Stimulation of Cu$^{2+}$-catalyzed Oxidation of Norepinephrine by Nucleic Acid Components

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Oxidation of norepinephrine catalyzed by Cu$^{2+}$ was variously regulated with nucleic acid components. The reaction proceeded by a mechanism of sequential random ordered reaction via formation of the mixed complex of nucleic acid component, Cu$^{2+}$ and aromatic reductone. Using norepinephrine as an aromatic reductone, the promoting activities of nucleic acid components on the oxidation of norepinephrine were compared and the effect of these components to the specific stage of the oxidation process was kinetically investigated. The results indicated that velocity of the oxidation was most remarkably stimulated in the presence of adenine. The velocity was followed by guanine, guanosine monophosphate, cytosine, cytidine, NAD$^+$, adenosine, cytidine monophosphate, uridine monophosphate, then adenosine monophosphate in that order. It was also discussed that adenine was the most plausible nucleic acid component which could participate in the in vivo oxidation of norepinephrine, taking into account the concentration of Cu$^{2+}$ and nucleic acid components in living tissues.

A variety of reductones such as ascorbic acid and polyphenols are found in foodstuff. Triose reductone is a degradation product of monosaccharides during food processing. Catechol amines, which belong to aromatic reductones, are synthesized in vivo from tyrosine or phenylalanine in foodstuff. Subsequently, catechol amine level in living tissues can be controlled by these amino acid contents in foods. On the other hand, concentration of these biologically active amines are also under the metabolic regulations. Cupric ion is one of the plausible regulatory factors of catechol amine concentration in vivo. Oxidation of catechol amines is catalyzed by Cu$^{2+}$ and this reaction is regulated by nucleic acid components. This may suggest that catechol amine level in many tissues and organs is balanced by the oxidation reaction.

The present paper deals with the comparison of accelerating activity among nucleic acid components in the Cu$^{2+}$-catalyzed oxidation of norepinephrine. For the purpose, in the presence of nucleic acid components, velocity and equilibrium constants in the oxidation circuit, which had been proposed by us, were calculated.

MATERIALS AND METHODS

Chemicals. Bases (Cyt, Thy, Ura, Ade and Gua), nucleosides (Cyd, dThd, Urd, Ado and Guo) and nucleotides (P-Cyd, P-Urd, P-Ado and P-Guo) were obtained from Wako Pure Chemical Co., Ltd. and Kojin Co., Ltd., respectively. NAD$^+$ was obtained from Kyowa Hakko Co., Ltd. NE was a product of Fuka Co., Ltd. Each chemical was confirmed to show a single UV-absorbing spot on paper chromatography or paper electrophoresis. The solvent systems used were isopropanol–HCl–H$_2$O (17: 4: 4) and n-
butanol–H₂O) (80: 14) for paper chromatography, and 0.1 M acetate buffer (pH 4.5) for paper electrophoresis, respectively.

Oxidation reaction of norepinephrine catalyzed by Cu²⁺ in the presence of nucleic acid components. The oxidation reaction was performed in 50 mM acetate buffer (pH 5.9) unless otherwise specified. Under a fixed concentration of Cu²⁺ (0.3 mM), a different concentration of NC or NE was mixed. The mixture was incubated in the buffer at 37°C. Then, the oxidation was chased by the augmentation of absorption at 490 nm. Concentration of the oxidation product of NE was calculated by use of the molar extinction of noradrenochrome (ε₄₉₀=3.58×10³) in the acetate buffer.³) Reaction time employed was 30 min, except for Ade. Since Ade extremely stimulated the oxidation at the initial period of reaction and then suppressed, the absorption of the oxidation product was measured after the 2 min-reaction. In those conditions, the oxidative reaction seemed to proceed by a zero-order reaction since the absorption increased linearly. The oxidation reaction of Gua, Thy, Ura or Urd did not proceed within 30 min. In this case reaction time was prolonged.

