Transketolase Activity of Blood Hemolysate, a Useful Index for Diagnosing Thiamine Deficiency

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Introduction

Transketolase (EC 2.2.1.1, sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycoaldehyde transferase), a thiamine pyrophosphate-dependent enzyme, catalyzes two reactions in the pentose phosphate pathway:

1. Xylulose-5-phosphate + ribose-5-phosphate → sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate
2. Xylulose-5-phosphate + erythrose-4-phosphate → fructose-6-phosphate + glyceraldehyde-3-phosphate

Erythrocytes contain much enzyme activity; serum or plasma contains negligible amounts. Measurement of the activity in the erythrocyte has been used to assess thiamine (vitamin B₁) nutrition in man. The easy availability of this tissue makes it the choice for clinical diagnosis of thiamine deficiency.

There are several methods for evaluating transketolase activity. Brin et al. (1) were perhaps the first to propose a method for clinical use, which has contributed much to the evaluation of thiamine nutrition. This method depends on the stimulation of the activity of a sample when exogenous thiamine pyrophosphate is added (appropriately called the "TPP effect"). Thus, it really reflects a stimulatory effect and not the true transketolase activity.

Dreyfus (2) provided another procedure, which is based on sedoheptulose formation. The original article was clear in its description of the incubation procedure, but the details of the color reaction were ambiguous. This, plus a dilution error [not corrected for six years (3)] caused a lack of interest in this method that is unfortunate, because the method is quite satisfactory when used properly.

By the modification (4) presented here of the method of Brin et al. (1), true transketolase activity can be more accurately estimated. Both substrate disappearance and product formation can be measured from the same color reaction, and the method takes less time than the Dreyfus method.

Principle

A hemolysate of whole blood is incubated at 37 °C for a timed interval with a substrate of ribose-5-phosphate, and the proteins are then precipitated with trichloroacetic acid. Although the true substrates for transketolase are xylulose-5-phosphate and ribose-5-phosphate, only ribose-5-phosphate is used, the xylulose-5-phosphate being derived from ribose-5-phosphate by the action of two enzymes, pentose phosphate isomerase (EC 5.3.1.6, D-ribose-5-phosphate ketol-isomerase) and pentose phosphate epimerase (EC 5.1.3.1, D-ribulose-5-phosphate-3-epimerase), which are present in erythrocytes in nonlimiting amounts. An equilibrium mixture of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate is formed in which the concentrations of ribose phosphate and xylulose phosphate are about equal. The remaining pentose and the sedoheptulose formed enzymatically in the presence of transketolase are measured in the supernatant fluid by conversion to furfural and furfural derivatives, by the action of concentrated hydrochloric acid, and condensation with a polyhydric phenol such as orcinol in the presence of metals ions to form colored complexes. Although the structures of the colored complexes are unknown, they have definite absorption maxima: 670 nm and 580 nm for the derivatives of ribose and sedoheptulose, respectively. The concentrations of these derivatives, measured by differential spectrophotometry (6), are an indirect measure of transketolase activity in the sample of erythrocytes. A subnormal activity is evidence of thiamine deficiency.

Materials and Methods

Reagents

1. Substrate, ribose-5-phosphate disodium salt dihydrate, C₉H₁₀O₅PNa₂·2H₂O, 12 mmol/liter. Dissolve 372 mg in 100 ml of distilled water.

2. Standard ribose-5-phosphate disodium salt dihydrate.

A. Stock standard, 4.0 mmol/liter. Dissolve 124 mg in 100 ml of distilled wter.
B. Working standard, 0.040 mmol/liter. Dilute 1 ml of the stock standard to 100 ml with distilled water.

   A. Stock standard, 4.0 mmol/liter. Dissolve 84.08 mg of sedoheptulose anhydride monohydrate in 100 ml distilled water.

Note: This material (2,7-anhydro-β-D-altro-heptulopyranose monohydrate) is often listed by suppliers as “se-
doheptulose anhydride.”

