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HUMAN ERYTHROCYTE PHOSPHOFRUCTOKINASE:
ITS PURIFICATION AND SOME PROPERTIES

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SUMMARY

1. Human erythrocyte phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was purified 15 000-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation, heating and column chromatography on Sepharose 6-B. The resulting enzyme preparation had a specific activity of 60 $\mu\text{moles Fru-1,6-}P_2$ formed per min per mg protein at 25 °C.

2. With cellulose-acetate electrophoresis only one band was observed after detection of the enzyme activity with the fluorescent technique.

3. Citrate and 2,3-diphosphoglycerate do not inhibit. The inhibition by ATP is pH dependent. Cyclic AMP is able to reverse the inhibition by ATP to some extent.

4. With GTP, ITP and UTP no inhibition is observed. At saturating concentrations of GTP, ATP still inhibits phosphofructokinase.

5. The variation of the activity of phosphofructokinase at various ATP and Fru-6-*P* concentrations was studied. In the reaction mechanism a ternary complex is involved.

INTRODUCTION

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the conversion of Fru-6-*P* to Fru-1,6-*P*₂. Control of glycolysis is usually explained in terms of the properties of three enzymes: hexokinase (EC 2.7.1.1), phosphofructokinase and pyruvate kinase (EC 2.7.1.40). Layzer *et al.*¹ demonstrated that phosphofructokinase from human muscle and erythrocytes may be distinguished electrophoretically, chromatographically and immunologically. In patients with muscle phosphofructokinase deficiency clinical features for non-spherocytic hemolytic anemia were demonstrated by Tarui *et al.*². It was found that erythrocyte phosphofructokinase activity was about half that of the normal controls, in contrast to the almost complete absence of muscle phosphofructokinase activity. In view of the control of glycolysis by phosphofructokinase and the reports on phosphofructokinase deficiency we started an investigation on normal erythrocyte phosphofructokinase.

MATERIALS AND METHODS

Chemicals

ATP, Fru-6-*P*, NADH, fructose diphosphate aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1), glycerol 1-phosphate dehydrogenase (EC 1.1.1.8), pyruvate kinase, glutathione reductase (EC 1.6.4.2), lactate dehydrogenase (EC 1.1.1.28) and phosphorylase *a* (EC 2.4.1.1) were obtained from Boehringer. All the other reagents used were of analytical grade. DEAE-cellulose (DE 11) from Whatman and Sepharose 6-B and Sephadex G-100 were purchased from Pharmacia, Uppsala. Concentration of the protein solutions was carried out with Diaflo ultrafiltration cells from Amicon with X M-50 membranes. Human erythrocytes were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands).

Determination of enzyme activity

Phosphofructokinase activities were measured by spectrophotometric assay at 25 °C by coupling the system with fructose diphosphate aldolase, triose phosphate isomerase and glycerol 1-phosphate dehydrogenase. The oxidation of NADH was followed spectrophotometrically at 340 nm. Phosphofructokinase activity was determined in 0.2 M Tris-HCl (pH 8.1) in a final volume of 3 ml containing 1 mM Fru-6-*P*, 2 mM ATP, 5 mM MgSO₄, 6 mM KCl, 0.05 ml dialyzed auxiliary enzyme solution (fructose diphosphate aldolase, 10 mg/ml; triose phosphate isomerase, 2 mg/ml; glycerol 1-phosphate dehydrogenase, 2 mg/ml), 0.25 mM NADH and enzyme. A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mole of Fru-1,6-*P*₂ per min at 25 °C. The specific activity is defined as units per mg of protein. The protein content was determined by the method of Lowry *et al.*⁴ using crystalline bovine serum albumin as a standard.

Electrophoresis

Cellulose-acetate electrophoresis was carried out with the Beckman microzone electrophoresis system. The buffer used was 0.1 M Tris-HCl, pH 8.2. Enzyme activity was detected according to the method of Layzer *et al.*¹.

RESULTS

Isolation of phosphofructokinase

The whole purification procedure was carried out at 4 °C; all the buffers used contained 0.01% Fru-6-*P*, 0.01% EDTA, and 0.01% β -mercaptoethanol.

