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HUMAN ERYTHROCYTE PYRUVATE KINASE

ITS PURIFICATION AND SOME PROPERTIES*

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SUMMARY

1. Human erythrocyte pyruvate kinase (EC 2.7.1.40) was purified 30 000-fold by successive $(NH_4)_2SO_4$ precipitation and column chromatography with blue dextran 2000. The resulting enzyme preparation had a specific activity of 150 μmoles NADH · $min^{-1} \cdot mg^{-1}$ at 25°.

2. The molecular weight as determined by gel filtration with Sephadex G-200 was 205 000 $\pm$ 5000.

3. With starch gel electrophoresis only one band was observed after detection of the enzyme activity with the fluorescent technique.

4. The variation of activity of pyruvate kinase at various ADP and phosphoenolpyruvate (PEP) concentrations was studied.

5. The stimulatory effect of Fru-1,6-P_2 as well as the inhibitory effect of ATP on the activity of pyruvate kinase were pH dependent.

6. Phosphorylated hexoses (Fru-1,6-P_2 and Glc-6-P), P_i and the substrate PEP overcame the inhibition of the activity of pyruvate kinase by ATP at physiological pH.

7. Phosphorylated hexoses and P_i stimulated the activity of pyruvate kinase.

INTRODUCTION

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate with regeneration of ATP and is a key enzyme in the glycolysis. Deficiency of pyruvate kinase in human erythrocytes results in a haemolytic anaemia. Until now attempts to purify human erythrocyte pyruvate kinase have had limited success because of its instability[1,2]. Recently HUNSLEY AND SUELTER[3] reported that yeast pyruvate kinase was stabilized by the addition of glycerol. HAECKEL *et al.*[4] reported

Abbreviation: PEP, phosphoenolpyruvate.

* Some of the results have already been presented by one of us (G.S.) at the colloquium on "La Biochimie Moleculaire du Globule Rouge", Paris, April 10–11th, 1970.

that yeast pyruvate kinase could be purified by applying the property of the enzyme to bind specifically to blue dextran.

TANAKA *et al.*[5] showed that pyruvate kinase in the liver exists in two forms, L(liver) type and M(muscle) type. The L type is under hormonal and dietary control. The kinetic properties of the two types are different. For example, the sensitivity of type L to inhibition by ATP is much greater than the sensitivity of type M. The L type can be stimulated by Fru-1,6-P_2, whereas this phosphorylated hexose has no influence on the M type. ROZENGURT *et al.*[6] have shown that the effects of ATP and Fru-1,6-P_2 on rat liver L-type enzyme are pH dependent: at pH < 7 the enzyme follows Michaelis–Menten kinetics and cannot be activated by Fru-1,6-P_2. At higher pH (7.2) the PEP saturation curve is sigmoid, and Fru-1,6-P_2 is able to transform these sigmoid curves into normal Michaelis–Menten curves. Although the liver contains both types of enzyme, the erythrocyte contains only the L type[5], but it is claimed that the L type of erythrocytes is not identical with the liver L type[5].

In order to explain the decreased pyruvate kinase activity of patients with a haemolytic anaemia, we started an investigation on the normal enzyme. This paper deals with the purification of, and some investigations on, human erythrocyte pyruvate kinase.

MATERIALS AND METHODS

Chemicals

ADP, ATP, NADH, Tricyclohexylammonium PEP, phosphorylated hexoses, lactate dehydrogenase, glutathione reductase, pyruvate kinase and glycerokinase were obtained from Boehringer and Soehne. $(NH_4)_2SO_4$, glycerol, β-mercaptoethanol and amino caproic acid were of analytical grade purity. Amfotericine B came from Squib (Brussels, Belgium) and chloramphenicol from Nogepha, Amsterdam (The Netherlands). DEAE-Sephadex A-50, capacity 3.5 ± 0.5 mequiv/g, particle size 40–120 μ, Sephadex G-200, particle size 40–120 μm, and blue dextran 2000 were purchased from Pharmacia, Uppsala. Concentration of the protein solutions were carried out with Diaflo ultrafiltration cells from Amicon with XM-50 membranes. Human erythrocytes were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands).

