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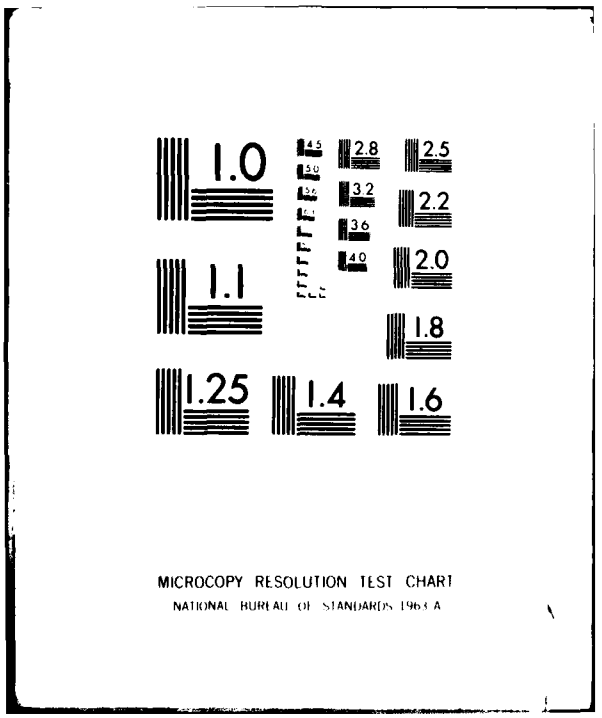
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(6) PURIFICATION OF TRANSKETOLASE FROM HUMAN ERYTHROCYTES.
I. Using Solvent Denaturation as the Initial Step.

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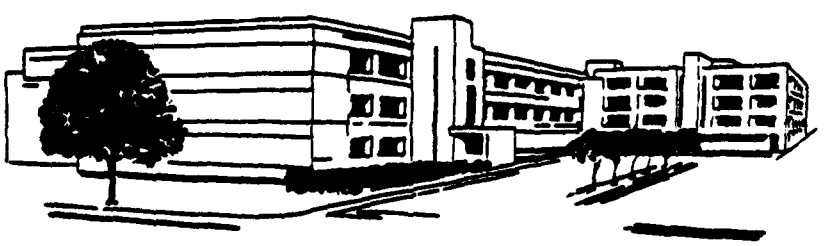

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An attempt was made to purify human erythrocyte transketolase (EC 2.2.1.1) by the use of solvent denaturation as the initial isolation step. This involved the use of 95% ethanol solution (-20 C) to denature a large amount of the hemoglobin and other non-transketolase proteins. Although the data obtained from this study did not contribute to sound statistical values because of the small sample size, they tended to support the application of the method as an effective initial step in the purification of human erythrocyte transketolase. The mean recovery of the enzyme for five trials conducted with blood obtained from

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20. (cont) three donors was 61 ± 3%. The purification ranged from 21 to 23. The method permitted the removal of nearly 95% of the hemoglobin initially present.

PURIFICATION OF TRANSKETOLASE FROM HUMAN ERYTHROCYTES

I. Using Solvent Denaturation as the Initial Step

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ABSTRACT

An attempt was made to purify human erythrocyte transketolase (EC 2.2.1.1) by the use of solvent denaturation as the initial isolation step. This involved the use of 95% ethanol solution (-20 C) to denature a large amount of the hemoglobin and other non-transketolase proteins. Although the data obtained from this study did not contribute to sound statistical values because of small sample sizes, they tended to support the application of the method as an effective initial step in the purification of human erythrocyte transketolase. The mean recovery of the enzyme for five trials conducted with blood obtained from three donors was 61 ± 3%. The purification ranged from 21 to 23. The method permitted the removal of nearly 95% of the hemoglobin initially present.

INTRODUCTION

In 1967, Sauberlich (1) reviewed extensively the various assay methods used for assessment of thiamin adequacy in man. He concluded that the best of these methods was the measurement of erythrocyte transketolase (TK) activity. Since 1950, it has been known that the enzyme transketolase requires thiamin pyrophosphate (TPP) as a co-factor in the metabolism of pentose (2,3). The use of red cell TK in the functional evaluation of thiamin nutritional status in man was not proposed until after 1960.

In the last two decades, several methods have been described for the determination of TK activity in man: measurement of (a) formation of sedoheptulose-7-phosphate (S-7-P) (7,8), (b) formation of glyceraldehyde-3-phosphate (GAP) (9-13), (c) formation of hexose phosphates

(4,14,15), and (d) decrease of pentose phosphates (14,15). Since TK values obtained from these attempts have little diagnostic usefulness for patients with severe liver or other related disease, there has been a considerable controversy in recent years as to whether or not the activity of TK, as presently measured, truly reflects thiamin nutritional status in man (1, 16-18). Figure 1 shows the pentose phosphate pathway.

