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Properties of Phosphofructokinase from Rat Liver and their Relation to the Control of Glycolysis and Gluconeogenesis

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1. Phosphofructokinase from rat liver has been partially purified by ammonium sulphate precipitation so as to remove enzymes that interfere in one assay for phosphofructokinase. The properties of this enzyme were found to be similar to those of the same enzyme from other tissues (e.g. cardiac muscle, skeletal muscle and brain) that were previously investigated by other workers. 2. Low concentrations of ATP inhibited phosphofructokinase activity by decreasing the affinity of the enzyme for the other substrate, fructose 6-phosphate. Citrate, and other intermediates of the tricarboxylic acid cycle, also inhibited the activity of phosphofructokinase. 3. This inhibition was relieved by either AMP or fructose 1,6-diphosphate; however, higher concentrations of ATP decreased and finally removed the effect of these activators. 4. Ammonium sulphate protected the enzyme from inactivation, and increased the activity by relieving the inhibition due to ATP. The latter effect was similar to that of AMP. 5. Phosphofructokinase was found in the same cellular compartment as fructose 1,6-diphosphatase, namely the soluble cytoplasm. 6. The properties of phosphofructokinase and fructose 1,6-diphosphatase are compared and a theory is proposed that affords dual control of both enzymes in the liver. The relation of this to the control of glycolysis and gluconeogenesis is discussed.

Glucose metabolism in liver and kidney tissue poses the question of how the two opposing pathways of glucose degradation and glucose synthesis are controlled. One limiting step for glycolysis is the activity of phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11); and one limiting step for gluconeogenesis is the activity of fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) (Wu, 1963; Cahill, Ashmore, Renold & Hastings, 1959; Krebs, 1963, 1964). These two enzymes, PFK and FDPase,* will therefore control the rates of both pathways at the same point in metabolism: the interconversion of fructose 6-phosphate and FDP. The properties of these two enzymes, particularly those that could change the activity of either enzyme, could be of importance for understanding the mechanism of control at this step in metabolism.

Some properties of rat-liver FDPase that may be relevant to metabolic control have been reported by Taketa & Pogell (1963) and Underwood & Newsholme (1965). The enzyme is inhibited by low concentrations of AMP, and by its own substrate, FDP. These properties have provided some of the basis for a theory of control of gluconeogenesis (Krebs, Newsholme, Speake, Gascoyne & Lund, 1964). As the activity of FDPase in liver extracts was greater than that of PFK, control of glucose metabolism could be achieved by maintaining a constant activity of PFK and varying the activity of FDPase, according to the requirements of the tissue and the organism. Such a control system has been suggested by Cahill *et al.* (1959) for the analogous relationship between glucokinase and glucose 6-phosphatase in the control of the blood sugar concentration by the liver. If a similar mechanism operated at the level of fructose 6-phosphate phosphorylation, liver PFK should not possess any properties that are specific for regulation of activity. In particular, the complex kinetics that are characteristic of the regulatory function of this enzyme in cardiac muscle, skeletal muscle and brain would be redundant (Mansour, 1963; Passonneau & Lowry, 1962, 1963). However, if the properties of liver PFK were similar to those of the enzyme from other tissues, this would suggest that both PFK and FDPase are normally subject to metabolic control. As these properties of PFK would be, to some extent, opposite to those of

* Abbreviations: PFK, phosphofructokinase; FDPase, fructose 1,6-diphosphatase; FDP, fructose 1,6-diphosphate.

FDPase, this could provide a system for the dual control of both enzymes. It therefore seemed important to ascertain whether or not hepatic PFK possessed similar properties to the enzyme from other tissues. This has been carried out and the results are reported and discussed in the present paper.

MATERIALS AND METHODS

Chemicals and enzymes. Glucose 6-phosphate (sodium salt), FDP (tetracyclohexylammonium salt), phosphoenolpyruvate (tricyclohexylammonium salt), $NADH_2$, ATP, ADP, AMP, phosphoglucose isomerase, aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The chemicals were used without any further purification. In the assay of PFK only very small concentrations (less than 0·5mM) of these chemicals were used, so that the concentration of any impurity in the assay would be negligible. In experiments with the partially purified extracts, ammonium sulphate was removed from the above enzymes either by dialysis or by passing the enzyme through a column of Sephadex G-25.