Determination of enzymic activity

Pyruvate kinase activities were measured at 25° by coupling the system with lactate dehydrogenase, according to the method of BÜCHER AND PFLEIDERER[7]. The oxidation of NADH was followed at 340 nm with a Zeiss spectrophotometer PMQ II in combination with a Photovolt-recorder model 43. Pyruvate kinase activity was determined in 0.2 M Tris–HCl (pH 7.2) in a final volume of 3 ml containing 5 mM tricyclohexylammonium PEP, 5 mM ADP, 65 mM KCl, 20 mM $MgSO_4$, 0.02 ml dialysed lactate dehydrogenase, 90 μM NADH and enzyme. A unit of activity is defined as the amount of enzyme required to oxidize 1 μmole of NADH per min. The specific activity is defined as units per mg of protein. The protein content was determined by the method of LOWRY *et al.*[8] using crystalline bovine serum albumin as a standard.

Molecular weight determination

The molecular weight was estimated according to ANDREWS[9]. The reference proteins used for calibration of the column are given in the corresponding figure.

Starch gel electrophoresis

Horizontal starch gel electrophoresis was performed at 4° during 16 h according to the method of BIGLEY *et al.*[10] in 0.1 M boric acid–NaOH buffer (pH 8.6). Pyruvate kinase activity was detected with the fluorescent technique according to the method of Von FELLEDBURG *et al.*[11].

RESULTS

Isolation of pyruvate kinase

The whole purification was carried out at 4°.

(1) About 0.5 l of erythrocytes was mixed with an equal volume of water containing 1 mM β-mercaptoethanol. The erythrocytes were haemolysed by freezing and thawing (3×). Approx. 1 l of haemolysate was obtained.

(2) To the haemolysate were added 2 l of DEAE-Sephadex suspension, prepared according to the instructions of the manufacturers. The mixture was equilibrated with 0.01 M phosphate buffer (pH 6.8) containing 1 mM β-mercaptoethanol and 1 mM ε-aminocaproic acid. To prevent microbial infection, chloramphenicol and amfotericine B were added to the buffer. The mixture of DEAE-Sephadex and haemolysate was stirred during 1 h. The Sephadex was repeatedly washed with 0.01 M phosphate buffer (pH 6.8) to remove the haemoglobin, about 10 l of buffer being used. The enzyme remained bound to the Sephadex. Pyruvate kinase was eluted with 4 l of 0.1 M phosphate buffer (pH 6.8) containing β-mercaptoethanol (1 mM), ε-aminocaproic acid (1 mM), chloramphenicol and amfotericine B.

(3) To the enzyme solution obtained in the previous step 22 g $(NH_4)_2SO_4$ per 100 ml solution were added. After 12 h the precipitate was collected by filtration with Whatman No. 1 paper and dissolved in 450 ml 0.05 M phosphate buffer (pH 6.8).

(4) To Fraction 3 22 g $(NH_4)_2SO_4$ per 100 ml enzyme solution were added. After 12 h the precipitate was collected by centrifugation (10 000 × *g* for 30 min) and suspended in 40 ml 0.1 M phosphate buffer (pH 6.8) containing 2 mM $MgSO_4$, 2 mM β-mercaptoethanol and the compounds mentioned under Fraction 2.

(5) After being heated during 2 min at 60°, the enzyme fraction was quickly cooled to 4°, and the precipitate was removed by centrifugation (15 min 10 000 × *g*). This procedure removed much of the protein but pyruvate kinase was not inactivated.

(6) The enzyme fraction was concentrated to about 4 ml as described in MATERIALS AND METHODS. Blue dextran 2000 was added (end concentration 0.5%). Any insoluble material was removed by centrifugation. The clear solution was placed on a Sephadex G-200 column (2.5 cm × 100 cm) equilibrated with 5 mM phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$, 2 mM β-mercaptoethanol, chloramphenicol and amfotericine B. The pyruvate kinase was eluted together with blue dextran 2000 as first described by HAECKEL *et al.*[4] for the yeast enzyme. The enzyme-blue dextran complex was concentrated to 4 or 5 ml as described in MATERIALS AND METHODS to give Fraction 7.