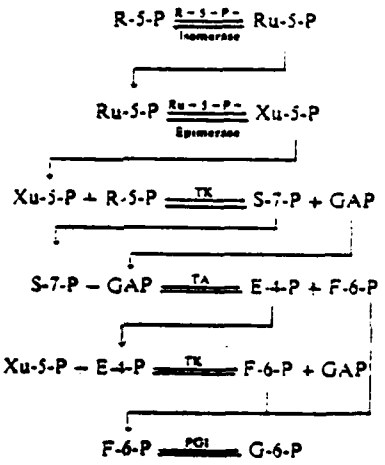

Figure 1. Pentose phosphate pathway.*†

*Abbreviations: R-5-P = ribose-5-phosphate, Ru-5-P = ribulose-5-phosphate, Xu-5-P = xylulose-5-phosphate, S-7-P = sedoheptulose-7-phosphate, GAP = glyceraldehyde-3-phosphate, E-4-P = erythrose-4-phosphate, PGI = phosphoglucoisomerase, G-6-P = glucose-6-phosphate, TK = transketolase, TA = transaldolase.

†*Reproduced from figure presented in reference 19 of this manuscript.*

In all the methods used heretofore to determine the activity of TK in erythrocytes, only ribose-5-phosphate (R-5-P) has been added to the assay system as a substrate. Since external substrate xylulose-5-phosphate (Xu-5-P) was not known or available when most of these enzyme assays were developed, this TK substrate normally was not used for carrying out the enzyme assay. It was instead assumed to be provided

internally from R-5-P (first two reactions outlined in Figure 1). According to Brin (19), the enzymes catalyzing for the first two reactions outlined above are not rate-limiting. There is, however, some evidence that this may not be a valid assumption (Waring, unpublished data).

In any event, to validate the measurement of TK activity in human erythrocytes, one must first demonstrate that in the hemolysate system TK is the rate-limiting enzyme. A second requirement is that R-5-P does, in fact, equilibrate with the ketopentoses. These, in turn, necessitate the isolation or purification of TK from human red cells. After purified TK from human red cells becomes available, one then can test the hypothesis that the activities of both the ribulose-5-phosphate (Ru-5-P) epimerase and R-5-P isomerase are much higher than the activity of TK. For example, various amounts of purified TK can then be added to induce the TK-related reactions in a known quantity of hemolysate to observe the enzymatic effect on the formation of GAP or S-7-P. If the activities of other related enzymes are, in fact, excessively higher, the induced reaction rate probably will not be limited, regardless of the amount of purified enzyme added. The purification of TK from human red cells can lead to an understanding of the true kinetic properties of the enzyme as well.

Transketolase has been isolated and purified from many sources (20-24), but in only one instance from human erythrocytes (25), to our knowledge. These investigators (25) used hydroxylapatite adsorption as the initial isolation step.

The process of using the hydroxylapatite adsorption method (25) as the possible initial isolation step is both laborious and time-consuming. Furthermore, we tried to use this method several times but had little success. This technical note describes the use of solvent denaturation as an initial step in the isolation or purification of TK from human erythrocytes. This method has been effective, is relatively simple to perform, and requires only about two hours to accomplish.

MATERIALS AND METHODS

Chemicals. Glycerin-3-phosphate-dehydrogenase/triosephosphate-isomerase (GPH/TIM) obtained from rabbit muscle was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Other chemicals including yeast R-5-P were products of Sigma Company (St Louis, Missouri).

Source of enzyme. Erythrocytes were prepared from outdated (4- to 5-week old) heparinized human blood. We separated the cells from plasma and washed them three times with 0.9% NaCl by centrifugation at 900 x G for 10 minutes. We then lysed the erythrocytes by adding one volume of 0.02 M K_2HPO_4 at pH 7.8 and two volumes of distilled water. After allowing the hemolysate to stand for several hours, we removed the stroma by centrifugation at 2,000 x G for 10 minutes and collected the

supernatant fraction for enzyme isolation. All steps subsequent to the separation of erythrocytes were carried out at 4 C, except where otherwise noted.

