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# PURIFICATION AND PROPERTIES OF PYRUVATE KINASE IN NORMAL AND IN PYRUVATE KINASE DEFICIENT HUMAN RED BLOOD CELLS

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SUMMARY

1. Pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) from normal and enzyme-deficient human red blood cells was purified 10 000-fold with an overall yield of 10%.

2. The molecular weight of the different variants of pyruvate kinase was determined by dextran gel filtration by Sephadex G-200 and was found to be 195 000 ± 6000.

3. In high-voltage electrophoresis the pattern of normal red blood cell pyruvate kinase shows three fractions, two being interconvertible. In enzyme-deficient material one of the three fractions was not detectable because of its pronounced instability.

4. A stepwise purification of the three electrophoretic fractions can be achieved by $(NH_4)_2SO_4$ precipitation. The kinetic constants of the fractions from all phenotypes do not differ significantly with respect to ADP and phosphoenolpyruvate. A striking difference was found in enzymic activation by the allosteric effector fructose 1,6-diphosphate.

## INTRODUCTION

Hereditary deficiency of erythrocyte pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) was shown by VALENTINE *et al.*[1] to cause nonspherocytic hemolytic anemia. Considerable heterogeneity in clinical severity and in biochemical abnormality has been described. There is no strict correlation between the degree of reduced enzymic activity and the severity of clinical manifestation. FUSCO *et al.*[2], HANEL AND BRAND[3] and MIWA *et al.*[4] even found cases with increased enzymic activity under conventional assay conditions, the presence of abnormal enzymes being revealed by biochemical studies. BUSCH AND PELZ[5] described the interfamilial variability with respect to residual pyruvate kinase activity in homozygotes: he found a variant "A" (residual activity 0–35%) and a variant "B" (residual activity 40–70%). Both phenotypes showed the clinical picture of a nonspherocytic hemolytic anemia and the typical pattern of increased concentrations of 2,3-diphosphoglycerate, 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate in the deficient cells as well as in most cases diminished ATP content.

This paper deals with the question whether there are only quantitative differences in respect to overall activities or qualitative differences between the pyruvate kinase proteins.

## MATERIALS AND METHODS

All biochemicals and enzymes used were products of C.F. Boehringer, Mannheim. Blue Dextran, Sephadex G-25, G-100, G-200 and the columns K 50/100 and K 15/90 were purchased from Pharmacia, Uppsala. Cellogel strips for electrophoresis were obtained from Chemetron, Milano.

*Preparation of hemolyzates*

Red blood cells were isolated from freshly drawn venous blood by the method of BUSCH AND PELZ[6]. Hemolysis was performed by digitonin and rapid freezing and thawing. $2.0 \cdot 10^{-6}$ M 2-mercaptoethanol and $4.0 \cdot 10^{-4}$ M EDTA (magnesium dipotassium salt) were added to the hemolyzates. Stroma was removed by centrifugation for 20 min. at 40 000 × *g*. The hemoglobin concentration in the supernatant was about 10 g/100 ml.

*Purification procedures*

(1) The following buffer was used throughout the procedure: $1.5 \cdot 10^{-1}$ M KCl, $5.0 \cdot 10^{-3}$ M $MgCl_2$, $1.0 \cdot 10^{-2}$ M $Na_2HPO_4$–$KH_2PO_4$ (pH 5.6), $2.0 \cdot 10^{-6}$ M 2-mercaptoethanol, $4.0 \cdot 10^{-4}$ M EDTA (magnesium dipotassium salt). 20 ml of hemolyzate were chromatographed on a Sephadex G-100 column (5 cm × 90 cm, $V_0 = 565$ ml, flow rate 8.5 ml·cm$^{-2}$·h$^{-1}$, ascending chromatography).

(2) The yield of enzyme solution was 200 ml. The fraction precipitating between 25 and 60% $(NH_4)_2SO_4$ saturation was collected by centrifugation (40 000 × *g* for 20 min at 0°) and suspended in the buffer.

(3) The enzyme solution was diluted with buffer to a protein concentration of 0.5–1.0 mg/ml and was heated for 20 min at 53°. The precipitate was removed by centrifuging for 20 min at 40 000 × *g*.

(4) The enzyme was precipitated by the addition of $(NH_4)_2SO_4$ solution (25–60% saturation) and centrifugation for 20 min at 40 000 × *g*. The sediment was suspended in the buffer, containing 1% Blue Dextran. The enzyme was completely absorbed by the colored dextran.

