Kinetic Studies on the Autoxidation of Cysteine Catalyzed by Copper Complexes: Catecholamines Stimulate the Autoxidation

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(Received April 15, 1999)

Catecholamines including epinephrine, norepinephrine, dopamine, and dihydroxyphenylalanine accelerated copper-catalyzed oxidation of cysteine under aerobic conditions, while they had no effect under anaerobic conditions. The efficiency of the catecholamines was arranged in the decreasing order as follows; epinephrine ≒ norepinephrine ≧ dopamine > dihydroxyphenylalanine. On the contrary, phenolic amines such as tyramine and tyrosine neither accelerate nor inhibit the autoxidation. The reaction can be elucidated by a "sequentical mechanism" which is composed of Eqs. I, II, III, IV, V, and VI:

$$\text{Cu(II)–L} + \text{CyS}^- \rightleftarrows \text{L–Cu(II)(CyS}^-) \quad \text{(I)}$$

$$\text{L–Cu(II)(CyS}^-) \rightarrow \text{Cu(I)–L} + \cdot\text{CyS} \quad \text{(II)}$$

$$\text{Cu(I)–L} + \text{CyS}^- \rightleftarrows \text{Cu(I)(CyS}^-) + \text{L} \quad \text{(IIa)}$$

$$\text{Cu(I)(CyS}^-) + \text{O}_2 \rightleftarrows \text{(CyS}^-)\text{Cu(I)–O}_2 \quad \text{(III)}$$

$$\text{(CyS}^-)\text{Cu(I)–O}_2 + \text{CyS}^- + 2\text{H}^+ \rightarrow \text{Cu(II)(CyS}^-) + \cdot\text{CyS} + \text{H}_2\text{O}_2 \quad \text{(IV)}$$

$$\text{(CyS}^-)\text{Cu(I)–O}_2 + \text{Cat}^- + 2\text{H}^+ \rightarrow \text{Cu(II)(CyS}^-) + \cdot\text{Cat} + \text{H}_2\text{O}_2 \quad \text{(V)}$$

$$\text{CyS}^- + \cdot\text{Cat} \rightarrow \text{Cat}^- + \cdot\text{CyS} \quad \text{(VI)}$$

where L is glycylglycine used as the buffer, and CyS^- and Cat^- denote cysteinate and catecholamine ions, respectively. The reactions (IV), (V), or both are considered to be rate-determining. The optimum pH for the autoxidation was observed at pH 7.5—7.6, and below pH 7.2 reaction (V) does not participate in the autoxidation system.

Dioxygen, which is biologically essential to any aerobic organism, functions as the terminal electron acceptor in the respiratory chain. It accepts either two-electrons or four-electrons from the electron donors and is reduced to hydrogen peroxide or to water, respectively. The electron donors primarily undergo stepwise one-electron oxidation. In the course of the electron transfer, superoxide ion (O_2^-), hydroxyl radical (·OH), or both, is likely to leak; the former is one-equivalent reduction product from dioxygen and the latter is one-equivalent reduction product from hydrogen peroxide.[1] Those intermediates, being chemically reactive and probably damaging biopolymers or biomembranes, have been considered to be toxic to living organisms and thereby have been named "active oxygen species".[2,3]

In the chemical reaction, the oxidation by dioxygen, i.e., autoxidation, can be achieved by catalyzing with redox-active metal ions such as copper and iron. The reaction is primarily a two-equivalent oxido-reduction, and the catalyst is turned over between the oxidized and reduced states:

$$2\text{AH} + \text{O}_2 \rightarrow 2\text{A} + \text{H}_2\text{O}_2$$

where AH and A represent the electron donor (reductant) and its oxidation product, respectively. The superoxide would be produced as a transient, which has been considered as a candidate for the active species toward the autoxidation.[4] However, formation and participation of O_2^- in the reaction have remained uncertain. If the O_2^- were formed as an intermediate, it could be quenched by several techniques. Superoxide dismutase (SOD) and epinephrine have been known as capable quenchers for O_2^-. SOD can disproportionate rapidly the O_2^- to O_2 and H_2O_2.[5] Epinephrine is known to accept rapidly one-electron from O_2^- forming a pigment named adrenochrome.[6]

In a previous paper, we showed that addition of SOD to the reaction system consisting of Cu(II) and cysteine (electron donor) did not affect the autoxidation of cysteine. This suggested that the O_2^- was not generated or, even if produced,

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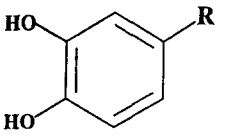
did not act as an oxidant.[7] We attempted now to study the effects of epinephrine and related catecholamines on the autoxidation system referring to those of phenolic amines. The catecholamines examined are shown in Chart 1. The reactions were carried out under dioxygen (aerobic condition) and under nitrogen (unaerobic condition). In the former, the dioxygen is involved in the oxidation. If a superoxide ion is produced in the system, catecholamines trap it and expels it from the medium. As a result, the autoxidation of cysteine would be retarded. Unexpectedly, however, the catecholamines examined did not retard the autoxidation and otherwise enhance the reaction system relative to their concentrations. In this paper we describe results from kinetic studies which show that the catecholamines probably react with the Cu(I)–O_2 species, forming a radical species (·Cat) which subsequently reacts with cysteinate ion (CyS^-) accelerating the autoxidation. Under anaerobic condition, the catecholamines did neither enhance nor retard the oxidation of cysteine.

