M, [glycylglycine]$=1.6\times10^{-2}$M, $pO_2=100\%$ (760 mmHg)

The rate of oxidation with hydrogen peroxide depends on pH. The rate, depending on the concentrations of both cysteine and hydrogen peroxide, was difficult to be determined

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[5] G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

[6] A. Weissler, *Ind. Eng. Chem., Anal. Ed.*, **17**, 695 (1945).

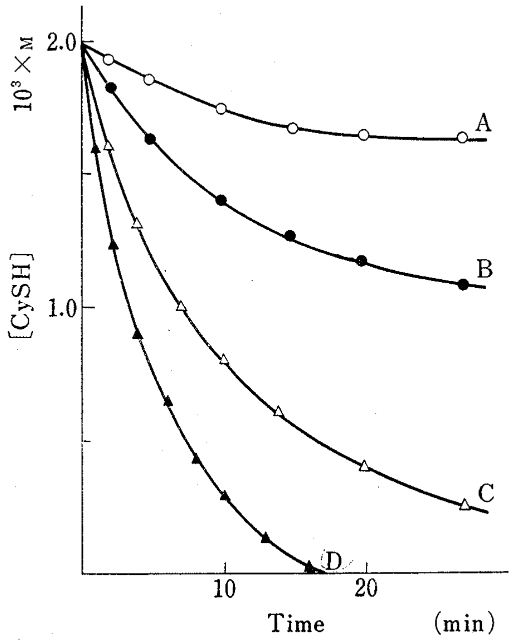

Fig. 1. Time Course of Cysteine Oxidation with Hydrogen Peroxide at pH 7.4

$[CySH]_0 = 2.00 \times 10^{-3}$ M, $[Cu(II)] = 1.45 \times 10^{-6}$ M
[glycylglycine] $= 1.6 \times 10^{-2}$ M, $[H_2O_2]_0$: curve A: 2.11×10^{-4} M
curve B: 5.27×10^{-4} M; curve C: 1.05×10^{-3} M, curve D: 2.11×10^{-3} M

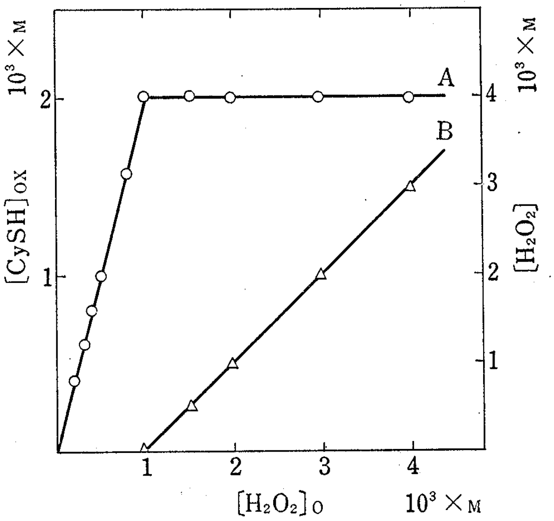

Fig. 2. Stoichiometric Relation of Cysteine Oxidation at pH 7.4

$[CySH]_0 = 2.00 \times 10^{-3}$ M, $[Cu(II)]_0 = 1.45 \times 10^{-6}$ M
[glycylglycine] $= 1.6 \times 10^{-2}$ M
curve A: $[H_2O_2]_0$ *vs* $[H_2O_2]_0$; curve B: $[H_2O_2]_0$ *vs* $[CySH]_{ox}$

Table II. pH Dependence of the Half Life in the Oxidation of Cysteine with Hydrogen Peroxide under Nitrogen

pH	Half life (min)	(Half life)$^{-1}$ (min^{-1})
6.94	11	0.09
7.16	6.3	0.16
7.32	4.0	0.24
7.56	2.7	0.37
7.78	2.0	0.50
8.12	1.1	0.90
8.40	0.8	1.25

