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PURIFICATION AND SOME PROPERTIES OF PHOSPHOFRUCTOKINASE FROM EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from Ehrlich ascites tumor cells has been purified about 150-fold with a 30% yield. The purified enzyme migrates as a single band when subjected to disc electrophoresis on polyacrylamide gel and has an average specific activity of 150 units per mg of protein.

2. The molecular weight of the purified enzyme has been estimated to be $3.0 \cdot 10^5$ by gel filtration. Optimum pH is in the vicinity of 7.1. In contrast to most enzymes from other sources, K^+ is strongly inhibitory at higher concentrations despite being essential for the enzymic activity.

3. This enzyme is chromatographically distinguishable from phosphofructokinases of other mouse organs such as muscle and erytrhocytes.

INTRODUCTION

Phosphofructokinases (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from a variety of biological sources exhibit highly complicated allosteric properties which are generally believed to reflect their important roles as a key enzyme in controlling glycolytic activities in these cells. Whereas extensive studies have been made of phosphofructokinases purified from mammalian tissues with high glycolytic rates such as skeletal muscle[1–5] and heart[6–9], essentially no attempt has been made so far to purify the enzyme from ascites tumor cells, which are also characterized by high glycolytic activities (see ref. 10 for 30-fold purification). As a result, no information is available as to the physicochemical properties or the detailed kinetic characteristics of phosphofructokinase from tumor cells. Our preliminary experiments showed that glycolytic rates of Ehrlich ascites tumor cells were not essentially affected by changing the pH of the incubation medium bathing the cells, in sharp contrast to the muscle glycolytic system which had been shown to be pH dependent[11]. Since evidence was previously presented that the pH-dependent regulation of muscle glycolysis resulted solely from a kinetic property characteristic of muscle phospho-

fructokinase[11,12], it is highly probable that the kinetic behaviour of phosphofructokinase from Ehrlich ascites tumor cells is somewhat distinct from those of the muscle enzyme.

In the present study, we have attempted to purify phosphofructokinase from mouse Ehrlich ascites tumor cells. Several properties unique to the tumor enzyme are also presented. Detailed analysis of kinetic data along with a proposal of a reaction model useful for elucidating the kinetic patterns will be the subject of the accompanying paper[13].

MATERIALS AND METHODS

Chemicals

Disodium salts of ATP, fructose 6-phosphate (Fru-6-*P*) and NADH, Tris, imidazole, aldolase, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Company. The following marker proteins of known molecular weights were purchased from Mann Reseach Laboratory; myoglobin, albumin (bovine), γ-globulin (human) and apo-ferritin (horse). DEAE-Sephadex A-50 and Sephadex G-200 were obtained from Pharmacia. Bovine serum albumin was from Armour Pharmaceutical Company. All other chemicals used were analytical grade reagents from commercial sources.

Enzyme assay

At each step of the purification procedure, phosphofructokinase activity was measured in a coupled assay system by following the decrease of absorbance at 340 nm due to the oxidation of NADH in Gilford Model 2400 recording spectrophotometer. The reaction was initiated by adding phosphofructokinase in an amount to give a change of absorbance less than 0.05 absorbance unit per min to the reaction mixture containing 0.5 mM ATP, 1.0 mM Fru-6-*P*, 1.5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 10 mM mercaptoethanol, 0.05 mM NADH, 1.0 unit of aldolase, 0.8 unit of triosephosphate isomerase, 0.4 unit of α-glycerophosphate dehydrogenase and 50 mM Tris–HCl (pH 7.5) in a total volume of 3.0 ml at 28 °C. One unit of phosphofructokinase activity was defined as the amount of enzyme which catalyzed the formation of 1 μmole of fructose 1,6-diphosphate (Fru-1,6-P_2) per min under the above conditions.

