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284. Transketolase from Human Erythrocytes Purification and Properties

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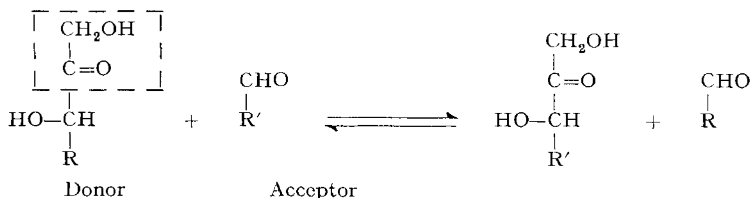
(3. VIII. 71)

Summary. Human erythrocyte transketolase (sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehyde-transferase) was purified 8200-fold by adsorption onto hydroxylapatite, DEAE-cellulose treatment, acetone fractionation, and chromatography on Sephadex G-100. The purified transketolase could not be separated from glyceraldehyde-3-phosphate dehydrogenase, whereas the latter enzyme could be isolated in a pure state. Its homogeneity is suggested by sedimentation velocity, sedimentation equilibrium, and acrylamide electrophoresis. A molecular weight of 136000 was found. The physicochemical properties of glyceraldehyde-3-phosphate dehydrogenase and transketolase are very similar. A molecular weight of 136000 is suggested for transketolase, although gel filtration with Sephadex G-100 gave only 104000 ± 10%. This discrepancy is a reflection of an interaction of transketolase with the gel filtration medium. The isoelectric point for transketolase as well as for glyceraldehyde-3-phosphate dehydrogenase, as determined by isoelectric focussing, was found to be around 8.5. The activity of the enzyme is close to the maximum for pH 7.5 to pH 8.6. Additions of thiamine pyrophosphate or other cofactors do not influence the activity. Several divalent cations were tested. Sulfate and phosphate inhibit transketolase approximately to 50% between 50 and 100 mM concentration. Thiamine was present in transketolase, as shown by a microbiological assay and by the thiochrome reaction. The activation energy for the formation of sedoheptulose-7-phosphate from xylulose-5-phosphate was estimated from rate measurements to be 11.2 kcal/mole in the temperature range from 5° to 55°.

Introduction. – Transketolase (EC 2.2.1.1) catalyzes the transfer of a ketol group to a suitable aldehyde acceptor:



Transketolase from baker's yeast as well as from spinach requires both Mg^{++} and thiamine pyrophosphate for activity [1] [2].

In contrast, no cofactor requirement of transketolase of dialyzed stroma-free hemolysate from human erythrocytes could be demonstrated by *Brownstone & Denstedt* [3]. Experiments of *Brin* [4], on the other hand, showed that an activation of enzymatic activity is observed when thiamine pyrophosphate is added to the hemolysate of red blood cells from patients with beriberi and heart disease [5].

In order to demonstrate the coenzyme function of thiamine pyrophosphate in transketolase from human red blood cells and to study the interaction between apoenzyme and coenzyme it was necessary to isolate the enzyme. As until now no attempts have been made to purify transketolase from human red blood cells.

Experimental

1. Materials. – DEAE-cellulose with a capacity of 0.50 mequiv./g was obtained from *Serva* (*Entwicklungslabor*, D-69 Heidelberg, Germany). The resin was treated with 1 M K_2HPO_4 (pH 9,1) before use as described by *Hennessey* [6]. Hydroxylapatite was prepared as described by *Levin* [7]. Sephadex G-100 was purchased from *Pharmacia* (Uppsala, Sweden). Acrylamide, bisacrylamide, N,N,N',N'-tetramethylethylenediamine and 2-mercaptoethanol were obtained from *Fluka AG*, Buchs (St. Gallen, Switzerland). Amido Black 10 B was from *Merck AG* (D-61 Darmstadt, Germany). Triosephosphate isomerase, glycerol-1-phosphate dehydrogenase, aldolase (muscle), alcoholdehydrogenase (yeast), acid phosphatase, DL-glyceraldehyde-3-phosphate (as Ba-diethylacetal), NAD^+, and NADH were from *Boehringer Mannheim GmbH* (D-68 Mannheim, Germany). Ribose-5-phosphate (disodium salt) was obtained from *Sigma Chem. Corp.* (St. Louis, USA). Ovalbumin and γ-globulin (7 S) (human) were purchased from *Calbiochem AG* (Lucerne, Switzerland). Human blood was obtained from the Blutspendezentrum Basel-Stadt (Basle, Switzerland). All other reagents were commercial products of analytical grade. Water was bidestilled.