Experimental

Materials. Epinephrine was a product from E. Merck & Co. (Darmstadt, Germany). Norepinephrine, dopamine, 3-hydroxytyrosine (dopa), tyrosine, and tyramine were obtained from Wako Pure Chemicals Co. (Osaka, Japan).[8] 6,6′-Dinitro-3,3′-dithiodibenzoic acid (DTNB) was obtained from Wako Pure Chemicals Co. (Osaka, Japan). L-Cysteine, abbreviated as CySH, and *N*-acetyl-L-cysteine, ACySH, were purchased from Sigma Chem. Co. (Mo., USA). Copper chips (99.999%) were from Kishida Chem & Co. (Tokyo, Japan). Other chemicals were the purest available from standard suppliers. Those reagents were used as received.

All the solutions were prepared from deionized and doubly distilled water; the first distillation was from alkaline permanganate. A stock solution of Cu(II), which was prepared by dissolving the chips in a small amount of concd HNO_3, was diluted to a desired concentration with purified water. The concentration of the Cu(II) solution was checked by titration with standardized 0.01 M EDTA (1 M = 1 mol dm^{-3}). Cysteine solutions were prepared freshly just prior to use and determined with DTNB.[9]

Kinetic Procedure for the Autoxidation. The autoxidation of cysteine was conducted at 20 °C and *I* (ionic strength) = 0.1 M $NaNO_3$ using a spectrophotometric method. Measurements were done over the range from pH 6.9 to 8.4 under oxygen and oxygen/nitrogen mixtures. The Cu(II) solution in 0.015 M glycylglycine/0.01 M phosphate buffers was equilibrated at 20 °C under aerobic (O_2 or O_2/N_2 purged) conditions. Glycylglycine was abbreviated hereafter as GlyGly. After equilibration for 15 min, either a catecholamine or a phenolic amine was added. Subsequently, the cysteine solution was added and the reaction was started. The initial concentration of Cu(II) was routinely 1.38×10^{-6} M. During the reaction an O_2 or O_2/N_2 gas mixture was purged at the rate of 100 ml min^{-1}. Aliquots were withdrawn at periodic intervals from the reaction mixtures and were subjected to the spectrophotometric determination of either CySH by DTNB or H_2O_2 by $TiCl_4$.[10] The concentration of dissolved dioxygen was checked both before and after the kinetic runs with a Beckman Fieldlab oxygen analyzer, which had been calibrated against air-saturated water. An absorbance-time plot gave a straight line for over 60% reaction. The rate of autoxidation (v), which was an average of at least four runs, was obtained from the initial slope, and kinetic data were analyzed by the Lineweaver–Burke treatment.[11]



Epinephrine ; R = $CH(OH)\text{-}CH_2\text{-}NHCH_3$

Norepinephrine ; R = $CH(OH)\text{-}CH_2\text{-}NH_2$

Dopamine ; R = $CH_2\text{-}CH_2\text{-}NH_2$

Dopa ; R = $CH_2\text{-}CH(COOH)\text{-}NH_2$

Chart 1.

Kinetic Procedure for the Anaerobic Oxidation. Stopped-flow kinetic analysis was conducted at 25 °C and under N_2 over the range from pH 6.2 to 8.6 under pseudo first-order conditions using a large excess of cysteine. Solutions of 1.25×10^{-4} M Cu(II)$(Gly)_2$ and of 4.00×10^{-3} M CySH in 0.01 M phosphate/0.01 M borate buffers, which were equilibrated for 20 min under N_2, were mixed under N_2 (8 kg cm^{-2}) to start the reaction, and subsequent changes in the absorbance at 330 nm were recorded on a Union RA-401 stopped-flow spectrophotometer. The apparent rate constant, k_{obsd}, was determined by the Gugenheim method.[12]

Results

Addition of epinephrine did stimulate the autoxidation system composed of Cu(II) and CySH in the GlyGly/phosphate buffer, but did not increase the absorbance at 485 nm (A_{485}) due to adrenochrome, as long as cysteine remained in the medium. After cysteine had been consumed completely, the A_{485} increased gradually with time. The time courses of decay of cysteine and increase of A_{485} are shown in Fig. 1, which suggests that O_2^- is not released for initial 20 min, or was consumed rapidly by cysteine if it were produced.

All of the catecholamines examined stimulate the autox-

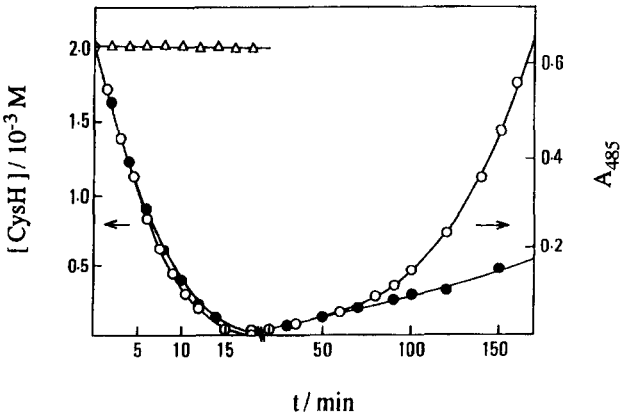


Fig. 1. Time courses for the consumption of cysteine and increase of absorbance at 485 nm under oxygen. (○), $[epinephrine]_0 = 2.00\times10^{-2}$ M with Cu(II), (●), $[epinephrine]_0 = 2.00\times10^{-3}$ M with Cu(II), and (△), $[epinephrine]_0 = 2.00\times10^{-2}$ M without Cu(II). $[Cu(II)]_0 = 1.38\times10^{-6}$ M, $[CySH]_0 = 2.00\times10^{-3}$ M, pH 7.5 (0.015 M GlyGly/0.01 M phosphate buffer), T = 20°C.