(1) Erythrocytes were washed with 0.9% NaCl solution and made free of leukocytes. About 1 l of packed erythrocytes was mixed with an equal volume of water containing 4 g saponin. The mixture was stirred for 1 h.

(2) To the 2 l of haemolysate were added 4 l DEAE-cellulose suspension equilibrated with 0.005 M phosphate buffer (pH 7.0). The mixture of DEAE-cellulose and haemolysate was stirred for at least 1 h and then centrifuged. The DEAE-cellulose was repeatedly washed with 0.005 M phosphate buffer (pH 7.0) to remove the haemoglobin. About 40 l of buffer was used. The enzyme remained bound to the DEAE-

cellulose. Phosphofructokinase was eluted batch wise with 0.5 M phosphate buffer (pH 8.0).

(3) To the enzyme solution obtained in the previous step $(\text{NH}_4)_2\text{SO}_4$ (40% saturation) was added. The pH was adjusted to pH 8.0 with 50% KOH. After a few hours the precipitate was collected by filtration on Whatman No. 1 paper and the residue dissolved in 0.1 M phosphate buffer (pH 8.0).

(4) When necessary the pH of the enzyme solution was adjusted to pH 8.0 with KOH and β -mercaptoethanol added (1 mM). After being heated for 30 s at 60 °C, the enzyme fraction was quickly cooled to 4 °C and the precipitate removed by centrifugation (15 min at $10\,000 \times g$).

(5) The enzyme solution was concentrated to 5 ml as described under Materials and Methods and placed on a Sephadex G-100 column (3 cm \times 100 cm) equilibrated with 0.01 M phosphate buffer (pH 8.2). The enzyme was eluted with the void volume and concentrated to 5 ml as described under Materials and Methods.

(6) The enzyme solution, obtained in the previous steps, was placed on a DEAE-cellulose column (2 cm \times 10 cm), equilibrated with 0.005 M phosphate buffer (pH 7.0). The column was washed with 50 ml 0.005 M phosphate buffer (pH 7.0) and then with 30 ml 0.05 M phosphate buffer (pH 8.2). Under these conditions little phosphofructokinase, but much haemoproteins eluted. The enzyme was eluted with 50 ml 0.5 M phosphate buffer (pH 8.2).

(7) The enzyme solution from Step 6 was concentrated in the Amicon ultrafiltration cell to 3 ml and placed on a Sepharose 6B column (3.5 cm \times 50 cm), equilibrated with 0.1 M phosphate buffer (pH 8.1). The enzyme was eluted in two fractions: the first with a high specific activity, the second with a lower specific activity. The yield obtained with the first fraction is 10%. This yield can be increased by putting the second fraction again on a Sepharose 6B column. With this procedure the total yield amounted 20%.

The whole purification procedure is summarized in Table I.

TABLE I

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM HUMAN ERYTHROCYTES

Step of purification	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Cumulative purification (-fold)
1	2000	$310 \cdot 10^3$	1250	$4 \cdot 10^{-3}$	100	1
2	2900	5945	1190	$2 \cdot 10^{-1}$	95	50
3	156	2824	1134	$4 \cdot 10^{-1}$	90	100
4	140	1120	1060	$9.5 \cdot 10^{-1}$	85	240
5	80	360	690	1.9	55	480
6	50	157	626	4.0	50	1000
7	2	2.06	124	60	10	15000

pH optimum

The activity of phosphofructokinase has been studied in the pH range from 6.0 to 9.2. The optimum is at about pH 8.2 and is rather sharp. The physiological pH of the erythrocyte (intracellular) is 7.1–7.2 (ref. 3). In this pH range the activity of phosphofructokinase is only about 40% of the activity at pH 8.2.

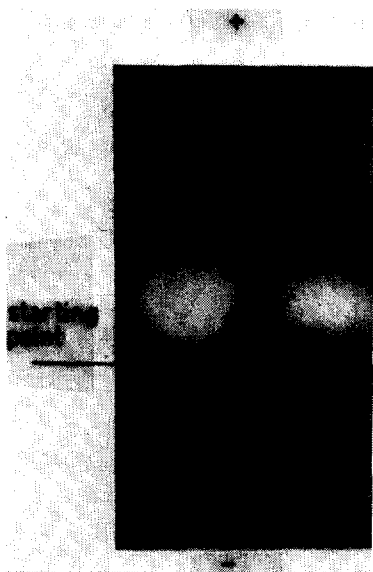


Fig. 1. Photograph of the cellulose-acetate electrophoresis for the purified enzyme. For conditions see Materials and Methods.