Preparation of crude extracts. The liver and kidney cortex from a freshly killed rat were cooled on ice, weighed, cut into small pieces and homogenized in 3vol. of a solution comprising tris (20mM), $MgSO_4$ (5mM) and EDTA (0·1mM), pH8·0, in a manually operated glass Potter–Elvehjem homogenizer. These extracts were used to determine total PFK activity in kidney cortex and liver.

Cell fractionation. Liver tissue was fractionated according to the method of Getz & Bartley (1961). The liver was homogenized in 0·25M-sucrose and the fractions were separated by centrifugation. The mitochondria were resuspended and lysed in the original volume of 0·1M-potassium phosphate buffer, pH7·0. The microsomes and the cell-debris fraction were resuspended in the original volume of 0·25M-sucrose.

Partial purification of PFK. The liver of a freshly killed rat was passed through a chilled Fischer mincer, homogenized in 3vol. of 20mM-tris–5mM-$MgSO_4$–0·1mM-EDTA medium, pH8·0, and centrifuged at 30000*g* for 60min. at 2°. The clear supernatant was brought up to 40% saturation of ammonium sulphate by the addition of a saturated solution of ammonium sulphate at pH8·0 and at 0°. After 20min. mixing at 0° the precipitate was centrifuged at 9000*g* for 10min., then the supernatant was discarded and the precipitate dissolved in the original volume of 20mM-tris–5mM-$MgSO_4$–0·1mM-EDTA medium, pH8·0. This process of precipitation, centrifugation and dissolution was repeated twice. Each precipitation resulted in a further loss in α-glycerophosphate-dehydrogenase activity. From the original crude homogenate this procedure yielded a 35-fold increase in the specific activity of PFK, and 50% of the original activity was recovered. The final supernatant fraction was used for the assay of PFK either directly, or after dialysis or Sephadex treatment. Dialysis was carried out for approx. 16hr. against three changes of the 20mM-tris–5mM-$MgSO_4$–0·1mM-EDTA medium, pH8·0. Alternatively, the supernatant was passed down a column of Sephadex G-25 that had been equilibrated against the above buffer solution. Either of these procedures resulted in the complete removal of ammonium sulphate from the extract. In the presence of ammonium sulphate the enzyme activity could be preserved for several days at 4°. In the absence of this salt the enzyme was relatively unstable: 33% of activity was lost in 2·5hr. at 0°. This instability could account for the low activity of PFK after Sephadex treatment or dialysis. The addition of ammonium sulphate always increased the activity, probably as a result of a combination of stabilization and decrease of ATP inhibition (see the Results section). Except for this effect on ATP inhibition, ammonium sulphate did not change qualitatively the properties of PFK. Nor did the means of removal of ammonium sulphate change the properties to any noticeable degree.

Assay of PFK activity. The activity was assayed by two methods depending on which preparation was being investigated. For crude extracts activity was measured by following the formation of FDP in the presence of 2mM-AMP, which maximally activates PFK and inhibits FDPase. The activity of PFK cannot be accurately measured by this assay in the absence of AMP (see the Discussion section). For partially purified extracts PFK activity was assayed by following the formation of ADP. Such preparations have been used to investigate the properties of PFK; in all experiments in which the properties of the enzyme were to be compared the concentration of the extract in the cuvette was constant throughout the experiment. This was necessary because the response of the enzyme to ATP inhibition varied according to the extract concentration (see below). The crude extract was used to determine maximum PFK activity only.

Method 1. With crude extracts and cell fractions FDP formation was followed by converting it with aldolase into triose phosphates, which oxidized $NADH_2$ in the presence of α-glycerophosphate dehydrogenase and triose phosphate isomerase. The decrease in extinction at 340mμ at 25° was followed in a Gilford recording spectrophotometer when 0·05ml. of extract was added to 1·95ml. of assay medium contained in the cuvette. The assay medium comprised imidazole (20mM), $MgCl_2$ (5mM), $NADH_2$ (0·1mM), glucose 6-phosphate (2mM), ATP (1mM), AMP (2mM), KCN (0·3mM), KCl (200mM), aldolase (50μg.), α-glycerophosphate dehydrogenase (5μg.), phosphoglucose isomerase (5μg.) and triose phosphate isomerase (5μg.), at pH7·0. Glucose 6-phosphate was maintained in equilibrium with fructose 6-phosphate by the presence of excess of phosphoglucose isomerase. The KCN was present at a concentration that inhibited most of the $NADH_2$-oxidase activity (L. V. Eggleston, personal communication), but did not affect the activity of PFK.

