A Micromethod for the Determination of Ferrooxidase (Ceruloplasmin) in Human Serums

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A sensitive method for the detection of ceruloplasmin (ferrooxidase) in human serums, using Fe(II) as the substrate, is described. Initial Fe(II) concentrations of 30 and 120 μM may be used, depending on the sensitivity of the spectrophotometer, for the determination of the rate of Fe(III)-transferrin formation following the oxidation of Fe(II) by ceruloplasmin (ferrooxidase). Advantages of this method over other methods employing p-phenylenediamine as a substrate and the standardization of enzyme activity in terms of international enzyme units are discussed.

Since 1948, when Holmberg and Laurell (1) first isolated the blue alpha₂ globulin from plasma, which they called ceruloplasmin, various methods have been employed for the determination of its enzymatic activity and/or concentration in human serum. These methods include: the determination, in a Warburg apparatus or spectrophotometer, of the oxidase activity of the enzyme toward p-phenylenediamine or its derivatives; the determination of light absorption at 610 mμ by ceruloplasmin; the determination of ceruloplasmin-bound copper; and the determination by immunochromatographic assay using specific anticeruloplasmin serums.

In 1956, Ravin (2) reported the first colorimetric method using p-phenylenediamine. Many modifications subsequently have been published based on the use of p-phenylenediamine or its derivatives (3–13). More recently, Rice (14, 15) proposed a colorimetric method suitable for standardization in terms of international enzyme units based on...
"Bandrowski's Base" as simulating the major oxidation product of p-phenylenediamine. Articles pertaining to other methods of ceruloplasmin assay and physical and chemical properties of ceruloplasmin are summarized elsewhere (16, 17).

Colorimetric technics, using p-phenylenediamine or related compounds, are perhaps the most convenient and widely used methods currently employed for ceruloplasmin determination. However, this reaction is complicated by the formation of several colored products (18–20). The amount of each product formed changes during the reaction, producing a nonlinear absorbance versus time relationship. Thus, considerable variation of estimated reaction rates can result because of this nonlinearity.

Recently, we studied the enzymic activity of ceruloplasmin toward Fe(II) as a substrate and proposed the more suitable name "ferroxidase" instead of ceruloplasmin (21, 22). While the name ceruloplasmin, coined by its discoverers, Holmberg and Laurell (1), will always retain its historical import, we anticipate that the name ferroxidase may be more useful than designating this enzyme as a sky-blue substance from plasma.

The fact that the molecular activity of ceruloplasmin toward Fe(II) is many times greater than any known substrate (18), formed the basis for an investigation of a sensitive and rapid assay of ferroxidase activity, described in this paper.

The basic principle for our new method, reproduced from Osaki et al. (21), is shown as follows:

\[
\begin{align*}
\text{H}_2\text{O} + & \text{ceruloplasmin} \\
\text{O}_2 & \rightarrow \text{reduced ceruloplasmin} \\
& 2 \text{Fe}^{2+} \\
& \rightarrow 2 \text{Fe}^{3+} \\
& \text{apo transferrin} \\
& \text{transferrin}
\end{align*}
\]

**Materials**

*Ferroxidase* Crystalline ceruloplasmin (ferroxidase) was obtained by the method of Deutsch (23) as slightly modified by Osaki et al. (24). Stock solutions, 2–166 \(\mu\)M, were passed through a column of Chelex-100 (Bio-Rad) to remove nonenzymic Cu ion.

*Serum* A sufficient volume of blood was collected to permit the recovery of at least 10 \(\mu\)l. of serum for each assay. The ferroxidase activity in serum is stable for several weeks at 0° or in the frozen state.