Solvent denaturation. Our method is based on the one described by Oh, et al (26) who used the procedure to purify red cell glutathione peroxidase. To every 100 ml of hemolysate, we stirred in 82 ml of 95% ethanol (-20 C); and then we added slowly 35 ml of carbon tetrachloride (25 C) over a 90-second interval. (Ethanol can be cooled to -30 C or lower by simply placing the flask containing the solvent in a dry ice/acetone bath.) After most of the precipitate had formed, we added 300 ml of distilled water (per 100 ml hemolysate) and removed the bulk of the precipitate by filtering through cheese cloth. Centrifugation at 2,000 x G for 15 minutes removed the remaining precipitate and excessive carbon tetrachloride.

Transketolase assay. The reaction mixture contained the following components: 2.90 ml assay solution; 0.04 ml of hemolysate; and 0.76 ml of mixture containing enzyme extract and/or TRIS buffer in preselected proportions to obtain a standard curve (Figure 2). The 2.90 ml assay solution was composed of 2.80 ml NADH/GPH/TIM solution and either 0.10 ml R-5-P or, in the case of a blank, 0.10 ml TRIS buffer. The reaction was monitored at 37 C by the decrease in absorbance at 340 nm. At 340 nm the optical density will change in proportion to the amount of NADH oxidized to NAD^+. This oxidation of NADH is attributed to the activity and availability of GAP accumulated in the assay system. (The GAP is synthesized through a series of pentose-phosphate-related reactions and in the presence of TK. All required co-factors and other related enzymes were assumed to be in excess following the hemolysate addition.) Solution of assay reagents was prepared as follows: 10 mg NADH per 100 ml TRIS buffer plus 0.5 ml GPH/TIM; 500 mg yeast R-5-P per 100 ml TRIS; and 0.05 M TRIS buffer at pH 7.6 with two to three drops of TRITON-X405.

Enzyme activity. In the present procedure, enzyme activity was expressed in terms of change in optical density per minute at 340 nm and 37 C. (Note: specific activity = enzyme activity per milligram of protein present.) The enzyme activity of the extract was determined by an indirect method, i.e., the difference between the TK activity attributed to hemolysate alone and that attributed to the hemolysate-extract mixture (Figure 2).

Protein determination. Protein content of each sample (hemolysate or extract) was determined by the method of Lowry, et al (27). Human serum albumin was used as the standard and the results were expressed as milligrams per milliliter of hemolysate or extract solution.

RESULTS AND DISCUSSION

Before we used the solvent denaturation as the initial step in the isolation of TK from human erythrocytes, we also attempted other methods in this laboratory. These methods included hydroxylapatite

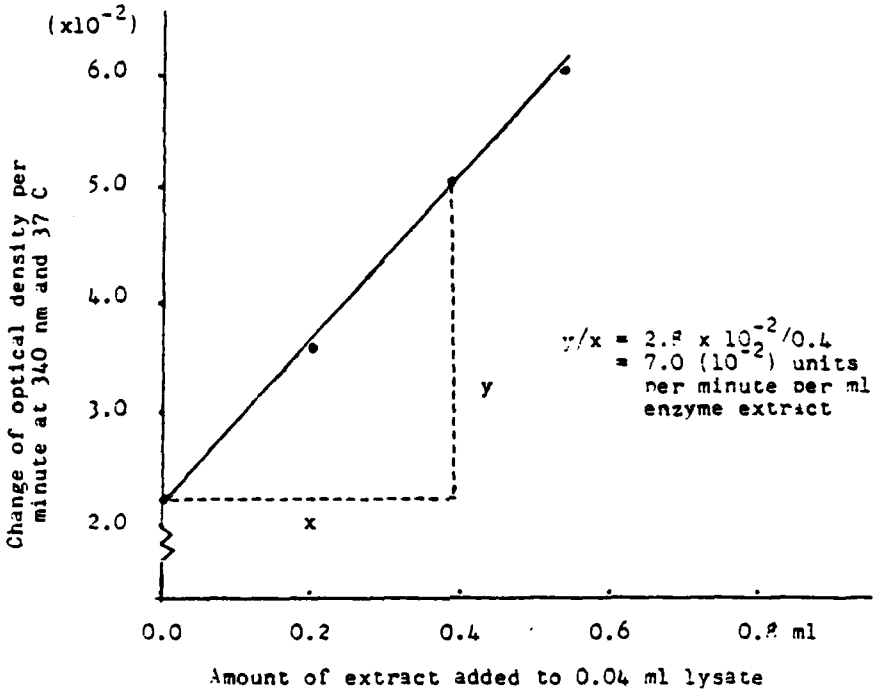

Figure 2. Determination of the activity of transketolase derived from Hemolysate 1107A.