Calculation of velocity and equilibrium constants. Kinetic analyses have shown that oxidation of NE catalyzed by Ade-Cu²⁺ proceeds via formation of a mixed complex of Ade-Cu²⁺-NE by a sequential random ordered reaction mechanism.²) The process was illustrated in Fig. 1. Symbols Kₐ, Kₜ, Kₐ', Kₜ' and KᵦKₜ'=Kᵦ'Kₜ are dissociation equilibrium constants in corresponding pathways and k indicates velocity constant of the oxidative reaction. Then, oxidation velocity (v) of NE was represented by the equation,

\[
\frac{1}{v} = \frac{1}{k[Cu^{2+}]} \left\{ \frac{K_a' + K_aK_t'}{[NC]} \right\} \frac{1}{[NE]} + \frac{K_t'}{[NC]} + 1
\]  

(1)

If I₀ was plotted versus 1/[NC] in equation (2), the intercept with vertical axis was 1/[Cu²⁺] and with abscissa was −1/Kₜ'. On the abscissa, value of the intersecting point met by a group of lines which were drawn using the concentration of nucleic acid component as a parameter was given by −1/Kₐ. By the similar treatment using the concentration of norepinephrine as a parameter, −1/Kₐ' was obtained from the intercept of abscissa of the Lineweaver-Burk plot. Values Kₐ, Kₜ, Kₐ', Kₜ' and KᵦKₜ'=Kᵦ'Kₜ were also calculated with respect to each nucleic acid component, respectively. These values were computed by FACOM 230–75 using the programing described elsewhere.⁴)

RESULTS

Oxidation of norepinephrine catalyzed by Cu²⁺ in the presence of nucleic acid components

Effect of bases. In the presence of 0.3 mM Cu²⁺, the velocity of Cyt dependent NE oxidation was measured in different concentration of NE (0.1, 0.3 and 0.6 mM) and NE dependent NE oxidation was also tested using various concentration of Cyt (1, 3 and 9 mM). The results were shown in Fig. 2-a. The oxidation rate was enhanced depending on both concentration of Cyt and NE. This result indicated that as was the case of Ade, Cyt stimulated NE oxidation by composing the mixed complex, Cyt-Cu²⁺-NE. Though NE oxidation by Cu²⁺ alone was so small that absorbance at 490 nm of the reaction mixture attained 0.01 after a 30 min incubation even in the presence of the highest concentration of NE, but the reaction was promoted 30 to 35 folds by addition of Cyt.

Ade showed a remarkable effect on oxidation of NE at initial 2 min, followed by a decreasing rate. The initial reaction rate markedly depend on the concentration of Ade and NE. In order to measure the oxidation under the
zero-order reaction, the concentrations of Ade and NE were lowered to 0.6 to 0.1 mm and 3 to 0.1 mm, respectively. By addition of Ade, oxidation of NE was enhanced 5 to 85 folds than by Cu²⁺ alone (Fig. 2-b).

It was difficult to estimate the oxidation rate of Gua under the same condition as other NC, since Gua was scarcely soluble in the buffer solution used on account of its intramolecular hydrogen bondings. Then, the concentration of both Cu²⁺ and NE was modified to 0.3 mm. Even though NE was incubated with various concentration of Gua (0.0667, 0.02 or 0.00667 mm Gua) for longer time the oxidation was not effective. This result was in contrast to that of Ade, which remarkably promoted the oxidation of NE even at 0.1 mm. Thy or Ura never promoted the oxidation.

**Effect of nucleosides.** The activity of Cyd dependent NE oxidation was not so remarkable as that of Cyt. Ado showed the NE oxidation activity, but the effect was not followed by the suppressive one as Ade. Since Guo was poorly soluble, the concentration of Guo used was 1.67 ~ 0.167 mm. Although Gua did not show oxidation activity to NE, Guo remarkably promoted the oxidation. These results are shown in Fig. 3.

To investigate the oxidative activity of dThd and Urd, each nucleoside (9 mm) was added to the mixture of NE-Cu³⁺ (contained 3 mm NE and 0.3 mm Cu²⁺). The reaction conditions were the same as those used for the assay of oxidative activity of their bases. The results indicated that these nucleosides had little effect on the oxidation of NE.