idation system. The rate of autoxidation depended on the concentration of the catecholamine and on pH. Plots of the rate against the concentrations at pH 7.5 are shown in Fig. 2. Increasing concentrations of the catecholamine caused the autoxidation to accelerate. The maximal velocity (V_{max}) was the same in each of the catecholamines and evaluated as $(3.45 \pm 0.05) \times 10^{-6}$ M s^{-1} at [catecholamine] = 1 M. But $V_{0.5}$ is different among the catecholamines, where $V_{0.5}$ is the concentration of catecholamine at one-half the maximal velocity.[13] Referring to the $V_{0.5}$, the efficiency of the catecholamines could be arranged as follows;

epinephrine (6.0×10^{-5} M) ≒ norepinephrine (6.0×10^{-5} M) ≧ dopamine (7.5×10^{-5} M) > dopa (1.5×10^{-4} M),

where the $V_{0.5}$ values were shown in parentheses. On the contrary, phenolic amines such as tyrosine and tyramine had little effect, as shown in Table 1. The activation toward the system appeared to necessitate the catechol skelton.

The catecholamines appeared to stimulate the autoxidation system above pH 7.4. pH plots of the rates in the absence and presence of dopamine are shown in Fig. 3, which exhibited bell-shaped curves. In the range below pH 7.4, the rate increased relative to $(K_a/K_a+[H^+])$, where K_a denoted the proton-ionization constant of cysteine. This informs us that cysteinate ion, Cys^-, is an active species.[14] However, since the cysteine is likely to inhibit the reduction of Cu(II) as stated later, the rate of autoxidation decreased proportionally to the increasing pH values. Dopamine clearly accelerated the autoxidation above pH 7.4, while below pH 7.4 it neither accerelated nor inhibited the autoxidation. The optimum pH tended to shift to alkaline region with increasing concentrations of dopamine. The relative rate v/v_0 could express more clearly the effects of dopamine, where v and v_0 denoted the rates in the presence and absence of dopamine, respectively. The pH–v/v_0 plots are shown in Fig. 4, which indicates definitely that dopamine stimulates the reaction system above

Table 1. Effect of Phenolic Amines on the Autoxidation of Cysteine at 20 °C and pH 7.5[a)]

[Phenolic amine]	Rate/10^{-6} M s^{-1}	
M	Tyramine	Tyrosine
2.00×10^{-3}	2.10 (0.03)	2.16 (0.04)
2.00×10^{-4}	2.10 (0.03)	2.17 (0.06)
2.00×10^{-5}	2.17 (0.07)	2.18 (0.06)
2.00×10^{-6}	2.14 (0.04)	2.18 (0.07)
Null	2.11 (0.04)	

$[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M, and under O_2. a) A values in parenthesis represents standard deviation from an average of five runs.

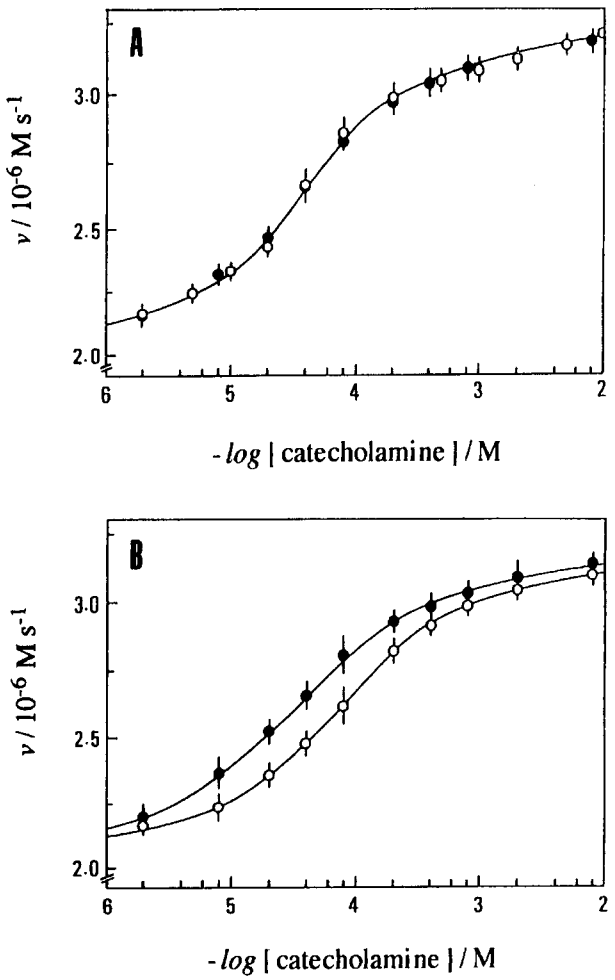


Fig. 2. Plot of the autoxidation rate of cysteine against the concentration of catecholamines under oxygen. A: (○), epinephrine, (●), norepinephrine, and B: (○), dopa, (●), dopamine. $[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M, pH 7.5 (0.015 M GlylGly/0.01 M phosphater buffer), $T = 20$°C. Vertical line in the figure indicates the standard deviation from an average of five runs.