2. Methods. – 2.1. *Preparation of pentose-5-phosphate equilibrium mixture.* A mixture of ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase was prepared from calf spleen following *Ashwell & Hickman* [8]. The equilibrium mixture of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate, used as substrate in the transketolase assay, was obtained by incubation of disodium ribose-5-phosphate with ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase [8]. The pentose-5-phosphates were precipitated by the addition of barium acetate and ethanol. The barium salts were stored at $-20°$.

2.2. *Sedoheptulose-7-phosphate* was prepared using a combination of the procedures of *Sokatch & McFadden* [9] and *Horecker* [10]. The material contained 79% barium sedoheptulose-7-phosphate as judged from the phosphate liberated after incubation with acid phosphatase at pH 5.4 and 37° for 1 h.

2.3. *Sedoheptulose-7-phosphate determination* was effected by means of the sulfuric acid reaction according to *Dische* [11] with several modifications. A mixture containing 0.60 ml of an aqueous solution of sedoheptulose-7-phosphate in 0.02 M Tris/HCl, pH 7.6, 50 μl of a 0.03 M solution of $MgCl_2$ and 0.05% thiamine pyrophosphate, 10μl of 0.06 M solution of the pentose-5-phosphate equilibrium mixture was incubated at 37°. After 30 min, 0,75 ml of trichloroacetic acid (10%) was added and 0.5 ml of this solution was mixed vigorously with 2.5 ml of sulfuric acid (92%). After heating to 100° for 3 min, the test tubes were cooled in ice water. Readings were taken at 405 and 350 nm in a *Zeiss* PMQ II spectrophotometer.

2.4. *Transketolase activity* was determined by estimating the sedoheptulose-7-phosphate accumulated in the mixture described under section 2.3, during 30 min of incubation at 37°. Mg^{++} and thiamine pyrophosphate were added although they did not influence the enzyme activity. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmole sedoheptulose-7-phosphate per minute at 37°. At 37° the rate was 2.5 times greater than

at 25°. Specific activity is expressed as units of enzyme activity per mg protein. The relation between μmoles of sedoheptulose-7-phosphate accumulated and time of incubation is linear in the range from 0.003 to 0.03 transketolase units under the conditions employed. In some experiments the formation of glyceraldehyde-3-phosphate from xylulose-5-phosphate in the presence of ribose-5-phosphate as acceptor aldehyde was measured by following the oxidation of NADH at 340 nm in the presence of triosephosphate isomerase and glycerophosphate dehydrogenase [12].

2.5. *Glyceraldehyde-3-phosphate dehydrogenase activity* was measured by the initial rate of reduction of NAD^+ at 25°. The increase in absorbance at 366 nm was recorded. The standard assay mixture [13] was as follows: 0.25 mM NAD^+, 0.20 mM glyceraldehyde-3-phosphate, 1 mM β-mercaptoethanol, 4 mM sodium arsenate, 50 mM Tris/HCl buffer, pH 8.6, and enzyme in a final volume of 1.10 ml.

2.6. *Protein determination* was effected by the method of *Lowry et al.* [14] with bovine serum albumin as protein standard. After Sephadex G-100 gel filtration the absorbance at 280 nm of the protein solutions was measured: a solution containing 1 mg of protein per ml was assumed to present an absorbance of 1.0 at 280 nm.

2.7. *Analytical acrylamide disc gel electrophoresis* was used routinely to follow the progress of purification. The electrophoresis was performed according to the technique of *Davis* [15] with Tris/glycine buffer with a running pH of 9.5. The upper gel was omitted. Bromophenol blue was used as tracking dye. Electrophoresis with a constant current of 2 mA per tube was carried out until the dye had almost reached the bottom of the gel. The gels were stained with Amido Black (1% *w/v* in 7% *v/v* acetic acid), destained and stored in 7% *v/v* acetic acid.

2.8. *Densitometry* was conducted by use of a Pherogramm-Auswerter (*"Eppendorf"* 2602).

2.9. *Concentration of protein solutions* was performed by filtration on *Amicon* ultrafiltration cells with Diaflow ultrafilters PM-10 (*Amicon*, The Hague, Holland).