B. Working standard, 40 μmol/liter. Dilute 1 ml of stock standard to 100 ml with distilled water.

4. Trichloroacetic acid, 150 g/liter. Dissolve 15 g of trichloroacetic acid in enough water to make 100 ml of solution.

5. Orcinol reagent. Dissolve 100 mg in 1.0 ml of absolute ethanol. Prepare freshly each day.

The orcinol should be recrystallized as follows: Dissolve the compound in warm benzene. Separate the supernate from the insoluble syrup. Add two volumes of chloroform to three volumes of supernate. Cool, decant the supernatant fluid from the crystals. Repeat the procedure. Filter the crystals and dry them.

6. Ferric chloride · HCl. Dissolve 100 mg of FeCl₃ · 6H₂O in 100 ml of concentrated HCl.

7. Orcinol–FeCl₃ · HCl reagent. Add 1 ml of reagent 5 to 10 ml of reagent 6.

8. Buffer, pH 7.4. Mix the following and adjust to pH 7.4 with dilute HCl (1 mol/liter):
   - 20 ml of NaCl solution (9 g/liter)
   - 515 ml of KCl solution (11.5 g/liter)
   - 100 ml of K₂HPO₄ solution (17.5 g/liter)
   - 5 ml of MgSO₄ · 7H₂O solution (38 g/liter)

Apparatus

The most satisfactory method of heating the color reaction mixture is with an aluminum heating block. Boiling water baths are satisfactory, but condensate on the marbles used to cap the tubes drains into the sample and effects a small dilution.

For determining the absorbance at the two wavelength, a spectrophotometer that can be read to the third decimal place is necessary. We used a dual-wavelength recording spectrophotometer with an automatic reference compensator (Gilford Model 2400; Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074).

Note: Evaluator A.P. used a Coleman Jr. Spectrophotometer. Although this instrument is satisfactory, an instrument with which absorbance can be more precisely read will improve the results.

Collection of Samples

Whole blood is collected in Vacutainers (Becton-Dickinson, Rutherford, N. J. 07070) that have disodium ethylenediaminetetraacetate as the anticoagulant. The samples are kept refrigerated until the erythrocytes have been counted, but then can be stored at −40 °C for as long as five weeks without significant change in activity.

Use of heparin as the anticoagulant is less satisfactory; it causes some color development that may result in errors as great as 5%.

Procedure

1. Macroscale procedure:
   (a) Add 0.5 ml of whole blood to 0.5 ml of buffer (reagent 8), and alternately freeze and thaw the mixture three times to facilitate hemolysis.
   (b) Bring the temperature of the hemolysate to 37 °C and add 1.0 ml of ribose-5-phosphate substrate (reagent 1). Continue the incubations at 37 °C.
   (c) Precisely 10 and 40 min after the substrate is added, remove a 0.2-ml sample, place it in a tube containing 0.1 ml of the trichloroacetic acid (reagent 4), and allow the mixture to stand at room temperature for 10 min. Adjust the volume to 3 ml with distilled water and centrifuge. Save the supernatant fluid for pentose and sedoheptulose determinations as described below.

2. Microscale procedure:
   (a) Using calibrated capillary pipets, collect at least two 50-μl samples of whole blood by finger prick.
   (b) Add each sample to 50 μl of buffer (reagent 8).
   (c) Alternately freeze and thaw this blood–buffer mixture three times by use of a methanol–solid carbon dioxide slush.
   (d) Bring the temperature of the resulting hemo-

lysates to 37 °C, add 100 μl of ribose-5-phosphate substrate (reagent 1), and continue the incubation at 37 °C.
   (e) Add 100 μl of trichloroacetic acid (reagent 4) to one tube after 10 min of incubation and 100 μl to the second tube after 40 min of incubation.
   (f) Allow the mixture to stand for 10 min at room temperature and adjust the volumes of each tube to 3 ml with distilled water.
   (g) Centrifuge and save the supernate for pentose and sedoheptulose determinations as described below.