(5) The pyruvate kinase–Blue Dextran complex was chromatographed on a Sephadex G-200 column (1.5 cm × 90 cm, $V_0 = 49$ ml, flow rate 6.4 ml·cm$^{-2}$·h$^{-1}$, ascending chromatography). All enzymic activity appeared with the void volume. Precipitation was performed with solid $(NH_4)_2SO_4$ (25–60% saturation) in the presence of $5.0 \cdot 10^{-4}$ M fructose 1,6-diphosphate. The sediment was collected in the usual buffer and was re-chromatographed on the same column. For this step the elution buffer contained fructose 1,6-diphosphate $5.0 \cdot 10^{-4}$ M. Blue Dextran appeared again with the void volume, well separated from pyruvate kinase. The specific activity is defined in units/mg of protein. The protein concentration was determined according to LOWRY *et al.*[7], using crystallized bovine serum albumin as a standard.

*Molecular weight determination*

The molecular weight was determined by ascending gel chromatography on Sephadex G-200 equilibrated with the buffer mentioned above. Chromatography was performed with the same column as described for the separation of Blue Dextran from the pyruvate kinase preparation. The following reference proteins were used: 1, ovalbumin (45 000); 2, rabbit muscle phosphoglycerate mutase (64 000); 3, hemoglobin (65 000); 4, bovine serum albumin, monomer (67 000); 5, human erythrocyte glucose phosphate isomerase (92 000); 6, bovine serum albumin, dimer (134 000); 7, rabbit muscle aldolase (158 000); 8, rabbit muscle pyruvate kinase (237 000).

*Electrophoresis of pyruvate kinase*

Electrophoresis was performed on Cellogel strips (170 mm × 25 mm × 0.3 mm) which were incubated for 1 h in the following buffer: 0.01 M $KH_2PO_4$–$Na_2HPO_4$ (pH 5.3), $4.0 \cdot 10^{-4}$ M EDTA (magnesium dipotassium salt), $2.0 \cdot 10^{-6}$ M 2-mercaptoethanol. This buffer was used throughout the electrophoretic procedure.

Hemolyzates or enriched preparations (dialyzed against this buffer) were applied at the middle of the strips using a semi-micro-sample applicator. The run took 100 min at 3 mA/strip, at 50 V/cm and −4°. The staining procedure was performed according to VON FELLENBERG *et al.*[8]. After incubation for 10 min at 37° the bands could be observed under ultraviolet irradiation.

*Fractionation of pyruvate kinase*

After Sephadex G-100 filtration the enzyme was fractionated at different $(NH_4)_2SO_4$ concentrations: 25–38, 38–45 and 45–60%. For the kinetic studies the precipitates were chromatographed on Sephadex G-25, and eluted with $1.5 \cdot 10^{-1}$ M KCl. For electrophoretic studies the fractions were dialyzed for 6 h against the buffer system used for electrophoresis.

*Kinetic studies of pyruvate kinase fractions*

The influence of the various ligands was studied under the following conditions.

(1) Phosphoenolpyruvate concentration varied: 0.1 M Triethanolamine–EDTA (pH 7.5), $8.2 \cdot 10^{-2}$ M KCl, $6.5 \cdot 10^{-3}$ M $MgCl_2$, $7.0 \cdot 10^{-4}$ M ADP, $2.0 \cdot 10^{-4}$ M NADH, 0.01 mg crystallized lactate dehydrogenase (free from pyruvate kinase), 0.2 ml enzyme solution, total volume 2.0 ml.

(2) Phosphoenolpyruvate concentration varied in the presence of $5.0 \cdot 10^{-4}$ M fructose 1,6-diphosphate: Preincubation with fructose 1,6-diphosphate, 5 min at 25°.

(3) ADP concentration varied in the presence of $2.3 \cdot 10^{-3}$ M phosphoenolpyruvate.

(4) Fructose 1,6-diphosphate concentration varied in the presence of $5.0 \cdot 10^{-5}$ M phosphoenolpyruvate. Preincubation with fructose 1,6-diphosphate, 5 min at 25°. $K_{1/2}$ refers to the molar concentration of a ligand at which half maximal activity is observed. HILL exponents ($nH$) are calculated from the equation:

$$nH = \frac{\Delta \lg \frac{v}{V - v}}{\Delta \lg [L]}$$

where [L] = concentration of the ligand.

