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RABBIT RETICULOCYTE PHOSPHOFRUCTOKINASE

PURIFICATION AND SOME PROPERTIES

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SUMMARY

1. Rabbit reticulocyte phosphofructokinase (EC 2.7.1.11) was purified by $(NH_4)_2SO_4$ precipitation, heat treatment and chromatography on Sepharose 4B. The specific activity of the enzyme was close to 170 units/mg.

2. The comparison of reticulocyte phosphofructokinase and phosphofructokinase obtained from mature red cells has shown the differences in the protective action of AMP and ATP, and in the inhibitory effect of citrate and the excess of ATP on phosphofructokinase activity.

3. In reticulocyte hemolysate two activity bands were detected by means of polyacrylamide gel electrophoresis. In the mature red cell one of these activity bands was considerably diminished.

4. It can be postulated that by cell maturation some modifications of the phosphofructokinase molecule may take place.

INTRODUCTION

The young and mature red cell differ substantially in their metabolism. The developing red cell is capable of synthesing protein, nucleic acids, carbohydrate and lipids. To meet the energy requirement it has a highly active tricarboxylic acid cycle, oxidative phosphorylation and glycolysis. With the loss of the nucleus and mitochondria the mature cell has lost its ability to synthesize most of the compounds. The tricarboxylic acid cycle and oxidative phosphorylation are no longer functional. Only the carbohydrate metabolism through the Parnas–Embden–Meyerhof and pentose phosphate pathways still persist, although it is not so active as in young cells. The studies concerning levels and activities of glycolytic enzymes demonstrated significant changes taking place during cell maturation. Studying glutathione reductase (EC 1.6.4.2) Fornaini *et al.*[1] have found that some properties of the enzyme: its affinity for the substrate and coenzyme, heat stability and pH optimum were different

in young and mature red cells. Authors concluded that some modifications in the enzyme molecule are taking place during cell aging.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is believed to play an essential role in the regulation of glucose metabolism in the red cell. In this view it seemed possible that this enzyme may undergo some modification during the cell maturation, affecting the entire glycolytic activity of the erythrocyte.

It is the purpose of the present work to study the properties of phosphofructokinase obtained from reticulocytes and to compare them with those from mature erythrocytes.

MATERIALS AND METHODS

Reticulocytosis was induced in rabbits by means of phenylhydrazine injections. 40 mg of phenylhydrazine in 4 ml of saline were injected subcutaneously. The injection was repeated after 48 h. On the next day after the second injection, the blood was drawn by venipuncture. Total red cell count consisted of 98–100% of reticulocytes.

Purification of phosphofructokinase

The purification procedure was always carried out on freshly obtained blood and started immediately after its drawing. When the blood was collected from untreated animals for the preparation of mature erythrocyte enzyme, the top layer of red cells was discarded. The initial steps of the purification procedure: hemolysate preparation, DEAE-cellulose batch, $(NH_4)_2SO_4$ precipitation and heat treatment were performed according to Layzer *et al.*[2], except that the material was heated in the presence of 0.5 mM Fru-6-P instead of 1 mM AMP used in the original procedure. Fru-6-P was found to be the most active protective agent at 65 °C. The material was then applied to a column of Sepharose 4B, equilibrated with 0.1 M triethanolamine phosphate buffer (pH 8.0), containing 1 mM EDTA and 1 mM dithiotreitol. Elution was carried out with the same buffer, containing also 0.5 mM Fru-6-P. Phosphofructokinase was eluted as the single peak with the front of the solvent. The fractions with maximal phosphofructokinase activity were combined and the enzyme precipitated with solid $(NH_4)_2SO_4$ at 50% saturation. After standing overnight the precipitate was centrifuged and dissolved in 0.1 M triethanolamine phosphate buffer, containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM AMP.

Assay of the enzyme activity

The activity was measured by following the increase in absorbance at 340 nm in a mixture containing: 1 mM Fru-6-P, 0.5 mM ATP, 3 mM $MgSO_4$, 5 mM sodium arsenate, 1 mM NAD, 2 units of rabbit muscle aldolase, (EC 4.1.2.13), 10 units of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and 50 mM Tris–HCl buffer (pH 7.4) (ref. 3). The enzyme was first treated with Sephadex G-25 or dialysed against 50 mM Tris–HCl buffer, containing 1 mM EDTA and 1 mM dithiothreitol to remove $(NH_4)_2SO_4$, phosphate and AMP. Approximately the same amounts of enzyme (1–5 μg) were used in studies on erythrocyte and reticulocyte phosphofructokinase. One unit of enzyme activity is defined as the amount of the enzyme catalysing the phosphorylation of 1 μmole of Fru-6-P per minute.