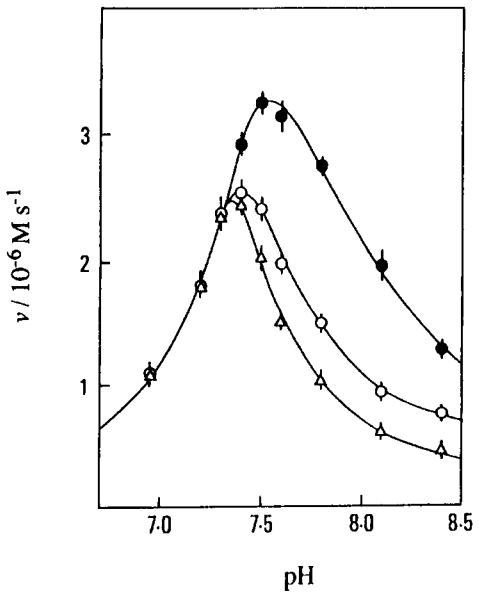


Fig. 3. pH dependence of the autoxidation rate for cysteine. $[dopamine]_0$: (○), 2.00×10^{-5} M, (●), 2.00×10^{-4} M, and (△), null. $[Cu(II)]_0 = 1.38 \times 10^{-6}$ M, $[CySH]_0 = 2.00 \times 10^{-3}$ M, 0.015 M GlyGly/0.01 M phosphate buffer, $T = 20$°C. Vertical line in the figure indicates the standard deviation from an average of at least four runs.

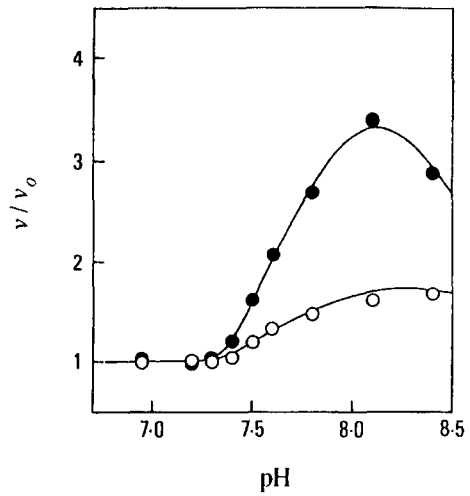


Fig. 4. pH dependence of the relative rate for the autoxidation of cysteine. [dopamine]$_0$: (○), 2.00×10^{-5} M, (●), 2.00×10^{-4} M. Conditions same as under Fig. 3.

pH 7.4.

A Cu(II)–L complex reacts with cysteine, yielding sequentially a mixed-ligand complex, L–Cu(II)(CyS^-), and a binary complex, Cu(II)(CyS^-)$_2$, which exhibit the S→Cu(II) charge transfer (LMCT) absorptions around 330 nm;[15,16]

$$\text{Cu(II)–L} \rightarrow \text{L–Cu(II)(CyS}^-\text{)} \rightarrow \text{Cu(II)(CyS}^-\text{)}_2 \rightarrow \text{Cu(I) species.}$$

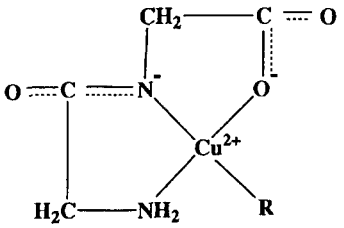
The ligand L denotes glycylglycine and the structures for Cu(II)–L and L–Cu(II)(CyS^-) are shown in Chart 2. The cysteine-containing complexes suffered rapid oxido-reduction. Those sequential reactions can be quantitatively elucidated by analyzing the time plot of A_{330}.[17]

The pathway for the reaction of cysteine with Cu(II)$(Gly)_2$, which was labile toward the ligand-exchange, was simple:

$$\text{Cu(II)(Gly)}_2 \rightarrow \text{Cu(II)(CyS}^-\text{)}_2 \rightarrow \text{Cu(I) species.}$$

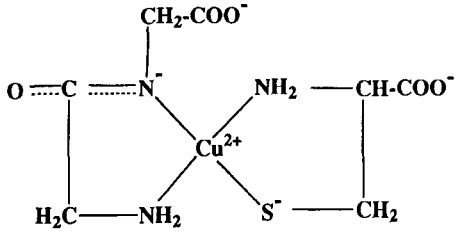
Under the anaerobic condition, the Cu(I) species were not oxidized back. Then, the rates for the reduction of Cu(II) species can be easily assessed. The rate was determined under the pseudo first-order condition using a stopped-flow spectrophotometric technique at 330 nm. The pH dependence of the rate constant, k_{obsd}, is shown in Fig. 5. The transients were labile at a lower pH, undergoing rapid oxido-reduction. As the pH increased, the rates decreased proportionally, probably because of the stabilization of transients. Strong affinity of CyS^- for Cu(II) is likely to inhibit the electron tansfer.[17]