Electrophoresis

Electrophoresis with cellulose acetate at pH 8.2 shows, after detection of the enzyme with the fluorescent technique, a single band (Fig. 1). This was also found when the enzyme was investigated after the different steps of purification. These data suggest that human erythrocytes contain only one type of phosphofructokinase.

Influence of several ligands

The activity of phosphofructokinase from various sources is influenced by several ligands^{5,6}. The influence of ADP, cyclic AMP, AMP and P_i is pH dependent.

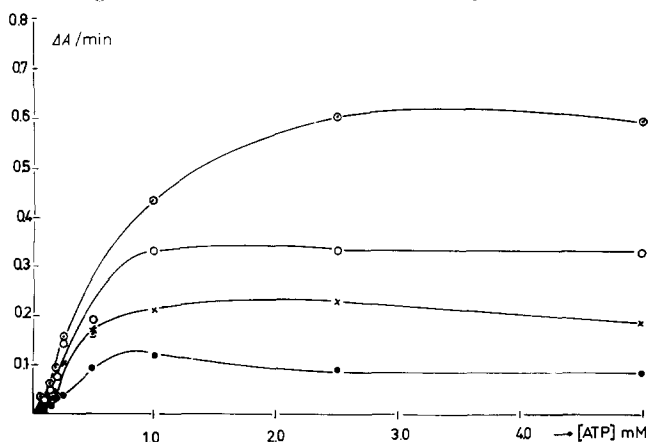


Fig. 2. The effect of ATP on the phosphofructokinase reaction at pH 8.1. Buffer 0.2 M Tris-HCl (pH 8.1), $[KCl] = 6 \text{ mM}$, $[Mg^{2+}] = 2 \times [ATP]$. $\bullet-\bullet$, 0.025 mM Fru-6-P; $\times-\times$, 0.05 mM Fru-6-P; $\circ-\circ$, 0.1 mM Fru-6-P; $\odot-\odot$, 0.5 mM Fru-6-P. Temp., 25 °C.

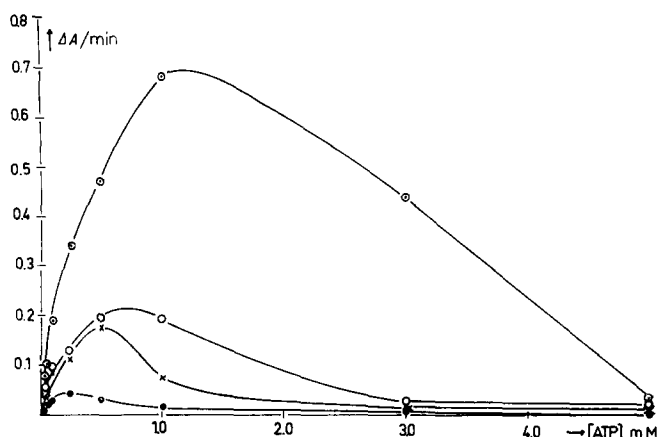


Fig. 3. The effect of ATP on the phosphofructokinase reaction at pH 7.1. Buffer 0.2 M Tris-HCl (pH 7.1), $[KCl] = 6$ mM, $[Mg^{2+}] = 2 \times [ATP]$. ●—●, 0.025 mM Fru-6-P; ×—×, 0.05 mM Fru-6-P; ○—○, 0.1 mM Fru-6-P; ○—○, 0.5 mM Fru-6-P. Temp., 25 °C.

At acid pH they activate the enzyme, but at alkaline pH this activation is much less.

Other ligands tested in the same way have little or no influence on phosphofructokinase activity; these include 2-phosphoglycerate, 3-phosphoglycerate, phosphoenolpyruvate, 2,3-diphosphoglycerate, Glc-1-P, 6-phosphogluconate and citrate. The fact that citrate and 2,3-diphosphoglycerate do not inhibit human erythrocyte phosphofructokinase, make the results different from those obtained with enzyme isolated from other sources^{6,7}.