(7) The concentrated pyruvate kinase-blue dextran solution was placed on a

Sephadex G-200 column (2.5 cm × 100 cm) equilibrated with 0.2 M phosphate buffer (pH 6.8) containing 5% $(NH_4)_2SO_4$ (w/v) and the compounds mentioned under Fraction 2. Under these conditions pyruvate kinase was separated from the blue dextran. The active fractions were collected in 50% glycerol (w/v), to stabilize the enzyme, and further concentrated with the Amicon ultrafiltration cell to 1 or 2 ml. After concentration, the enzyme was dialysed overnight against 0.01 M Tris–maleic acid (pH 6.8) containing 50% glycerol (v/v). When the separation between pyruvate kinase and blue dextran was not complete, a DEAE-Sephadex column (1 cm × 3 cm) equilibrated with 0.2 M phosphate buffer (pH 6.8) containing 5% $(NH_4)_2SO_4$ (w/v) and 50% glycerol (v/v) was used. Under these conditions blue dextran 2000 is adsorbed at the DEAE-Sephadex while pyruvate kinase is not.

The whole purification procedure is summarized in Table I.

TABLE I

PURIFICATION OF PYRUVATE KINASE FROM NORMAL HUMAN ERYTHROCYTES

Step of purification	*Total vol. (ml)*	*Total Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Cumulative purification (-fold)*
1	1000	$150 \cdot 10^3$	725	$5 \cdot 10^{-3}$	100	1
2	4000	$1.76 \cdot 10^3$	580	$33 \cdot 10^{-2}$	80	66
3	450	$1.66 \cdot 10^2$	500	3	69	600
4	40	87.6	456	5.2	63	1 040
5	40	26	300	11.5	40	2 300
6	—	—	—	—	—	—
7	2	0.38	58	150	8	30 000

Properties of the enzyme

Molecular weight

From Fig. 1 a mol. wt. of 205 000 (± 5000) was calculated using the method of ANDREWS[9]. This value is the same as found for the yeast enzyme by HAECKEL *et al.*[4]

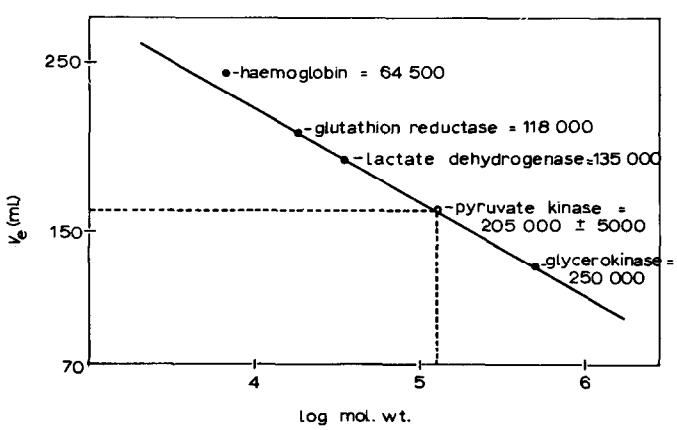

Fig. 1. Estimation of the molecular weight by gel filtration on Sephadex G-200. Column size 3.0 cm × 55 cm. Buffer: 0.2 M phosphate (pH 6.8) containing 5% $(NH_4)_2SO_4$.

