A NADH-DEPENDENT TRANSKETOLASE ASSAY IN ERYTHROCYTE HEMOLYSATES

E. H. J. SMEETS, H. MULLER
Biochemical Laboratory, Neurological Department, State University, Utrecht
AND J. DE WAEL
Biochemical Laboratory, Internal Department, State University, Utrecht (The Netherlands)
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SUMMARY

A simple and specific method for the assay of transketolase activity in erythrocyte hemolysates based on the measurement of NADH consumption is described. The test conditions are discussed and the results are compared with a colorimetric method. Normal values are given for the transketolase activity and the thiamine pyrophosphate effect.

Most parenchymal organs contain transketolase (TK) (E.C. 2.2.1.1). The enzyme activity in these organs and in the red blood cells decreases readily, when a deficiency of thiamine develops[1]. Brin and associates[2–4] have demonstrated, that the erythrocyte TK activity and specially the relative enhancement of TK activity in erythrocyte homogenates after saturation with the coenzyme thiamine pyrophosphate (TPP effect) *in vitro* is a sensitive index for the detection and evaluation of thiamine deficiency. Thiamine depletion experiments on human test subjects[5–9] and on test animals[5,6,10,11] have shown, that an increase of the TPP effect can be observed even before clinical symptoms are perceptible. After a prolonged deficiency also the saturated enzyme activity *in vitro* is decreased[6].

The value of these assays is restricted somewhat by the observation, that patients suffering from hepatic cirrhosis—both with[12] and without[13] neuropathies caused by vitamine B_1 deficiency—showed a diminished erythrocyte TK activity in combination with a normal TPP effect. Besides, the improvement of the liver function and the erythrocyte TK activity were found to be related. In patients with thiamine phosphorylating defect TK activities remained deficient (TPP effect increased) after thiamine treatment[14]. Erythrocyte TK activities exceeding the normal values with TPP effects within the normal ranges were reported in patients with Addisonian pernicious anemia[15].

TK catalyses the following metabolic steps in the metabolism of pentoses[16]:

(1) xylulose-5-*P*+ribose-5-*P* → sedoheptulose-7-*P*+glyceraldehyde-3-*P*
(2) xylulose-5-*P*+erythrose-4-*P* → fructose-6-*P*+glyceraldehyde-3-*P*

TK activities in RBC have been assayed by measuring the recovery of $^{14}CO_2$ from [2-^{14}C]glucose with intact erythrocytes in the presence of methylene blue[3]. In most studies colorimetric estimation of sugars in red blood cell (RBC) hemolysates is employed; originally the disappearance of ribose -5-*P* using the orcinol method or the formation of hexose by the anthrone reaction[5].

Dreyfus[17] introduced a method based on the determination of sedoheptulose-7-*P* with the cysteine–sulfuric acid reaction, which method was modified by Schouten *et al.*[18]. This color reaction is highly susceptible to slight changes of circumstances[9,15], is not very specific, difficult to reproduce and cumbersome to perform.

Horecker *et al.*[19] employed for the assay of TK activity in preparations from rat liver and spinach a test system, where the glyceraldehyde-3-*P* formed during the TK reaction (1) after conversion to dihydroxyacetone-*P* was reduced by NADH to glycerol-1-*P* in the presence of excess of the enzymes triosephosphate isomerase (TIM) and glycerolphosphate dehydrogenase (GDH). The rate of NADH oxidation, measured at 340 nm, is proportional to the TK activity. In the present paper this system is adapted for the assay of TK in RBC hemolysates for the evaluation of vitamin B_1 deficiency. As substrate ribose-5-*P* is used, a part of which is converted via ribulose-5-*P* in xylulose-5-*P* by the enzymes ribosephosphate isomerase and ribulosephosphate-3-epimerase from the erythrocytes.

APPARATUS AND REAGENTS

Spectrophotometer Bausch and Lomb Spectronic 600 E (bandwidth 5 nm, 1-cm light path) with cuvette house thermostated at 37° and recorder Bausch and Lomb VOM 8 E.