Influence of ATP

Layzer *et al.*¹ reported that phosphofructokinase of erythrocytes is inhibited by high ATP concentrations at pH 8.0. We have investigated the ATP inhibition at two different pH values (pH 8.1 and pH 7.1). Fig. 2 shows the influence of ATP on phosphofructokinase activity at pH 8.1 at different Fru-6-P concentrations. Only at high

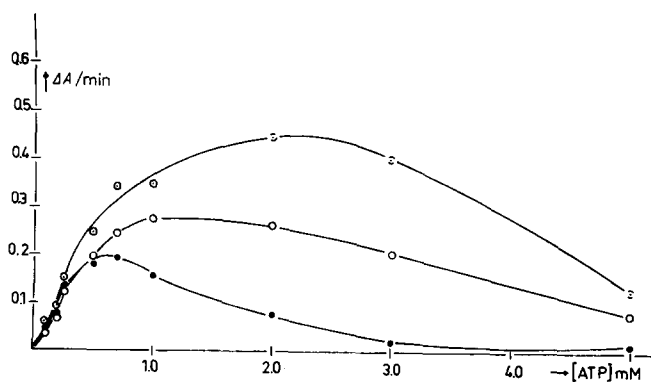


Fig. 4. The effect of cyclic AMP and AMP on the inhibition of phosphofructokinase by ATP. Buffer 0.2 M Tris-HCl (pH 7.1). Control: $[Fru-6-P] = 0.1$ mM, $[KCl] = 6$ mM, $[Mg^{2+}] = 2 \times [ATP]$. ●—●, control; ○—○, 0.5 mM AMP; ○—○, cyclic AMP. Temp., 25 °C.

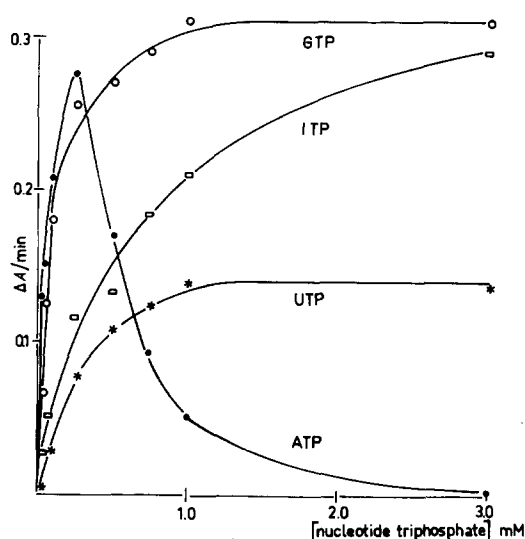


Fig. 5. The phosphofructokinase reaction with different types of nucleotide triphosphate at pH 7.2 at a Fru-6-P concentration of 0.1 mM. $[Mg^{2+}] = 3$ mM in excess of the nucleotide, $[KCl] = 6$ mM and 0.2 M Tris-HCl buffer. ●—●, ATP; ○—○, GTP; □—□, ITP; *—*, UTP.

ATP concentrations and at low Fru-6-P concentrations a small inhibition by ATP is observed. However, at pH 7.1 (Fig. 3) the inhibition by ATP is much more pronounced. The inhibitory concentration of ATP is shifted to higher values by increasing the concentration of Fru-6-P. However, cyclic AMP and AMP is able to overcome the inhibition by ATP to some extent, as shown in Fig. 4. This means that the presence of