$[CySH]_0 = 2.00 \times 10^{-3}$ M, $[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[H_2O_2]_0 = 2.09 \times 10^{-3}$ M, [glycylglycine] $= 1.6 \times 10^{-2}$ M

accurately and easily. Accordingly, the half life of the substrate, estimated under a definite condition, was used as a kinetic parameter. The pH dependence of the half life, as well as the reciprocal of the life, is given in Table II. The reaction was advanced clearly with increasing pH. Those findings support that, as pH is raised, hydrogen peroxide produced by two equivalent reduction of oxygen is rapidly reduced by cysteine to yield water. In the autoxidation, it is shown that the peroxide is little detected in the medium over pH 7.5 and the oxidation appears to couple with four equivalent reduction of oxygen to water.[3]

The next problem is to ascertain whether Cu (II) participates positively in the oxidation with hydrogen peroxide. As for the role of the peroxide, following two mechanisms, *i.e.*, direct and indirect mechanisms, can be considered.

direct mechanism: $2CySH + H_2O_2 \longrightarrow CyS\text{-}SCy + 2H_2O$ (i)

indirect mechanism: $2Cu^+ + H_2O_2 \longrightarrow 2Cu^{2+} + 2H_2O$ (iia)

$2CySH + 2Cu^{2+} \longrightarrow 2Cu^+ + CyS\text{-}SCy + 2H^+$ (iib)

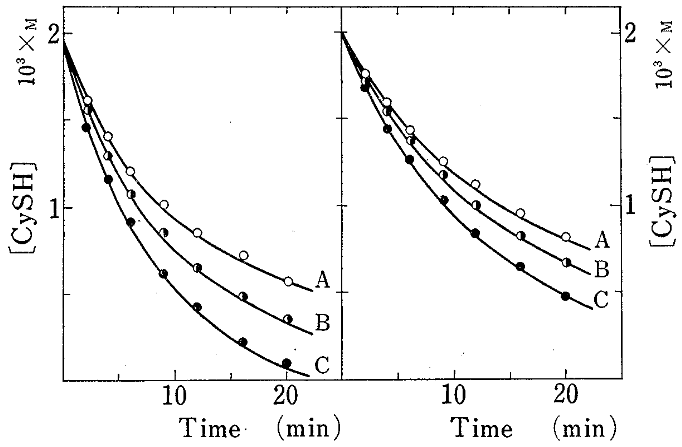

Fig. 3. Effect of Cu(II) Concentration upon Oxidation of Cysteine at pH 7.0 (right) and at pH 7.4 (left)

$[CySH]_0=2.00\times10^{-3}$M, $[H_2O_2]_0=1.03\times10^{-3}$M, $[glycylglycine]=1.6\times10^{-2}$M
$[Cu(II)]_0$: curve A: copper free; curve B: 6.90×10^{-6}M, curve C: 2.76×10^{-5}M

A key to differenciate above two mechanisms is to examine an effect of Cu(II) upon the acceleration of oxidation. The reaction profile in the presence of various concentrations of Cu(II) ion at different pHs is shown in Fig. 3. In the copper-free system, was added 2.00×10^{-3}M EDTA to mask an effect by trace amount of Cu(II) contaminated. The substrate was oxidized at a fairly rapid speed even in the absence of Cu(II) ion. Addition of Cu(II) promoted the reaction according to the increase of its concentration. Addition of EDTA under the corresponding condition hindered the oxidation as shown in Fig. 4. Those observations inform that Cu(II) ion at a fairly high concentration participates in the promotion of the oxidation with hydrogen peroxide under nitrogen and that the peroxide participates positively in the oxidation even in the absence of Cu(II) ion. Fe(II) ion accelerated the oxidation markedly. Neville demonstrated that addition of Fe(II) to oxygen-free dilute acid or alkaline solution of cysteine containing hydrogen peroxide advanced the oxidation more than fifty times as compared with the iron-free system.[7]

