Colorimetric Measurement of Ornithine Carbamoyl Transferase Activity in Plasma, and Results for a Supposedly Healthy Population

Alain Bagrel, Gérard Museur, and Gérard Siest

Determination of ornithine carbamoyl transferase (EC 2.1.3.3) activity in plasma is important for detection of liver diseases. The assay established in this paper has been made optimum. A blank is needed containing both substrates, carbamoyl phosphate and ornithine. We used a new colorimetric assay, based on a complex with a phosphoferric-antipyrine reagent and diacetyl monoxime, to measure the citrulline formed. The highly sensitive assay permits low activities to be determined accurately. Values for blood plasma from 425 supposedly healthy people, varied from 0 to 16 U/liter (95th percentile), and 27% of this population showed an activity of less than 2 μmol of citrulline formed per minute per liter, 2 U/liter being the limit of the method's sensitivity.

Additional Keyphrases: reference values • enzyme activity • liver disease

Ornithine carbamoyl transferase (OCT; EC 2.1.3.3) is located almost exclusively in the liver (1), intramitochondrially (2, 3). In liver disease, this enzyme activity is considerably increased in the blood. Reicher (4) stated that OCT is more sensitive than are L-aspartate aminotransferase (EC 2.6.1.1) or alkaline phosphatase (EC 3.1.3.1) for detecting liver diseases. However, the published or commercial methods (reviewed in 5) are sensitive only to above-normal activities and have generally not been adequate for the values in supposedly healthy subjects. We therefore attempted to develop a more sensitive technique for measuring such activity in healthy subjects.

Consequently, we studied all the variables of the enzymatic and the color reaction and especially interference by pre-existing citrulline and by the color of the substrates, ornithine and carbamoyl phosphate.

Materials and Method

Triethanolamine buffer, 0.4 mol/liter; pH adjusted to 7.7 with HCl (1 mol/liter) at 25 °C.

Urease powder (Biolyon, 6 Rue de La Barre, Lyon, France). A 2 g/liter solution in triethanolamine buffer was prepared just before use. The intrinsic OCT activity of the solution must be checked for each batch.

Carbamoyl phosphate, lithium salt (Fluka AG Chemische Fabrik, CH-9470 Buchs, Switzerland), 50 mmol/liter of urease solution, prepared just before use.

Ornithine hydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio) 5 mmol/liter of triethanolamine buffer.

Phosphoferric-antipyrine reagent: 65 mmol of antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one), 12.5 mmol of FeCl₃, 625 ml of H₃PO₄, and 375 ml of distilled water.

Diacetyl monoxime (Merck, Darmstadt, R.F.A.), 100 mmol/liter of distilled water.

Citrulline (Fluka), 20 mmol/liter of distilled water.

1. Three tubes are prepared for each determination of OCT activity, the protocol being as follows:

<table>
<thead>
<tr>
<th>Tubes</th>
<th>1 OCT activity</th>
<th>2 Blank OCT</th>
<th>3 Pre-existing citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine buffer, ml</td>
<td>—</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Ornithine solution, ml</td>
<td>0.4</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>Serum or plasma, ml</td>
<td>0.1</td>
<td>—</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Incubate tubes (37 °C, 5 min).
2. Add 0.4 ml of carbamoyl phosphate solution to tubes 1 and 2 and 0.4 ml of urease solution to tube 3 (warm solutions to 37 °C before use).

3. Carefully mix and incubate at 37 °C for 20 min. Stop the enzymatic reaction with 1.1 ml of trichloroacetic acid (100 g/liter) and centrifuge the tubes (5000 rpm, 10 min).

4. Add 4 ml of the phosphoferric-antipyrin reagent and 1 ml of the diacetyl monoxime solution to 1 ml of the supernates. Heat the tubes at 100 °C for 20 min, and then place them in the dark, in cooled water, for 10 min.

5. Measure the absorbance at 460 nm. Subtract the absorbance of tubes 2 and 3 from that of tube 1, and calculate the citrulline formed according to a standard plot performed with citrulline.

Express OCT activities in U/liter (micromoles of citrulline formed per minute per liter of the biological fluid).

Results and Discussion

Conditions of the Enzymatic Reaction

**Buffer.** The optimal pH of the enzymatic assay under the conditions for our method is 7.7 (Figure 1). This optimal pH, with the triethanolamine buffer we used, was identical to the optimal pH observed with an EDTA buffer (6). However, it was reported (7) that a barbital acetate buffer led to an optimal pH of 7.0. Figure 1 shows that with our method we obtained 30% more citrulline at pH 7.7 than at pH 7. The Michaelis constant for ornithine for the three following buffers was:

- Triethanolamine buffer, $K_m = 0.38$ mmol
- EDTA buffer (6), $K_m = 1.95$ mmol
- Barbital acetate buffer (7), $K_m = 1.66$ mmol

In triethanolamine buffer the $K_m$ was lowest, and therefore the yield of citrulline was clearly better in the conditions we chose.