TABLE II

COMPARISON OF THE KINETIC CONSTANTS ($K_{1/2}$) AND HILL EXPONENTS ($nH$) FOR THE LIGANDS PHOSPHOENOLPYRUVATE, ADP AND FRUCTOSE DIPHOSPHATE IN $(NH_4)_2SO_4$ PRECIPITATES OF PYRUVATE KINASE FROM NORMAL ERYTHROCYTES AND FROM ERYTHROCYTES OF PYRUVATE KINASE DEFICIENCY TYPES "A" AND "B"

| *Concentration of ligands varied* | *Constants of ligands varied* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Normal enzyme $(NH_4)_2SO_4$ precipitates* | | | | | | *Pyruvate kinase deficiency type "A" $(NH_4)_2SO_4$ precipitates* | | | | | | *Pyruvate kinase deficiency type "B" $(NH_4)_2SO_4$ precipitates* | | | | | |
| | *I (20–38%)* | | *II (38–45%)* | | *III (45–60%)* | | *I (20–38%)* | | *II (38–45%)* | | *III (45–60%)* | | *I (20–38%)* | | *II (38–45%)* | | *III (45–60%)* | |
| | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ | $K_{1/2}$ | $nH$ |
| Phosphoenol pyruvate varied; ADP, $7.0 \cdot 10^{-4}$ M; fructose diphosphate, 0 | $4.8 \cdot 10^{-4}$ | 1.2 | $3.0 \cdot 10^{-4}$ | 1.0 | $1.0 \cdot 10^{-4}$ | 0.6 | $2.4 \cdot 10^{-4}$ | 1.1 | $1.7 \cdot 10^{-4}$ | 0.7 | $3.6 \cdot 10^{-4}$ | 1.1 | $2.9 \cdot 10^{-4}$ | 0.8 | $1.9 \cdot 10^{-4}$ | 0.8 | $3.1 \cdot 10^{-4}$ | 1.1 |
| Phosphoenol pyruvate varied; ADP, $7.0 \cdot 10^{-4}$ M; fructose diphosphate, $5.0 \cdot 10^{-4}$ M | $0.6 \cdot 10^{-4}$ | 0.8 | $0.7 \cdot 10^{-4}$ | 0.8 | $0.8 \cdot 10^{-4}$ | 0.9 | $1.2 \cdot 10^{-4}$ | 0.9 | $2.1 \cdot 10^{-4}$ | 0.9 | $5.0 \cdot 10^{-4}$ | 1.3 | $1.1 \cdot 10^{-4}$ | 0.8 | $0.5 \cdot 10^{-4}$ | 1.0 | $0.9 \cdot 10^{-4}$ | 0.8 |
| ADP varied; phosphoenol pyruvate $2.3 \cdot 10^{-3}$ M; fructose diphosphate 0 | $4.4 \cdot 10^{-4}$ | 1.1 | $2.4 \cdot 10^{-4}$ | 1.0 | $2.3 \cdot 10^{-4}$ | 0.9 | $6.0 \cdot 10^{-4}$ | 1.0 | $4.5 \cdot 10^{-4}$ | 0.8 | $2.3 \cdot 10^{-4}$ | 1.1 | $3.9 \cdot 10^{-4}$ | 0.9 | $1.7 \cdot 10^{-4}$ | 1.1 | $8.0 \cdot 10^{-4}$ | 1.0 |
| Fructose diphosphate varied; phosphoenol pyruvate, $5.0 \cdot 10^{-5}$ M; ADP, $7.0 \cdot 10^{-4}$ M | $0.6 \cdot 10^{-7}$ | 1.5 | $1.7 \cdot 10^{-7}$ | 1.6 | $1.1 \cdot 10^{-7}$ | 1.5 | $4.6 \cdot 10^{-7}$ | 1.2 | $3.8 \cdot 10^{-7}$ | 1.3 | $5.8 \cdot 10^{-7}$ | 1.1 | $1.5 \cdot 10^{-5}$ | 1.3 | $1.8 \cdot 10^{-5}$ | 0.9 | $5.0 \cdot 10^{-7}$ | 1.3 |

RESULTS

Pyruvate kinase from human red blood cells was purified 8300 to 11600-fold with a yield of 10–15% by Sephadex G-100 filtration, two precipitations with $(NH_4)_2SO_4$, heat denaturation and 2-fold Sephadex G-200 filtration with and without Blue Dextran. The purification procedure is summarized in Table I.