Kinetics of the purified enzyme were analyzed by incubating the dialyzed enzyme solution dissolved in 0.5% bovine serum albumin under the same conditions as above but with the omission of $(NH_4)_2SO_4$ from the reaction mixture containing 50 mM imidazole–HCl (pH 7.5) instead of Tris–HCl. Auxiliary enzymes were also made free from $(NH_4)_2SO_4$ by dialyzing in 0.5% albumin overnight against a large volume of 50 mM Tris buffer, pH 8.0.

Other analytical procedures

The method of Warburg and Christian[14] was used for the determination of protein except in the case of column effluents which were analyzed by measuring the absorbance at 280 nm, an extinction coefficient of unity being assumed for 1 mg/ml. Polyacrylamide gel disc electrophoresis was performed as described by Ornstein[15]

and Davis[16]. Estimation of molecular weight was carried out on a Sephadex G-200 column by the method of Andrews[17].

RESULTS

Purification

Ehrlich diploid ascites tumor cells were harvested 7–8 days after inoculation in albino mice (SWJ/mk). Cells from ascitic fluids of 50 mice were combined and washed once by suspending and centrifuging in 500 ml of ice-cold 0.9% NaCl. All manipulations were performed at 4 °C. The analytical details of a typical purification of phosphofructokinase from approx. 100 ml of packed cells are shown in Table I.

TABLE I

SUMMARY OF PURIFICATION OF EHRLICH ASCITES TUMOR PHOSPHOFRUCTOKINASE

Fractions	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Purification*	*Yield*
Crude extract	3620	3800	1.0	1	100
35–65% $(NH_4)_2SO_4$	3160	803	3.9	4	87
DEAE-Sephadex	1160	15	80	80	32
Sephadex G-200	1030	7	150	150	29

Step 1: Preparation of crude extract. The once-washed packed cells, approx. 100 ml, were suspended in 500 ml of 0.1 M sodium and potassium phosphate buffer (pH 8.0) containing 1 mM EDTA (this solution is henceforth referred to as Buffer A) and centrifuged at 1600 × *g* for 10 min. The precipitated cells were then disrupted by grinding with 100 g of sea sand and 30 ml of Buffer A in a mortar. The mixture, suspended in 500 ml of Buffer A, was centrifuged at 4500 × *g* for 30 min and the precipitate was re-extracted with 200 ml of Buffer A followed by centrifugation. The volume of the combined extract was about 800 ml.

Step 2: Fractionation with $(NH_4)_2SO_4$. To the crude extract from Step 1, fine powder of $(NH_4)_2SO_4$ was added slowly with stirring to give a final saturation of 35%, and the solution, brought to pH 7.5 with 2.0 M NaOH, was allowed to stand for 4–6 h. The precipitate was removed by centrifugation at 13 000 × *g* for 30 min and discarded. Additional solid $(NH_4)_2SO_4$ in an amount sufficient to achieve 65% saturation was added to the supernatant, which was then readjusted to pH 7.5 and kept for 4–6 h before the precipitate containing phosphofructokinase activity was collected by centrifugation at 13 000 × *g* for 30 min.

Step 3: DEAE-Sephadex A-50 column chromatography. The precipitate obtained from Step 2 was dissolved in 50 ml of 20 mM phosphate buffer (pH 7.8) containing 80 mM $(NH_4)_2SO_4$, 25 mM mercaptoethanol and 1 mM EDTA (Buffer B) and was dialyzed for 12 h against 2 l of the same buffer with change of buffer after 6 h. The dialyzed enzyme solution was clarified by centrifugation at 12 000 × *g* for 30 min and was applied to a column (3 cm × 25 cm) of DEAE-Sephadex A-50 which had been equilibrated with Buffer B. The column was first washed with the same buffer

