with increase in the apparent degree of conjugation of endogenous coenzyme with the enzyme, and a prompt action by added pyridoxal phosphate.

The participation of factors besides pyridoxal phosphate and α-ketoglutarate can be inferred from the faster and greater activation occurring in homogenates, and the occurrence of activation only in supernatant fractions that are concentrated. A similar activation of crude rat liver transaminase to the extent of 10 to 30% over the initial activity has been reported. This occurred during incubation of soluble *plus* particulate fractions with a complex mixture that included α-ketoglutarate[5].

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Department of Biological Chemistry,
Harvard Medical School, and
Cancer Research Institute,
New England Deaconess Hospital,
Boston, Mass. (U.S.A.)

NICOLAS S. CONSTANTSAS
W. EUGENE KNOX

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Purification of human erythrocyte pyruvate kinase

Hereditary non-spherocytic hemolytic anemias in man may be classified on the basis of several different enzyme defects. The red cells of one such group of patients has a low ATP content and a specific deficiency of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (refs. 1–6). This report is concerned with purification and properties of this enzyme which catalyzes interconversion of ATP and pyruvate to ADP and phosphoenolpyruvate (PEP) in the presence of Mg^{2+} and K^+.

Pyruvate kinase was assayed by coupling the reaction to the DPNH-dependent reduction of pyruvate to lactate by lactate dehydrogenase (free of pyruvate kinase). Decrease in absorbance at 340 mμ provided a measure of pyruvate kinase activity. The assay system (3 ml) contained, in addition to the solution being assayed, the following reagents at 31°, pH 7.25: 0.04 M Tris, 0.075 M KCl, 0.008 M $MgSO_4$, 0.001 M

Abbreviation: PEP, phosphoenolypyruvate.

EDTA, 0.0002 M mercaptoethanol, 0.002 M ADP, 17 μg lactate dehydrogenase per ml, 0.00013 M DPNH, and 0.00065 M PEP. All reagents except PEP were mixed and incubated 5 min at 31°. PEP was then added and immediately thereafter decrease in absorbance at 340 mμ was recorded at 2-min intervals. A unit of activity is that amount of enzyme which leads to dehydrogenation of 1 μmole DPNH per min.

Cells from several pints of recently outdated blood from the blood bank were used. All steps were performed at 4° unless otherwise specified. The cells were washed 4 times with an equal vol. of 0.85% NaCl and once with an equal vol. of 1.08% KCl. An equal volume of distilled water was mixed with cells packed by centrifugation at 2000 × *g*, and the mixture was frozen over dry ice to complete hemolysis and preserve the product until ready for Step 1.

Step 1: The hemolysate was diluted with maleate buffer (0.05 M potassium maleate, pH 6.6 containing 0.001 M EDTA and 0.1% (v/v) mercaptoethanol) to give a hemoglobin concentration of about 5 g/100 ml. To this was added an equal vol. of satd. $(NH_4)_2SO_4$ soln., and the mixture was allowed to stand overnight. Half of its volume of celite preparation was added, and the mixture was filtered. (Celite was prepared by washing several times with distilled water. To the packed celite was added 2/3 its vol. of satd. $(NH_4)_2SO_4$ soln. The mixture was stirred, allowed to settle, and the supernatant was discarded. An equal vol. of 50% satd. $(NH_4)_2SO_4$ in maleate buffer was then added, and this material was stored at 4°.) The insoluble material was then mixed with 2 times its volume of 45% satd. $(NH_4)_2SO_4$ soln. in maleate buffer and the mixture was again filtered. The washing was repeated a second time; the filtrate was discarded, and the filter pads were stored at 4° until ready for Step 2.

Step 2: The solids were slurried with an equal vol. of 0.05 M citrate buffer (containing 0.001 M EDTA and 0.1% (v/v) mercaptoethanol), pH 5.3, and filtered. The process was repeated, washing the filter cake on the funnel with a little citrate buffer. The percent saturation with $(NH_4)_2SO_4$ was determined on the combined filtrates, and sufficient satd. $(NH_4)_2SO_4$ soln. was added to bring the mixture to 40% satn. The mixture was allowed to stand overnight. The precipitate was collected by centrifugation at 2000 × *g* for 30 min and stored in a small vol. of 50% satd. $(NH_4)_2SO_4$ soln. in

TABLE I

PURIFICATION AND RECOVERY OF PYRUVATE KINASE

Step	*Purification*		*% Recovery*	
	Stepwise	*Overall*	*Stepwise*	*Overall*
Hemolysate	—	—	100	—
Step 1	60 ×	60 ×	86	86
Step 2	3.2 ×	191 ×	67	58
Step 3	2.7 ×	515 ×	77	45
Step 4	1.8 ×	911 ×	75	34

0.1 M phosphate buffer (containing 0.001 M EDTA, 0.1% (v/v) mercaptoethanol, 1.0 M KCl, and 0.01 M $MgCl_2$), pH 6.6.

Step 3: The precent saturation of the above mixture with $(NH_4)_2SO_4$ was determined, and sufficient phosphate buffer was added to give 20% $(NH_4)_2SO_4$ satn. This