Electrophoresis

Polyacrylamide disc gel electrophoresis of the enzyme was performed in 7.5% gel with Tris–glycine buffer (pH 8.4) according to Davis[4]. It was carried out at 4 °C, using 5 mA of current per gel for 2 h. Following electrophoresis, the activity of phosphofructokinase was located by incubating the gel at 37 °C for 1 h in a mixture containing 2 mM Fru-6-P, 2 mM ATP, 3 mM Mg^{2+}, 5 mM sodium arsenate, 1 mM NAD, 50 mM Tris–HCl buffer (pH 7.4), 1 unit of aldolase, 10 units of glyceraldehyde-3-phosphate dehydrogenase, 0.25 mg/ml phenazine methosulfate and 0.5 mg/ml nitrotetrazolium blue. Proteins were detected by staining with Amido black.

RESULTS

Activity

Results of representative erythrocyte and reticulocyte phosphofructokinase purification procedure are presented in Table I. The activity of phosphofructokinase

TABLE I

PURIFICATION OF THE RABBIT RED CELL PHOSPHOFRUCTOKINASE

	Activity					
	Erythrocyte			*Reticulocyte*		
	Units/mg	*Total*	*Yield, %*	*Units/mg*	*Total*	*Yield, %*
Hemolysate	0.006	840	100	0.012	620	100
DEAE-cellulose batch and $(NH_4)_2SO_4$ precipitation	4.1	588	72	6.2	470	75
Heat treatment	16.1	520	61	23.9	372	60
Sepharose 4B	115.2	324	38	165.0	254	41

measured in hemolysates from reticulocytes was higher than in erythrocytes in all preparations performed. Also the specific activity of purified reticulocyte enzyme, which amounted to 160–170 units/mg was higher than that of phosphofructokinase obtained from mature erythrocytes (110–120 units/mg).

Electrophoretic behavior

In polyacrylamide gel electrophoresis of the hemolysate and crude preparations of erythrocyte phosphofructokinase, 1 main activity band and traces of a second activity band were detected (see Fig. 1A). The purified enzyme does not enter the lower gel. These results are consistent with these obtained by Tarui *et al.*[5] in their electrophoretic, sedimentation and sucrose gradient centrifugation studies on the rabbit erythrocyte enzyme. The authors suggested that in erythrocytes the enzyme exists as a "monomer" molecule, which readily aggregates in purified preparation. As seen in Fig. 1B, in reticulocyte hemolysate, the second activity band is close in intensity to the band found as the main band in mature erythrocytes. In the purification procedure the aggregation of the reticulocyte enzyme probably also takes place, because the purified enzyme does not enter the lower gel.