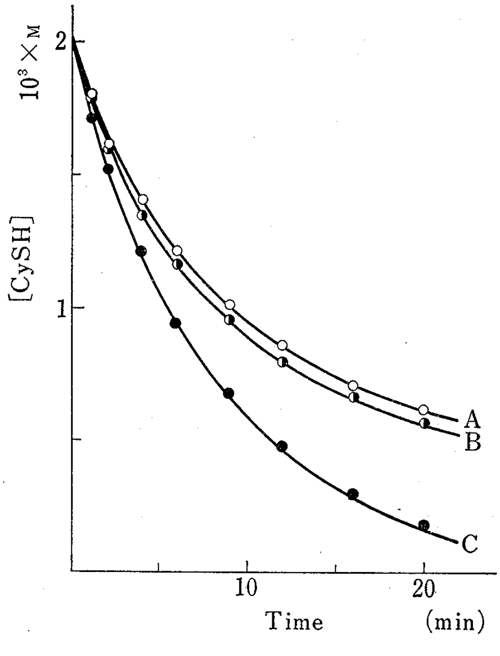

Fig. 4. Acceleration of Cysteine Oxidation by Cu(II) Ion at pH 7.4

$[CySH]_0=2.00\times10^{-3}$M, $[Cu(II)]_0=2.76\times10^{-5}$M
$[H_2O_2]_0=1.03\times10^{-3}$M, $[EDTA]=2.00\times10^{-3}$M
$[glycylglycine]=1.6\times10^{-2}$M, curve A: copper free system
curve B: complete system, curve C: EDTA free system

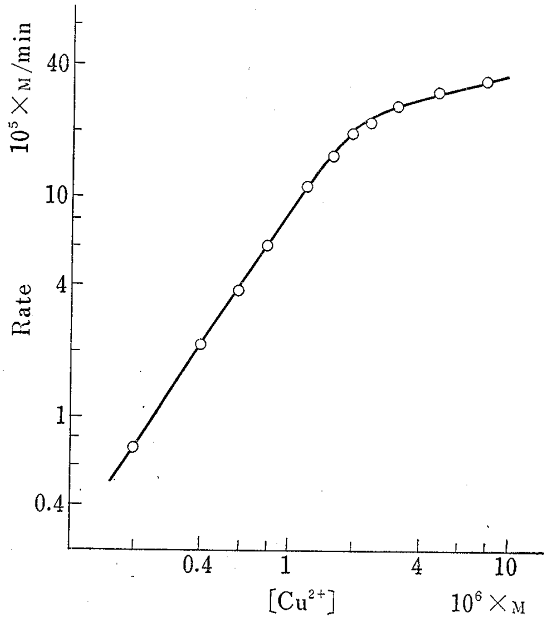

Fig. 5. Effect of Cu(II) Concentration upon Autoxidation of Cysteine at pH 7.4

$[CySH]_0=2.00\times10^{-3}$M, $[glycylglycine]=1.6\times10^{-2}$M
$pO_2=100\%$ (760 mmHg)

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7) R.G. Neville, *J. Am. Chem. Soc.*, **79**, 2456 (1957).

Addition of Cu (II) promotes the autoxidation remarkably. In the absence of Cu (II) ion, cysteine was not autoxidized. The effect of various concentrations of Cu (II) on the rate of autoxidation is presented in Fig. 5. In the autoxidation the substrate may be attacked by Cu (II), while in the oxidation with hydrogen peroxide it is attacked by hydrogen peroxide and/or Cu (II) ion. The rates of cysteine oxidation may depend on the concentrations of cysteine and the oxidant as indicated in the steps (i) and (iib). Under the standard conditions in the autoxidation and in the anaerobic oxidation with hydrogen peroxide, the total concentration of copper was very low, approximately 10^{-6}M. On the contrary, the concentration of the peroxide may be relatively high; it was approximately 10^{-3}M under the standard condition in the anaerobic oxidation and probably more than 10^{-4}M in the autoxidation as predicted from Table I. Accordingly, provided that the rate constant in the step (i) is not different remarkably from that of step (iib), the rate of cysteine oxidation will be more rapid in the step (i). It means that, in the copper ion-catalyzed autoxidation of cysteine, molecular oxygen oxidizes indirectly, *i.e.*, catalytically, the substrate in the first step and the peroxide generated from oxygen is utilized to oxidize directly, *i.e.*, non-catalytically, the substrate in the second step.