Substrate solution: ribose-5-phosphate. Na 0.015 M in tris–HCl buffer 0.1 M, pH 7.6; 1 g ribose-5-phosphate. Ba (Boehringer Mannheim GmbH, contains 58% ribose-5-phosphate) is converted to the sodium salt by dissolving in 2 ml aqua dest. and adding 1.0 M Na_2SO_4 until all Ba ions are precipitated. The precipitate is discarded and the supernatant is diluted to 190 ml with tris–HCl buffer 0.1 M, pH 7.6.

Thiamine pyrophosphate chloride (Calbiochem, A grade): 0.010 M in tris–HCl buffer 0.1 M, pH 7.6. NADH (Boehringer Mannheim GmbH): 0.010 M in tris–HCl buffer 0.1 M, pH 7.6. GDH–TIM suspension 2 mg/ml (Boehringer Mannheim GmbH). Sterox SE (Hartman–Leddon Company): 5 g/l in aqua dest. The NADH solution is prepared freshly before use; the other reagents are kept at −20°.

PROCEDURE

Freshly drawn heparinized blood is centrifuged at 1500 rev./min for 3 min and again for 5 min at 3000 rev./min for better separation of erythrocytes and leucocytes. The plasma and buffy coat are sucked off and the erythrocytes washed with saline 9 g/l. The RBC are hemolyzed by mixing equal parts of cells and sterox. The TK activity is then assayed immediately or the hemolysate is stored at −20°.

Before the TK assay the hemolysate is diluted 3 times with saline 9 g/l until a hemoglobin concentration between 45 and 50 g/l is reached. The hemoglobin concentration is determined by the cyanmethemoglobin method[20]. The TK activity is evaluated in the following way. To 2.5 ml substrate, kept at 37°, is added 0.1 ml hemolysate,

0.01 ml GDH–TIM and 0.05 ml NADH. After mixing the solution is incubated at 37° for 15 min and then the reaction rate is measured by recording the absorbance (A) at 339 nm for 15 min. A potassium dichromate solution of about 0.4 g/l is used as reference.

For the assay of the saturated enzyme activity *in vitro* 2.5 ml substrate is replaced by 2.4 ml substrate plus 0.1 ml TPP. The assays are performed in duplo.

CALCULATIONS

The erythrocyte TK activity is expressed in International Units (U) per gram hemoglobin (Hb), equivalent to the number of micromoles glyceraldehyde-3-*P* formed per min per g Hb under the described conditions.

$$\text{TK activity} = \frac{A}{15} \times \frac{1000}{6.22 \times 10^6} \times 26.6 \times \frac{1}{\text{Hb conc.}} \text{U/g Hb}$$

A = decrease in absorbance at 339 nm per 15 min.
6.22×10^6 = molar extinction coefficient of NADH at 339 nm.
26.6 = dilution factor of the hemolysate in the incubation mixture.
Hb conc. = hemoglobin concentration in the hemolysate in g/l.

$$\text{TPP effect} = \frac{\text{TK sat.} - \text{TK}}{\text{TK sat.}} \times 100\%$$

TK sat. = TK activity after saturation with TPP *in vitro*.
TK = TK activity of the hemolysate without addition of TPP.

DISCUSSION AND RESULTS

Preparation of the hemolysate

Plasma and leucocytes are removed as thoroughly as possible, because especially at high leucocyte counts enzyme activities can be found in whole blood hemolysates considerably higher than those in hemolysates of washed RBC from the same samples (Table I). However the absolute difference in enzyme activity before and after saturation with TPP is equal for hemolysates of whole blood and washed cells. After

TABLE I

TK ACTIVITIES IN U/g Hb IN THE HEMOLYSATE OF 10 DIFFERENT SAMPLES ASSAYED IN DUPLO IN WHOLE BLOOD, AFTER CENTRIFUGATION AND SUCTION OF PLASMA AND BUFFY COAT, AND AFTER WASHING OF THE RBC

