Citric Acid as the Principal Serum Inhibitor of Ceruloplasmin*

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During an investigation of the effects of various compounds on the oxidase activity of ceruloplasmin, a strong inhibitory activity of undialyzed human serum was observed. Dialysis removed this inhibitor of ceruloplasmin from serum as shown in Figs. 1 and 4. However, dialysis did not diminish the strong inhibition by serum of the oxidation of ascorbate by Cu++, possibly due to the presence of serum albumin. The dialysate had no effect on ascorbate oxidation catalyzed by Cu++, indicating that: (a) there is a specific inhibitor(s) of ceruloplasmin in serum; and (b) the inhibitor(s) is dialyzable. When the dialysate was added back to the serum, the inhibition was restored as shown in Fig. 4. While this work was being completed, Walsh also reported the presence of an unidentified dialyzable inhibitor of ceruloplasmin in serum (1).

In order to identify the inhibitor in human serum, fresh samples of 80 to 100 ml of whole blood from five donors were collected and allowed to clot. The serum was separated by centrifugation and was treated according to the method of Huehns and Shooter (2) to obtain the ultrafiltrate. Each sample was shown to inhibit the ascorbate oxidase activity of purified ceruloplasmin (ultracentrifugally homogeneous and treated with Chelex 100). When a very small volume of serum ultrafiltrate (2.0 μl) was added to 3 μl of reaction mixture in the presence of 3.8 × 10⁻⁵ M ascorbate and 2 × 10⁻⁷ M ceruloplasmin, the rate was inhibited 25 to 50%. Preliminary experiments showed that the inhibitor(s) could pass through a column of CG-120, a strong anion resin, suggesting its acidic nature. Therefore, each ultrafiltrate was treated with a small column of CG-120. The strongly acidic solution obtained was then concentrated to a very small volume and subjected to examination by paper chromatography, with propionic acid-butanol-water (2:2:1) as the solvent. Two major spots and a minor spot were detected.

The strongly acidic solution obtained was then chromatographed, with propionic acid-butanol-water (2:2:1) as the solvent. Two major spots and a minor spot were detected. The mobility of the two major spots corresponds to that of authentic citric acid (Rf 0.38) and lactic acid (Rf 0.68) which were spotted on the same paper sheet. A major part of the inhibitory activity of the original dialyzed serum was associated with eluants from the two major spots. The ultrafiltrate of fresh human serum was examined by two other criteria to establish the presence of citric acid. Citrate was specifically detected by the formation of a pentaboromocresol-thiourea complex, described by Natelson et al. and modified by Elliot (3). Confirmatory data was also suggested by its conversion to ammonium citrazinate which gave strong fluorescence in the ultraviolet (4). We are investigating the identity of any other inhibitors, although they are not in sufficient concentration to contribute significantly to ceruloplasmin inhibition in normal human serum.

The effect of citrate on the oxidation of ascorbate and other substrates by ceruloplasmin was tested, and the strong inhibitory activity of citrate, under appropriate conditions, was confirmed. Among the common organic acids tested as reported in Table I, citrate is the only acid which is sufficiently inhibitory and in sufficient concentration to account for the inhibition in serum. The amount of citrate in human serum is reported to be 1.4 to 3.2 mg/100 ml (3) or 0.7 to 1.7 × 10⁻⁴ M. This has been con-

![Fig. 1. Effect of dialysis on ceruloplasmin (CP) activity in serum. At zero time, fresh serum (S) and dialyzed serum (Sd) were added to 3.5 × 10⁻⁴ M ascorbic acid in 0.2 M acetate buffer, pH 5.2. No addition was made to a third sample containing the same reaction mixture. At 2 minutes, 1.93 × 10⁻⁶ M ceruloplasmin was added to all three reaction mixtures. As indicated ceruloplasmin can oxidize ascorbate only in serum which had been dialyzed.](http://www.jbc.org/)

**Table I**

**Inhibition of ceruloplasmin by organic acids**

No inhibition was observed by the following related acids at the indicated molarities: oxaloacetic, 32; glycevic, 660; malonic, 660; pyruvic, 660; α-ketoglutaric, 660; dihydroxymaleic, 330; malic, 660; fumaric, 330; ascorbic, 660.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>DL-Isocitric acid</td>
<td>100</td>
<td>95</td>
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<td>Oxalate acid</td>
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<td>Oxaloacetic acid</td>
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<td>80</td>
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<td>cis-Aconitic acid</td>
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<td>25</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>660</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>&gt;1000</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ascorbate was 3.8 × 10⁻⁴ M in a 3.0 ml volume containing 0.2 M acetate buffer at pH 5.2; temperature, 30°C. Ceruloplasmin (homogeneous and treated with Chelex 100) concentration, 2.0 × 10⁻⁶ M.

† This concentration corresponds to that of citrate in human blood.
firmed in our laboratory; the estimated citrate concentration in 60 normal human serums was 0.4 to 2.6 \times 10^{-4} \text{ M}, as determined by the method of Elliot as described by Stern (3). Thus, the normal citrate concentration of serum is several hundred times greater than the K_I value of 4 \times 10^{-3} \text{ M} reported below. Presumably, the oxidation of ascorbate and other ceruloplasmin substrates in the plasma will be strongly inhibited by citrate. The amount of serum isocitric acid, another powerful ceruloplasmin inhibitor (see Table I), was determined enzymically.