3. Determination of pentose and sedoheptulose:
   (a) Add 0.1 ml of the orcinol–ferric chloride solution (reagent 7).
   (b) Add 1 ml of the orcinol–ferric chloride solution (reagent 7).
   (c) Place the mixture in a heating block set at 100 °C for 40 min. (Large marbles should be placed on the tubes to minimize evaporation.)
   (d) Cool the samples to room temperature.
   (e) Transfer the samples to cuvets having a light path of 1 cm and measure the absorbance at 580 nm and at 670 nm with the instrument set on a reagent blank consisting of 1 ml of water and 1 ml of reagent 7. Carry 1-ml samples of ribose-5-phosphate (reagent 2) and sedoheptulose (reagent 3) standards through
the color reactions by adding 1.0 ml of orcinol–ferric chloride solution.

Calculations

From the absorbance (A) readings of the ribose and sedoheptulose standards, determine the following values:

\[
W = \frac{0.65 \times (A \text{ for ribose-5-phosphate standard at 670 nm})}{0.040}
\]

\[
X = \frac{A \text{ for sedoheptulose standard at 670 nm}}{0.040}
\]

\[
Y = \frac{0.65 \times (A \text{ for ribose-5-phosphate standard at 580 nm})}{0.040}
\]

\[
Z = \frac{A \text{ for sedoheptulose standard at 580 nm}}{0.040}
\]

To find the amount of pentose and sedoheptulose in each sample, the above values are substituted into the following equations:

pentose per tube, \( \mu \text{mol} = \frac{[Z \times (A \text{ for sample at 670 nm})] - [X \times (A \text{ for sample at 580 nm})]}{WZ - XY} \)

sedoheptulose per tube, \( \mu \text{mol} = \frac{[W \times (A \text{ for sample at 580 nm})] - [Y \times (A \text{ for sample at 670 nm})]}{WZ - XY} \)

Table 1. Mean Erythrocyte Transketolase Activity, by Sex and Race, of Blood from Adolescents

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pentose disappearance/h/ml</th>
<th>Pentose disappearance/h/10^9 erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \mu \text{mol} )</td>
<td>( \mu \text{mol} )</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>181</td>
<td>12.58 ± 1.46</td>
<td>2.37 ± .335</td>
</tr>
<tr>
<td>Negro</td>
<td>26</td>
<td>11.28 ± 1.59</td>
<td>2.10 ± .376</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>178</td>
<td>11.96 ± 1.59</td>
<td>2.42 ± .316</td>
</tr>
<tr>
<td>Negro</td>
<td>32</td>
<td>10.53 ± 1.24</td>
<td>2.09 ± .372</td>
</tr>
<tr>
<td><strong>Whites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>181</td>
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<td>178</td>
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<td>2.09 ± .372</td>
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<td>2.39 ± .327</td>
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<tr>
<td>Negro</td>
<td>58</td>
<td>10.87 ± 1.44</td>
<td>2.10 ± .372</td>
</tr>
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</table>

All subjects 417 12.08 ± 1.54 2.35 ± .348

Table 2. Reproducibility of Analysis

<table>
<thead>
<tr>
<th>Day</th>
<th>n</th>
<th>Pentose utilized, ( \mu \text{mol/h/ml} )</th>
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<tr>
<td>1</td>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>6</td>
<td>6</td>
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</tr>
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<td>7</td>
<td>13.1</td>
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<td>10</td>
<td>13.1</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Mean, 12.93 SD, 0.237

The dilution factor to determine amounts for 1 ml of whole blood for both procedures is 600. Thus, micromoles per tube \( \times 600 = \mu \text{mol/ml whole blood} \). The change in substrate or product concentration between 10 and 40 min is a measure of the enzyme activity over a 30-min period. This can be expressed in micromoles per milliliter of whole blood by multiplying by two or, if one desires, as international (IUB) units per liter by the following expression:

\[
U/\text{liter} = \frac{(\mu \text{mol in 40-min tube} - \mu \text{mol in 10-min tube}) \times 1000 \times 600}{30}
\]

If erythrocyte counts are done, a more meaningful base is established as activity per hour per billion erythrocytes.