*Apo transferrin* A 2.0% (w/v) stock solution (2.2 \(\times\) 10\(^{-4}\) M) of iron-free transferrin (code no. 8547, Hoechst Pharmaceuticals, Inc.) was prepared in Chelex-100 treated water. This solution is stable at 0° for several weeks.
The substrate Fe(II) A solution of crystalline reagent grade Fe(NH₄)₂(SO₄)₂·6H₂O (J. T. Baker Co.) in deoxygenated water was prepared just prior to assay. Prepurified grade nitrogen gas, purity, 99.997% minimum (The Matheson Co., Inc.), was used for this purpose. For 30 µM Fe(II) as substrate, a 1.0 × 10⁻⁴ M (0.0392 gm./L.) stock solution was prepared and a 4.0 × 10⁻⁴ M (0.1568 gm./L.) stock solution for 120 µM Fe(II). These solutions are stable up to 8 hr., provided a low oxygen concentration is maintained.

Buffer A 1.2 M (147.7 gm./L.) acetate buffer of pH 6.0 was used after passing through a Chelex-100 column to reduce metal ion contamination. The final concentration of the buffer in the reaction mixture was 0.20 M. Although the optimum pH value observed was pH 6.5, pH 6.0 was chosen to reduce nonenzymic oxidation and to retain sufficient enzymatic activity and adequate buffering capacity.

Methods

One of the two optimal substrate concentrations may be used in the assay, depending upon the sensitivity of the spectrophotometer used.

A Cary recording spectrophotometer, Model 15, capable of presenting 0.1 absorbance unit on a 10-in. chart scale, was used for an initial Fe(II) concentration of 30 µM in the reaction mixture. The time course of the absorbance change at 460 mµ was recorded on the chart for a period of 1–3 min. or until an absorbance change of 0.01–0.02 units occurred, in order to allow adequate precision in the estimation of the initial reaction rate.

An initial Fe(II) concentration of 120 µM may be used for determining rates with other spectrophotometers, e.g., a Beckman DU, that provides a reliable measurement of an absorbance change of 0.3.

Procedure for 30 µM Fe(II) using a spectrophotometer equipped with 0.1 absorbance expansion Spectrophotometer cuvettes, 10 mm. in path length, that provide a working volume of 1.0 ml. were used (e.g., S18-260 1013, Pyrocell Manufacturing Co.). The cuvette contained 0.170 ml. buffer, 0.270 ml. Chelex-100 treated water, 0.250 ml. apotransferrin stock solution, and 2–10 µl. of serum. A 0.2-ml pipet (e.g., Kimble 37022, Mohr, long tip, color-coded) may be used for delivering the buffer to the cuvette after calibrating for 0.170-ml. delivery. A 0.5-ml pipet of similar design may be used for delivering apotransferrin and Chelex-100 treated water to the cuvette after calibrating for delivery of the volumes indicated. For transfer of 2–10 µl. of enzyme solution or serum to the cuvette, Kimble 37065 Lang-Levy microliter pipets (calibrated to deliver stated volume ± 5%) may be used. After the solution was mixed by inverting several times, the cuvettes were placed in the thermostat-equipped cell holder of the
spectrophotometer and equilibrated for 3 min. at 30°. Stock solution of Fe(II) was added (300 μl of 1.0 × 10⁻⁴ M), and the cuvette was inverted several times again to insure thorough mixing, and immediately replaced in the spectrophotometer.

**Procedure for 120 μM Fe(II)**  The reaction mixture was prepared as above except that 300 μl of a 4.0 × 10⁻⁴ M stock solution of Fe(II) was used. After mixing and replacement in the spectrophotometer, the initial absorbance value at 460 mμ was determined, and again after a change of 0.1 ± 0.02 absorbance units due to the reaction.

**Control**  A control for each of the above procedures was prepared by omitting only the serum. The absorbance change that occurred during the same time interval as the sample was determined and used in the calculation of enzyme concentration.