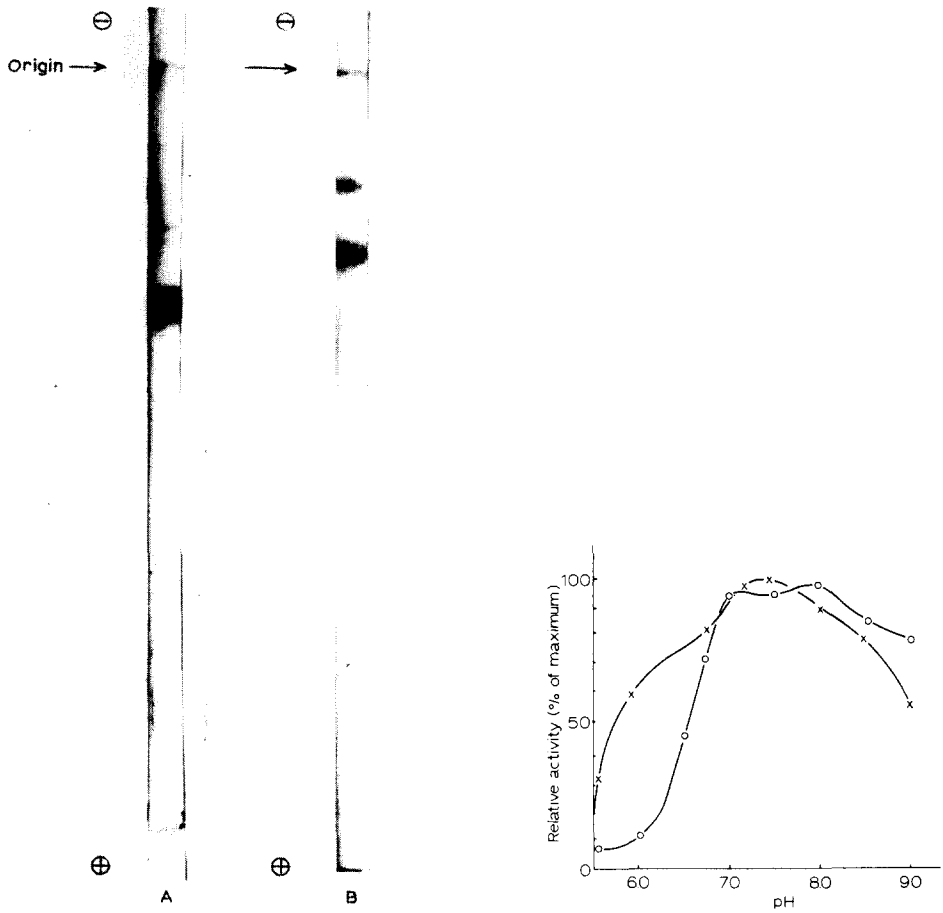

Fig. 1. Polyacrylamide gel electrophoresis of reticulocyte and mature red cell phsophofructokinase. For conditions see Materials and Methods. A: Mature erythrocyte hemolysate. B: Reticulodyte hemolysate. In A and B the activity of phosphofructokinase is visualized.

Fig. 2. Phosphofructokinase activity as a function of pH. Buffers: 50 mM veronal–acetate in the pH range 5.5–6.5 and 50 mM Tris–HCl in the pH range 7.0–9.0. For other conditions see Materials and Methods. ×—×, erythrocyte phosphofructokinase; ○—○, reticulocyte phosphofructokinase.

Stability

The purified reticulocyte enzyme, stored at 4 °C in the triethanolamine phosphate buffer (pH 8.0), containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM AMP, at protein concentrations above 5 mg/ml is stable for 2–3 weeks, similar to the erythrocyte enzyme[5]. At such protein concentrations and with addition of 0.5 mM Fru-6-P, the enzyme is thermostabile and can be heated at 65 °C for 15 min without activity loss.

In more diluted solutions at the protein concentration less than 1 mg/ml the activity is completely lost after 12 h at 4 °C, even in the presence of protective factors, such as dithiothreitol, AMP, ATP or Fru-6-P. The diluted enzyme is also more labile to heat treatment. The enzyme preincubated for 10 min at 50 °C lost 50%, at 60 °C

—84%, and at 65 °C—100% of its initial activity. In reticulocyte enzyme, ATP does not serve as a protective agent in heat treatment, in contrast to the mature erythrocyte phosphofructokinase. AMP protected both enzymes at heating up to 60 °C, at 65 °C it showed no effect. At this temperature, Fru-6-P was found to be the best protective agent for both the erythrocyte and reticulocyte phosphofructokinase.

pH curve

The activity of both erythrocyte and reticulocyte phosphofructokinase has been studied in the pH range from 5.5 to 9.0 (see Fig. 2). For activity calculations, only the time of reaction, where activity was linear with time has been taken into consideration. The pH curve of the reticulocyte enzyme is characterized by a broad maximum ranging from pH 7.0 to 8.0. The enzyme loses its activity completely below pH 6.0. The activity of the reticulocyte enzyme, incubated at pH 5.5 for 15 min could not be reversed in higher pH neither in the absence nor in the presence of ATP. The mature erythrocyte enzyme is less susceptible to inhibition at acid pH. When after exposure to pH 5.5 the pH of the enzyme solution was adjusted to pH 8.0, partial restoration of the activity took place. This restoration was enhanced by preincubation with 1 mM ATP.