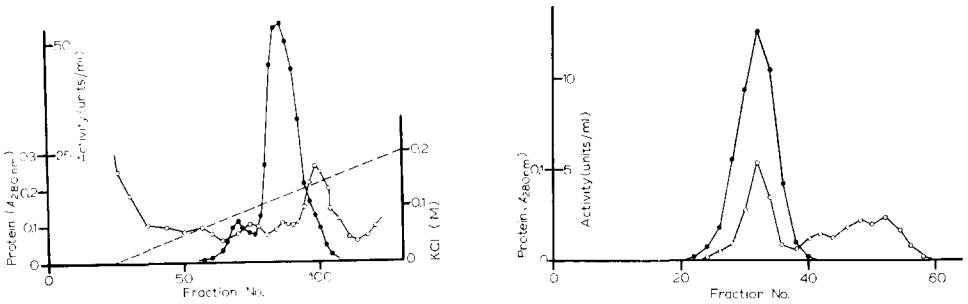
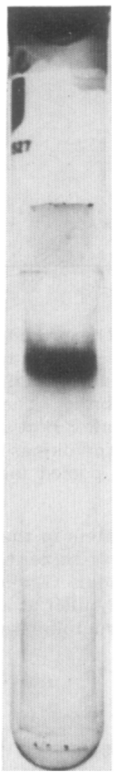


Fig. 1. Elution pattern of phosphofructokinase on DEAE-Sephadex A-50 column. The protein solution prepared from the $(NH_4)_2SO_4$ precipitate as described in the text was applied to a column of DEAE-Sephadex A-50 (25 cm × 3 cm) equilibrated with Buffer B. The unadsorbed protein having no phosphofructokinase activity was washed away with 400 ml of the same buffer. The adsorbed protein was then eluted with a linear gradient at pH 7.8 of 0–0.2 M KCl in Buffer B at a flow rate of 60 ml/h. The 20-ml fractions were collected and assayed for phosphofructokinase activity (●——●) and for absorbance at 280 nm (○——○). Fractions 80–90 were pooled for further purification.

Fig. 2. Elution pattern of phosphofructokinase on a Sephadex G-200 column. The protein in the eluate from DEAE-Sephadex A-50 column was once precipitated and dissolved in 4 ml of Buffer C as described in text. This protein solution was applied to a column of Sephadex G-200 (95 cm × 3 cm) equilibrated with Buffer C. The protein was then eluted with the same buffer at a flow rate of 15 ml/h. The 10-ml fractions were collected for assay of phosphofructokinase activity (●——●) and for absorbance at 280 nm (○——○).

(approx. 400 ml) until unadsorbed protein having no phosphofructokinase activity was washed away. Elution of the enzyme was then achieved with a linear gradient from 0 to 0.2 M KCl in Buffer B at a flow rate of 60 ml/h. As shown in Fig. 1, phosphofructokinase activity was eluted giving two peaks of activity with the bulk of the activity emerging in the second peak. Further purification was performed on the pooled eluate between Fractions 80–90 (215 ml) corresponding to the second peak. The active protein was precipitated by being dialyzed overnight against a dialysis solution prepared by adding 1100 g of $(NH_4)_2SO_4$ to 1 l of 0.1 M phosphate buffer (pH 7.8) containing 1 mM EDTA. $(NH_4)_2SO_4$ remaining undissolved under saturated solution when prepared was dissolved during dialysis.

Step 4: Fractionation on Sephadex G-200 column. The precipitate from Step 3 was collected by centrifugation at 25 000 × *g* for 30 min and dissolved in 4 ml of 0.1 M phosphate buffer (pH 7.8) containing 0.5 M $(NH_4)_2SO_4$, 25 mM mercaptoethanol and 1 mM EDTA (Buffer C). The enzyme solution was applied to a Sephadex G-200 column (3 cm × 95 cm) which had been equilibrated with Buffer C. Upon being eluted with the same buffer, phosphofructokinase activity emerged as the first of two protein boundaries as shown in Fig. 2. Fractions containing phosphofructokinase activity (Fractions 26–36, 110 ml) were combined and dialyzed overnight against 1 l of 80% saturated $(NH_4)_2SO_4$ in 0.1 M phosphate buffer (pH 7.8) containing 0.1 mM ATP and 1 mM EDTA to precipitate the enzyme. The volume of the solution was reduced to about one-third during dialysis, and the resultant enzyme suspension was stored as such at 3 °C without loss of activity for at least 1 month. The presence of ATP was essential; approx. 50% of the activity was lost after 1 month if ATP was omitted from the stock solution.