The ultraviolet spectrum of the purified enzyme exhibited a maximum at 275 nm and a minimum at 249 nm. For comparison, the rabbit muscle pyruvate kinase is shown together with the absorbance spectrum of the purified human red cell pyruvate kinase in Fig. 1.

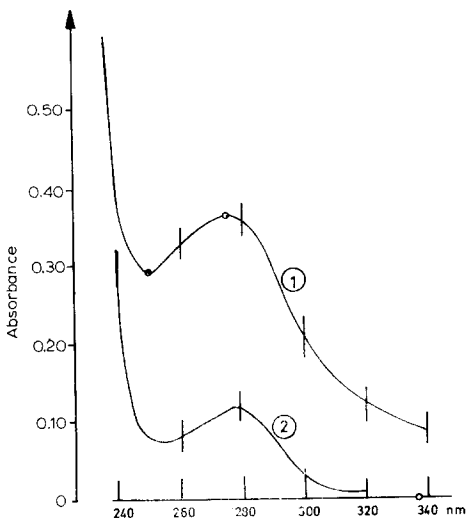


Fig. 1. Ultraviolet spectrum of purified red cell pyruvate kinase (1) and of crystallized rabbit muscle pyruvate kinase (2).

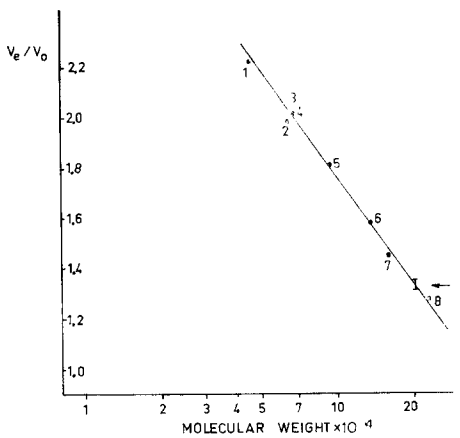


Fig. 2. Determination of molecular weight of red cell pyruvate kinase from normal and pyruvate kinase deficient erythrocytes. Arrow: range of 8 determinations of pyruvate kinase from normal and pyruvate kinase deficient red blood cells. The figure shows the calibration curve, established with the following reference proteins: 1, ovalbumin; 2, rabbit muscle phosphoglyceromutase; 3, hemoglobin; 4, bovine serum albumin, monomer; 5, red cell glucosephosphate isomerase, (32); 6, bovine serum albumin, dimer; 7, rabbit muscle aldolase; 8, rabbit muscle pyruvate kinase.

By dextran gel filtration on Sephadex G-200 the molecular weight of pyruvate kinase from all three phenotypes was found to be 195 000 ± 6000. The determinations were performed with hemolyzates and enriched preparations. The calibration curve for molecular weight determination is shown in Fig. 2.

High-voltage electrophoresis separated pyruvate kinase from normal human erythrocytes into three fractions. Fraction I was converted to Fraction II by adding fructose 1,6-diphosphate ($5.0 \cdot 10^{-4}$ M) to the enzyme preparations and to the buffer system. Aging of the enzyme had the same effect. Fraction III from normal erythrocytes was not affected by this treatment. In homozygous cases studied from both variants Fraction III could not be detected after electrophoresis of hemolyzates, as can be seen from Fig. 3.

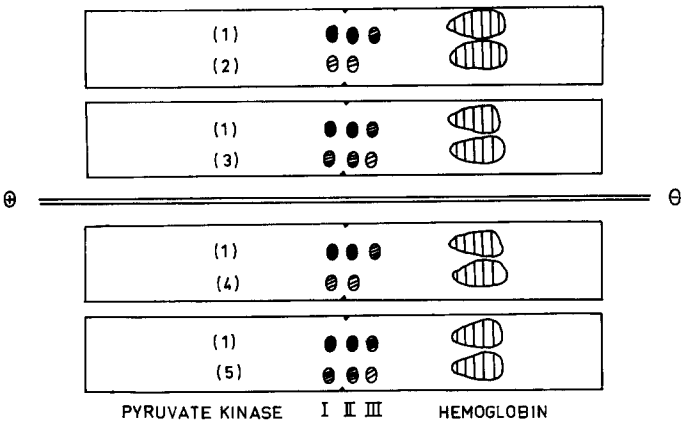


Fig. 3. High-voltage electrophoresis of pyruvate kinase from normal and pyruvate kinase deficient red blood cells. Normal red cell pyruvate kinase (1); pyruvate kinase deficiency type A, homozygous (2); pyruvate kinase deficiency type A, heterozygous (3); pyruvate kinase deficiency type B, homozygous (4); pyruvate kinase deficiency type B, heterozygous (5).