Method 2. The partially purified preparation of liver was free from enzymes that hydrolysed ATP to ADP in the absence of any added substrate, and from aldolase, α-glycerophosphate dehydrogenase, adenylate kinase and glyceraldehyde phosphate dehydrogenase (all of which could have interfered with the PFK assay). The preparation did, however, contain FDPase at slightly higher activity then PFK: the activities assayed under optimum conditions were 45 and 52μmoles/g. of liver/hr. for PFK and FDPase respectively. PFK was assayed by following the formation of ADP with a coupled system containing commercial pyruvate kinase and lactate dehydrogenase and observing the oxidation of $NADH_2$ at 340mμ in a Gilford recording spectrophotometer at 25°. The assay was usually initiated

by addition of 0·025 or 0·05 ml. of extract to 2·975 or 2·95 ml. respectively of assay medium in the cuvette. The assay medium comprised imidazole (20 mM), $MgCl_2$ (5 mM), KCl (67 mM), $NADH_2$ (0·1 mM), phosphoenolpyruvate (0·5 mM), ATP and glucose 6-phosphate (various concentrations), lactate dehydrogenase (5 μg.), pyruvate kinase (5 μg.) and phosphoglucose isomerase (5 μg.), at pH 7·0. After an initial period of approx. 5 min., during which time a steady state with respect to the subsidiary enzymes was reached, the reaction was linear for at least 10 min. After this linear period the activity often increased slowly, in contrast with the findings of Passonneau & Lowry (1962) with the enzyme from skeletal muscle. The effect of extract concentration on the assay for PFK activity is discussed in the Results section. Three points of importance in this assay system should be stressed: (1) the substrate, fructose 6-phosphate, is maintained at a fairly constant concentration by being in equilibrium with glucose 6-phosphate; (2) the other substrate, ATP, is also maintained at a constant concentration by being regenerated from ADP in the assay system; (3) as FDP is a potent activator of FDPase (see below), any large accumulation of this during the assay could complicate the interpretation of results. This problem was avoided in this assay because the presence of FDPase in the extract removed most of the FDP as soon as it was formed. After a typical PFK assay only 2–5 mμmoles of FDP were detected in the cuvette. Under the conditions of the assay this concentration would not change PFK activity.

All assays were compared with controls in which glucose 6-phosphate was omitted. Factors that modified the rate of $NADH_2$ oxidation in this assay had no effect on the rate of $NADH_2$ oxidation due to the addition of commercial ADP. The rate of oxidation of $NADH_2$ due to the addition of commercial ADP was always much greater than the maximum activity of PFK. Therefore it has been assumed that the rate of $NADH_2$ oxidation is a measure of PFK activity under the conditions of the assay. In all cases the activity is expressed as μmoles of ADP (or FDP) produced/g. wet wt. of tissue/hr. In experiments with purified preparations no account is taken of activity loss or dilution that occurred during dialysis or Sephadex treatment.

Assay of FDPase. Activity in crude extracts of liver and kidney, and in cell fractions of liver, was measured by a technique described by Underwood & Newsholme (1965), except that increased quantities of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase were used (20 μg. of each enzyme/cuvette). This was done to compete effectively with any glucose 6-phosphatase activity that might be present in the crude extracts or cell fractions. In these experiments added commercial glucose 6-phosphate produced an increment in NADP reduction that was similar to the increment produced in a control cuvette to which the extract was not added.

RESULTS

Effects of ATP and hexose monophosphate. The activity of PFK is inhibited by one substrate, ATP, and experiments show that this is due to lowering the affinity of the enzyme for the second substrate, fructose 6-phosphate. Maximum activity could be obtained by providing a high concentration of hexose monophosphate and a low concentration of ATP, provided that the latter was sufficiently high to saturate the substrate site. In Fig. 1 the activity of PFK is plotted against the concentration of hexose monophosphate, at two different concentrations of ATP. At an ATP concentration of 0·01 mM the maximum activity of PFK remained very low, probably because ATP was limiting for the substrate site. At a concentration of 0·05 mM the maximum activity was increased; but, in comparison with the activity in the presence of AMP, FDP or ammonium sulphate, this activity was low. Only at high concentrations of hexose monophosphate did the activity in the absence