Effect of metabolites

5 mM glucose 6-phosphate, 5 mM 2-phosphoglycerate, 5 mM 3-phosphoglycerate and 5 mM pyruvate have had no effect on the activity of reticulocyte phosphofructokinase at pH 7.4. 5 mM fructose 1,6-diphosphate inhibited, at this pH, the enzyme to 50%, and 5 mM ADP and 5 mM phosphoenolpyruvate to 70% of the initial activity. This was valid at various Fru-6-P concentrations. One mM AMP, 5 mM phosphate and 5 mM NH_4Cl inhibited the enzyme at pH 7.4 at 0.25 mM Fru-6-P concentration. At 0.5 mM and 1 mM Fru-6-P concentration, the activity was not influenced by phosphate and AMP. NH_4^+ showed some activating effect, but only at saturating Fru-6-P concentrations. Similar effects were observed with rabbit erythrocyte enzyme.

Effect of urea and high salt concentration

The activity of both reticulocyte and erythrocyte enzyme dropped to 50% at 2 M, and dropped fully at 4 M urea concentration. The reticulocyte enzyme is more susceptible to the inhibition by high NaCl concentration than the erythrocyte enzyme, as shown in Fig. 3.

Inhibition by ATP

At pH 7.4, both the reticulocyte and erythrocyte phosphofructokinase was inhibited by high ATP concentration (see Fig. 4). This inhibition is dependent on the Fru-6-P concentration. It is less pronounced at higher Fru-6-phosphate concentrations.

It is known that the inhibition of phosphofructokinase obtained from various mammalian tissues, including human erythrocytes can be reversed by AMP and inorganic phosphate[6–8]. This is not the case with the rabbit mature erythrocyte enzyme. Only at an ATP concentration above 15 mM, is the enzyme partly protected by 1 mM AMP and 5 mM P_i added simultaneously. On the contrary, with the reti-

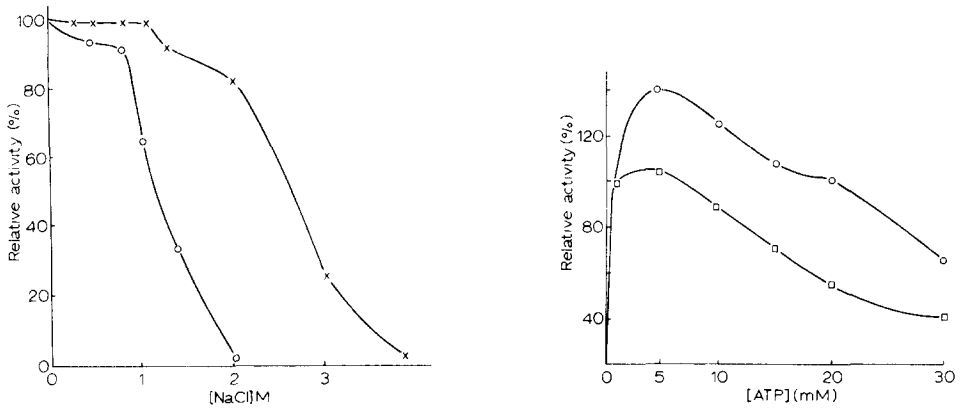

Fig. 3. The effect of NaCl on the activity of phosphofructokinase. For assay see Materials and Methods. The data are expressed as a percentage of the activity measured in the absence of NaCl, at ionic strength $I = 0.17$. ×—×, erythrocyte phosphofructokinase; ○—○, reticulocyte phosphofructokinase.

Fig. 4. The effect of ATP on the activity of reticulocyte phopshofructokinase at pH 7.4. Buffer: 50 mM Tris–HCl (pH 7.4). The data are expressed as a percentage of the activity measured at 1 mM ATP concentration. □—□, [Fru-6-P] = 0.5 mM; ○—○, [Fru-6-P] = 1 mM.

culocyte phosphofructokinase, 1 mM AMP was able to overcome the inhibition at lower ATP inhibitory concentrations, and had no effect when larger amounts of ATP were added (see Fig. 5). Also inorganic phosphate showed some protective effect on the reticulocyte enzyme.

At pH 8.0 the mature erythrocyte phosphofructokinase was not inhibited by ATP. As shown in Fig. 6, the inhibitory effect of ATP on reticulocyte phosphofructokinase was apparent at this pH. The inhibitory concentration of ATP was shifted to lower values by decreasing the concentration of Fru-6-P.