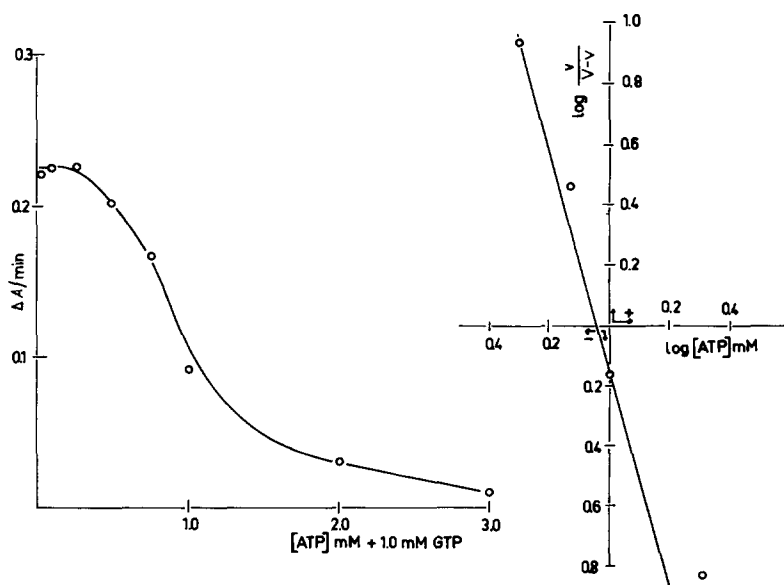


Fig. 6. The influence of ATP on the phosphofructokinase reaction in the presence of 1 mM GTP. Insert shows the Hill plot.

cyclic AMP rendered the enzyme less susceptible to inhibition by ATP. It is clear, that the ATP inhibition is strongly pH dependent and this is in contrast with the results reported by Layzer *et al.*¹. The difference in inhibition by ATP at different pH values indicates that there are different binding sites for ATP. This is further supported by investigating the influence of other nucleoside triphosphates such as GTP, UTP and ITP. Fig. 5 shows the influence of these nucleotides on the activity of phosphofructokinase at pH 7.2, compared to the influence of ATP. No inhibition at all is observed by GTP, ITP or UTP in contrast to the results obtained with ATP. The same result is obtained with CTP (not shown). It may be concluded therefore, that GTP, ITP and UTP are able only to react at the catalytic site but that the inhibitory site is highly