Discussion

Probably the most reliable, accurate method for measuring transketolase activity in clear solutions would be to use the coupled reaction with glyceralde-
hyde-3-phosphate dehydrogenase [EC 1.2.1.12, d-glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating)] and to follow the reaction by measuring spectrophotometrically (at 340 nm) the oxidation of the NADH. However, when working with erythrocytes, such a procedure cannot be used because the color of the hemoglobin is too great to blank out. With erythrocytes or whole blood, one is thus compelled to use a separate incubation followed by precipitation of the protein and subsequent determination of substrate and products.

Values for Enzyme Activity

Transketolase activity can be expressed either in terms of pentose disappearance or sedoheptulose formation. The sedoheptulose formation is not stoichiometric with the pentose disappearance, because the sedoheptulose formed is acted upon by transaldolase, the next step in the pentose phosphate pathway. Thus, a separate range of values is required for each way of assessing the activity.

Normal values: (a) Pentose disappearance. Table 1 shows normal values (6) for pentose disappearance. These values were established on 417 high-school student volunteers. When activities are expressed per milliliter of whole blood (Table 1), a difference becomes apparent in the mean values for young men and young women. Basing the enzyme activity on erythrocyte count (Table 1) abolishes this sex-related difference; a difference between Caucasians and Negroes remains significant statistically but is of little, if any, physiological significance.

Note: Evaluator V.F. assayed 18 samples, all from white men 19 to 25 years old, and obtained the following results:

Pentose utilized: 11.09 ± 2.90 mol/h per milliliter of blood, or 2.30 ± .59 μmol/h per billion erythrocytes.
Evaluator A.P. assayed 12 samples, all from women 20-28 years old, with the following results:

Pentose utilization: 12.63 ± 3.04 μmol/h per milliliter of blood.

These values are in general agreement with those in Table 1. The rather large standard deviation of the Evaluators, as compared to those in Table 1, may reflect the smaller sample size, 12 and 18 vs. 417.

(b) Sedoheptulose formation. The normal values for sedoheptulose formation, obtained from assays of 89 samples from hospital employees, are 2.44 ± 0.54 (SD) μmol/h per milliliter of whole blood, or 0.45 ± 0.03 μmol/h per billion erythrocytes.

Note: Evaluator V.F. obtained a value of 2.33 ± 0.91 (SD) μmol/h per milliliter of blood, and Evaluator A.P. obtained 2.34 ± 1.81 (SD). Again, the large standard deviation may result from the small sample used by the Evaluators.

Abnormal values: Only a few samples from patients known to be showing signs of thiamine deficiency have been examined. The blood of these patients did have 30-50% of the normal transketolase activity (about 5 μmol pentose disappearance and 1 μmol sedoheptulose formation/h per milliliter whole blood). Studies on rats indicate that they have a higher normal range [10-17 μmol/h per milliliter and in deficiency values of less than 7.5 μmol/h per milliliter (4)].

Reproducibility

Aliquots of one hemolysate were kept frozen at -70 °C. These aliquots were thawed and assayed each day for five days. Results for 11 samples are shown in Table 2.

References


Appendix

\[ w = A(\text{pentose}) \text{ at } 670 \text{ nm} \]
\[ x = A(\text{heptose}) \text{ at } 670 \text{ nm} \]
\[ y = A(\text{pentose}) \text{ at } 580 \text{ nm} \]
\[ z = A(\text{heptose}) \text{ at } 580 \text{ nm} \]
\[ \alpha = \text{Concn std., in } \mu\text{mol} \]
\[ H = \text{Concn of heptose unknown} \]
\[ P = \text{Concn of pentose unknown} \]