**Method of Calculation**  The number of international units per milliliter of serum or ferroxidase is calculated as described below:

\[
\frac{(a - a_0) - (c - c_0)}{D \cdot t \cdot \gamma} = \mu \text{M Fe}(III)\text{-transferrin} \text{formed/min./µl. of serum.}
\]

Since the reaction volume is 1 ml.,

\[
\text{I.U./ml.} = \frac{(a - a_0) - (c - c_0)}{D \cdot t \cdot \gamma} = \mu \text{moles Fe}(III)\text{-transferrin} \text{formed/min./ml. of serum.}
\]

where:

\[
\begin{align*}
\text{I.U.} & = \text{international units of serum or ceruloplasmin (ferroxidase) solution.} \\
D & = \epsilon_{460 \text{ mμ}} \mu \text{M Fe}(III)\text{-transferrin} = 0.00250. \text{ This micromolar epsilon value is} \\
& \text{based on the iron concentration (25).} \\
t & = \text{reaction time in minutes.} \\
\gamma & = \text{microliters of serum or enzyme solution added to the reaction mixture.}
\end{align*}
\]

"One unit of any enzyme is that amount which will catalyze the transformation of one micromole of the substrate per minute under defined conditions" (26). From Fig. 1, 0.00357 μmole of ferroxidase will catalyze the transformation of 1 μmole of Fe(II) to Fe(III) in the presence of 120 μM Fe(II) under specified conditions. Or, 1 ml. of serum or enzyme solution containing 3.57 nmoles of ferroxidase contains 1 I.U.*

*The definition of an international unit used here is based on 120 μM Fe(II). If the 30 μM Fe(II) method is used, then 0.00476 μmole of ferroxidase will catalyze the transformation of 1 μmole of Fe(II) to Fe(III) instead of 0.00357 (Fig. 1). Therefore, "international units" obtained in the 30 μM Fe(II) method multiplied by 1.33 will produce values in terms of 120 μM Fe(II), the basis of an international unit used in this calculation.
Since 3.57 nmoles per milliliter of ferroxidase is equivalent to 3.57 μmolar and also to 1 I.U./ml, then μmolar enzyme concentration is calculated by multiplying international units per milliliter by 3.57:

\[ [E] = (\text{I.U. per milliliter}) \times (3.57) \]

**Fig. 1.** Oxidation of Fe(II) by crystalline human ferroxidase at pH 6.0 at 30°. Formation of Fe(III)-transferrin, using 30 μM and 120 μM concentrations of Fe(II) as substrate, was followed spectrophotometrically at 460 mμ. Reaction mixture contained 3.3-66 nM ferroxidase, 55 μM apo-transferrin, 30 or 120 μM ferrous ammonium sulfate, and acetate buffer at pH 6.0. Nonenzymic rates for low and high substrate concentrations were 0.28 and 1.1 μM Fe(III)-transferrin per minute, respectively.

Under these assay conditions the molecular activity of the enzyme is 280 units per μmole. In a previous report (16), a molecular activity of 550 was estimated from an extrapolated \( V_m \) value at pH 6.5. However, it is neither convenient nor practical to use these conditions for the microestimation of ferroxidase activity in serum.

**Results and Discussion**

Standard curves of activity versus concentration of crystalline human ferroxidase, dissolved in 0.2 M Chelex-100 treated sodium acetate buffer, pH 6.0, for low (30 μM) and high (120 μM) initial Fe(II) concentrations are presented in Fig. 1. The nonenzymic rate for low and
high iron was 0.28 μM Fe(III)-transferrin per minute and 1.1 μM, respectively.

A comparison of the results of determining serum ferroxidase concentrations by both high and low initial Fe(II) concentrations (Table 1) indicates that comparable values are obtained. The standard deviation of the enzyme concentration values of individual replicates was approximately ± 5% for crystalline human ferroxidase and also for the serum samples presented. The range of ferroxidase concentrations observed in several human groups is presented in Table 2. These values agree well with values previously determined using p-phenylenediamine as a substrate (27, 28). A very strong positive linear correlation has been observed (Fig. 2) in normal as well as in pathological serums (except for lyophilized serum from patients with Wilson’s disease) between p-phenylenediamine oxidase activity and ferroxidase activity (18). As expected, ferroxidase activity is elevated in pregnancy serum and reduced in cord blood. A somewhat higher level was also observed for "disturbed" patients, but this could be due to a response to a drug