**Effect of ribonucleotides and NAD⁺.** Oxidation of NE by ribonucleotides was also investigated. P-Cyd was effective for the oxidation of NE as well as Cyt and Cyd. The effect of P-Ado on the oxidation of NE was similar to that of Ado, but not of Ade. P-Ado exhibited the stimulative effect. P-Guo also remarkably promoted the oxidation as Guo. P-Urd showed a significant effect on the oxidation of NE, though Ura or Urd had little effect. The results of oxidation of NE by these nucleotides are summerized in Fig. 4.
Oxidation of NE was measured after a 30 min-incubation. In both (a) and (b), the concentrations of NE in panel A were 0.1 mM, 0.3 mM, and 0.6 mM; and of nucleoside in panel B were 1 mM, 3 mM, and 9 mM. In the case of (c) Guo, the concentrations of NE in panel A were the same as those in (a) and (b), however the concentrations of Guo in Panel B were modified to 0.167 mM, 0.5 mM, and 1.67 mM.

NAD\textsuperscript{+} was also a potentially active reagent for the oxidation of NE as the nucleotides tested (Fig. 5).

**Calculation of equilibrium and velocity constants**

Equilibrium and velocity constants of the oxidation reaction in the presence of each NC

![Graphs showing the effect of Cyd, Ado, Guo, and NAD\textsuperscript{+} on the oxidation of NE.](image)

**Fig. 3.** Effect of Cyd, Ado and Guo on the Oxidation of NE.

**Fig. 4.** Effect of Ribonucleotides on the Oxidation of NE.
FIG. 5. Effect of NAD\(^+\) on the Oxidation of NA.
Both concentrations of NA in panel A and NAD\(^+\) in panel B were similar to those described in Fig. 3.

were computed from the results shown above and listed in Table I. When the concentrations of Cu\(^{2+}\) and NE were 0.3 mm and 1 mm respectively, the oxidation velocities \(v\) in the presence of NC were given by substituting the corresponding equilibrium and velocity constants in equation (1). Ade had the most significant \(v\) value and followed by Guo, P-Guo, Cyt, Cyd, NAD\(^+\), Ado, P-Cyd, P-Urd and P-Ado, which decreased in that order. Even though nucleotides had almost the same oxidation rate, their velocity and equilibrium constants largely deviated from each other. Ade also had extraordinarily large velocity constant \((k)\), which was followed by P-Guo, P-Cyd, Cyt, P-Ado, Cyd, NAD\(^+\), Guo, Ado and P-Urd in that order. P-Ado had a small \(v\), but showed relatively large \(k\). The value of KaKb' (Ka'Kb) indicated the facility of the mixed complex formation (the smaller the value, the easier to form the complex). The results showed that Ade formed the mixed complex easily. The facility was followed by Guo, P-Urd, Ado, NAD\(^+\), Cyt, P-Ado, Cyd, P-Guo and P-Cyd in that order. Although the mixed complex was easily formed (small KaKb') in the case of Ado or P-Urd, the \(k\) value was small. It was concluded that these NC formed the mixed complex easily, however, \(k\) was so small that the subsequent oxidation proceeded slowly. On the other hand, P-Guo or P-Cyd did not form the mixed complex so easily, but \(k\) values of each nucleotide was so large that the oxidation took place fast. Ratio of Kb/Ka indicated the predominancy between the two routes, in which the first product formed was Cu\(^{2+}\)-NE complex (route I) or NC-Cu\(^{2+}\) complex (route II). Since these