N-Acetyl-L-cysteine, which is a S-monodentate ligand, is able to form a mixed-ligand complex, L–Cu(II)($ACyS^-$), but the binary complex, Cu(II)($ACyS^-$)$_2$, is labile, undergoing prompt oxido-reduction.[18] Accordingly, the pseudo first-order rate constant, k_{obsd}, for the anaerobic oxidation was three orders of magnitude faster than that of cysteine ; k_{obsd} at pH 8.6 : 5.4×10^{-2} s^{-1} for Cys and 80 s^{-1} for ACySH. Under aerobic conditions, on the other hand, ACySH resisted the oxidation, and was hardly oxidized at all below pH 9. This is probably because of the weak affinity of Cu(I)($ACyS^-$)



Cu(II)(H_{-1}GlyGly)

R = H_2O, OH^- or HPO_4^{2-}



(CyS$^-$)Cu(II)(H_{-1}GlyGly)

Chart 2.

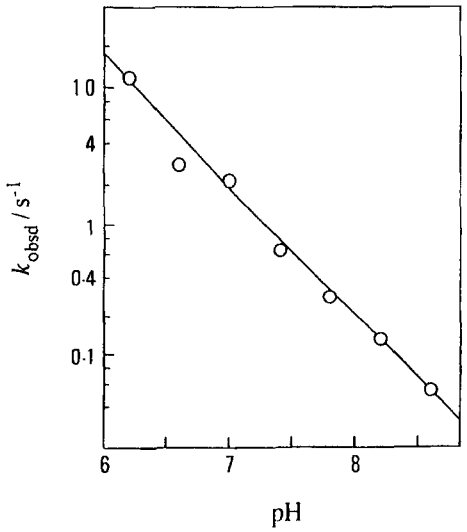


Fig. 5. pH vs. k_{obsd} plot for the anaerobic oxidation of cysteine by Cu$(Gly)_2$ under nitrogen. [Cu(II)]$_0$ = 6.30×10^{-5} M, [CySH]$_0$ = 2.00×10^{-3} M, 0.01 M phosphate/0.01 M borate buffer, T = 25°C.

for dioxygen as compared with that of Cu(I)(CyS^-). The coordination modes of Cu(I)–L, Cu(II)–L, or both, would controll the efficiency of the autoxidation system.

The autoxidation in the presence, as well as in the absence,[7] of catecholamines depended on the concentrations of dissolvd dioxygen. A double-reciprocal plot of v

against the concentration of the dissolved dioxygen, $[O_2]$ (Lineweaver–Burke plot), gave a straight line. This indicates that the autoxidation can be elucidated by the saturation kinetics, namely the "Michaelis–Menten" mechanism. Kinetic analyses for the reaction will be discussed later.

The Cu(I) species would be autoxidized as shown in Eq. 1.

$$Cu(I)\text{–}L + O_2 + 2CyS^- + 2H^+ \rightleftarrows Cu(II)\text{–}L + CyS\text{–}SCy + H_2O_2 \quad (1)$$

According to Eq. 1, one molar H_2O_2 is produced primarily on consumption of two molars cysteine. This stoichiometry was achieved limitedly in the initial stage for two or three minutes, probably because the peroxide upon forming was consumed in the oxidation. The relation of the autoxidation rate with $[H_2O_2]_0$ is shown in Table 2; where $[H_2O_2]_0$ denotes the total concentrations of the peroxide determined after the cysteine has been consumed, At pH 7.2, where dopamine did not affect the autoxidation system, increasing concentrations of dopamine did not change $[H_2O_2]_0$. At pH 7.6, on the contrary, increasing concentrations of dopamine caused increases in both the rate and $[H_2O_2]_0$.

Discussion

The first step in the oxidation of cysteine is formation of the mixed-ligand complex, L–Cu(II)(CyS^-), which is followed by the production of the binary complex, Cu(II)(CyS^-)$_2$. Those transients were identified by the S→Cu(II) LMCT band at 330 nm. The reactions are expressed by Eqs. 2a and 2b:

$$Cu(II)\text{–}L + CyS^- \rightleftarrows L\text{–}Cu(II)(CyS^-) \quad (2a)$$

$$L\text{–}Cu(II)(CyS^-) + CyS^- \rightleftarrows Cu(II)(CyS^-)_2 + L \quad (2b)$$

where L denotes glycylglycine. Generally, the formation of L–Cu(II)(CyS^-) was extremely fast; the rate constant could not be determined by the conventional stopped-flow technique. It was of the magnitude of 10^6 M^{-1} s^{-1} or bigger.[19] The ternary complex, upon forming, either changed to Cu(II)(CyS^-)$_2$ or suffered reduction.