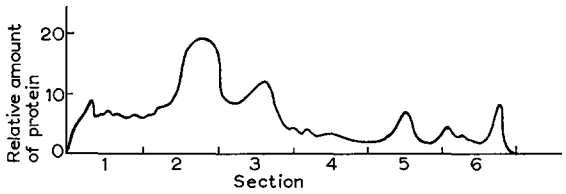

Fig. 1. Disc electrophoretic pattern of pyruvate kinase from Step 4. Enzyme activity was recovered only from Section 1 (of the abscissa). Recovery, 26%. Purification, 1.6 ×.

solution was allowed to stand 30 min with occasional mixing, and was centrifuged at 20 000 × *g* for 5 min. Sufficient satd. $(NH_4)_2SO_4$ soln. was added to the supernatant to make it 38% saturated. After 1 h the mixture was centrifuged at 20 000 × *g*, and the supernatant was discarded.

Step 4: The precipitate was dissolved in a small volume of the pH 6.6 phosphate buffer and was placed in a 53° water bath for 10 min. The mixture was centrifuged, the precipitate discarded, and the supernatant mixed with an equal vol. of satd. $(NH_4)_2SO_4$ soln. for storage.

The above steps generally resulted in a 900–1000-fold increase in the specific activity of pyruvate kinase with about 25% recovery. A summary of the degree of purification and recovery for each step is given in Table I. The initial hemolysate contained $1.81 \cdot 10^6$ pyruvate kinase units.

In the ultracentrifuge enzyme from Step 4 gave rise to 4 well-resolved sedimentation bands with (uncorrected) S values of 10.8, 7.6, 5.9, and 2.6. The heterogeneity of this preparation is further seen in the disc electrophoresis pattern of Fig. 1. Using a stained sample as a guide, the gel was cut and protein eluted. Pyruvate kinase activity was recovered only from Section 1 of the pattern, exhibiting a mobility about that of a serum γ-globulin. Despite the enzyme being confined to Section 1, specific activity was increased only 1.6 times, and the best recovery was 26%.

In several experiments protein from Step 3 was chromatographed on Sephadex G-200 (Fig. 2) with an ensuing 3–3.5-fold purification. Total recovery of enzyme wa 85% which corresponded to recovery of enzyme permitted to stand in solution for the same time interval. Pyruvate kinase was delayed on the Sephadex G-200 column, emerging significantly after the void volume.

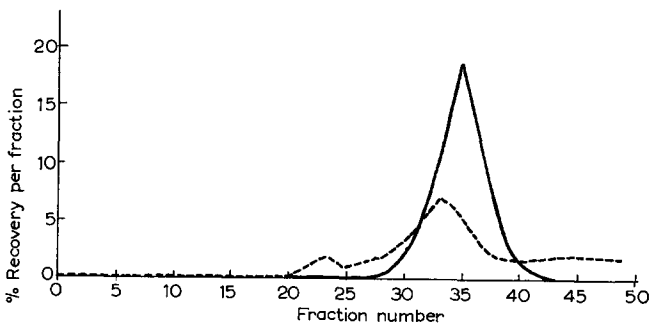

Fig. 2. Chromatogram on Sephadex G-200 of pyruvate kinase from Step 3. Flow rate, 9 ml/h. Total enzyme recovery, 85%. Purification, 3 ×. - - - - -, protein; ———, enzyme.

Michaelis constants of pyruvate kinase from Step 4 were determined and were compared with those for crystalline rabbit muscle enzyme. The assay method was that already described except that ADP and PEP concentration varied. Decrease in absorbance at 340 mμ was continuously recorded using a Beckman DB spectrophotometer and Sargent SRL recorder. Amounts of enzyme were chosen such that the maximum reaction velocity was equivalent to a decrease in absorbance of about 0.15 per min. Since phosphatases could affect the K_m determinations, phosphatases were sought under the pyruvate kinase assay conditions using PEP as substrate in the absence of ADP. Under these conditions no phosphatase activity was detected.

The K_m's for reactions at 31° in pH 7.25 Tris buffer are as follows:

Substrate	*Red cell enzyme*	*Rabbit muscle enzyme*
ADP	$4 \cdot 10^{-4}$ M	$3 \cdot 10^{-4}$ M
PEP	$5 \cdot 10^{-4}$ M	$6 \cdot 10^{-5}$ M

The constants obtained in this laboratory are nearly identical with the values reported by McQUATE AND UTTER[7] for rabbit muscle enzyme: K_m for ADP, $3 \cdot 10^{-4}$ M; K_m for PEP, $7 \cdot 10^{-5}$ M (pH 7.5, 30°). Although the K_m for ADP is essentially the same for the two enzymes, K_m for PEP is almost an order of magnitude larger for red cell pyruvate kinase than it is for rabbit muscle enzyme.

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Wadley Research Institute and Blood Bank, and the Graduate Research Institute of Baylor University, Dallas, Texas (U.S.A.)

MORTON D. PRAGER
WILLIAM R. WHIGHAM

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