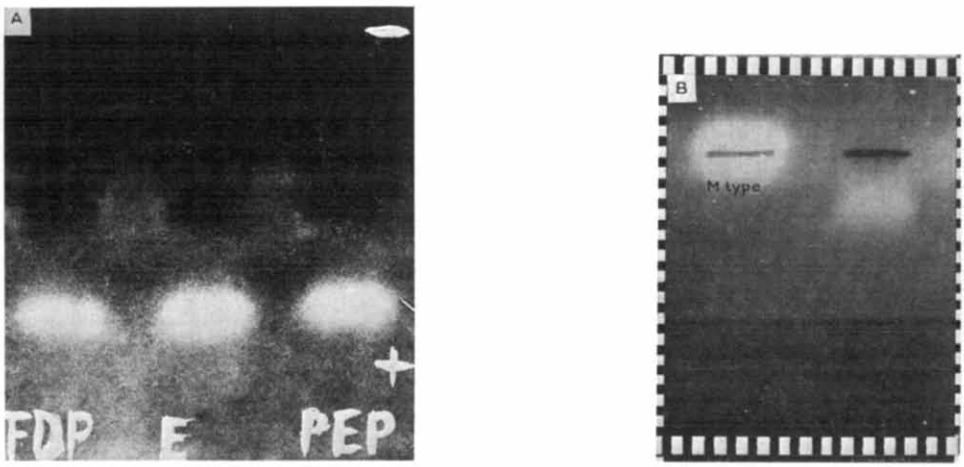

Fig. 2. (A) Photograph of the starch-gel electrophoresis for the purified enzyme in the presence of Fru-1,6-P_2 (FDP) or PEP and the control. The enzyme was previously incubated with Fru-1,6-P_2 or PEP. For conditions see MATERIALS AND METHODS. (B) Photograph of the starch-gel electrophoresis for the preparation before blue dextran treatment. This figure also shows the M type of rabbit muscle pyruvate kinase.

(200 000) and for the L type of rat liver by TANAKA *et al.*[5] (208 000), but differs from the value reported by KOLER *et al.*[12] for the human erythrocyte enzyme (150 000).

Electrophoresis

Electrophoresis with starch gel at pH 8.6 shows, after detection of the enzyme with the fluorescent technique, a single band (see Figs. 2A, B). This is in agreement with the results reported by BIGLEY *et al.*[10], but differs from the results described by BLUME *et al.*[13] and by TOWNES[14], who found two bands. Electrophoresis in the presence of PEP or Fru-1,6-P_2 had no influence on the electrophoretic mobility of the purified enzyme (Fig. 2A). Fig. 2B shows the electrophoresis of the preparation before the addition of blue dextran. One band was found, which means that before the blue dextran treatment only the L type was present, and the possibility that with this treatment the M type was lost can be excluded.

Kinetics

Figs. 3A, 3B show the Lineweaver–Burk plots. The $1/v$ *vs.* $1/[PEP]$ plots show curves concave upwards, which are the reflection of the sigmoid character. The $1/v$ *vs.* $1/[ADP]$ plot shows straight lines at different PEP concentrations; however, there is an indication of substrate inhibition by high ADP concentrations at low [PEP], which diminishes at higher PEP concentrations. The Hill plot at $[ADP] = \infty$ shows positive cooperativity ($n = 1.6$) with respect to PEP (Fig. 4); however, the positive cooperativity exists at all ADP concentrations used. From Fig. 4 a K_m value of 0.63 mM for PEP was calculated; a similar value is also obtained by extrapolation of the curve in the $1/v$ *vs.* $1/[PEP]$ plot. The K_m value of ADP at $[PEP] = \infty$ is 0.6 mM. With both substrates at infinite concentrations a v_{max} of 200 μmoles NADH/min per mg is obtained. With the Hill plot at the different ADP concentrations it is possible to calculate the various apparent K_m values for PEP. By plotting the apparent K_m values against the respective v_{max} values, Fig. 5 is obtained. In this K_m *vs.* v_{max} plot, it is shown that the line does not pass through the origin, which