The second step is electron transfers in the transients, which occur under both aerobic and anaerobic conditions. The process can be shown to be

$$L\text{–}Cu(II)(CyS^-) \rightarrow Cu(I)\text{–}L + \cdot CyS \quad (3a)$$

$$Cu(II)(CyS^-)_2 \rightarrow Cu(I)(CyS^-) + \cdot CyS \quad (3b)$$

where ·CyS represents the sulfanyl radical. The sulanyl radical, which has not been detected, would be rapidly combined to yield cystine.[20,21] The L–Cu(II)(CyS^-) was labile at a lower pH, undergoing rapid oxido-reduction without changing to Cu(II)(CyS^-)$_2$.[17] The rate of oxido-reduction of Cu(II)(CyS^-)$_2$ could be assessed by the k_{obsd} shown in Fig. 5. As the pH increased, the Cu(II)(CyS^-)$_2$ became proportionally stable. This caused a dramatic decrease in the autoxidation rate above pH 7.5 as shown in Figs. 3 and 4. The cysteine is likely to inhibit primarily the intramolecular electron tansfer in Cu(II)(CyS^-)$_2$. So the double reciprocal plot of the rate against the concentration of cysteine did not give a straight line.[7] Cysteine is considered as a suicide substance which works as both a substrate and an inhibiter.

Cu(I) is preferable to CyS^- rather than GlyGly,[22] and then the main species for Cu(I) is likely to be Cu(I)(CyS^-). The Cu(I)/Cu(II) ratio in the steady state of autoxidation was shown to be approximately 4/1.[7] The Cu(I) species may be oxygenated as shown in Eqs. 4a and 4b.

$$Cu(I)(CyS^-) + O_2 \rightleftarrows (CyS^-)Cu(I)\text{–}O_2 \quad (4a)$$

$$(CyS^-)Cu(I)\text{–}O_2 + Cu(I)(CyS^-) \rightleftarrows (CyS^-)Cu(I)\text{–}O_2\text{–}Cu(I)(CyS^-) \quad (4b)$$

Both the kinetic and thermodynamic data for the autoxidation of Cu(I)–Y have supported formation of the dioxygen-adducts, where Y denotes a ligand.[23–27] The equilibrium and rate constants for the formation of Y–Cu(I)–O_2, i.e, a superoxide, and of Y–Cu(I)–O_2–Cu(I)–Y, i.e., a peroxide, have been estimated.[28] The equilibrium constant for the formation of Y–Cu(I)–O_2–Cu(I)–Y is approximately five orders of magnitude bigger than that of Y–Cu(II)–O_2, though the rates of formation of those two species were very fast. This indicates that the Y–Cu(I)–O_2 adduct upon forming either reacts promptly with another Y–Cu(I)–O_2 adduct to yield Y–Cu(I)–O_2–Cu(I)–Y or dissociates to Cu(I)–Y and dioxygen. The Y–Cu(I)–O_2 is anbifunctional.

Table 2. Relation of the Autoxidation Rate and the Concentration of Hydrogen Peroxide Produced at pH 7.2 and pH 7.6[a)]

[Dopamine]/10^{-4} M	Rate/10^{-6} M s^{-1}	$[H_2O_2]_0/10^{-4}$ M	$[H_2O_2]_0/[CySH]_0^{b)}$
pH 7.2			
Null	2.00 (0.03)	5.85 (0.05)	0.29 (0.26)
0.20	2.00 (0.08)	5.80 (0.05)	0.29 (0.26)
2.00	1.96 (0.07)	5.85 (0.05)	0.29 (0.26)
pH 7.6			
Null	1.50 (0.03)	3.85 (0.05)	0.19 (0.45)
0.20	1.98 (0.03)	4.00 (0.05)	0.20 (0.43)
2.00	3.10 (0.07)	4.85 (0.05)	0.24 (0.35)

$[Cu(II)]_0 = 1.38\times10^{-6}$ M, $[CySH]_0 = 2.00\times10^{-3}$ M, and under O_2. a) A values in parenthesis represents standard deviation from an average of five runs. b) In parentheses is shown the calculated value for percentage of the oxidation by H_2O_2 produced in the autoxidation system.

The $(CyS^-)Cu(I)–O_2$ is capable of reacting preferably with the reductant, such as cysteine or catecholamines, to yield radical species, ·CyS or ·Cat, as shown in Eqs. 5 and 6:

$$(CyS^-)Cu(I)–O_2 + CyS^- + 2H^+ \rightleftarrows Cu(II)(CyS^-) + \cdot CyS + H_2O_2 \quad (5)$$

$$(CyS^-)Cu(I)–O_2 + Cat^- + 2H^+ \rightleftarrows Cu(II)(CyS^-) + \cdot Cat + H_2O_2 \quad (6)$$

where Cat^- denote the catecholamine anion, R–Ph–(OH)-(OH^-). As far as cysteine exists in the medium, the ·Cat spontaneously accepts one electron from CyS^- yielding the ·Cys, as shown in reaction (7), instead of forming an adrenochrome-like pigment. This is supported by results from Fig. 1.