From the absorbance readings, simultaneous equations are developed:

\[ A_{670} = \frac{x}{\alpha} + \frac{w}{\alpha} \] (1)
\[ A_{580} = \frac{z}{\alpha} + \frac{y}{\alpha} \] (2)

Rearrange (1) and (2) in terms of H:

\[ H = \frac{A_{670} - \frac{w}{\alpha}}{\frac{x}{\alpha}} \] (3)
\[ H = \frac{A_{580} - \frac{y}{\alpha}}{\frac{z}{\alpha}} \] (4)

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Setting (3) = (4) and solving for P:

\[
\frac{A_{670} - \frac{w}{\alpha}(P)}{\frac{x}{\alpha}} = \frac{A_{580} - \frac{y}{\alpha}(P)}{\frac{z}{\alpha}}
\]

\[
\frac{z}{\alpha}(A_{670}) - \frac{x}{\alpha}(A_{580}) = \frac{z}{\alpha}\left[\frac{w}{\alpha}(P)\right] - \frac{x}{\alpha}\left[\frac{y}{\alpha}(P)\right]
\]

\[
\frac{z}{\alpha}(A_{670}) - \frac{x}{\alpha}(A_{580}) = \frac{z}{\alpha}\left[\frac{w}{\alpha}(P)\right] - \frac{x}{\alpha}\left[\frac{y}{\alpha}(P)\right]
\]

\[
\left[\frac{z}{\alpha}\cdot\frac{w}{\alpha} - \frac{x}{\alpha}\cdot\frac{y}{\alpha}\right] = \frac{z}{\alpha}(A_{670}) - \frac{x}{\alpha}(A_{580})
\]

Substituting W, X, Y, Z:

\[
\frac{Z}{\alpha}(A_{670}) - \frac{X}{\alpha}(A_{580}) = P
\]

The heptose concentration calculation is derived in a similar manner. Rearrange (1) and (2) in terms of P:

\[
P = \frac{A_{670} - \frac{x}{\alpha}(H)}{\frac{w}{\alpha}}
\]

\[
P = \frac{A_{580} - \frac{z}{\alpha}(H)}{\frac{y}{\alpha}}
\]

Setting (5) = (6) and solving for H:

\[
A_{670} - \frac{x}{\alpha}(H) = A_{580} - \frac{z}{\alpha}(H)
\]

\[
\frac{w}{\alpha}\left[\frac{z}{\alpha}(H)\right] - \frac{y}{\alpha}\left[\frac{x}{\alpha}(H)\right] = \frac{w}{\alpha}(A_{580}) - \frac{y}{\alpha}(A_{670})
\]

\[
H(w \cdot z) - (y \cdot x) = w(A_{580}) - y(A_{670})
\]

Substituting W, X, Y, Z:

\[
\frac{w}{\alpha}(A_{580}) - \frac{y}{\alpha}(A_{670}) = \frac{w}{\alpha}\left[\frac{z}{\alpha}(H)\right] - \frac{y}{\alpha}\left[\frac{x}{\alpha}(H)\right]
\]

\[
\left[\frac{w}{\alpha} - \frac{y}{\alpha}\right] = \frac{w}{\alpha}(A_{580}) - \frac{y}{\alpha}(A_{670})
\]

\[
\frac{W}{\alpha}(A_{580}) - \frac{Y}{\alpha}(A_{670}) = H
\]

We remind our readers that the Editors do not regard any of the Selected Methods as the nearest plus ultra. They are methods that submitters have found to be useful and evaluators have found to be reproducible, which therefore seem especially worthy of the attention of a laboratory who is doing, or planning to do, such a determination or procedure. Any reader who adopts a Selected Method as it appears in this journal has the opportunity to offer his criticisms of it, and such criticism will receive attention before (and if) the method is finally incorporated into the next volume in the series Selected (previously Standard) Methods of Clinical Chemistry. For details, see the introductory editorial [Clin. Chem. 19, 1207 (1973)].

No reprints of this paper will be available.