By $(NH_4)_2SO_4$ precipitations at different saturations a relative purification of the three fractions observed in the electrophoretic studies was achieved. At 4° the half-life of Fraction III from normal red blood cells was 32–41 h, and that of the enzyme-deficient cells was 4–6 h.

The kinetic constants and *nH* values of the three $(NH_4)_2SO_4$ fractions of each of the three phenotypes were studied. They did not differ significantly with respect to ADP and to phosphoenolpyruvate (with and without fructose 1,6-diphosphate). However, the concentration of fructose 1,6-diphosphate required for half maximal enzymic activation of Fractions I and II from type "B" was increased 100-fold, compared with the normal enzyme and the "A" variant, respectively. The values of $K_{1/2}$ and of *nH* for the different fractions from the three phenotypes are summarized in Table II.

TABLE I

PURIFICATION OF PYRUVATE KINASE FROM NORMAL HUMAN ERYTHROCYTES

| *Fraction* | *Vol. (ml)* | *Protein (mg/ml)* | *Total protein (mg)* | *Specific activity (units/mg protein)* | *Total activity (units)* | *Purification (-fold)* | *Yield (%)* |
|---|---|---|---|---|---|---|---|
| Hemolyzate | 20 | 107 | 2140 | 0.0056 | 12.0 | 1.00 | 1.00 |
| Sephadex G-100 filtrate | 200 | 0.11 | 22 | 0.66 | 14.2 | 118 | 1.21 |
| Precipitate, $(NH_4)_2SO_4$ (25–60%) | 9.1 | 0.79 | 7.2 | 1.48 | 10.6 | 264 | 0.88 |
| Heat denaturation, 20 min, 53° | 9.0 | 0.21 | 1.88 | 3.51 | 6.61 | 626 | 0.55 |
| Precipitate, $(NH_4)_2SO_4$ (25–60%) | 2.4 | 0.19 | 0.45 | 12.9 | 5.80 | 2320 | 0.48 |
| Sephadex G-200 filtrate | 3.0 | 0.08 | 0.23 | 57.9 | 1.32 | 10 400 | 0.11 |

## DISCUSSION

Purification procedures for pyruvate kinase from human red blood cells have been described by PRAGER AND WHIGHAM[9], IBSEN *et al.*[10], DUBIN AND BERNARD[11], and STAAL[12]. The advantage of the method reported herein is the small amount of blood required for a rather high purification. A comparison of the normal enzyme with that from homozygous individuals suffering from hemolytic anemia is possible under the conditions of our method of preparation.

In the first step a 100–120-fold enrichment was achieved by removing the total hemoglobin content of the hemolyzates by Sephadex G-100 filtration as described by AEBI *et al.*[13]. In this step an increase of the yield from 1.00 to 1.20–1.30 was regularly observed, possibly resulting from the removal of inhibiting agents. By heat treatment and two $(NH_4)_2SO_4$ precipitations the purification was increased to 2300-fold. The concentration of $(NH_4)_2SO_4$ required for precipitation of all erythrocyte pyruvate kinase under our conditions was higher than that described by BIGLEY *et al.*[14]. Additionally about 10% of total red cell pyruvate kinase was precipitated at 45–60% saturated $(NH_4)_2SO_4$. As leukocyte contamination in the preparations is below 1 leukocyte in 40 000 erythrocytes the enzymic activity precipitated at these $(NH_4)_2SO_4$ concentrations cannot be explained from leukocytes present.

The use of Blue Dextran for isolation of pyruvate kinase was first described by HAECKEL *et al.*[15] for yeast pyruvate kinase. We found the interaction with Blue Dextran in all cases of normal pyruvate kinase and of heterozygotes and homozygotes of pyruvate kinase deficiency. This effect is probably dependent on the conformation of pyruvate kinase, since the complex can be separated by $(NH_4)_2SO_4$ precipitation in the presence of fructose 1,6-diphosphate.