<table>
<thead>
<tr>
<th>NC</th>
<th>(k) x 10(^2) (min(^{-1}))</th>
<th>(K_aK_b' (=K_a'K_b)) x 10(^8) (m(^2))</th>
<th>(K_a) x 10(^2) (m)</th>
<th>(K_b) x 10(^4) (m)</th>
<th>(K_b') x 10(^9) (m)</th>
<th>(K_b/K_a) x 10(^4) (m(^{-1})min(^{-1}))</th>
<th>(\psi) x 10(^4) (m(^{-1})min(^{-1}))</th>
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<tr>
<td>Cyt</td>
<td>1.28</td>
<td>0.746</td>
<td>0.072</td>
<td>14.4</td>
<td>1.38</td>
<td>19.2</td>
<td>1.10</td>
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<tr>
<td>Cyd</td>
<td>1.10</td>
<td>1.45</td>
<td>0.142</td>
<td>7.96</td>
<td>0.781</td>
<td>5.50</td>
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<td>P-Cyd</td>
<td>1.46</td>
<td>0.996</td>
<td>0.102</td>
<td>15.5</td>
<td>1.59</td>
<td>15.6</td>
<td>1.02</td>
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<tr>
<td>Ade</td>
<td>18.9</td>
<td>0.094</td>
<td>0.031</td>
<td>0.645</td>
<td>0.212</td>
<td>6.84</td>
<td>44.9</td>
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<tr>
<td>Ado</td>
<td>0.887</td>
<td>0.936</td>
<td>0.150</td>
<td>4.55</td>
<td>0.730</td>
<td>4.86</td>
<td>1.04</td>
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<tr>
<td>P-Ado</td>
<td>1.20</td>
<td>0.479</td>
<td>0.125</td>
<td>8.40</td>
<td>2.19</td>
<td>17.5</td>
<td>0.824</td>
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<tr>
<td>Guo</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guo</td>
<td>1.00</td>
<td>0.697</td>
<td>0.139</td>
<td>0.763</td>
<td>0.152</td>
<td>1.09</td>
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<tr>
<td>P-Guo</td>
<td>1.88</td>
<td>0.795</td>
<td>0.214</td>
<td>5.42</td>
<td>1.46</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Urn</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>P-Urd</td>
<td>0.877</td>
<td>0.792</td>
<td>0.102</td>
<td>6.66</td>
<td>0.857</td>
<td>8.41</td>
<td>0.996</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dTyd</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>NAD(^+)</td>
<td>1.00</td>
<td>0.925</td>
<td>0.052</td>
<td>17.8</td>
<td>0.847</td>
<td>16.3</td>
<td>1.06</td>
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</table>
equilibrium constants were dissociation constants which were adopted by Michaelis et al., the smaller the value, the more the deviation of equilibrium to give higher concentration of the product. Consequently, that \( K_b/K_a \) was more than 1 indicated that the product by route I was more abundant than that by route II. The ratio was more than 1 for all NC except Guo. This indicated that the equilibria in most cases deviated to the route I in which NC combined with \( Cu^{2+}-NE \) complex. Deviation to this route was the most significant in Cyt and followed by \( P-Ado, NAD^+, P-Cyd, P-Urd, Ade, P-Guo, Cyd \) and \( Ado \) in that order. In the case of Guo, the oxidation proceeded equally by both routes. Furthermore, that \( K_a' \) or \( K_b' \) was smaller than \( K_a \) or \( K_b \) implied that NC or NE preferred to bind to \( Cu^{2+}-NE \) or \( NC-Cu^{2+} \) complex to \( Cu^{2+} \).

**DISCUSSION**

Aromatic reductones such as catechol amines have an enediol group in their molecules. The enediol group relates to the development of their biological functions. By oxidation of the enediol group, their biological activities are invalidated. Aromatic reductones have an antitumor activity. These reductones cleave highly polymerized DNA, synthetic homopolyribonucleotides and cellular chromatin DNA. These activities are enhanced by coexistence of \( Cu^{2+} \). We have found that the oxidation of aromatic reductones catalyzed by \( Cu^{2+} \) is controlled by DNA and NC.

The oxidation proceeds by the mechanism that after the co-ordination of \( Cu^{2+} \) with the enediol group, the semiquinone radical and \( Cu^+ \) are formed by one electron transfer. By the kinetic analyses of the oxidation of NE in \( Ade-Cu^{2+} \) system, the stimulated oxidation by Ade is supposed to proceed by the formation of the mixed complex, \( Ade-Cu^{2+}-NE \). Analyses by ESR also support that this oxidation proceeds by one electron transfer.