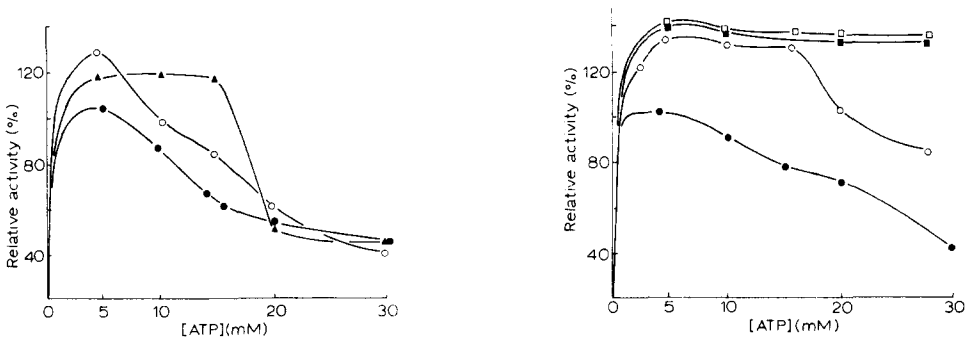

Fig. 5. The effect of AMP and inorganic phosphate on the inhibition of reticulocyte phosphofructokinase by ATP at pH 7.4. Buffer: 50 mM Tris–HCl (pH 7.4). [Fru-6-P] = 0.5 mM. The data are expressed as percentage of the activity measured at 1 mM ATP concentration. ●—●, control; ▲—▲, 1 mM AMP; ○—○, 5 mM P_i.

Fig. 6. The effect of ATP on the phosphofructokinase activity at pH 8.0. Buffer: 50 mM Tris–HCl, (pH 8.0). The data are expressed as a percentage of the activity measured at 1 mM ATP concentration. □—□, erythrocyte phosphofructokinase, [Fru-6-P] = 1 mM; ■—■, erythrocyte phosphofructokinase, [Fru-6-P] = 0.5 mM; ○—○, reticulocyte phosphofructokinase, [Fru-6-P] = 1 mM; ●—●, reticulocyte phosphofructokinase, Fru-6-P = 0.5 mM.

Inhibition by citrate

Unlike the mature erythrocyte enzyme, reticulocyte phosphofructokinase was inhibited by citrate (Fig. 7). This phenomenon was dependent on Fru-6-P concentration. The reticulocyte enzyme was more susceptible to the citrate inhibition at low Fru-6-P concentration.

Kinetic studies

The Michaelis constants for reticulocyte and mature erythrocyte phosphofructokinase were determined at pH 7.4. Only noninhibitory concentrations of ATP were used. When concentrations of one substrate were varied against several fixed concentrations of the other substrate, $1/v$ versus $1/s$ plots were linear and gave a series of paralell lines (see Fig. 8). As the concentration of ATP was raised, the apparent V and apparent K_m for Fru-6-P increased; an increase in Fru-6-P resulted in higher apparent V and apparent K_m for ATP.

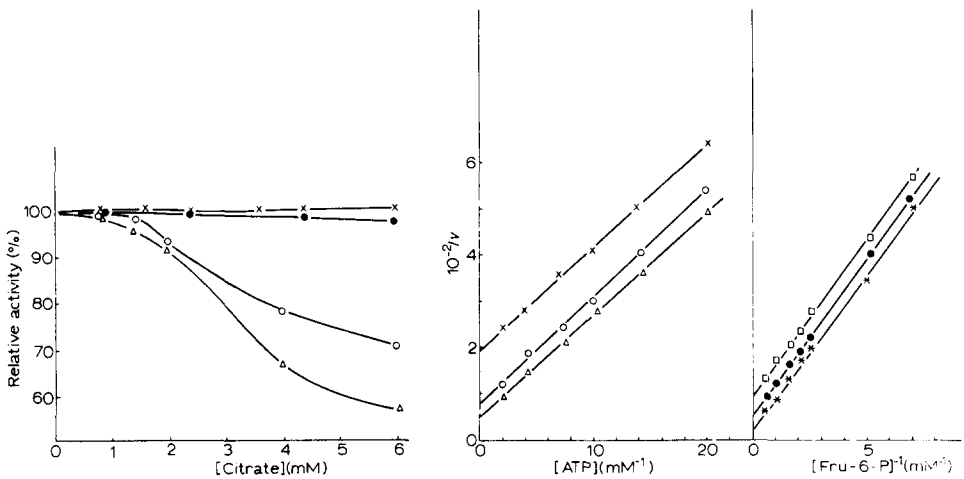

Fig. 7. The effect of citrate on the activity of phosphofructokinase. Buffer: 50 mM Tris–HCl (pH 7.4). The data are expressed as a percentage of the activity measured in the absence of citrate. ×—×, erythrocyte phosphofructokinase, [Fru-6-P] = 1 mM; ●—●, erythrocyte phosphofructokinase, [Fru-6-P] = 0.5 mM; ○—○, reticulocyte phosphofructokinase, [Fru-6-P] = 1 mM; △—△, reticulocyte phosphofructokinase, [Fru-6-P] = 0.5 mM.