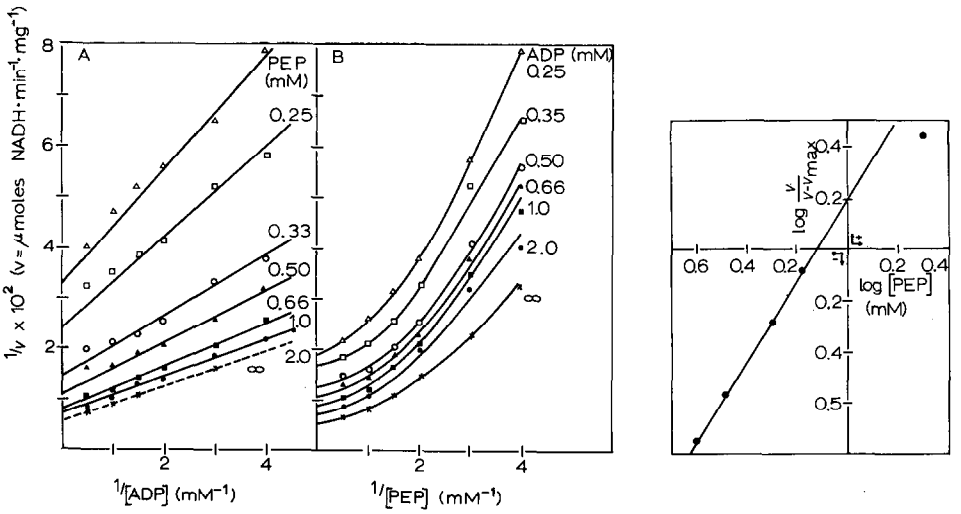

Fig. 3. (A) The Lineweaver–Burk plot of the variation of the activity of pyruvate kinase with [ADP] at various [PEP]. The various [PEP] are indicated in the figure. Buffer: 0.1 M Tris–HCl (pH 7.4). Further conditions are described in the text. (B) The Lineweaver–Burk plot of the variation of the activity of pyruvate kinase with [PEP] at various [ADP]. The various [ADP] are indicated in the Figure.

Fig. 4. The Hill plot of variation of the activity of pyruvate kinase with [PEP] at [ADP] = ∞. Conditions as for Fig. 3.

means that in the reaction mechanism a ternary complex is involved[15,16] (assuming that the phosphate donor is the first substrate).

It is known that the stimulation of rat-liver pyruvate kinase by Fru-1,6-P_2 is pH dependent[6,17]. ROZENGURT *et al.*[6] showed that a change in pH has a marked effect on the substrate cooperative interactions. At high pH (8.15) a sigmoid curve was obtained, while at pH 5.9 the curve was hyperbolic. The stimulation by Fru-1,6-P_2 disappeared at pH 5.9, and the same happened with the inhibition of pyruvate kinase activity by ATP. Our results with the purified erythrocyte pyruvate kinase closely

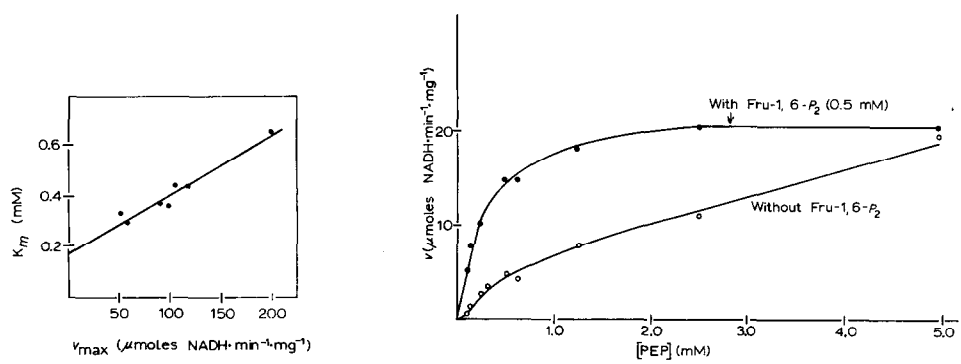

Fig. 5. The K_m *vs.* v_{max} plot. The K_m values are calculated from the Hill plots at various [ADP].

Fig. 6. The effect of Fru-1,6-P_2 on the activity of pyruvate kinase at pH 7.6. [ADP] = 2 mM. Buffer: 0.2 M Tris–maleate (pH 7.6). Further conditions as described in the text.