$$CyS^- + \cdot Cat \rightleftharpoons \cdot CyS + Cat^- \quad (7)$$

According to Eqs. 5, 6, and 7 one molar H_2O_2 is produced primarily on consumption of two molars cysteine. This stoichiometry existed at low concentrations of cysteine and under sufficient supply of dioxygen,[29] and was achieved limitedly in the initial stage of reaction. In the presence of Cu(I) species, H_2O_2 was able to oxidize cysteine by the Fenton-like mechanism.[30] Addition of catalase to the autoxidation system did partly retard the reaction. Such results indicate that the peroxide produced in the autoxidation system is utilized for oxidizing cysteine and consumed. Accordingly, the $[H_2O_2]_0/[CySH]_0$ ratio is generally less than 0.5. Provided that the half of the peroxide produced in the autoxidation is utilized further, the $[H_2O_2]_0/[CySH]_0$ ratio ought to formally be 0.17. The $[H_2O_2]_0/[CySH]_0$ ratios, which are determined under various conditions, is shown in the fourth column of Table 2. At pH 7.2, where dopamine did not affect the autoxidation system, the $[H_2O_2]/[CySH]_0$ ratio did not vary, remaining at 0.29 irrespective of [dopamine]. At pH 7.6, on the contrary, increasing concentrations of dopamine caused increases in both the rate and $[H_2O_2]_0/[CySH]_0$. Such results suggest that increasing pH augments the contribution of H_2O_2 to the overall oxidation and that increasing concentrations of dopamine undoubtedly enhance the autoxidation system at pH 7.6 but diminish the overall contribution of H_2O_2.

Catecholamines would take a role in the formation of radical species, probably semiquinone radical, which works at the production of ·CyS. Since the reactions (5) and (7) work in a synergic manner, increasing the concentration of catecholamines is convenient to the autoxidation system. In Table 3[31–33] is summarized V_{max} (the rate at [catecholamine] = 1.0 M), $V_{0.5}$, and the pK_a for one of the phenolic groups. Dopa, which is a diphenolic amino acid having one order of magnitude bigger pK_a, was less effective toward the reaction. A fairly good relationship existing between $V_{0.5}$ and pK_a would indicate that the catecholamine forms the ·Cat radicals via its monophenolate anion.

The mechanism for the copper-catalyzed autoxidation of cysteine has been elucidated by the "sequential mechanism".[7,34] Cysteine is oxidized not only by the Cu(II) species, but also by the $Cu(I)–O_2$ adducts. A sequence of the reactions in the presence of catecholamines can be kinetically expressed as follows:

Table 3. The Rate of Autoxidation and pK_a(OH) of Catecholamine

Catecholamine	$V_{max}/10^{-6}$M[a]	$V_{0.5}/10^{-5}$ M	pK_a(OH)[b]
Epinephrine	3.49	6.0	8.63[31]
Norepinephrine	3.49	6.0	8.63[32]
Dopamine	3.44	7.4	8.88[33]
Dopa	3.40	16	9.77[33]
Null	2.10[c]		

$[Cu(II)]_0 = 1.38\times10^{-6}$ M, $[CySH]_0 = 2.00\times10^{-3}$ M, and under O_2. a) The rate extraporated at [catecholamine] = 1.0 M. b) At 25 °C and $I = 0.1$ M KNO_3. c) Correspond to v_0.

$$Cu(II) + CyS^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Cu(II)(CyS^-) \quad (I)$$

$$Cu(II)(CyS^-) \xrightarrow{k_2} Cu(I) + \cdot CyS \quad (II)$$

$$Cu(I) + O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Cu(I)–O_2 \quad (III)$$

$$Cu(I)–O_2 + CyS^- + 2H^+ \xrightarrow{k_4} Cu(II) + \cdot CyS + H_2O_2 \quad (IV)$$

$$Cu(I)–O_2 + Cat^- + 2H^+ \xrightarrow{k_5} Cu(II) + \cdot Cat + H_2O_2 \quad (V)$$

$$CyS^- + \cdot Cat \rightarrow Cat^- + \cdot CyS \quad (VI)$$

For simplicity, Cu(I)–L and Cu(II)–L were abbreviated as Cu(I) amd Cu(II), respectively.

The rate expression for the autoxidation of cysteine without catecholamine has been shown by Eq. 8:[7]

$$\frac{V_{max}}{v} = 1 + \frac{K_{1.2}}{[CyS^-]} + \left(\frac{k_2}{k_4[CyS^-]}\right)\left(1 + \frac{K_{3.4}}{[O_2]}\right), \quad (8)$$

where V_{max}, $K_{1.2}$, and $K_{3.4}$ represent $(k_2+k_4)[Cu]_0$, $(k_{-1}+k_2)/k_1$ and $(k_{-3}+k_4[CyS^-])/k_3$, respectively. The $[Cu]_0$ denotes the total concentrations of copper as shown in Eq. 9

$$[Cu]_0 = [Cu(I)] + [Cu(I)–O_2] + [Cu(II)] + [Cu(II)(CyS^-)] \quad (9)$$

In the presence of catecholamines, the reaction (IV) works in a synergic way with the reaction (V) for the autoxidation of cysteine. Then, the rate expression can be described briefly by Eq. 10:

$$v = k_2[Cu(II)(CyS^-)] + [Cu(I)–O_2](k_4[CyS^-] + k_5[Cat^-]) \quad (10)$$

and rearranged to

$$\frac{V_{max}}{v} = 1 + \frac{K_{1.2}}{[CyS^-]} + \left(\frac{k_2}{k_4[CyS^-] + k_5[Cat^-]}\right)\left(1 + \frac{K_{3.4.5}}{[O_2]}\right), \quad (11)$$

where V_{max}, $K_{1.2}$, and $K_{3.4.5}$ are expressed respectively by Eqs. 12, 13, and 14.