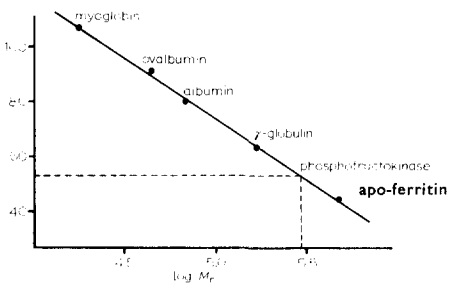


Fig. 3. Disc electrophoresis of purified phosphofructokinase. The purified enzyme (70 μg) was subjected to electrophoresis on 3.8% polyacrylamide gel containing 0.14 M Tris–HCl, pH 7.5. A constant current of 3 mA per column was applied for 80 min at 3–4 °C. Fixation and staining of protein were performed in a solution of 1% Amido Black 10 B in 7% acetic acid.

Fig. 4. Estimation of the molecular weight of phosphofructokinase by Sephadex G-200 column chromatography. Purified phosphofructokinase (100 units) and 5 mg each of non-enzymic marker proteins (myoglobin, ovalbumin, bovine serum albumin, γ-globulin and apo-ferritin) were dissolved in 3 ml of Buffer C. This protein solution was applied to a Sephadex G-200 column (2.6 cm × 100 cm) equilibrated with Buffer C and then eluted with the same buffer at a flow rate of 5 ml/h. Fractions of 4 ml were collected and assayed for the enzymic activity and for absorbance at 280 nm.

Properties of the purified enzyme

Electrophoresis. Purified phosphofructokinase migrates as a single band without noticeable contamination when subjected to disc electrophoresis in a polyacrylamide gel at pH 7.5 (Fig. 3).

Molecular weight. Commercial preparations of myoglobin, bovine serum albumin, γ-globulin and apo-ferritin, each about 3 mg, were mixed with 100 units of purified phosphofructokinase in Buffer C. The protein mixture was applied to a column of Sephadex G-200 equilibrated with Buffer C and eluted with the same buffer. Plotting the elution volume of each protein as a function of its molecular weight on a log scale gave a linear line as shown in Fig. 4. Thus, the molecular weight of phosphofructokinase was calculated as $3.0 \cdot 10^5$.

Optimum pH. Effect of pH on the enzymic activity is shown in Fig. 5.