<table>
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<tr>
<th>Sample</th>
<th>Estimated ferroxidase concentration in serum</th>
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<tbody>
<tr>
<td></td>
<td>30 μM Fe(II)</td>
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<tr>
<td></td>
<td>IU/ml</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>5</td>
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<tr>
<td>Female</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant, 6 mo.</td>
<td>0.92</td>
</tr>
<tr>
<td>Pregnant, 9 mo.*</td>
<td>1.51</td>
</tr>
<tr>
<td>Cord*</td>
<td>0.31</td>
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*Collected when the infant was delivered.

<table>
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<tr>
<th>Table 2. Ferroxidase Concentration in Human Serums Determined by Low Substrate Method</th>
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<td></td>
</tr>
<tr>
<td>Cord</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
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<tr>
<td>Pregnant, 9 mo.</td>
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<tr>
<td>Wilson's disease</td>
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<tr>
<th>Table 1. Values Obtained at 2 Different Substrate Concentrations</th>
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<td>Sample</td>
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<td></td>
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<tr>
<td></td>
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<tr>
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<tr>
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<tr>
<td>5</td>
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<tr>
<td>Female</td>
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<tr>
<td>Pregnant, 6 mo.</td>
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<tr>
<td>Pregnant, 9 mo.*</td>
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<tr>
<td>Cord*</td>
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</tbody>
</table>
treatment. It has been recognized that serums from some patients with Wilson's disease have almost no p-phenylenediamine oxidase activity. This is also demonstrated in the present study (Fig. 2). However, several serums that have considerably lower than normal p-phenylenediamine oxidase activity still have appreciable Fe(II) oxidase activity. A finding that indicates the possibility of the presence of a modified ferroxidase or an additional unknown catalyst.

This assay offers several advantages in comparison with the method reported by Rice (14, 15).

1. Only 2–10 µl. of serum, instead of 100 µl., is necessary.

Fig. 2. Correlation between p-phenylenediamine (PPD) and Fe(II) oxidation by human serum. Fe(II) oxidation by human serum is plotted against the corresponding PPD oxidase activity. These data give correlation coefficient, \( r = 0.725 \), indicating strongly positive correlation of the two activities by human serums with at least 98% probability of significance. Open square indicates male serum; open circle, female; solid circle, pregnant; half closed circle, cord blood; X, lyophilized and reconstituted, from patients with Wilson's disease; solid triangle, serum of "disturbed" patient; open triangle, anemia (probably caused by extensive blood loss). Test conditions were as follows: (1) PPD oxidation—200 µl. serum, 9.2 mM PPD and 0.2 mM neocuproine in 0.2 M acetate of pH 5.2 at 30° in total volume of 0.600 ml.; (2) Fe(II) oxidation—10 µl. serum, 30 µM ferrous ammonium sulfate, and 55 µM apotransferrin in 0.2 M acetate of pH 6.0 at 30° in total volume of 1.00 ml. Part of this figure, containing only results obtained with normal human serum, has been published previously (#1).
2. Approximately one-fifth the reaction time is required.

3. In the early stages of p-phenylenediamine oxidation, a negative rate results when hemoglobin is present owing to hemolysis. The subsequent emergence of a positive rate occurs after a reaction time that is proportional to the degree of hemolysis and inversely proportional to enzymic activity. Fe(II) oxidation by serum ferroxidase is unaffected by hemolysis.

4. Both the substrate and the product formed in this assay are physiologic.

5. Intermediates as well as products contribute to the observed absorbance in the p-phenylenediamine assay, resulting in nonlinear rates, whereas Fe(III)-transferrin formation is not complicated by this factor.

6. The calculation of enzyme concentration or international units per milliliter (Table 2) is simplified because of the formation of a single product.

7. The preparation of the substrate is simple and reproducible.

References