Fig. 8. Reticulocyte phosphofructokinase activity as a functon of Fru-6-P and ATP concentration. The reciprocal of the velocity is plotted on the ordinate, the reciprocal of substrate concentration on the abscissa. Buffer: 50 mM Tris–HCl (pH 7.4). ×—×, [Fru-6-P] = 0.25 mM; ○—○, [Fru-6-P] = 0.5 mM; △—△, [Fru-6-P] = 1 mM; □—□, [ATP] = 0.25 mM; ●—●, [ATP] = 0.5 mM; *—*, [ATP] = 1 mM.

K_m values for ATP for the reticulocyte enzyme were found to be 0.13 mM at 0.25 mM Fru-6-P; 0.29 mM at 0.5 mM Fru-6-P, and 0.5 mM at 1 mM Fru-6-P. The values found in a similar experiment, performed on the same amount of erythrocyte enzyme, were somewhat higher, being 0.3 mM, 0.5 mM and 0.8 mM, respectively. K_m values for Fru-6-P were similar in reticulocyte and erythrocyte enzyme; 0.6 mM at 0.25 mM ATP; 0.95 mM at 0.5 mM ATP and 1.3 mM at 1 mM ATP.

DISCUSSION

Results presented in this paper indicate that phosphofructokinase obtained from young red cells differs in some of its properties from the mature erythrocyte enzyme. The activity of the reticulocyte phosphofructokinase is higher than that of erythrocytes as well as in hemolysate after purification.

In polyacrylamide gel electrophoresis the reticulocyte enzyme showed two main activity bands in the hemolysate. In the mature erythrocyte hemolysate only one main activity band was found, with traces of the other band. On the basis of this study it can not be said whether the activity bands found in reticulocyte hemolysate represent various isoenzymic forms or molecules with different degrees of aggregation. The purified enzyme, obtained both from reticulocytes and erythrocytes did not enter the gel, probably due to aggregation of the molecules[5].

There are no significant differences in the stability of the enzyme from both sources in storage at 4 °C and with heat treatment. The reticulocyte enzyme is more susceptible to inactivation by acid pH and by high salt concentration.

The affinity for Fru-6-P was equal for the reticulocyte and erythrocyte enzymes. Fru-6-P was found to protect both enzymes with heat treatment and at high ATP concentration.

The differences were observed in the effect of ATP with respect to its action as a protective agent, substrate, and allosteric effector. The addition of ATP overcomes the inactivation by heat treatment and by acid pH of the erythrocyte, but not reticulocyte enzyme. The affinity for ATP of the reticulocyte enzyme is higher than that of erythrocyte phosphofructokinase, although the difference is not large.

At pH 7.4 the enzyme from both sources is inhibited by ATP (ref. 5), but in young cells, unlike in the mature ones, this inhibition can be reversed by AMP. We have found that the reticulocyte phosphofructokinase is inhibited by excess of ATP, also at pH 8.0. At this pH ATP has no inhibitory effect on the enzyme derived from mature rabbit erythrocytes, as well as from human red cells[6].

The mature erythrocyte phosphofructokinase was not influenced by citrate. These results are consistent with those of Staal *et al.*[6] on human red cell phosphofructokinase, but not with Layzer *et al.*[2]. The reticulocyte enzyme was inhibited by citrate, although this inhibition was less pronounced than in the enzyme from other sources[8–10].

The differences in ATP, citrate and AMP effect on the phosphofructokinase activity in young and mature red cells may be of interest in respect to the regulation of the enzyme, and with it of the entire glucose metabolism.

The data presented in this paper support the suggestion that by the aging of red cells some enzyme modifications can probably take place[1]. Further investigations are needed to elucidate the nature of these changes. They may concern the conformational state of the enzyme or its association-dissociation state. One of the characteristic features of red cell phosphofructokinase is its tendency to aggregate[5,11,12]. The degree of aggregation is dependent on pH, substrates and effectors concentration, and these factors change with the cell maturation. Also the appearance of another phosphofructokinase isoenzyme in young red cells cannot be excluded.

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