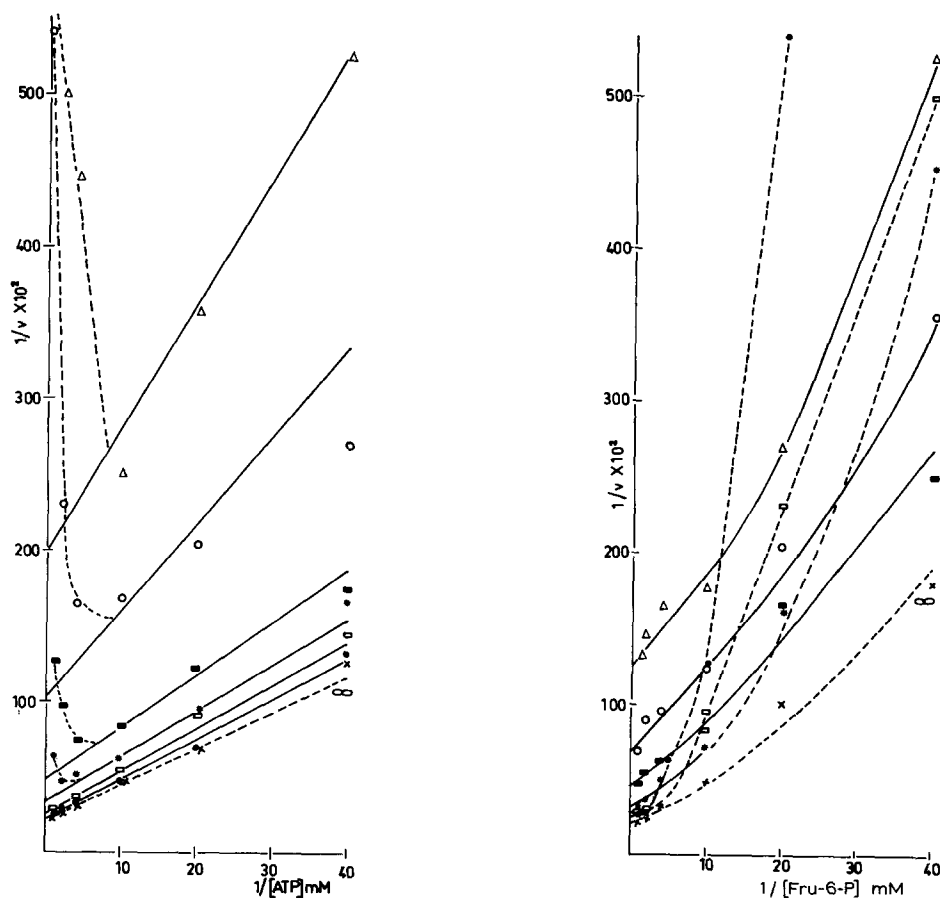


Fig. 7. The $1/v$ vs $1/[ATP]$ plot at pH 7.4 at various Fru-6-P concentrations. Further conditions: $[Mg^{2+}] = [ATP] + 3 \text{ mM}$, $[KCl] = 6 \text{ mM}$ and 0.2 M Tris-HCl buffer. Temp., 25°C . $\triangle-\triangle$, $[Fru-6-P] = 0.025 \text{ mM}$; $\circ-\circ$, $[Fru-6-P] = 0.05 \text{ mM}$; $\blacksquare-\blacksquare$, $[Fru-6-P] = 0.10 \text{ mM}$; $\square-\square$, $[Fru-6-P] = 0.25 \text{ mM}$; $\bullet-\bullet$, $[Fru-6-P] = 0.50 \text{ mM}$; $\times-\times$, $[Fru-6-P] = \infty$.

Fig. 8. The $1/v$ vs $1/[Fru-6-P]$ plot at pH 7.4 at various ATP concentrations. Further conditions see Fig. 7. $\triangle-\triangle$, $[ATP] = 0.025 \text{ mM}$; $\circ-\circ$, $[ATP] = 0.05 \text{ mM}$; $\blacksquare-\blacksquare$, $[ATP] = 0.10 \text{ mM}$; $\times-\times$, $[ATP] = 0.25 \text{ mM}$; $\square-\square$, $[ATP] = 0.50 \text{ mM}$; $\bullet-\bullet$, $[ATP] = 1.0 \text{ mM}$; $\times-\times$, $[ATP] = \infty$.

specific for ATP. Fig. 6 shows the activity of phosphofructokinase at a saturating concentration of GTP (1.0 mM) and at different ATP concentrations. The Hill plot derived from this figure shows an n value of 3.7 (insert of Fig. 6). The K_i for ATP calculated from the Hill plot is 0.1 mM. The plot of Fig. 6 shows that the inhibition by ATP starts at an ATP concentration of 0.3 mM. This makes it rather doubtful whether GTP only binds to the catalytic site and not to the regulatory site as has been concluded from Fig. 5.

Kinetics

Fig. 7 shows the Lineweaver-Burk plots at pH 7.4. The $1/v$ vs $1/[ATP]$ plot shows straight lines at different Fru-6-P concentrations; with the exception of a strong inhibition by ATP at low [Fru-6-P]. The $1/v$ vs $1/[Fru-6-P]$ plot (Fig. 8) shows concave upward curves, which are the reflection of the sigmoid character in the v vs [Fru-6-P]. The Hill plot at $[ATP] = \infty$ shows positive cooperativity ($n = 1.4$) with

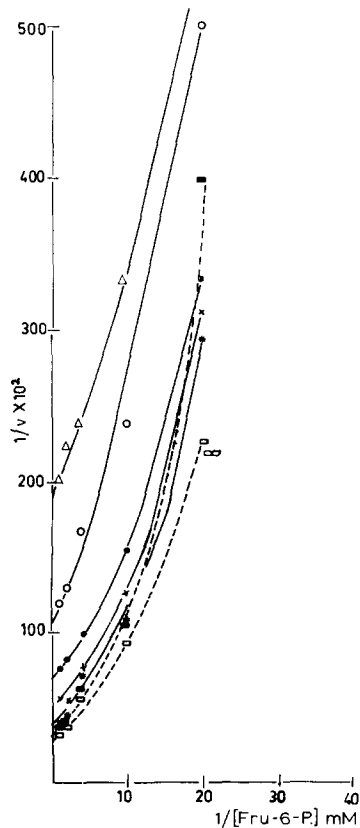
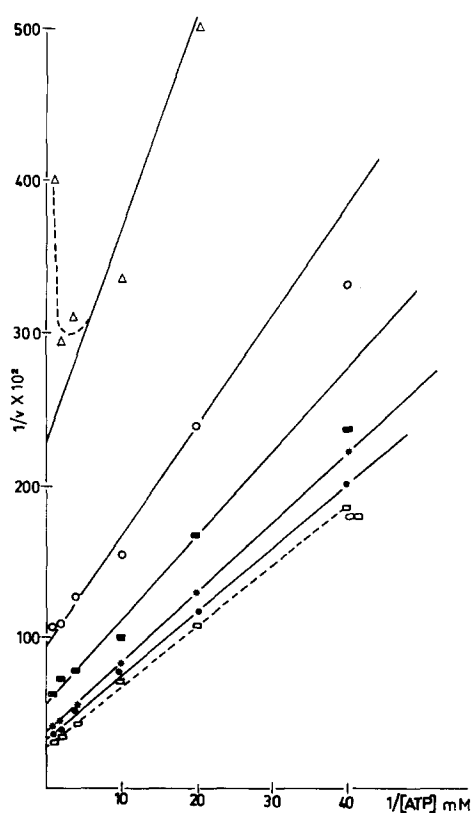