2.10. *Molecular weight determination by gel filtration* was achieved according to *Andrews* [16] and *Determann* [17].

2.11. *Ultracentrifugation studies* were carried out in a *Spinco* Model E ultracentrifuge. Sedimentation equilibrium experiments were performed by standard methods [18] in a six-channel *Yphantis* cell, using the ultraviolet absorption scanning system. Molecular weights were calculated using the relationship (1)

$$\overline{\text{M}}\text{w.(app)} = \frac{2\,R\,T}{(1 - V \cdot \varrho) \cdot w^2} \cdot \frac{\mathrm{d}\ln c}{\mathrm{d}\,x^2} \qquad (1)$$

where $\overline{\text{M}}$w.(app) is the apparent molecular weight, ϱ the density, V the partial specific volume of the protein, R the gas constant, T the absolute temperature, w the angular velocity of the rotor, x the distance from the axis of rotation and c the protein concentration, measured by the optical density at 280 nm. $\overline{\text{M}}$w.(app) was calculated with a partial specific volume of $V = 0.735$ cm³/g. These calculations were done with the aid of an *Olivetti*-Programma 101 calculator.

2.12. *Isoelectric focussing* was performed on an electrofocussing column (*LKB* 8100) with a capacity of 110 ml. A continuous sucrose density gradient of 0 to 50% (*w/v*) was formed above a cathode solution containing 2% (*v/v*) ethylene diamine in 55% (*w/v*) Ampholine (pH 7–9 range ampholytes) in H_2O with the use of a linear gradient generator. The gradient column was completed by layering 10 ml of 1% (*v/v*) phosphoric acid at the top (anode). The enzyme to be applied to the column was placed directly into the middle of the gradient. The column was kept at 4° by means of a circulating refrigerated bath. After 96 hours isoelectric equilibrium was attained, fractions of 2 ml were then collected from the bottom at a flow rate of 60 ml per hour, extensively dialyzed against 0.01 M Tris/HCl buffer pH 7.6, and tested for activity.

3. Purification of transketolase. – Except when otherwise mentioned, all manipulations were carried out at 0–4°.

3.1. *Source of enzyme.* Citrated human blood (500-ml bottles) was obtained from the blood bank. The blood had either reached the age (3 weeks) at which it could no longer be used for transfusion or had been discarded because of marginally abnormal serological tests.

3.2. *Purification procedure* (Table 1). – 3.2.1. *Step 1. Preparation of hemolysates.* 800–900 ml of human blood were centrifuged at 2000 × *g* for 10 min. The supernatant plasma and buffy coat

were removed, and the erythrocytes suspended in 3 times their volume of 0.15 M NaCl and centrifuged as before. This washing was repeated 3 times. After the last centrifugation as much of the supernatant fluid as possible was drawn off, the packed erythrocytes were mixed with 1 volume of water, and the hemolysate was prepared by freezing and thawing 3 times. The stromata of the lysed erythrocytes were removed by centrifugation at $13000 \times g$ for 30 min, following by careful decantation of the transparent hemolysate (approximately 1000 ml with a pH of 6.8–7.0).

3.2.2. *Step 2. Hydroxylapatite adsorption and elution.* 2000 ml (2 volumes) of hydroxylapatite suspension (50% settled bed), equilibrated with 0.001 M phosphate buffer, pH 7.0, were mixed with 1000 ml (1 volume) of hemolysate. After stirring for 30 min the suspension was placed on a *Büchner* funnel (diameter, 30 cm) and the liquid removed by suction. The hydroxylapatite was washed with 40 l of 0.01 M phosphate buffer, pH 7.6, until the filtrate was only slightly pink, and

Summary of purification procedure for human erythrocyte transketolase

Step	Total volume	Total protein	Total activity (units)[a]	Specific activity	accumulative yield	accumulative purification
	ml	mg		units/mg	%	
(1) Hemolysate	1100	121000	37.4	0.0003	100	–
(2) Hydroxylapatite adsorption	350	1225	23.6	0.0193	63	64
(3) DEAE-cellulose	345	628	21.4	0.0340	57	113
(4) Acetone fractionation	2.4	24.8	5.9	0.238	15.8	795
(5) Sephadex G-100 column chromatography	1.1	3.0	3.6	1.19	9.6	3960
(6) Sephadex G-100 rechromatography	1.0	1.1	2.6	2.38	6.9	8200

[a]) 1 unit = formation of 1 μmole of sedoheptulose-7-phosphate/minute at 37°

then eluted 3 times with a total of 250 ml of 1.0 M phosphate buffer, pH 7.6, by stirring for 20 min each time. After centrifugation at $13000 \times g$ for 15 min the supernatants were combined and dialyzed for 12 h against 4 l of 0.01 M Tris/HCl, pH 7.6, changed 4 times during dialysis.