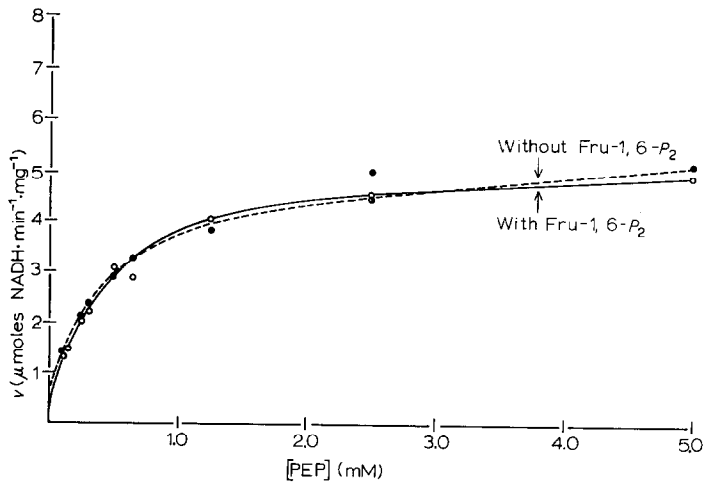

Fig. 7. The effect of Fru-1,6-P_2 on the activity of pyruvate kinase at pH 5.9. [ADP] = 2 mM. Buffer: 0.2 M Tris–maleate (pH 5.9).

resemble the results with the rat liver (L type). Fru-1,6-P_2 changed the kinetic behaviour from a sigmoid to a hyperbolic curve at pH 7.6 (Fig. 6), whereas at pH 5.9 the curves in the presence and absence of Fru-1,6-P_2 were identical (Fig. 7). At pH 7.6 the Hill coefficient n had a value of 1.4, while in the presence of Fru-1,6-P_2 this value decreased to 0.9. However, at pH 5.9 the Hill coefficient was not influenced by Fru-1,6-P_2 and had a value of 1.0. Fig. 8 shows that the inhibition of pyruvate kinase by ATP was pH dependent. At pH 8.2 a normal inhibition curve was obtained, while at pH 5.9 this was dramatically changed and showed a positive cooperativity. Similar results were obtained by ROZENGURT *et al.*[6] with rat-liver pyruvate kinase (type L).

With rat-liver pyruvate kinase (type L) P_i and the phosphorylated hexoses stimulate pyruvate kinase and reverse the ATP inhibition of the enzymic activities[18].

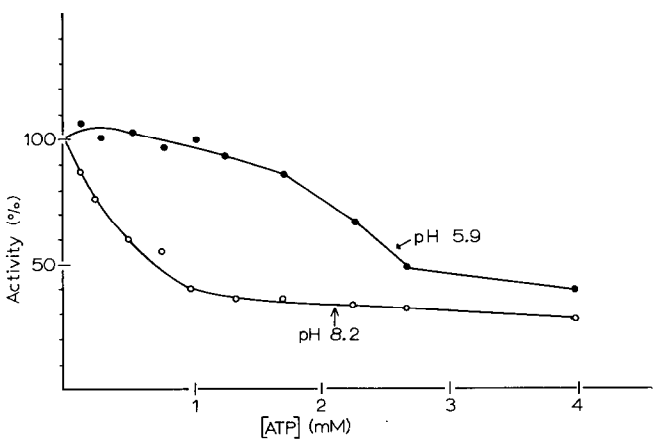

Fig. 8. The effect of ATP on the reaction of pyruvate kinase and the pH dependency. [PEP] = 0.5 mM; [ADP] — 0.3 mM, Buffer: 0.2 M Tris–maleate. ●—●, pH 5.9; ○—○, pH 8.2.

TABLE II

THE EFFECT OF PHOSPHORYLATED HEXOSES (0.5 mM) AND P_i (6.6 mM) ON THE ACTIVITY OF PYRUVATE KINASE IN 0.2 M TRIS–HCl BUFFER (pH 7.2) WITH PEP = 0.25 mM AND ADP = 0.5 mM

Addition	*Activity (%)*
Control	100
Glucose-1-*P*	100
Glucose-6-*P*	140
Galactose-1-*P*	100
Fructose-1-*P*	112
Fructose-6-*P*	112
Fructose-1,6-P_2	195
2,3-Diphosphoglycerate	123
P_i	173