No.	*Leucocyte count*	*Whole blood*	*Centr.*	*One washing*	*2 washings*
1	26500	1.72	0.95	0.98	0.96
2	16500	1.24	0.76	0.70	0.68
3	13000	1.03	1.13	1.11	1.02
4	10000	0.74	0.67	0.65	0.65
5	7900	0.76	0.77	0.65	0.65
6	7000	0.89	0.90	0.73	0.75
7	6800	1.02	0.99	0.91	0.90
8	4100	0.58	0.62	0.58	0.56
9	3700	0.86	0.95	0.92	0.87
10	2800	0.79	0.77	0.75	0.75

suction of plasma and buffy coat and washing of the RBC, the range of TK activities of different individuals is rather small. No further decrease of enzyme activity is effected after prolonged washing.

The RBC are hemolysed by mixing with an equal part of sterox, because this causes faster and more complete hemolysation than mixing with aqua dest. and freezing, as performed by Brin[5] and Schouten *et al.*[18]. The mean TK activities of 10 different samples assayed after hemolysis in a sterox solution (0.386 U/g Hb) and in aqua dest. (0.376 U/g Hb) showed a small, but significant difference ($p < 0.05$, Student test for paired observations).

The hemolysate can be kept frozen at −20° for at least 4 weeks without loss of enzyme activity or change of the TPP effect. After two times freezing and thawing a significant fall of TK activity can occur.

Reaction conditions

Temperature. The temperature optimum for the sequence of reactions was found near 40° (Fig. 1A). For practical purposes the conventional temperature of 37° was chosen for the enzyme assay.

pH Optimum. The pH optimum for the reaction in 0.1 M tris–HCl buffer is 7.6 as can be seen in Fig. 1B. The TK activity at pH 7.6 assayed in 5 different buffers was the highest in the tris–HCl buffer (Table II).

Ribose-5-P concentration. The relation between the TK activity and the concentration of ribose-5-*P* in the substrate solution is demonstrated in Fig. 1C. The

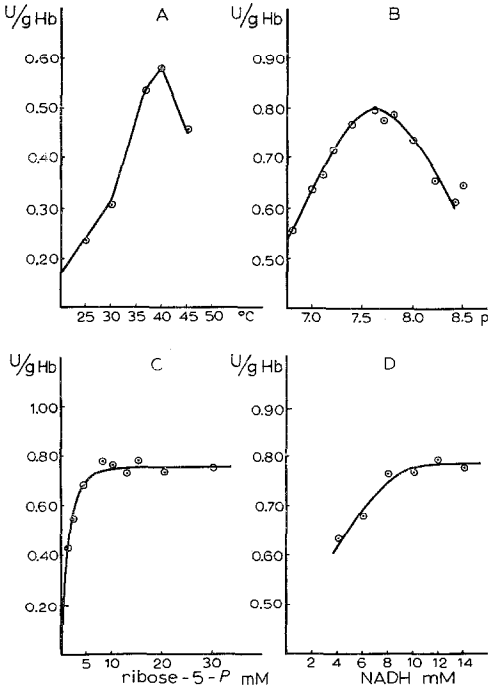

Fig. 1. Relation between the TK activity and several reaction conditions. All reaction circumstances, except that shown in the figure, are as described in the text.

TABLE II

TK ASSAY IN SEVERAL BUFFERS AT pH 7.6

TK activity of one hemolysate in buffers of 0.1 M concentration.

Buffer	*U/g Hb*
Tris–HCl	0.76
Triethanolamine–HCl	0.39
Glycine–NaOH	0.39
Na_2HPO_4–KH_2PO_4	0.24
Collidine–HCl	turbidity appears

reaction rate increases up to a ribose-5-*P* concentration of about 8 mM and remains nearly constant up to a concentration of at least 30 mM. Considering possible loss of ribose-5-*P* at the conversion of the barium salt in the sodium salt a substrate concentration of 15 mM, calulated from the barium salt, was chosen.