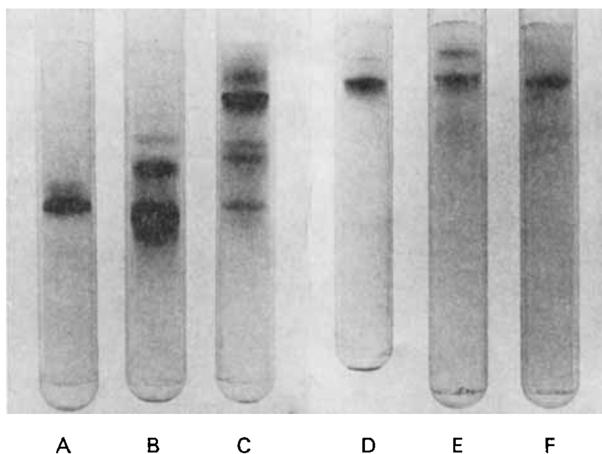
3.2.3. *Step 3. Batch purification on DEAE-cellulose.* 300–350 ml (1 volume) of the dialyzed enzyme solution of step 2 were stirred for 30 min with 1 volume of a DEAE-cellulose suspension (16 g per 100 ml), pH 7.0, which had been centrifuged before the additions in order to keep down the volume. No transketolase was bound to the DEAE-cellulose under these conditions. After filtration on a *Büchner* funnel the clear filtrate (300–350 ml) was used for acetone precipitation.

3.2.4. *Step 4. Acetone fractionation* was carried out with small quantities. 15 ml (0.75 volumes) of cold acetone (–20°) were added over a period of 5 min to 20 ml (1 volume) of the DEAE-filtrate placed in a –20° bath. The mixture was centrifuged immediately for 10 min at –15°, extreme care being taken to avoid raising of temperature. The precipitate was dissolved in 1.0 ml of 0.01 M Tris/HCl, pH 7.6. After 15 min at 0° the precipitated hemoglobin was removed by centrifugation at 0°. The combined acetone precipitates (approximately 16 ml) were dialyzed against 0.01 M Tris/HCl, pH 7.6, and concentrated to 2–3 ml (approximately 10 mg protein per ml) by ultrafiltration.

3.2.5. *Step 5. Sephadex G-100 column chromatography.* The final solution of 2.0 ml (10–20 mg protein) of the acetone fractionation was applied to a column of Sephadex G-100, 2.5×80 cm, equilibrated with 0.01 M Tris/HCl, pH 9.0. Flow rate 8.0 ml/h; fractions of 1.2 ml. Two protein peaks were observed, but only the first peak contained active material. The fractions from the first peak were combined and concentrated by ultrafiltration.

3.2.6. *Step 6. Sephadex G-100 rechromatography.* The final solution from step 5 (3–5 mg protein) was applied to a column as used in step 5. The fractions of the only protein peak witch appeared were combined and concentrated by ultrafiltration.

Results. – *Composition of the final preparation.* The various stages during the purification of transketolase are demonstrated in Fig. 1. The acrylamide gel electrophoresis of the preparation with the highest specific activity (Fig. 1, E) revealed the presence of two protein bands. The gel was cut into 2 mm slices, each homogenized and tested for transketolase activity. The activity was located in the upper band. Densitometric measurement showed the transketolase to be 15% of the total protein. Preparative acrylamide gel electrophoresis, isoelectric focussing and CM-cellulose chromatography failed to remove the accompanying protein component. However,



A B C D E F

Fig. 1. *Acrylamide-gel* (7.5%) *electrophoresis patterns during various stages of the purification of the enzyme*

Approximately 50 μg of protein were applied to each gel. Electrophoresis was carried out at 10° with a current of 2 mA per tube. The gels were stained with 1% Amido Black in 7% acetic acid.