Fig. 9. The $1/v$ vs $1/[ATP]$ plot at pH 8.2 at various Fru-6-P concentrations. Further conditions see Fig. 7. $\triangle-\triangle$, [Fru-6-P] = 0.05 mM; $\circ-\circ$, [Fru-6-P] = 0.10 mM; $\blacksquare-\blacksquare$, [Fru-6-P] = 0.25 mM; $*-*$, [Fru-6-P] = 0.50 mM; $\bullet-\bullet$, [Fru-6-P] = 1.00 mM; $\square-\square$, [Fru-6-P] = ∞ .

Fig. 10. The $1/v$ vs $1/[Fru-6-P]$ plot at pH 8.2 at various ATP concentrations. Further conditions see Fig. 7. $\triangle-\triangle$, [ATP] = 0.025 mM; $\circ-\circ$, [ATP] = 0.05 mM; $\bullet-\bullet$, [ATP] = 0.10 mM; $\times-\times$, [ATP] = 0.25 mM; $*-*$, [ATP] = 0.50 mM; $\blacksquare-\blacksquare$, [ATP] = 1.0 mM; $\square-\square$, [ATP] = ∞ .

respect to Fru-6-P (not given in the figure). From these plots a K_m value for ATP, at $[\text{Fru-6-P}] = \infty$, of 0.12 mM was calculated; a similar value is obtained for the K_m for Fru-6-P at $[\text{ATP}] = \infty$ (0.12 mM). Figs 9 and 10 show the Lineweaver-Burk plots at pH 8.2. The $1/v$ vs $1/[\text{ATP}]$ plot (Fig. 9) shows straight lines and only little inhibition by ATP is observed, in contrast with the results at pH 7.4. Fig. 10 shows the $1/v$ vs $1/[\text{Fru-6-P}]$ plot, the curve is less concave than at pH 7.4. The K_m values calculated from these plots are the following: the K_m value for ATP, at $[\text{Fru-6-P}] = \infty$, is 0.15 mM and the K_m value for Fru-6-P, at $[\text{ATP}] = \infty$, 0.27 mM. The latter value is somewhat higher than that found at pH 7.4. It is possible to calculate the various apparent K_m values for ATP at the different Fru-6-P concentrations. By plotting the apparent K_m values for ATP against the respective V values Fig. 11 is obtained. In

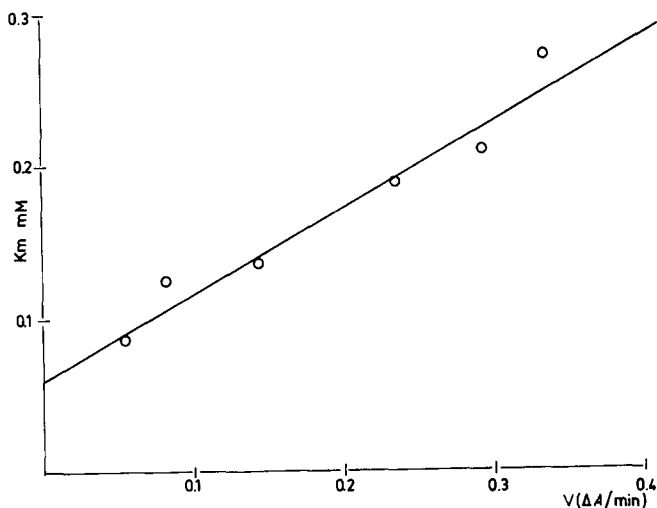


Fig. 11. The K_m vs V plot at pH 8.2. The K_m values are calculated from the Hill plots at various ATP concentrations.