The loss of activity during the two Sephadex G-200 filtration steps can be prevented by the addition of glycerol, as pointed out by STAAL[12]. In the presence of glycerol he obtained a preparation of pyruvate kinase from human red blood cells with a specific activity of about 150 units /mg of protein.

Absorption of pyruvate kinase preparations to CM-cellulose and subsequent elution of the enzyme by its allosteric effector fructose 1,6-diphosphate, as described by CARMINATTI *et al.*[16] for rat liver pyruvate kinase, has not till now been successful in red cell pyruvate kinase purification.

In order to define the state of purity, the activities of other enzymes of the glycolytic pathway and to the hexose monophosphate shunt were determined. Except for glutathione reductase (0.9%), phosphoglycerate kinase (1.2%) and lactate dehydrogenase (1.2%), all activities were below 0.1% of pyruvate kinase activity in the preparations. From the ultraviolet spectrum it can be assumed that pyruvate kinase from erythrocytes is a so-called "tyrosine enzyme", as described for rabbit muscle pyruvate kinase by BEISENHERZ *et al.*[17].

The molecular weight of pyruvate kinase from hemolyzates and enriched preparations from erythrocytes of all phenotypes is 195 000 ± 6000. This result is in good accord with the findings of STAAL[12], who reported a value of 200 000 of normal red cell pyruvate kinase by Sephadex G-200 filtration. KOLER *et al.*[18] found a value of 150 000 for red cell pyruvate kinase by ultracentrifugational studies. In the following the molecular weights of pyruvate kinase from various species and tissues are listed for comparison: yeast pyruvate kinase 200 000 by HAECKEL *et al.*[15], yeast

pyruvate kinase 166 000 by HUNSLEY *et al.*[19], rat liver pyruvate kinase 208 300 by TANAKA *et al.*[20], rabbit muscle pyruvate kinase 237 000 by WARNER[21] and 232 000–242 000 by COTTAM *et al.*[22], and rat muscle pyruvate kinase 250 000 by TANAKA *et al.*[20].

Upon electrophoresis we found three fractions of pyruvate kinase from normal hemolyzates. Two fractions were interconvertible under various conditions. Probably these two fractions represented different conformers. Fractions I and II were found in enzyme deficient red cells, too. Fraction III, however, could not be detected in enzyme deficient cells because of its instability. Special care was taken to avoid leukocyte contamination, since pyruvate kinase activity is about 300-fold higher in white cells than in red cells, as described by TANAKA *et al.*[23] and by BUSCH AND PELZ[6]. The existence of two components of the enzyme in red blood cells was reported by LÖHR[24], TOWNES[25], SALOMON[26], and STAAL[12].

The allosteric properties of pyruvate kinase were first described by HESS *et al.*[27], who found activation of yeast pyruvate kinase by the effector fructose 1,6-diphosphate. KOLER AND VANBELLINGHEN[28] described the mechanism of modulation of red cell pyruvate kinase by the effector fructose 1,6-diphosphate. CARTIER *et al.*[29], BOIVIN and associates[30], as well as MUNRO AND MILLER[31] reported on fructose 1,6-diphosphate activation of pyruvate kinase from enzyme-deficient red cells.

We performed kinetic studies with partially purified fractions from all phenotypes. The values for $K_{1/2}$ (phosphoenolpyruvate) did not differ significantly in the fractions from all phenotypes. In normal and type "B" red cells the affinity to phosphoenolpyruvate increased in the presence of fructose 1,6-diphosphate. All the values for $K_{1/2}$ (ADP) were similar. However, a striking difference was found in the activation kinetics of the enzyme by fructose 1,6-diphosphate. Whereas the concentrations of fructose 1,6-diphosphate required for half maximal enzyme activation in normal and type "A" red cells were of the order of $1 \cdot 10^{-7}$ M, Fractions I and II of type "B" enzyme showed $K_{1/2}$ values of the order of $1 \cdot 10^{-5}$ M. The Hill exponents sometimes indicated an increase and sometimes a decrease of cooperativity.

In summary, normal erythrocytes, types "A" and "B" pyruvate kinase deficient erythrocytes are quantitatively characterized by different overall pyruvate kinase activities. Qualitatively, a major difference between these three phenotypes affects the fructose 1,6-diphosphate concentrations required for half maximal activation of the enzymes and the stability of one of the electrophoretic fractions.

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