A. Hemolysate (step 1)
B. Hydroxylapatite adsorption (step 2)
C. Acetone fractionation (step 4)
D. Sephadex G-100 chromatography (step 5)
E. Sephadex G-100 rechromatography (step 6)
F. Sephadex G-100 chromatography at pH 7.5

if the Sephadex chromatography (step 5) was carried out at pH 7.5 and a flow rate of 12–15 ml per hour, this 'accompanying' protein was obtained in a pure state, as judged by gel electrophoresis (Fig. 1, F) and sedimentation in the analytical centrifuge. Sedimentation in the analytical ultracentrifuge was carried out at a rotor speed of 60,000 rev/min and 5.3° in a 12 mm aluminium double sector cell. The 'accompanying' protein appeared as a single symmetrical peak. Sedimentation equilibrium studies in order to determine its molecular weight were performed at 12,000 rev/min and 5.0° (Fig. 2) with solutions of 0.2 to 0.3 mg protein/ml in 0.01 M Tris/HCl buffer, pH 7.6. From the graphs of ln OD. against x^2 and equation (1) the molecular weight was calculated in several experiments as 136,000 ± 5%. There was no indication for any heterogeneity in the solution. No dependency of the molecular weight from the protein concentration could be detected in these ranges of low protein concentrations.

Very recently, *Oguchi* [13] reported about the physical properties of glyceraldehyde-3-phosphate dehydrogenase (GPDH) from human erythrocytes. The molecular weight was found to be 137,000. The assay for glyceraldehyde-3-phosphate dehydrogenase showed unambiguously this enzyme to be the 'accompanying' protein. The following studies on the properties of transketolase were carried out with the preparation containing 85% glyceraldehyde-3-phosphate dehydrogenase.

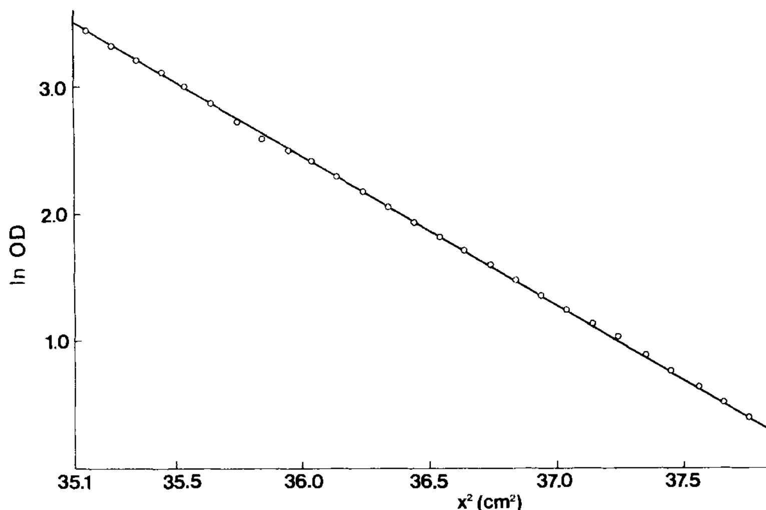


Fig. 2. *Sedimentation equilibrium of glyceraldehyde-3-phosphate dehydrogenase in 0.01* M *Tris/HCl, pH 7.6*

Data obtained after 24 h at 12,000 rev/min

Molecular weight. If the mixture of 15% transketolase and 85% glyceraldehyde-3-phosphate dehydrogenase is subjected to ultracentrifugation the same molecular weight of 136,000 ± 5% is obtained. This means that the two proteins must have the same molecular weight.

A molecular weight of 104,000 ± 10% was estimated by gel filtration on Sephadex G-100 (Fig. 3). The plot of V_E versus log Mw. in Fig. 3 did not give a straight line. This is in agreement with the V_E–log Mw. graph for Sephadex G-100 given by *Andrews* [19].

The discrepancy of the molecular weights obtained by low speed sedimentation equilibrium technique and gel filtration on Sephadex G-100 is probably due either to dissociation into subunits [20] or to an interaction of transketolase with the gel filtration media, leading to an increased elution volume. Such anomalous gel filtration behaviour has been observed with lysozyme [21] and with glycoproteins [22]. The fact that the molecular weight for transketolase obtained by gel filtration using only 10 mM Tris/HCl buffer, pH 7.6, instead of 50 mM Tris/HCl and 0.1 M KCl, pH 7.0, was found to be 88,000 ± 10% also indicates an interaction of transketolase with Sephadex. Furthermore, 25–30% inhibition was found when Sephadex G-100 was added to the transketolase assay mixture. 0.6 volumes of the incubation mixture

consisting of settled Sephadex G-100 gel bed and 0.6 volumes of Tris/HCl were used as blank instead of Sephadex.

