STUDIES ON THE ACCELERATING EFFECT OF CYANIDE ON ASCORBIC ACID OXIDATION BY INTESTINAL HOMOGENATE OF RATS

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It has been known since the early works on the respiratory systems of animal tissues (1-5), that cytochrome oxidase operates as the terminal system of respiratory chain and the oxidative activity is completely inhibited by cyanide as well as by azide. Stotz et al. (6) and Smith (7) reported that ascorbic acid is oxidized by cytochrome oxidase system in animal tissues.

In the course of the studies on the tissue respiration of rat intestine, it was found that respiration of small intestinal homogenate of rats without substrates was not inhibited by cyanide, and when ascorbic acid or cytochrome c and ascorbic acid were added as substrates, the respiration increased by 20 to 40 per cent in the presence of cyanide (8). This accelerating effect of cyanide on the respiration did not take place in other tissues of rats and various tissues of guinea pigs. Therefore, it can be said that this may be characteristic of rat small intestine. The purpose of the present work is to clarify the cause of this phenomenon.

MATERIALS AND METHODS

Small and large intestine of rats were used for this work. The rats were sacrificed by decapitation. The intestinal tract was removed and washed with cold 0.9 per cent NaCl and then, ground in an ice cold motar and suspended with 0.9 per cent NaCl. When the mucosa and muscle of intestine were used separately, the intestine washed with 0.9 per cent NaCl was opened lengthwise and washed again. The mucosal surface was removed by scraping with the blade of a knife and placed in cold 0.9 per cent NaCl and homogenized in an ice cold mortar. The muscle without the mucosal surface was also homogenized in similar way.

Particulate fraction was prepared from the homogenate of intestine with 0.25 M sucrose according to the principle of Schneider's method (9, 10). The homogenate was centrifuged for 10 minutes at 1,000 g, in a refrigerated centrifuge and the supernatant was again centrifuged for 10 minutes at 10,000 g. The sediment was washed twice with 0.25 M sucrose solution by centrifugation and suspended in the solution. It was examined by microscope that the collected fraction by centrifugation was mostly consisted of mitochondrial particles.
Oxygen consumption was determined in the Warburg apparatus. The respiration was carried out in phosphate buffer (pH 7.2) at 37°C, with the air as the gas phase. Ascorbic acid and cyanide were used after neutralization. Substrates were previously kept in the side arm and poured into the main flask after thermoequation. The rate of the autoxidation of ascorbic acid was measured by using homogenate which was boiled for 5 minutes.

The content of iron in homogenates was determined spectrophotometrically by o-phenanthroline method (11). Homogenates were treated with 5 per cent trichloroacetic acid and centrifuged. The precipitate was hydrolyzed by concentrated sulfuric acid and the hydrolysate was diluted with water and adjusted to pH 4.5 by concentrated NH₄OH. After the addition of 1 ml of 1 M acetate buffer (pH 4.5) to 1 ml of the diluted hydrolysate solution, 1 ml of 5 per cent hydroquinone was added, followed by 0.5 ml of 1.5 per cent o-phenanthroline. The mixture was shaken at each addition and diluted to 5 ml with water and kept at room temperature for 30 minutes and read at 510 mμ. As the blank, 0.9 per cent NaCl instead of homogenate was used. Glass-distilled water was also used to dissolve all reagents and to wash tubes or pipettes.

Nitrogen analysis was performed by micro-Kjeldahl method.

RESULTS

1. Effect of Cyanide on Cytochrome Oxidase System of Intestinal Homogenate

To investigate the oxidative activity of cytochrome system of rat intestine, ascorbic acid, hydroquinone and p-phenylenediamine were used as substrates. In the presence of cyanide, the oxidation of hydroquinone and p-phenylenediamine by homogenate of small intestine was reduced by 28 per cent and 29 per cent respectively, as shown in Table 1. The endogenous respiration of the intestine was not affected in the presence of cyanide whereas the oxidation of ascorbic acid was accelerated. The oxidation of these substrates in large intestine was inhibited in the presence of the cyanide and the endogenous respiration was also strongly inhibited.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Small intestine (μl/30 min)</th>
<th>Large intestine (μl/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21.1</td>
<td>12.1</td>
</tr>
<tr>
<td>10^{-4} M KCN</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>0.02 M Ascorbate</td>
<td>38.8</td>
<td>22.0</td>
</tr>
<tr>
<td>0.02 M Ascorbate + 10^{-4} M KCN</td>
<td>46.2</td>
<td>7.7</td>
</tr>
<tr>
<td>0.02 M Hydroquinone</td>
<td>26.0</td>
<td>28.8</td>
</tr>
<tr>
<td>0.02 M Hydroquinone + 10^{-4} M KCN</td>
<td>7.2</td>
<td>0.6</td>
</tr>
<tr>
<td>0.02 M p-Phenylenediamine</td>
<td>58.8</td>
<td>30.7</td>
</tr>
<tr>
<td>0.02 M p-Phenylenediamine + 10^{-4} M KCN</td>
<td>17.4</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The small intestinal homogenate containing 2.79 mg of protein N.