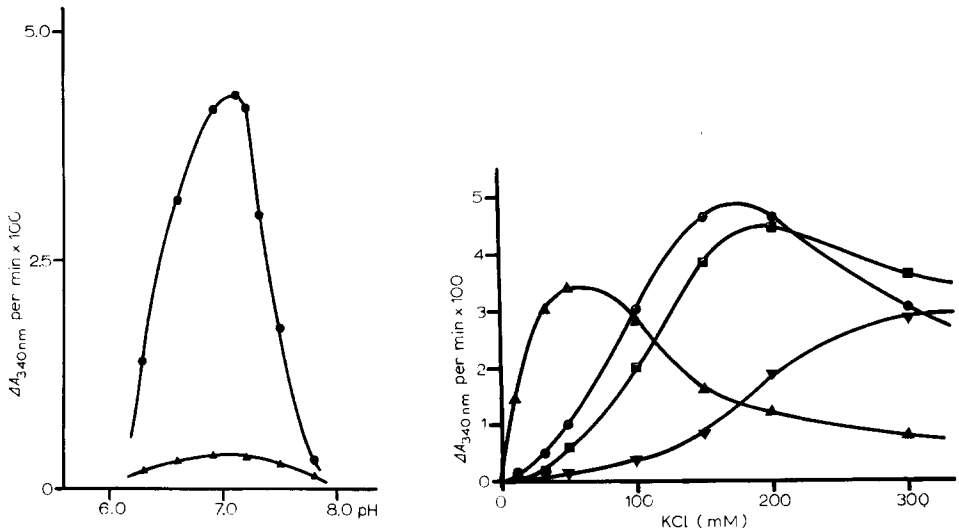


Fig. 5. Influence of pH on the initial velocity of ascites tumor phosphofructokinase. The purified phosphofructokinase was assayed as described in text at various pH values in the presence of 0.1 mM Fru-6-*P* and 5 mM NH_4Cl. ●——●, 0.02 mM ATP; ▲——▲, 0.3 mM ATP.

Fig. 6. Effect of K^+ on the initial velocity of ascites tumor phosphofructokinase. The purified enzyme was assayed as described in text in the presence of 0.5 mM Fru-6-*P* and KCl at the concentrations indicated on the abscissa. ▲——▲, 0.01 mM ATP; ●——●, 0.05 mM ATP; ■——■, 0.1 mM ATP; ▲——▲, 0.3 mM ATP.

In sharp contrast to 7 the muscle phosphofructokinase [11,12], the optimum pH of the tumor enzyme was maintained in the vicinity of 7.1 regardless of the concentration of ATP in the reaction mixture. SO_4^{2-} and K^+ were found to shift the optimum pH slightly toward alkaline side.

Dual function of K^+. Similar to phosphofructokinase from other sources [18–21], K^+ was essentially required for the tumor phosphofructokinase. Upon further increasing the concentration of K^+, however, an inhibition instead of an activation was observed as shown in Fig. 6. Inspection of Fig. 6 makes it clear that K^+ at high concentrations not only acts as an inhibitor by itself but also can overcome the inhibition induced by inhibitory levels of ATP. Conversely, the K^+-induced inhibition is reversed by increasing the concentration of ATP. In other words, K^+ decreases the apparent affinity of the inhibitory site of ATP and *vice versa*, though an actual mechanism of this apparent competition remains to be elucidated.

Though a variety of cations and anions has been reported to modify phosphofructokinase activity, no other papers, except for one dealing with bacterial enzyme[22], have mentioned so far the inhibition by K^+. Hence, it appeared that the dual response to K^+ was one of the unique properties that characterize phosphofructokinase from Ehrlich ascites tumor cells among the mammalian enzymes.

Separation of ascites tumor phosphofructokinase from muscle and erythrocyte enzymes on DEAE-Sephadex column

Differential affinity for DEAE-cellulose has been reported for human phosphofructokinases from muscle and erythrocytes. An attempt was made, therefore, to dis-