NADH concentration. The reaction rate at a ribose-5-*P* concentration of 15 mM reaches a constant value at a NADH concentration of about 10 mM, as is shown in Fig. 1D. This concentration was chosen for the assay to obviate a too high absorbance.

GDH–TIM. The GDH–TIM suspension added to the reaction mixture was tested for the presence of sufficient excess of these enzymes and the complete absence of TK activity using the following procedure. The TK activity of one hemolysate was assayed in 5 fold after addition of 0.01 ml of a GDH–TIM suspension containing 2 mg enzyme/ml, as well as after addition of 0.01 ml of a 10-fold dilution of the same suspension with saline 9 g/l.

There was no significant difference between the means (0.832 U/g Hb and 0.846 U/g Hb) of both tests ($p > 0.05$, Student's t test). Addition of more than 0.01 ml GDH–TIM 2 mg/ml had no effect, application of greater than 10-fold dilutions of the GDH–TIM suspension resulted in lower reaction rates. It may be concluded therefore, that no measurable TK activity is present in the suspension and that addition of 0.01 ml GDH–TIM 2 mg/ml is satisfactory.

Blank. Blank determinations of hemolysates with all reagents, except GDH–TIM, showed that no more NADH consumption takes place after 15 min incubation at 37°. Activation of the apoenzyme by the added TPP requires 6 min at room temperature[19], so the recording of the reaction rate can be started after 15 min incubation.

Magnesium ions. TK from spinach requires Mg^{2+} for full activation, while no such requirement is observed for liver TK[19]. Because several authors add Mg^{2+} ions to the buffer for the TK assay, the enzyme activity was assayed at several Mg^{2+} concentrations in the buffer. In Table III can be seen, that addition of Mg^{2+} to the buffer is not necessary nor desirable.

TABLE III

EFFECT OF ADDITION OF Mg^{2+} IONS OR MONOIODACETATE ON THE TK ACTIVITY

TK was assayed in triplo as described in the procedure with the following concentrations of Mg^{2+} or monoiodacetate in the substrate solution. Mg^{2+} was added as $MgCl_2$.

M conc. of Mg^{2+}	*U/g Hb*	*M conc. of monoiodacetate*	*U/g Hb*
0	0.77	0	0.85
0.0003	0.78	0.0001	0.82
0.003	0.75	0.001	0.81
0.03	0.68	0.01	0.74

Monoiodacetate. Monoiodacetate 0.001 M inhibits for 95% glyceraldehyde-3-*P*-dehydrogenase from erythrocytes[21]. This enzyme can convert the glyceraldehyde-3-*P* formed at reaction (1) to diphosphoglycerate, which would result in less oxidation of NADH at the reaction of dihydroxyacetone phosphate to glycerol phosphate. The reaction rates at various concentrations of monoiodacetate (Table III) indicate, that there is no evidence for conversion of glyceraldehyde-3-*P* in the test system via this way.

Assay time. For most TK determinations the decrease in *A* at 339 nm is about 0.10 in 15 min. The reaction rate remains constant for at least 30 min.

Comparison with other methods

Most authors report the TK activity per volume RBC or whole blood, eventually after adjusting the hematocrit to 35%, when the determination is done in whole blood[18].

In the proposed method the activity is expressed in U/g Hb instead of per volume erythrocytes to avoid errors due to swelling or shrinkage of RBC during washing of the erythrocytes.

The reproducibility of the method was assayed by a 10-fold determination of one hemolysate. The mean TK activity was 0.65 U/g Hb, S.D. = 0.017 U/g Hb, which corresponds to a coefficient of variation (C.V.) = 2.6%. The mean TPP effect was 13.0%, S.D. = 1.75%, C.V. = 13%. For a 10-fold determination following the colorimetric estimation of sedoheptulose-7-*P*[18] the S.D. was 0.013 U/g Hb at a TK activity of 0.46 U/g Hb, corresponding to C.V. = 2.8%. The mean TPP effect was 8.5%, S.D. = 4.2%, C.V. = 51%. These data demonstrate, that particularly for the estimation of the TPP effect the U.V. method has a better precision than the colorimetric method.