Similar results were obtained with the erythrocyte pyruvate kinase (Table II). The most potent activator of the erythrocyte pyruvate kinase was Fru-1,6-P_2, followed by P_i and Glc-6-*P*. It is interesting that 2,3-diphosphoglycerate was able, at least to some extent (23%), to stimulate the pyruvate kinase activity. In pyruvate kinase deficiency the concentration of 2,3-diphosphoglycerate is increased[19]. The phosphorylated hexoses and P_i were able to overcome the inhibition by ATP, as shown in Fig. 9. In the presence of these compounds there was an increase in cooperativity. Not only these compounds, but also the substrate PEP, reversed the inhibition by ATP. At low concentration of PEP there was no cooperative effect, but at a concentration of 1 mM homotropic interaction of ATP occurred (Fig. 10).

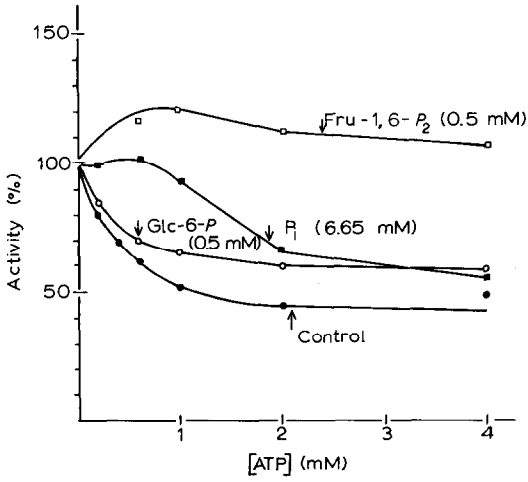

Fig. 9. The effect of P_i and phosphorylated hexoses on the inhibition of pyruvate kinase by ATP. The concentration of the phosphorylated hexoses and P_i are indicated in the figure. [PEP] = 0.25 mM; [ADP] = 0.5 mM. The 100% value is the activity measured in the presence of various additions and at [ATP] = 0 mM. Buffer: 0.2 M Tris–HCl (pH 7.2). Further conditions as described in the text.

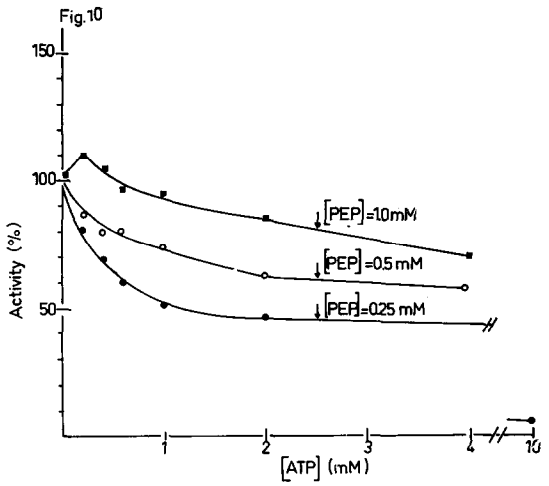

Fig. 10. The effect of ATP on the activity of pyruvate kinase at three different [PEP], as indicated in the figure. [ADP] = 0.5 mM. 100% value as in Fig. 9. Buffer: 0.2 M Tris–HCl (pH 7.2).

DISCUSSION

The best preparations of human erythrocyte pyruvate kinase described in the literature[1,2], appear to have a specific activity of 10–20 μmoles NADH $\cdot$ min^{-1} $\cdot$ mg^{-1}. Our preparations had a 10–20 times higher activity. This is mainly due to the use of high concentrations of glycerol to stabilize the enzyme, a procedure used by HUNSLEY AND SUELTER[3] for the yeast enzyme. Furthermore, the property of the enzyme to combine with blue dextran 2000, as discovered by HAECKEL *et al.*[4] for the yeast enzyme, was a great advance in the purification. The addition of ε-aminocaproic acid during the whole purification procedure to prevent hydrolysis of the enzyme is also important.