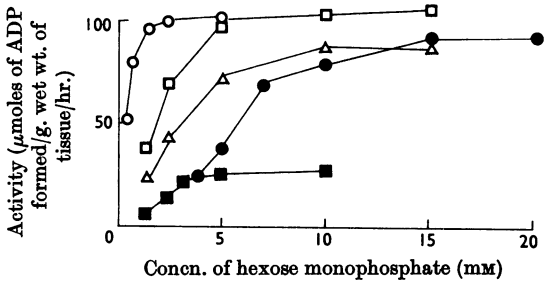

Fig. 1. Effect of hexose monophosphate concentration on partially purified liver PFK activity. The ammonium sulphate in the extract was removed with Sephadex treatment. The activities reported were corrected for activity loss during the experiment. ■, 0·01 mM-ATP; ●, 0·05 mM-ATP; △, 0·05 mM-ATP and 1·25 mM-FDP; □, 0·05 mM-ATP and 1·0 mM-ammonium sulphate; ○, 0·05 mM-ATP and 0·375 mM-AMP.

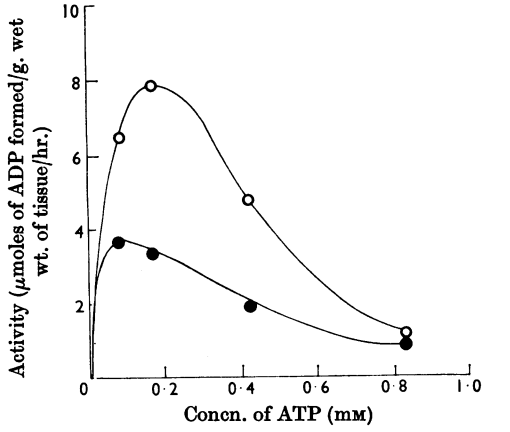

Fig. 2. Inhibition of partially purified liver PFK activity by ATP. Ammonium sulphate was removed from the extract by Sephadex treatment. ●, 3·3 mM-Hexose monophosphate; ○, 6·7 mM-hexose monophosphate.

Table 1. *Effect of increasing concentrations of citrate, AMP, FDP and ammonium sulphate on partially purified liver PFK activity*

The glucose 6-phosphate (G6P) and ATP concentrations were different in each case to provide conditions that were favourable for the action of the particular compound being tested. Both the citrate and the AMP were added in the form of the sodium salt; FDP was added in the form of the tetracyclohexylammonium salt. Ammonium sulphate solution was neutralized before use. Activity is expressed as μmoles of ADP formed/g. wet wt. of tissue/hr. Experimental details are given in the text.

G6P (5 mM) + ATP (0·01 mM)		G6P (3·3 mM) + ATP (0·16 mM)		G6P (5 mM) + ATP (0·1 mM)		G6P (3·3 mM) + ATP (0·16 mM)	
Concn. of citrate (mM)	Activity	Concn. of AMP (mM)	Activity	Concn. of FDP (mM)	Activity	Concn. of $(NH_4)_2SO_4$ (mM)	Activity
0·0	8·3	0·0	0·0	0·0	1·7	0·0	0·0
0·25	6·1	0·1	0·8	0·3	4·7	6·6	5·1
0·5	4·2	0·2	2·6	0·6	6·5	13·3	6·5
0·75	2·6	0·3	4·4	1·0	6·9	26·6	5·7
1·0	1·9	0·4	5·4				
		0·5	4·2				

Table 2. *Effect of ATP on the citrate inhibition of partially purified liver PFK activity*

The glucose 6-phosphate concentration was 3·3 mM. Activity is expressed as μmoles of ADP formed/g. wet wt. of tissue/hr. Experimental details are given in the text.

	Activity		
Concn. of ATP (mM)	No addition	Citrate (0·5 mM) added	Inhibition by citrate (%)
0·3	0·0	0·0	—
0·16	6·0	0·0	100
0·08	9·7	7·7	21
0·04	11·0	10·0	9
0·02	7·9	7·9	0

of these factors approach the same maximum (the $V_{max.}$ of the enzyme).