A comparison of the TK activities in hemolysates of washed RBC assayed by the estimation of sedoheptulose-7-*P*[18] and the U.V. method on 18 samples is shown in Fig. 2. A statistical study of these data showed, that the correlation between the colorimetric method (COL) and the U.V. method (UV) was: COL = 0.367 UV+0.14 (orthogonal regression coefficient). The coefficient of correlation between the methods

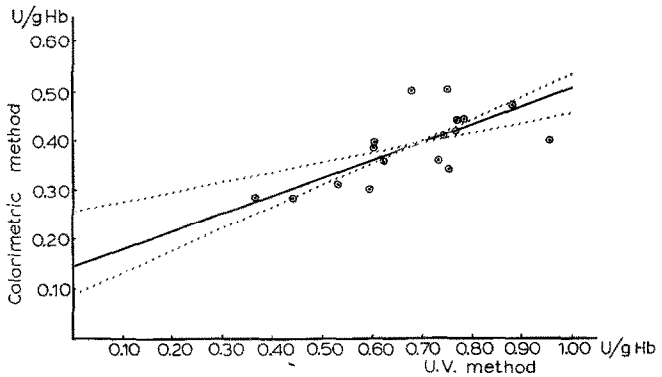

Fig. 2. Comparison of TK assays by the colorimetric determination of sedoheptulose-7-*P* and the U.V. method measuring NADH oxidation. Each assay was done in duplo. The dotted lines are the 95% confidence limits for the relation between the two methods.

was 0.70, which is significant at 0.1% level. The 95% confidence limits for the relation were: COL = 0.200 UV+0.26 and COL = 0.446 UV+0.09. This comparison shows, that the measured enzyme activity in the U.V. assay is much higher than in the colorimetric method. For the greater part this is caused by the use of a tris buffer instead of a phosphate buffer employed in other methods.

The specificity of the colorimetric methods that are used for the assay of TK activity in biological material, as the orcinol reaction for pentoses, the anthrone reaction for hexoses and the cysteine–sulfuric acid reaction for heptoses, has been questioned when relative large amounts of other sugars are present[23–25]. With the U.V. method no such interference can be expected, since the glyceraldehyde-3-*P* formed during the TK reaction is assayed enzymatically and there no measurable blank enzyme activity is found.

As all usually employed TK assays, the described method is assumed to have two non-thiamine-dependent enzymes present in adequate amounts, *viz.* ribose phosphate isomerase and ribulose phosphate-3-epimerase. As far as we know, no low TK activities have been shown to be caused by a lack of these enzymes. Possibly some of the non-thiamine-dependent low activities of the apoenzyme, that are occasionally encountered, are caused by a deficiency of these enzymes.

The TK activity assayed for 47 blood donors had a mean value of 0.77 U/g Hb and ranged from 0.59–1.06 U/g Hb. The mean TPP effect of this group was 11%, S.D. = 4.5% and range 2–20%. In Table IV a survey is presented of normal values for the TPP effect given by several authors and the present method.

TABLE IV

NORMAL VALUES FOR THE TPP EFFECT

Reference	*Number of subjects*	$\bar{x}$	*S.D.*	*Range*	*Determination*
Markkanen[15]	30	20	14	0–53	sedoheptulose
Schouten *et al.*[18]	22	22.5	18		sedoheptulose
Bamji[9]	26	13			sedoheptulose
Baker[13]	6	15		6–22	hexose
Brin *et al.*[22]				0–15	ribose
this paper	47	11	4.5	2–20	NADH

Comparing the U.V. method and the colorimetric method based on the sedoheptulose-7-*P* estimation the following advantages can be observed. The U.V. method is simple, rapid and more specific, has a better precision and does not use aggressive reagents. A difficulty of the U.V. method is the high *A* of hemoglobin in the reaction mixture, which requires a photometer of sufficient stability and sensitivity in the U.V. range.

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