Isoelectric point. Using the pH 7–9 range ampholytes the isoelectric point of transketolase was found to be 8.5.

Stability. Enzyme solutions during all stages of purification gradually lose activity. Freezing and thawing does not affect the enzymatic activity.

Effect of pH on stability. An enzyme preparation (1.2 mg/ml) with a specific activity of 0.8 units/mg was incubated at 4° for 15 hours in 0.1 M citrate, 0.1 M phosphate, 0.1 M Tris/HCl and 0.1 M glycine/NaOH buffers at different pH values. No loss of activity occurred between pH 7.5 and 10. At pH 5.0 90% and at pH 11.0 20% of the initial activity were lost.

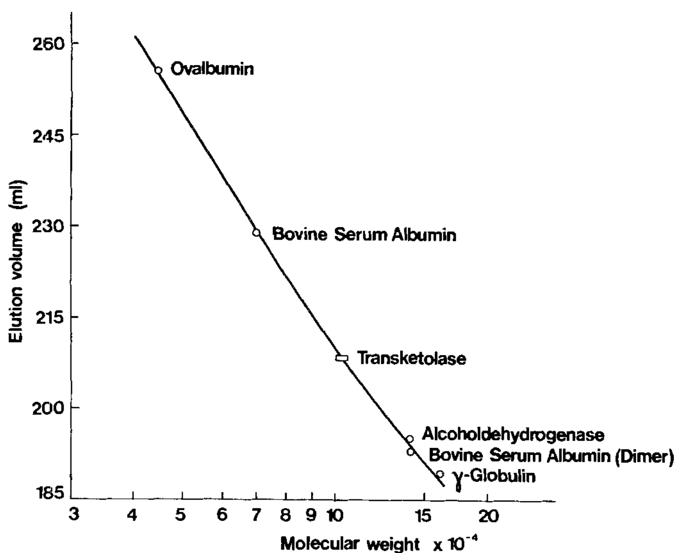


Fig. 3. *Estimation of molecular weight by gel filtration on Sephadex G-100*

Heat inactivation. The extent of inactivation as a function of temperature is shown in Fig. 4. In the range between 37° and 50° an activation was observed. The addition of Mg^{++} (30 mM), Mg^{++} (30 mM) and thiamine pyrophosphate (TPP) (1 mM) prevents this activation. Addition of thiamine pyrophosphate (1 mM) causes a pronounced decrease of transketolase activity.

Effect of concentration of substrate. The pentose phosphate equilibrium mixture prepared according to *Ashwell & Hickman* [8] was used as substrate, because pure xylulose-5-phosphate is at present not available. The K_m estimated from a *Lineweaver & Burk* plot was 1.7×10^{-3} M. No inhibition by substrate was evident at the highest concentration tested and the reaction appeared to follow *Michaelis-Menten* kinetics (Fig. 5).

Cofactors and inhibitors. There was no indication of a cofactor requirement for the enzyme. There is a slight activation by Ca^{++} and Mg^{++}. Other metals either had no effect or inhibited because of precipitating the pentose-5-phosphate substrate. Trans-

ketolase is not inhibited by iodoacetate or *p*-chloromercuribenzoate. However, sulfate and phosphate ions depress transketolase activity at concentrations above 10 mM. The enzymatic activity, decreased in the presence of mercaptoethanol, is restored by dialysis. Thiamine pyrophosphate had no effect on the enzymatic activity, although its function seems very probable (see below). It was not possible to resolve the enzyme by dialysis against EDTA-KCl [2] or by gel filtration on Sephadex G-25.