adsorption (25), ammonium sulfate fractionation (28), DEAE-cellulose adsorption (25,28), and acetone fractionation (28). None of these methods were completely satisfactory; the best was hydroxylapatite adsorption which gave a 14-fold purification and a yield of 47%. At one time we attempted to measure the activity of crudely isolated TK by simply estimating the GAP accumulated through the interaction between commercial preparations of Xu-5-P and R-5-P. However, the commercial yeast Xu-5-P was not in a sufficiently pure form (contaminated with TK) to warrant the attempt. Since commercially prepared Xu-5-P is rather expensive, the preparation of a pentose-5-phosphate equilibrium mixture in laboratory as a source of Xu-5-P was proposed but was not attempted because of the successful development of the present indirect method for measuring TK activity.

Table 1 shows the distribution of enzyme activity and protein content before and after the use of solvent denaturation. The data provided in this table represent the results obtained from five trials. Accordingly, the isolations were attempted on five different occasions with five hemolysates derived from blood donated by three individuals. As shown, the recovery of TK ranged from 57% to 64% with a mean of

Table 1. Summary of isolation of human erythrocyte transketolase (using solvent denaturation as the initial step) from blood of three donors

Isolation Step	Total Volume ml	Protein Content mg/ml	Protein Content total	Enzyme Activity unit*/ml	Enzyme Activity total	Enzyme Activity sp. act.†	Enzyme Yield,%	Purification
Hemolysate 1107A	100	77.00	7700	55.05	5505	0.715	100	1
Enzyme extract	450	0.44	198	7.00	3150	15.909	57	22
Hemolysate 1107B	100	72.00	7200	57.60	5760	0.800	100	1
Enzyme extract	430	0.44	189	8.10	3479	18.409	60	23
Hemolysate 1107B	100	69.00	6900	64.15	6417	0.930	100	1
Enzyme extract	450	0.43	194	8.40	3790	19.535	59	21
Hemolysate 1107C	100	73.00	7300	59.50	5950	0.815	100	1
Enzyme extract	440	0.45	198	8.45	3718	18.778	63	23
Hemolysate 1107C	100	74.00	7400	60.00	5994	0.811	100	1
Enzyme extract	450	0.46	207	8.55	3848	18.587	64	23
Extract Mean ± S.D.							61 ± 3	21 ± 1

*1 unit = change of optical density (in arbitrary units) x 100 per minute at 340 nm and 37 C.
†specific activity (units per milligram).

61%. A range of 21- to 23-fold purification was achieved. Although these data do not contribute to sound statistical values primarily because of the small sample size, they tend to support the use of solvent denaturation as an effective initial step in the isolation of TK from human red cells. The 61% enzyme yield appears to be comparable with that obtained by Heinrich and Wiss (25) who, as mentioned before, used hydroxylapatite adsorption as the initial isolation step. Their recovery from using hydroxylapatite adsorption was 63% and their corresponding purification was 64-fold. However their recovery and purification values cannot be readily compared to the data presented in this table since the activity of their enzyme extract was measured without the addition of any hemolysate to the assay system. That is, the activity of their enzyme extract was determined by the estimation of the S-7-P accumulated in the assay system in the absence of potential interference by erythrocyte transaldolase and other hemolysate contaminants. It can be argued, therefore, that the higher yield of enzyme activity of their extract may not be due entirely to a specific adsorption capacity of hydroxylapatite. It is possible that the removal of transaldolase and other contaminants provided by the hemolysate can also account for such higher yield. For in the absence of transaldolase, both S-7-P and GAP may not be convertible to erythrose-4-phosphate (E-4-P) and fructose-6-phosphate (F-6-P) (Figure 1); this would result in the accumulation of more S-7-P or GAP.

The use of solvent denaturation as the initial step for TK isolation is relatively simple to perform and requires only about two hours to accomplish. Another seemingly important feature is that upon the completion of this process, over 90% of the hemoglobin has been removed. This may be an important contribution since it has been proposed that the determination of erythrocyte TK activity for the clinical evaluation of thiamin nutritional status should be estimated in the absence of hemoglobin if optical density measurement is involved. This argument is based upon the fact that a colored solution often contributes an unnecessarily high optical density background. If the hydroxylapatite adsorption method is used as the initial step for TK isolation, another procedure such as DEAE-cellulose adsorption will be required for the separation of the enzyme from the large amount of hemoglobin present(25). Preliminary findings indicate that the enzyme isolated by the use of solvent denaturation can be stored at 4 C for at least three days without losing any of its activity.

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