Determinations of the molecular weights of both types of liver pyruvate kinase by TANAKA *et al.*[5] give values of 208 000 and 250 000 for the L and M types, respectively. From the molecular weight as determined by us (205 000), together with the results of Fig. 2, it can be concluded that human erythrocytes contain one type of enzyme. TANAKA *et al.*[5] and BIGLEY *et al.*[10] also found one type of enzyme in rat erythrocytes, but BLUME *et al.*[13] and TOWNES[14], using the same method, found two bands and concluded that two forms of human erythrocyte pyruvate kinase are present. It is possible that these preparations are contaminated with leucocyte pyruvate kinase, which according to HANEL[20] has a much higher activity than the erythrocyte pyruvate kinase.

From kinetic studies[21] it is known that Fru-1,6-P_2 and PEP have a great influence on the activity. However, the addition of these compounds during starch-gel electrophoresis does not change the mobility of the protein.

From the results described in this paper it can be concluded that the human erythrocyte pyruvate kinase very much resembles the hepatic pyruvate kinase (type L). An important difference between these two enzymes is that the hepatic enzyme is under hormonal and dietary control, whereas the erythrocyte enzyme is not[5]. The erythrocyte pyruvate kinase belongs to the group of enzymes with allosteric proper-

ties, at least at physiological pH, properties that are affected by changes in the pH of the medium. Fru-1,6-P_2 behaves as an allosteric activator, whereas ATP is an allosteric inhibitor. The phosphorylated hexoses and P_i behave more or less like Fru-1,6-P_2.

To explain the kinetic behaviour of the regulatory enzymes, various models have been proposed[22–25]. Although the kinetic data are insufficient for distinguishing between the alternative models, in a qualitative way the results presented can be explained by the model of MONOD *et al.*[23] and RUBIN AND CHANGEUX[24]. With erythrocyte pyruvate kinase, in accord with rat-liver pyruvate kinase (type L), the substrate PEP and Fru-1,6-P_2 cooperate in shifting the equilibrium towards the R form, whereas the T form is favoured by ATP. From the results obtained it is concluded that the R form is catalytically more active than the T form. From the observations that the phosphorylated hexoses and P_i stimulate the pyruvate kinase activity and are able to reverse the ATP inhibition, it can be concluded that these compounds shift the equilibrium between the two forms to the R state. The results at low pH (5.9), at which Fru-1,6-P_2 does not change the shape of the saturation curve and ATP exhibits positive cooperativity, indicate that at this pH the enzyme exists in the R form. Conversely, at the higher pH, Fru-1,6-P_2 does change the saturation curve from sigmoid to hyperbolic, ATP gives an inhibition curve and the equilibrium between the two forms shifts to the T form. Although the data can be explained by the model of MONOD *et al.*[23] and RUBIN AND CHANGEUX[24] in a qualitative way, it is doubtful whether PEP, phosphorylated hexoses, P_i or ATP are bound preferentially to one of the two conformations. For instance the stimulatory effect of low [ATP] in the presence of Fru-1,6-P_2 (Fig. 9) cannot be explained by this model. ATP inhibition studies are complicated by the fact that this nucleotide is a reaction product. The ATP inhibition is biphasic, which is in contrast to the results with rat liver pyruvate kinase[6,18].

ROSE AND WARMS[26] have shown that the glycolysis of the erythrocyte is stimulated by P_i. They explained this result by counteraction of the inhibitors of the first two irreversible steps: Glc-6-*P* inhibition of hexokinase[27] and ATP inhibition of phosphofructokinase[21,28]. The necessary increase in activity of pyruvate kinase is explained by an increase in PEP concentration in the cell, which seems reasonable because pyruvate kinase is operating far below saturation[26]. From our results it is clear that P_i can stimulate the pyruvate kinase activity and is able to reverse the inhibition by ATP to some extent. It is reasonable to assume that *in vivo* probably a combination of stimulation and the increased PEP concentration results in an increased turnover number.

How far the stimulation of pyruvate kinase by 2,3-diphosphoglycerate is of physiological importance, is rather doubtful. However, with pyruvate kinase-deficient patients the concentration in the erythrocytes of this compound increases[19]. Studies are in progress on properties of the pyruvate kinase isolated from erythrocytes of enzyme-deficient patients.

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