**Fig. 2.** The competitive inhibition of ceruloplasmin by citrate as measured spectrophotometrically in a Beckman model DK-1 recording spectrophotometer at 30°. The velocity (v) of ascorbate oxidation by ceruloplasmin with or without presence of the inhibitor, citrate, at various concentrations of ascorbate (AA) were plotted against v/ascorbate concentration; v in terms of absorbance change at 265 nm per minute. O---O, no citrate; \( \bullet \bullet \bullet \), 2.0 \times 10^{-7} \text{ M} citrate; \( \circ \circ \circ \), 5.0 \times 10^{-7} \text{ M} citrate.

**Fig. 3.** Competitive inhibition by citrate of the ceruloplasmin-catalyzed oxidation of \( N,N \)-dimethyl-\( p \)-phenylenediamine (DMPPD) as measured spectrophotometrically at 30°. The velocity (v) of \( N,N \)-dimethyl-\( p \)-phenylenediamine oxidation as measured in terms of the linear absorbance change at 550 nm per minute by ceruloplasmin with and without citrate, at various concentrations of \( N,N \)-dimethyl-\( p \)-phenylenediamine was plotted against v/(DMPPD). O---O, no citrate; \( \circ \circ \circ \), 2.0 \times 10^{-7} \text{ M} citrate; \( \bullet \bullet \bullet \), 5.0 \times 10^{-7} \text{ M} citrate; \( \triangle \triangle \triangle \), 10 \times 10^{-7} \text{ M} citrate.

1 It is recognized that the treatment of the data usually implicit in Figs. 2 and 3 is obviously oversimplified because the concentrations of inhibitor and enzyme are approximately equal. The full appropriate kinetic treatment will be given in a subsequent paper.

Correlation of the total ceruloplasmin activity and the copper content of human serum precludes the possibility of a significant amount of a nondialyzable ceruloplasmin inhibitor in normal human serum. This is in agreement with Walshe (1), who reported a nondialyzable ceruloplasmin inhibitor only in serum from patients with Wilson's disease.

Further kinetic studies on the inhibition by citrate of the oxidase activity of ceruloplasmin are shown in Figs. 2 and 3. The inhibition is competitive for both ascorbate and \( N,N \)-dimethyl-\( p \)-phenylenediamine oxidation with \( K_I \) values of 4 \times 10^{-7} \text{ M} and 7 \times 10^{-3} \text{ M}, respectively. Citrate also competetively inhibits \( p \)-phenylenediamine oxidation by ceruloplasmin with a \( K_I \) value of 8 \times 10^{-4} \text{ M}. With the use of conventional Warburg techniques at 30° in a 3-ml reaction mixture containing 0.002 \text{ M} ascorbate, 9.0 \times 10^{-3} \text{ M} ceruloplasmin, 1.7 \times 10^{-3} \text{ M} neocuproine, and 0.2 \text{ M} acetate buffer (pH 5.2), 50% inhibition of the initial rate of oxygen uptake was produced by 10^{-4} \text{ M} citrate. Greater concentrations of citrate are required to inhibit ceruloplasmin activity when determined by oxygen uptake, probably because the ascorbate concentration (0.002 \text{ M}) is 100 times greater than in the spectrophotometric method. The partial inhibition of ceruloplasmin by high concentrations of citrate (10^{-4} \text{ M}) first reported by Holmberg and Laurell (7) was considered essentially an anionic effect and was not associated with the presence of citrate in serum beforehand. Broman (8) and Curzon (9) have reported earlier on the inhibition of ceruloplasmin by numerous dibasic acids, none of which inhibited as strongly as the tribasic acids reported here.

In Fig. 4, the amount of citric acid that will restore the inhibition to dialyzed serum to give the same amount of inhibition as undialyzed serum is estimated to be 1.2 \times 10^{-4} \text{ M} citrate. The undialyzed serum used in this experiment contained 1.3 \times 10^{-4} \text{ M} citrate as measured by the formation of pentabromo-
acetone-thiourea complex (3); 1 ml of the same serum was dialyzed against 1 liter of 0.1 M acetate buffer, pH 5.5, and then checked for activity. The results of Fig. 4 show that the concentration of citric acid which gives the same amount of inhibition is very close to $1.3 \times 10^{-4}$ M. Both ceruloplasmin and the inhibitor of the serum were diluted 30-fold on addition to the final reaction mixture. Therefore, the original concentration of citrate in the serum anticipated from this kinetic study is $4 \times 10^{-4} \times 30$ or $1.2 \times 10^{-3}$ M, which is in good agreement with the value, $1.3 \times 10^{-4}$ M, obtained by the pentabromoacetone-thiourea method. This result also strongly suggests that the major inhibitor in the blood is citric acid.

In a previous paper (10), we have discussed the ascorbate oxidase activity of ceruloplasmin and emphasized that ascorbate oxidation could not be due to Cu++ contamination as suggested by Morrell, Aisen, and Scheinberg (11). For example, it was pointed out that several compounds which completely inhibited the Cu++ catalysis of ascorbate, e.g. neocuproine and serum albumin, did not affect ceruloplasmin catalysis of ascorbate oxidation. Citrate inhibition of ceruloplasmin action provides a converse situation, since $10^{-4}$ M citrate does not affect ascorbate oxidation as catalyzed by Cu++, but inhibits ceruloplasmin oxidation over 85%.

Finally, it should be emphasized that tests for ceruloplasmin activity in serum or plasma are valid only under conditions in which serum inhibitors have been removed. Obviously, anticoagulants such as citrate, oxalate, or EDTA, which are ceruloplasmin inhibitors, should be avoided or eliminated through exhaustive dialysis or other procedures. The implications of a common metabolite such as citrate in participating in the control of the activity of this serum enzyme are intriguing. The possible biological and clinical importance of the serum inhibitors of ceruloplasmin is now under further investigation.

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