The large intestinal homogenate, 2.57 mg of protein N. Autoxidations of substrates were subtracted.
Cyanide can be replaced by NaN₃ and ethylenediamine tetraacetate (EDTA), but not AgNO₃, as presented in Table 2. This fact suggests that the acceleration of ascorbic acid oxidation takes place as a result of chelating combination of these reagents with a certain substance contained in homogenate.

2. Influences of Concentrations of Cyanide and Homogenate on Ascorbic Acid Oxidation

The effect of various concentrations of cyanide on ascorbic acid oxidation of small intestinal homogenate was examined. Table 3 shows the result of the experiment. No remarkable change in oxidative activity of ascorbic acid by homogenate was found in the range from $10^{-3}$ M to $5 \times 10^{-4}$ M cyanide concentration, while the autoxidation of ascorbic acid was stimulated in the presence of $2 \times 10^{-3}$ M to $5 \times 10^{-4}$ M. Consequently, the net oxidation of ascorbic acid in the homogenate was inhibited by more than $5 \times 10^{-4}$ M cyanide, and stimulated by $10^{-4}$ M cyanide. These effective concentrations of cyanide were varied with the concentration of homogenates used. The results were presented in Fig. 1. When homogenate containing 1.9 mg of protein nitrogen was used, the optimal concentration of cyanide for acceleration was about $5 \times 10^{-4}$ M, and with homogenate containing 2.98 mg of protein nitrogen the optimal concentration was $10^{-3}$ M. When homogenate was used in high concentration (5 mg of protein nitrogen), no optimal concentration of cyanide

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**TABLE 2. Effects of azide, ethylenediamine tetraacetate and AgNO₃ on ascorbic acid oxidation of rat small intestine.**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>O₂ Uptake (µl)</th>
<th>O₂ rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>NaN₃ $10^{-4}$ M</td>
<td>34.9</td>
<td>-28</td>
</tr>
<tr>
<td>NaN₃ $5 \times 10^{-4}$ M</td>
<td>36.8</td>
<td>-35</td>
</tr>
<tr>
<td>EDTA $10^{-4}$ M</td>
<td>31.9</td>
<td>-17</td>
</tr>
<tr>
<td>AgNO₃ $10^{-4}$ M</td>
<td>6.3</td>
<td>77</td>
</tr>
<tr>
<td>AgNO₃ $10^{-3}$ M</td>
<td>5.1</td>
<td>81</td>
</tr>
</tbody>
</table>

Substrate: 0.02 M Ascorbic acid

**TABLE 3. Effect of cyanide concentrations on ascorbic acid oxidation of rat small intestine.**

<table>
<thead>
<tr>
<th>Concentration of KCN</th>
<th>O₂ Uptake in 30 min</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A Homogenate (µl)</td>
<td>Boiled homogenate (µl)</td>
</tr>
<tr>
<td>0</td>
<td>40.8</td>
<td>11.6</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$ M</td>
<td>49.2</td>
<td>17.3</td>
</tr>
<tr>
<td>$2 \times 10^{-3}$ M</td>
<td>52.6</td>
<td>32.9</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td>48.0</td>
<td>32.2</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$ M</td>
<td>36.7</td>
<td>28.8</td>
</tr>
</tbody>
</table>

Substrate: 0.02 M Ascorbic acid
was obtained.