this K_m vs V plot it is shown that the line does not pass through the origin, which means that in the reaction mechanism a ternary complex is involved^{8,9} (assuming that the phosphate donor is the first substrate). If it is assumed that Fru-6-P is the first substrate and the apparent K_m for Fru-6-P plotted against the V , the line also does not pass through the origin. Whatever the first substrate may be, a ternary complex is involved in the reaction mechanism, at both pH values. This is in contrast with the results obtained by Layzer *et al.*¹ The K_m values, at $[\text{Fru-6-P}] = \infty$, for GTP at pH 7.4 and pH 8.2 are in the same order as the K_m values for ATP.

DISCUSSION

Until now little attention has been directed towards human erythrocyte phosphofructokinase. The reason is probably the instability of the enzyme and the low activity in erythrocytes. Studies were carried out by Layzer *et al.*¹ with preparations with a specific activity of 8.8 units per mg protein. Our preparations have a 6 times

higher activity. This is mainly due to the use of Sepharose 6B in the purification method.

At pH 7.2 phosphofructokinase shows allosteric properties towards the substrate Fru-6-P ($n = 1.4$), while towards the substrate ATP straight lines (except for the substrate inhibition) in the Lineweaver-Burk plot are obtained. These data show that Fru-6-P is an activator of the reaction and that there are at least two binding sites. At pH 8.2 the allosteric properties have practically disappeared ($n = 1.2$). From the effect of GTP and ATP (Fig. 6), it can be concluded that GTP also binds to the inhibitory site of ATP. The kinetics with GTP, ATP, UTP, CTP and ITP show that the pyrimidine ring is already enough to cause binding at the catalytic site. GTP and ATP have a greater affinity for the enzyme than the other nucleotides. Probably the NH_2 group enhances the affinity for the enzyme. In the inhibitory site, ATP binds at a two-point unit, one with the phosphate group and the other with the adenosine group, whereas GTP can only bind with the phosphate group. The guanosine group cannot bind, probably due to steric hindrance. At pH 8.2, ATP no longer inhibits, this means that withdrawal of a proton blocks the binding of ATP at the inhibitory site. The obtained results can be explained with the model proposed by Lowry and Passonneau¹⁰. Our findings show that red blood cell phosphofructokinase is not inhibited by citrate; this is in contrast to the results obtained by Layzer *et al.*¹. The citrate inhibition is also reported for phosphofructokinase from other sources⁵. What the physiological meaning of this inhibition is, is rather obscure. The citrate synthesis occurs in the mitochondria and this intermediate can be transported out of the liver mitochondria by an exchange carrier mechanism. In heart mitochondria this exchange does not occur¹¹⁻¹³ and there citrate accumulates in a compartment different to the compartment where phosphofructokinase is localized. In liver, during fasting, when gluconeogenesis is active and glycolysis inactive the liver citrate concentration decreases^{14,15} and therefore citrate does not contribute to a slowing down of glycolysis at the phosphofructokinase level. In heart, during fasting, the mitochondrial citrate concentration increases, but this also cannot contribute to a slowing down of glucose metabolism. Here the lack of insulin may limit glucose utilization. Perhaps in liver the citrate inhibition of phosphofructokinase in the non-fasted state is necessary to regulate the rate of glycolysis in order to prevent an overflow of pyruvate into the citric acid cycle. In this context it may of interest to note that NADH does not inhibit phosphofructokinase^{16,17}.

Finally, the effect of cyclic AMP in overcoming the ATP inhibition of phosphofructokinase is also difficult to appreciate. During glycolysis the cyclic AMP concentration is rather low and is increased during gluconeogenesis^{18,19}, when glycolysis is slow.

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