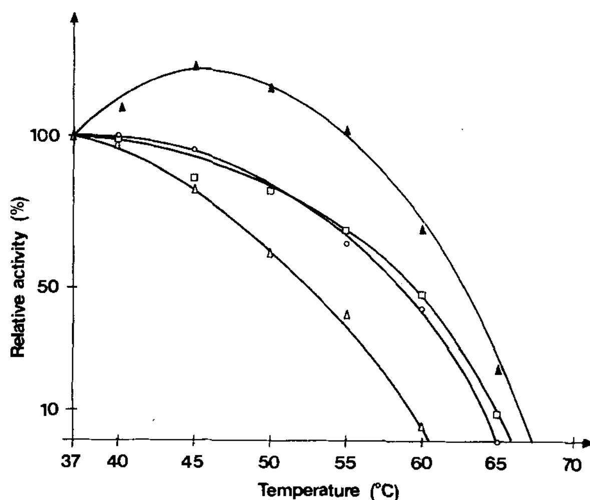


Fig. 4. *Heat inactivation of transketolase*

Enzyme (2 mg/ml, specific activity: 1.2 units/mg) was incubated for 5 min in 0.01 M Tris/HCl buffer, pH 7.6, rapidly chilled in ice, and assayed for transketolase activity

▲▲, No additions; □□, 30 mM Mg^{++}; ○○, 30 mM Mg^{++} and 1 mM TTP; △△, 1 mM TTP

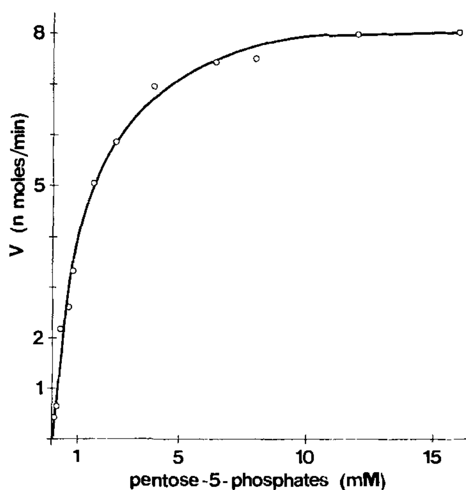


Fig. 5. *Effect of substrate concentration on transketolase activity*

Experimental details are given under "Materials and Methods"

Detection of thiamine. The presence of thiamine was revealed by thiochrom determination [23] as well as by microbiological assay with *Lactobacillus viridescens* [24]. The values obtained by the two methods were in good agreement. The thiamine pyrophosphate-transketolase stoichiometry was calculated on the basis of the following assumptions: 1. The transketolase content was estimated by densitometry to be 15% of the total protein, 2. the protein was estimated by the biuret method with a standard curve for bovine serum albumin, and 3. a molecular weight of 136,000 was used.

1.9 μmoles of thiamine pyrophosphate per μmole of transketolase were found.

pH optimum. The pH optimum for transketolase is around 7.75 (Fig. 6). Some uncertainty is introduced by the inhibitory effect of phosphate.

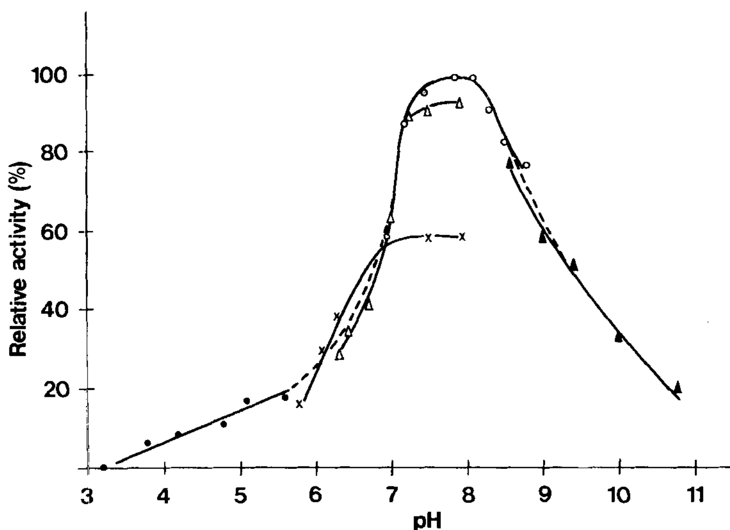


Fig. 6. *Effect of pH on activity of purified transketolase*
Assays run with the following buffers (concentration: 66 mM)
●, citrate; ×, phosphate; △, imidazole/HCl; ○, Tris/HCl; ▲, glycine/NaOH

Influence of temperature on transketolase activity was measured from 5° to 55°. The *Arrhenius* plot was linear. From the slope of the curve the activation energy was calculated to be 11.2 kcal/mole.