The kinetic analyses were performed on the oxidation of NE in which participation of bases, nucleosides and nucleotides was investigated. As shown in Table I, \( v, k \) and equilibrium constants were so available according to the kind of NC. On the formation of \( NE-Cu^{2+}-NC \) complex, NC or NE preferred to bind to \( Cu^{2+}-NE \) or \( Cu^{2+}-NC \) to \( Cu^{2+} \). This was explained by the fact that the \( Cu^{2+} \) complex co-ordinated to pyridine- or imidazole-nitrogen tended to combine with the ligand which had anionic oxygen atoms in the structure.

The relation of \( v \) and \( k \) versus equilibrium constant \( (K_aK_b') \) to form the mixed complex was graphically shown in Fig. 6. Ade had the most predominant effect in NC examined. Positive correlation was observed to a certain extent between \( v \) and \( k \), however that was not necessarily detected between \( v \) and \( K_aK_b' \). It was characteristic that \( K_aK_b' \) of Guo was the smallest except that of Ade. As suggested in Table I, in spite that the oxidation activity of Guo was very poor, Guo and \( P-Guo \) had the remarkable oxidation potentiality. These results suggested that a ribose or a ribose phosphate moiety takes part in the stimulation. The \( k \) values were not so different among Cyt, Cyd and \( P-Cyd \), however their \( K_b/K_a \) was very different from each other; Cyd showed the smallest value. Values of \( K_a/K_b \) indicated that the oxidation via route I shown in Fig. 1 was generally more preferred to nucleotides than nucleosides.

Acid dissociation constant \( (pK_a) \) of Ura or
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Thy which make a complex with Cu²⁺ in alkaline solution is 9.45 or 9.94. On the other hand, the pKa value of Cyt or Ade which makes chelate of Cu²⁺ in neutral solution is 4.5 or 4.45. These facts indicate that base dissociated at pH 5.9 such as Cyt and Ade can promote the NE oxidation but Ura, Urd, Thy or dTThd is inert.

In spite of these results, P-Urd significantly promoted the oxidation, suggesting that phosphate of this nucleotide took a part in the stimulation of the oxidation. However, Ado-5'PP or Ado-5'PPP rather suppressed the oxidation. ESR-spectral analyses also verified that P-Ado promoted the formation of P-Ado-Cu²⁺-NE complex, however Ado-5'PP or Ado-5'PPP inhibited the formation of Cu²⁺-NE complex. These facts suggested that the conformation of phosphate(s) in their nucleotides could play an important role in the regulation of the oxidation of aromatic reductones such as NE. Kinetically, the oxidation of NE by NAD⁺ seemed to proceed by the same way as by other NC, and in this case contribution of route I was as much as by Cyt. However, as it has been shown, NAD⁺ altered the ESR spectrum of epinephrine-Cu²⁺ complex and such an effect of NAD⁺ was distinct from that of Cyt or P-Ado. Then, nicotinamide ring in NAD⁺ could be attributable to an ESR-spectral change of Cu²⁺-NE complex.

As explained above, it is evident that in the presence of Cu²⁺, various NC can regulate the oxidation of NE in various levels.

Since biological functions of catechol amines such as adenylyl cyclase activation are remarkably abolished by the oxidation, regulation of the oxidation can govern the function of these amines. Cupric and cuprous ions are considerably distributed in liver and brain. Cupric ion concentration in human blood was 0.02~0.025 mm. Cupric ion incorporated via foodstuffs exists as Cu²⁺-albumin in blood, then it is transported to many tissues. NC content in living tissues are fairly so high that it is not unusual that the content of NC locally goes up to 0.1~0.3 mm. It is also known that exogenously administered catechol amines are attainable to not only cellular cytoplasm of rats but also nuclei of cultured mammalian cells. These results plausibly suggest that NC can participate in regulation of intracellular catechol amine level in co-existence of Cu²⁺. Especially Ade is expected as the most important factor in the metabolic regulatory system. Since the oxidation of NE by Ade is so remarkable that this function is obviously detected even at low pH or temperature, the regulatory phenomenon by this base should be conceivable in living tissues.

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