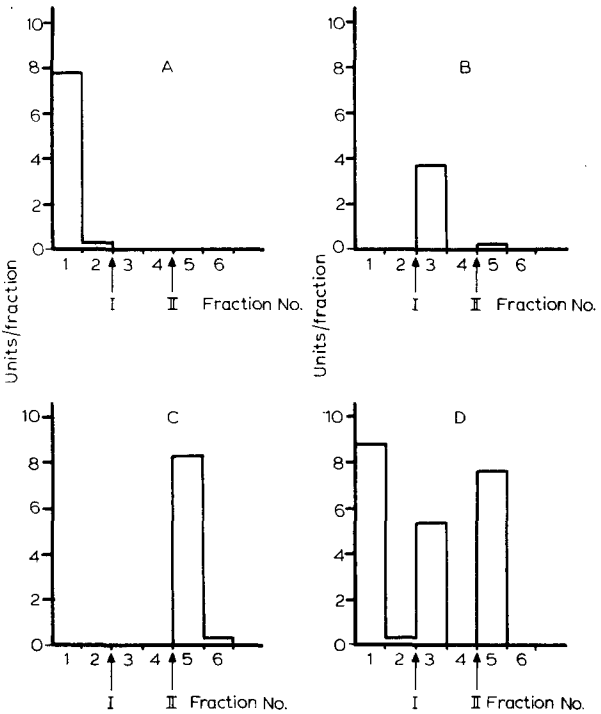


Fig. 7. Affinity of mouse muscle, erythrocyte and ascites tumor phosphofructokinase for DEAE-Sephadex A-50. The enzymes partially purified from mouse muscle, erythrocytes and ascites tumor cells as described in text were applied singulary or as a mixture to the columns of DEAE-Sephadex A-50 (10 cm × 0.8 cm) previously equilibrated with Buffer B. Step-wise elution was done by applying 40 ml each of Buffer B at three KCl concentration levels (0.01, 0 and 0.2 M). The addition of the KCl-containing buffer is indicated by arrows (Arrow I: 0.1 M KCl, Arrow II: 0.2 M KCl). The 20-ml fractions were collected and assayed for phosphofructokinase activity. Panel A: elution pattern of 10 units of muscle enzyme, B: 6 units of erythrocyte enzyme, C: 10 units of tumor enzyme, D: mixture.

tinguish the tumor phosphofructokinase from the enzymes of other mouse tissues on the basis of differences in affinity for DEAE-Sephadex A-50. Mouse skeletal muscle and ascites tumor cells were separately extracted with Buffer A and dialyzed against Buffer B. In the case of mouse erythrocytes, the protein containing phosphofructokinase activity was first precipitated by adding $(NH_4)_2SO_4$ to hemolysate at a saturation of 70% before dialysis against Buffer B. These dialyzed crude enzyme preparations thus prepared for muscle, tumor cells and erythrocytes were clarified by centrifugation and applied, separately or as a mixture, to columns (0.8 cm × 10 cm) of DEAE-Sephadex A-50 which had been equilibrated with the same buffer. It is seen in Fig. 7 that the muscle enzyme was readily eluted with Buffer B while erythrocyte and tumor enzymes were retained on the columns. A separation of the latter two enzymes was made by applying Buffer B containing 0.1 M KCl to the columns, which was effective in eluting erythrocyte enzyme only; a buffer of higher ionic strength being required for the tumor enzyme to be eluted.

Thus, phosphofructokinase from Ehrlich ascites tumor cells was characterized by its greater affinity for DEAE-Sephadex than enzymes from other mouse tissues.

DISCUSSION

Phosphofructokinase from Ehrlich ascites tumor cells purified by the procedure described is homogeneous as judged by disc electrophoresis on polyacrylamide gel. The purified enzyme preparation has an average specific activity of 150 units per mg of protein, which is within the range of specific activities of 52 to 190 units per mg of protein reported for purified phosphofructokinases from yeast[25–27], rabbit skeletal muscle[1,2], sheep heart[6], *Escherichia coli*[28] and *Clostridium pasteurianum*[22]. The molecular weight estimated to be $3.0 \cdot 10^5$ is comparable to $3.5 \cdot 10^5$ reported for rabbit muscle phosphofructokinase[29].

The inhibition by relatively high concentrations of ATP is one of the most striking kinetic features common to almost all of the phosphofructokinases so far examined (but see ref. 22). In this regard, ascites tumor phosphofructokinase was no exception, as shown in Figs 5 and 6. The ATP-induced inhibition of phosphofructokinase from various sources is known to be profoundly affected by a variety of effectors; activators such as Fru-6-*P*, ADP and AMP overcome the inhibition while inhibitors such as citrate and H^+ are effective in increasing the inhibition. Mostly similar relationships have been noted in the case of ascites tumor phosphofructokinase; the ATP inhibition was found to be overcome by Fru-6-*P*, AMP and ADP[13,30] and to be increased by citrate[30] in a fashion somewhat similar to other mammalian enzymes. A striking feature apparently unique to the tumor enzyme was that the ATP inhibition was not significatnly affected by a change of H^+ concentration. Instead, it responded to high concentrations of K^+ in decreasing the inhibition.