Discussion. – Red blood cells were selected for the isolation of transketolase because the highly differentiated erythrocyte contains only the enzymes of glycolysis and the pentose phosphate pathway. In general, the enzymes isolated from red blood cells are separated from the large amount of hemoglobin by adsorption onto DEAE-cellulose. Unfortunately at pH 7 (the pH chosen for purification procedures) transketolase as well as hemoglobin are not absorbed on DEAE-cellulose. But a batch treatment with DEAE-cellulose according to the procedure of *Hennessey et al.* [6] could be used. Finally, a preparation of transketolase (15%) accompanied by GPDH (85%) was obtained. Our data for pH optimum, heat stability, and activation energy

agree well with the data determined by *Bruns et al.* [25] and *Brownstone & Denstedt* [3] on dialyzed hemolysates. The similar physicochemical properties of transketolase and GPDH as well as the limited amount of starting material are responsible for the fact that these enzymes could not be separated. As *Kochetov* [26] pointed out for the yeast enzymes, there is evidence for a complex of transketolase and GPDH, two functionally bound enzymes. While the yeast enzyme can be easily resolved into apoenzyme and thiamine pyrophosphate, no such dissociation of the red blood cell transketolase could be demonstrated. By use of two analytical methods we have unambiguously shown that transketolase contains thiamine, as expected from *in vivo* studies demonstrating thiamine pyrophosphate as its coenzyme. GPDH bound to transketolase might be responsible for the fact that thiamine pyrophosphate cannot be removed from its apoenzyme by use of the common methods. A covalent bond between thiamine pyrophosphate and apotransketolase has not been ruled out. This linkage may be formed enzymatically in the intact red blood cell. From the fact that thiamine pyrophosphate is bound so tightly, a high turnover of the holoenzyme has to be considered to explain the decrease of enzymatic activity under thiamine depletion. The question whether the apoenzyme concentration is decreased in vitamin B_1-deficiency can only be answered when apotransketolase is available in order to study its recombination with thiamine pyrophosphate.

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285. Human Milk Lysozyme: Unpublished Data Concerning the Establishment of the Complete Primary Structure; Comparison with Lysozymes of Various Origins[1])

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(16. IX. 71)

Summary. Details concerning the establishment of the complete primary structure of human milk lysozyme (previously published in a preliminary note) are presented. The chymotryptic peptides obtained from the reduced alkylated enzyme were purified and their amino acid sequences determined chiefly by the '*Edman*-dansylation' procedure, and in two cases by partial acid or peptic hydrolyses. The tryptic peptides are alined into a single chain containing 129 amino acid residues, on the basis of overlapping peptides. Two labile glutamine residues easily converted into glutamic acid residues were characterized. Human milk lysozyme is compared with other human lysozymes (from normal and leuchaemic individuals) prepared by our group. The structure proposed is identical with the sequence of human leuchaemia lysozyme (from the urine of a patient with chronic monocytic leuchaemia) reported by *Canfield*. Human milk lysozyme is also near by related to several bird egg-white lysozymes (and bovine α-lactalbumin): identical positions of Cys and Trp residues and of the residues essential for the catalytic activity or involved in some hydrogen bonds; several identical regions, especially in the β-sheet region; between 71 and 77 identical amino acid residues. It is suggested that by an insertion and a deletion in the sequence of human milk lysozyme, sequences homologous to those of bird lysozymes can be obtained.

1. Introduction. – The establishment of the primary sequences of the N-terminal moiety (72 amino acid residues) and of the C-terminal end (23 amino acid residues) of human milk lysozyme (EC 3.2.1.17) (129 residues) was reported in detail in 1969 by *Jollès & Jollès* [2], who proposed simultaneously a tentative structure of the enzyme. The complete sequence was shortly published by the same authors in 1971 in a general review devoted to the evolution of proteins [2a]. In the present paper, evidence will be provided (a) for the sequence of the unique tryptic peptide which was not yet described in detail (residues No. 70–97 of the enzyme [2]); (b) for the alignment of all the tryptic peptides into a single chain, from chymotryptic overlapping peptides. Human milk lysozyme is compared with other human lysozymes as well as with lysozymes from various origins.

2. Materials and methods. – 2.1. Human milk lysozyme was prepared according to *Jollès & Jollès* [3] from pooled milk by ion-exchange chromatography on Amberlite CG-50.

2.2. Reduction, alkylation, tryptic hydrolysis, and separation of the tryptic peptides: for these procedures see [2].

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[1]) 80th communication on lysozymes; 79th communication, [1].