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, Walshe 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 ultrafiltrate of fresh human serum was examined by two other criteria to establish the presence of citric acid. 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 (5) or 0.7 to 1.7 × 10⁻⁴ M. This has been confirmed in 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.

![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 × 10⁻⁵ 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

<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>
</tr>
<tr>
<td>Oxalic acid</td>
<td>66</td>
<td>30</td>
</tr>
<tr>
<td>Oxalosuccinic acid</td>
<td>330</td>
<td>80</td>
</tr>
<tr>
<td>Lactic acid</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 × 10⁻⁷ 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 × 10⁻⁴ 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 Kᵢ value of 4 × 10⁻³ 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 on five different serums with the use of isocitric acid dehydrogenase (6) and was estimated to be less than 10⁻⁸ M.

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 with Kᵢ values of 4 × 10⁻⁷ M and 7 × 10⁻⁷ M, respectively. Citrate also competitively inhibits p-phenylenediamine oxidation by ceruloplasmin with a Kᵢ value of 8 × 10⁻⁹ M. With the use of conventional Warburg techniques at 30° in a 3-ml reaction mixture containing 0.002 M ascorbate, 9.0 × 10⁻² M ceruloplasmin, 1.7 × 10⁻³ M neocuproine, and 0.2 M acetate buffer (pH 5.2), 50% inhibition of the initial rate of oxygen uptake was produced by 10⁻⁴ M citrate. Greater concentrations of citrate are required to inhibit ceruloplasmin activity when determined by oxygen uptake, probably because the ascorbate concentration (0.002 M) is 100 times greater than in the spectrophotometric method.

The partial inhibition of ceruloplasmin by high concentrations of citrate (10⁻² 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 heretofore. 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.

Fig. 4. Restoration of serum ceruloplasmin inhibition by the addition of both citrate and serum ultrafiltrate (UF). Rates were determined with a Beckman model DR-1 recording spectrophotometer at a constant temperature of 30°. To a reaction mixture of 3.0 × 10⁻⁴ M ascorbate in 0.2 M acetate buffer, pH 5.2, 100 µl of serum (S) or dialyzed serum (SD) were added to a total volume of 3.0 ml. The activity of undialyzed serum is increased by dialysis and is inhibited by both citrate and 100 µl of serum ultrafiltrate (addition indicated by the arrow) obtained from the same serum. In the assay, 1.2 × 10⁻⁴ M citrate, on dilution, gives a final concentration of 4 × 10⁻⁸ M in the reaction mixture.
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.

REFERENCES
Citric Acid as the Principal Serum Inhibitor of Ceruloplasmin
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