$$V_{max} = (k_2 + k_4 + k_5)[Cu]_0, \quad (12)$$

$$K_{1,2} = \frac{(k_{-1} + k_2)}{k_1}. \quad (13)$$

$$K_{3,4,5} = \frac{(k_{-3} + k_4[CyS^-] + k_5[Cat^-])}{k_3}. \quad (14)$$

Equation 11 indicates that rate v is a function of $[Cat^-]$, as well as $[CyS^-]$ and $[O_2]$. When dioxygen is arranged as the variable substrate and either cysteine or catecholamine as the changing fixed substrate, a family of lines in the reciprocal plot should intersect at a point. The double-reciprocal plots of v against $[O_2]$ at various concentrations of dopamine are shown in Figs. 6 and 7, where $[CySH]_0$ is fixed at 2.00×10^{-3} M. A family of lines at pH 7.2 overlapped within experimental errors giving a single straight line; it indicated that dopamine did not affect the autoxidation system. At pH 7.6, in contrast, a family of lines intersected at a point, which fitted the "sequential mechanism".[11,34] Such results clearly support the participation of dopamine in the autoxidation system at pH 7.6.

The Cu(I)–O_2 could oxidize cysteine directly in reaction (IV) or indirectly via formation of ·Cat in reactions (V) and (VI). The Cu(I)–O_2 adduct, a superoxide complex of Cu(I), is capable of donating one electron to catecholamines, splitting itself into Cu(II) species and H_2O_2, and catecholamines would be oxidized to semiquinone radicals. Kinetic data on the catecholamine autoxidation have supported the formation of free radicals, based on the following evidence that O-methylated catecholamines are less susceptible to the autoxidation than their nonmethylated compounds and that melatonin, (*N*-acetyl-5-methoxytryptamine), which is a powerful antioxidant, is capable of scavenging free radical pruduced during the autoxidation.[35] ESR studies on the reaction of dopamine with ascorbate revealed production of dopamine radicals under alkaline conditions.[36] The dopamine radical which is produced first is likely to be replaced by the ascorbate radicals.[36] The produced radical would be either oxidized or reduced. As far as cysteine exists in the medium, the catecholamine radical would be reduced back to catecholamines. Thus, the redox properties of catecholamines should be biologically important.[37] Cysteine functions as both the ligand and the substrate. The cysteine coordinating firmly with Cu(II) hinders the electron transfer from CyS^- to Cu(II) and works as the suicide substrate, while the cysteine coordinating with Cu(I) assists the metal in the O_2 adduct formation and promotes the autoxidation. The coordination mode of Cu(I) in the O_2 adduct modifies the efficiency of the autoxidation system for cysteine.

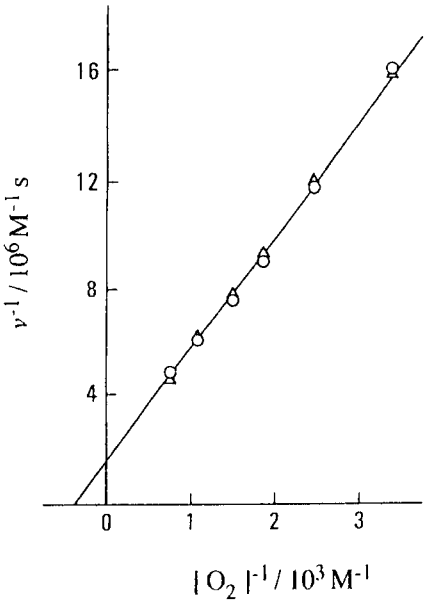


Fig. 6. Double reciprocal plot of the autoxidation rate for cysteine against the concentration of dissolved oxygen at pH 7.2. $[dopamine]_0$: (○), 4.00×10^{-6} M, (△), 1.00×10^{-4} M. Conditions same as under Fig. 2.

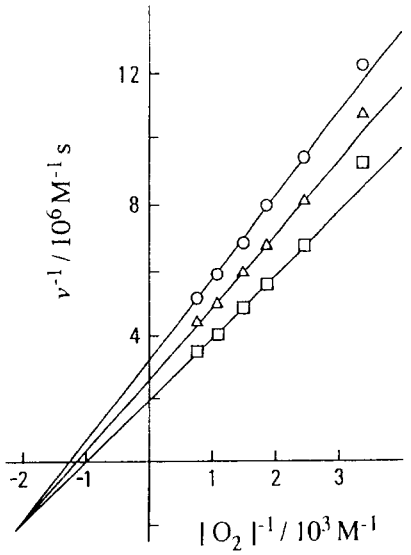


Fig. 7. Double reciprocal plot of the autoxidation rate for cysteine against the concentration of dissolved oxygen at pH 7.5. $[dopamine]_0$: (○), 4.00×10^{-6} M, (□), 2.00×10^{-5} M, (△), 1.00×10^{-4} M. Conditions same as under Fig. 2.

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8 Abbreviation: epinephrine; (*R*)-4-(2-(aminoethyl)-1,2-benzenediol, norepinephrine; (*R*)-4-(2-amino-1-hydroxyethyl)-1,2-benzenediol, dopamine; 4-(2-aminoethyl)-1,2-benzenediol, dopa; 3-(3,4-dihydroxyphenyl)alanine,

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