3. The Influence of Cyanide on Homogenates Differently Treated

For obtaining further informations on the above mentioned phenomenon, the particulate fraction, the supernatant and the sediment of homogenates treated by 5 per cent trichloroacetic acid and boiling homogenates were used. The acceleration by cyanide was found also in oxidation of ascorbic acid in particulate fraction as shown in Table 4. Table 5 shows that the acceleration was more remarkable in the oxidation of the sediment than that of the supernatant. It is apparent from Table 4 and 5 that the ascorbic acid oxidizing system accelerated by cyanide is, at least, not in soluble state in homogenate, but combined with particulate fraction of homogenate or homogenate protein and hard to be released by trichloroacetic acid treatment or boiling.

<table>
<thead>
<tr>
<th>Addition</th>
<th>$O_2$ Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.2</td>
</tr>
<tr>
<td>0.02 M Ascorbate</td>
<td>11.6</td>
</tr>
<tr>
<td>0.02 M Ascorbate + $10^{-2}$ M KCN</td>
<td>24.4</td>
</tr>
</tbody>
</table>

Numerals refer to $\mu$l of consumed oxygen for 30 min per mg of protein N.

### Table 4. Effect of cyanide on ascorbic acid oxidizing system of particulate fraction from rat small intestine.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Homogenate ($\mu$l)</th>
<th>Supernatant ($\mu$l)</th>
<th>Sediment ($\mu$l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 M Ascorbate</td>
<td>29.6</td>
<td>40.6</td>
<td>35.7</td>
</tr>
<tr>
<td>0.02 M Ascorbate + $10^{-2}$ M KCN</td>
<td>41.6</td>
<td>43.7</td>
<td>51.1</td>
</tr>
</tbody>
</table>

5% TCA treatment

<table>
<thead>
<tr>
<th>Addition</th>
<th>Supernatant ($\mu$l)</th>
<th>Sediment ($\mu$l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 M Ascorbate</td>
<td>40.2</td>
<td>42.0</td>
</tr>
<tr>
<td>0.02 M Ascorbate + $10^{-2}$ M KCN</td>
<td>48.7</td>
<td>54.6</td>
</tr>
</tbody>
</table>

Numerals refer to $\mu$l of consumed oxygen for 30 min.

TCA : Trichloroacetic acid.

4. Effects of Cyanide on Ascorbic Acid Oxidation in Mucosal or Muscular Homogenates

Mucosa or muscle of small intestine was used separately to examine the localization of the ascorbic acid oxidizing system accelerated by cyanide. As shown in Table 6, cyanide stimulated the oxidation of ascorbic acid in mucosal homogenate, while it had no effect on the autoxidation of ascorbic acid by the boiled homogenate. On the contrary, the oxidation in muscular homogenate was not affected, but the autoxidation of ascorbic acid was accelerated in the presence of cyanide. As a result, cyanide accelerated the net oxidation of ascorbic acid in mucosa and it inhibited the oxidation in muscle.

5. The Iron Levels of Intestinal Homogenates

It is obvious that ferric iron and copper are substances oxidizing ascorbic acid in vitro. Granik (12) reported that organic ferric iron in the form of ferritin exists in various
organs. Endicott et al. (13) suggested that ferritin also exists in gastrointestinal tract of rats. The content of iron in protein precipitated with 5 per cent trichloroacetic acid was determined, although the value did not manifest the true iron of ferritin as it showed total iron in protein of homogenates. In Table 7, total iron per milligram of nitrogen in small intestine showed low level as compared with that of large intestine. And, in the small intestine, iron levels in mucosa were found to be higher than that in the muscle.

**DISCUSSION**

Ascorbic acid is oxidized by cytochrome-cytochrome oxidase system in animal tissues as described above. If this oxidative system of rat small intestine is accelerated by cyanide, then, the oxidation of hydroquinone and \( p \)-phenylenediamine also must be accelerated by it. However, as shown in Table 1, the acceleration is restricted to ascorbic acid. The phenomena mentioned above, therefore, suggest that the acceleration of the oxidation of ascorbic acid by cyanide in rat small intestine is not due to the enzymatic system of organs. Endicott et al. (13) suggested that ferritin also exists in gastrointestinal tract of rats.

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ascorbic acid oxidation.