Other buffers have been used, such as glycyglycine (8, 9), phosphate (10), and tris(hydroxymethyl)aminomethane (11). However, the first led to a highly colored blank with a nonenzymatic formation of citrulline increasing according to the incubation time (12), and inhibition was observed with the latter two (5, 12).

**Urease quality—Blank OCT.** Commercially available urease contains OCT activity. The use of urease in OCT determination is necessary to eliminate urea from the assay, because it interferes with the determination of citrulline. When OCT activity of urease is determined by using a special blank (Table 1) this activity does not influence the final result. However, we prefer urease exhibiting the least OCT activity. Tube 2 in our method allowed us to avoid this interference caused by urease. This blank also contained both substrates, which enabled their own color and nonenzymatic citrulline formation to be eliminated, since the blank is large when old carbamoyl phosphate solutions are used, because of its hydrolysis (8). Tube 3 in the proposed method is used as a blank to eliminate pre-existing plasma citrulline.

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### Table 1. Measurement of Plasma OCT Activity with Different Types of Urease

<table>
<thead>
<tr>
<th>Urease</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>A (0.415)</td>
</tr>
<tr>
<td>Tube 2</td>
<td>B (0.054)</td>
</tr>
<tr>
<td>Tube 3</td>
<td>C (0.020)</td>
</tr>
<tr>
<td>1 (2+3)</td>
<td>A (0.341)</td>
</tr>
<tr>
<td></td>
<td>B (0.362)</td>
</tr>
<tr>
<td></td>
<td>C (0.359)</td>
</tr>
<tr>
<td>Activity calculated, U/liter</td>
<td>60</td>
</tr>
</tbody>
</table>

Urease from A, Biolyon; B, Merck, batch No. 8489; and C, BD Merieux.

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![Fig. 1. Determination of optimum pH in a triethanolamine medium](image1.png)

![Fig. 2. Michaelis plot for ornithine with human plasma OCT](image2.png)
We assessed the effect of the different components of the enzymatic reaction on the final result. Buffer, urease solution, or ornithine solution produce only a slight color, but carbamoyl phosphate yielded an intense color because of the urea it always contains. This clearly shows the need for a "blank OCT" made up of the entire set of enzymatic reaction reagents.

Optimal substrate concentrations. To avoid substrate inhibition (12), we studied the influence of
substrate concentration on enzymatic activity. Plasma OCT activity is inhibited by ornithine concentrations $>5$ mmol/liter (Figure 2). The same phenomenon occurred with carbamoyl phosphate, with a maximum enzymatic activity when 50 mmol/liter were used (Figure 3). The $K_m$ values were $0.38$ for ornithine and $1.02$ for carbamoyl phosphate (Figures 4 and 5).

**Incubation time and temperature.** For very low and moderate OCT activities the appearance of citrulline is a rectilinear function of incubation time (Figure 6). Figure 7 shows that a temperature of $37^\circ C$ is preferable to 20 or $25^\circ C$ because it leads to more citrulline formation.

**Citrulline Measurement**

Accurate determination of low OCT activities by a simple colorimetric method requires a very sensitive assay for citrulline. We used diacetyl monoxime in a phosphoferric-antipyrine medium (5, 13–16). The colored complex formed absorbed maximally at 464 nm. This method is more sensitive than the usual thiosemicarbazide/diacetylmonoxime assay and linearity is better at low concentrations of citrulline or urea (17). Color is completely developed after 20 min at $100^\circ C$ and remains stable in laboratory light as well as in the dark if one stores the tubes in the dark for the first 10 min after the color-developing reaction.

**Conditions for Applying the Proposed Method**

**Serum–plasma comparison.** The earliest publications concerned with determination of OCT activity dealt with assays of serum, but more and more analyses are being done on plasma. We checked that our technique was suitable for use on serum as well as plasma. The results obtained for 22 patients showed no significant differences between OCT activity in plasma and serum.

**Sensitivity and repeatability.** The method permits determination of serum OCT activities greater than 2 µmol of citrulline/min per liter. The range 0–2 U/liter corresponds to the limit of sensitivity of the method. By repeating the determination 20 times on a plasma rich in OCT, we obtained a mean of $61.4$ U/liter, with a standard deviation of $1.96$ and a coefficient of variation of 3.2%.

**Enzyme preservation.** A temperature of $-40^\circ C$ is preferable to preserve OCT activity (Figure 8). OCT activity in blood is reported to be stable for 24 h at $+4^\circ C$ (11, 18). When the serum is preserved at $-15^\circ C$, OCT activity is stable for a year (18–20). At $27^\circ C$, Ceriotti (7) observe no variation after eight days, but we found that at a temperature of $22^\circ C$ (room temperature), the activity decreases by about 25% after four days of storage.

**Reference Values**

We tested 425 supposedly healthy subjects. We did not observe a gaussian distribution (Figure 9). For 26.8% of the subjects we obtained results that were in the range 0–2 units. Consequently the OCT activity of plasma from supposedly healthy subjects is excessively low, approaching the lower limits of the technique. A few authors have indicated a zero value for OCT in the absence of hepatic disorder (8, 18, 21); others have obtained histograms which appear to be skewed to the right (7, 11). We think that our method permits the determination of low activities.
References