Our previous report has provided evidence that the decrease of the ATP inhibition of muscle phosphofructokinase activity observed upon raising the environmental pH forms a basis for the pH-dependent regulation of the overall rate of muscle glycolysis[11]. In this regard, it should be noticed that the glycolytic rate of intact Ehrlich ascites tumor cells is not significantly affected by a change of the pH of the incubation medium (to be published) in accord with the kinetic property of phosphofructokinase. These observations may confirm a close association of kinetic properties of phosphofructokinase with the glycolytic rate control mechanism in mammalian cells, and also are in keeping with a generally accepted view that phosphofructokinase is a key enzyme controlling the overall rate of glycolysis.

Effectiveness of K^+ at high concentrations in inhibiting phosphofructokinase by itself as well as in releasing the ATP inhibition is a very unique property of the tumor enzyme, because no mammalian enzymes have been reported to be subjected to the inhibition by K^+. The function of K^+ as an inhibitor as well as its function as an essential cofactor is similar to that of ATP. This similarity, when coupled with an abundance of K^+ in the cytosol of mammalian cells, might make it plausible to postulate that phosphofructokinase in Ehrlich ascites tumor cell is submitted to the dual basic regulation involving the ATP and K^+ inhibition, by which the primary kinetic feature of the enzyme is defined. Since several potential de-inhibitors of the ATP inhibition such as P_i and AMP fail to reverse the K^+ inhibition to a significant degree (see ref. 13), it would appear that this dual regulation operates in a rather separated manner in response to different physiological demands in the cell. But, "separated manner" does not mean that the effects of ATP and K^+ are completely independent from each other, because it was clearly shown that ATP reversed the K^+ inhibition and,

inversely, K^+ reversed the ATP inhibition (Fig. 6). Accordingly, it is concluded that in addition to their mutually independent functions as the regulators of the enzyme, ATP and K^+ may act as de-inhibitors of each other in the cell.

Several investigators have recently suggested that phosphofructokinase of mammalian tissues exists in multiple forms; muscle and erythrocyte types in human[23,24,31] as well as muscle and liver types in rat[32] and rabbit[21] have been distinguished chromatographically, electrophoretically or in some kinetic properties. In addition, two isozymes were separated from human platelet[31]. The present finding is also suggestive of the multiple forms of phosphofructokinase of mouse tissues. They differ in affinity for DEAE-Sephadex and can be separated by column chromatography (Fig. 7). Moreover, ascites tumor phosphofructokinase showed a kinetic behaviour apparently different from the enzyme of other tissues as discussed above. Hence, it is very likely that phosphofructokinase from Ehrlich ascites tumor cells differs from muscle and erythrocyte enzymes in protein structure. It should be noticed that DEAE-Sephadex column chromatography gave one extra peak, which, though very small in protein content, showed phosphofructokinase activity (Fig. 1). Therefore, it can not be stated with confidence that only one form of enzyme exists in Ehrlich ascites tumor cells, though it is difficult to rule out a possibility that such a minor component involves an artifact resulting from the fractionation procedure.

In any case, the present results reveal that the protein fraction possessing phosphofructokinase activity migrates in a relatively narrow peak during two types of column chromatography and finally gives only one band on disc electrophoresis with a good yield (roughly 30% of the activity of the original extract). This may suggest that phosphofructokinase purified here represents the major, if not total, fraction of Ehrlich ascites tumor phosphofructokinase which exists in a single form in the cell. Thus, it can be concluded that properties of this enzyme reported in the present and accompanying papers as being distinct from the enzyme of other tissues are those characteristic of phosphofructokinase of mouse Ehrlich ascites tumor cells.

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