Interesting fact is that the effect of cyanide varied with both its concentration and homogenate concentration. This was illustrated in Fig. 1. Cyanide of $10^{-4}$M stimulated greatly the oxidation of ascorbic acid and higher concentration of cyanide inhibited it. Here the homogenate contained about 3 mg of protein nitrogen. With a lower concentration of homogenate (1 to 2 mg of nitrogen) the optimum of cyanide for ascorbic acid oxidation shifted toward lower side, and no optimal concentration of cyanide could be obtained, when higher concentration of homogenate (about 5 mg of nitrogen) was used. From these facts, it is suggested that, in the rat small intestine, there is an inhibitor or inhibiting system which suppresses ascorbic acid oxidation and is removed by cyanide. Therefore, cyanide in relatively lower concentration, as compared with homogenate concentration, may combine specifically with only the inhibitor or inhibiting system; this combination may result in stimulation of ascorbic acid oxidation, on the contrary, cyanide in relatively higher concentration may attack not only the inhibitor or inhibiting system, but also ascorbic acid oxidizing system, so that the oxidation of ascorbic acid may be inhibited.

The results from Table 4 and 5 showed that the ascorbic acid oxidizing system affected by cyanide exists in particulate fraction, at least in protein part. And also, as shown in Table 2, in which azide and EDTA brought out the same effect as cyanide, it is apparent that this effect takes place as a result of chelating combination of cyanide, azide or EDTA with the inhibitor or inhibiting system in protein part. Consequently, it is expected that a part of this inhibitor or inhibiting system consists of heavy metals. From the results of the determination of iron content, it was found that the content of iron in the mucosa of small intestine was higher than that in the muscle (Table 7), and the cyanide effect of ascorbic acid oxidation occurred only in mucosal homogenate as shown in Table 6. Nevertheless, Table 7 presented that iron levels in small intestine are not higher than that in large intestine, in spite of the fact that the cyanide effect was restricted by small intestine. Gabrio and Solomon (14) reported the formation of ferritin in the mucosal cells, especially in the duodenal region and Granik (15) found that the formation of ferritin is marked in the duodenal region of the mucosa and diminishes in the lower portion of the intestinal tract. It is suggested from these reports and the results from Table 6 and 7 that the bulk of iron in the large intestine may exist in a form distinct from ferritin.

Granik (12) reported that ferritin takes part in the regulation of iron absorption in mucosal cell, and ferric hydroxide micelles of ferritin are converted to ferrous iron by a reducing system such as SH compounds and by ascorbic acid, and ferrous iron then diffuses into blood stream. If, in the small intestine, ascorbic acid is oxidized by ferritin and the oxidation is stimulated in the presence of cyanide, its stimulating effect has to occur also in liver which has relatively huge amount of ferritin (12). No acceleration in liver, however, was found in the present experiment. It seems therefore that ascorbic acid is oxidized not merely by free ferritin, but by the metabolic system containing fer-
ritin in the small intestine. Schlutze et al. (16) have found that glutathione and fixed SH compounds of the tissues are responsible for the reduction of added dehydroascorbic acid unless a large excess of ascorbic acid exists. Furthermore, it has been known that glutathione protects ascorbic acid against oxidation by ascorbic acid oxidase. From the facts described above it is suggested that in rat small intestine SH compounds contained in reducing system of ferritin act as an inhibitor of ascorbic acid oxidation and the stimulation of ascorbic acid oxidation by cyanide is due to its effect on the metabolic processes including ferritin and its reducing system, even though exact definition of the nature of the accelerating effect of cyanide must require further investigation.

**SUMMARY**

It was found that the oxidation of ascorbic acid by rat small intestine was accelerated by $10^{-4}$M cyanide which inhibited the oxidation of hydroquinone and $p$-phenylene-diamine. The oxidation of ascorbic acid was accelerated also by azide and ethylenediamine tetraacetate. The degree of acceleration was dependent on the concentration of cyanide and the tissue homogenate. The ascorbic acid oxidizing system accelerated by cyanide combined with particulate fraction (mitochondrial fraction) of the intestine, and was localized mainly in the intestinal mucosa. Total iron levels in the mucosa were found to be higher than that in the muscle. The results obtained suggest that the acceleration is not due to the direct effect of cyanide on cytochrome-cytochrome oxidase of the small intestine but due to its indirect effect on a given system, naturally occurring in the rat intestine and inhibiting the oxidation of ascorbic acid. From the results obtained, the role of iron transporting system in the intestine on the ascorbic acid oxidation and the effect of cyanide on its system were discussed.

**Acknowledgement**: I wish to express my sincere appreciation to Prof. T. Suzuki, Department of Pharmacology, Fukushima Medical College for his kind encouragement and advice during course of the present study.

**REFERENCES**

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