A plot of ATP concentration against activity at a fixed hexose monophosphate concentration produced a curve having a maximum activity at a specific ATP concentration (Fig. 2). At concentrations of ATP above this optimum the activity decreased to very low values. An increase in the concentration of the hexose monophosphate increased the maximum activity of the enzyme, and the curve was displaced to the right. This indicates that the ATP inhibition was relieved by increasing the concentration of the other substrate.

Effects of AMP and FDP. The inhibition by ATP can be relieved by the presence of either AMP or FDP. In Fig. 1 the presence of AMP or FDP displaced the curve to the left, and maximum activity ($V_{max.}$) was obtained at a much lower concentration of hexose monophosphate. However, the activities at 0·05 mM-ATP in the absence or the presence of AMP, FDP or ammonium sulphate approached the same $V_{max.}$ value. In the presence of these activators the curve obtained when plotting activity against ATP concentration was increased and displaced to the right (Figs. 3*a* and 3*b*). But increasing the ATP concentration diminished and finally removed the activation affects of either AMP or FDP. The range of concentrations of AMP and FDP that reversed the inhibition by one concentration of ATP are shown in Table 1.

Effect of citrate. The inhibition of PFK activity by increasing concentrations of citrate is shown in Table 1. The inhibition by citrate was increased as the ATP concentration in the assay system was raised (Table 2). Thus a sixfold increase in ATP concentration led to an increase in percentage inhibition from 28 to 71, by citrate. The relief of ATP inhibition by AMP or FDP was much less marked in the presence of 0·5 mM-citrate (Figs. 3*a* and 3*b*), and conversely the inhibition by this concentration of citrate was markedly lower in the presence of AMP or FDP.

Effect of intermediates of the tricarboxylic acid cycle. Some of the intermediates tested were found to inhibit PFK activity (Table 3), e.g. α-oxoglutarate, succinate and malate, but at concentrations much higher than that at which citrate was inhibitory. Acetoacetate at a high concentration (in comparison with citrate) was also inhibitory. No inhibition was observed with fumarate or acetyl-CoA. The chelating agent EDTA (1·0 mM) did not inhibit PFK activity (Table 3).

Effects of ammonium sulphate. Two effects of ammonium sulphate on the activity of PFK were observed.

(1) The enzyme was protected from inactivation by the presence of this salt. The enzyme as prepared

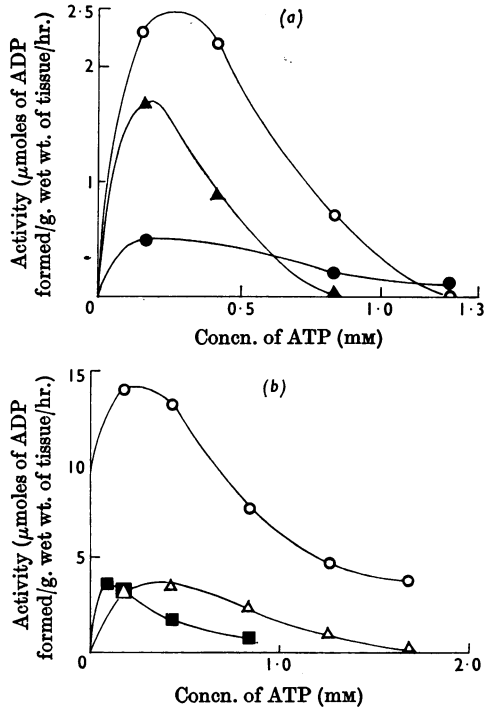

Fig. 3. Inhibition of partially purified liver PFK activity by ATP and the effect of AMP and FDP in the presence and absence of citrate. Ammonium sulphate was removed from the extract by Sephadex treatment. The hexose monophosphate concentration was 3·3mM. (*a*) ●, No addition; ○, 0·6mM-AMP; ▲, 0·6mM-AMP and 0·5mM-citrate. (*b*) ■, No addition; ○, 0·2mM-FDP; △, 0·2mM-FDP and 0·5mM-citrate. Experiments (*a*) and (*b*) were performed with different extracts at different times.

Table 3. *Effects of intermediates of the tricarboxylic acid cycle, acetoacetate and EDTA on partially purified liver PFK activity*

The activity is expressed as μmoles of ADP formed/g. wet wt. of tissue/hr. If necessary compounds were adjusted to pH7·0 before addition to the cuvette. Experimental details are given in the text.

Addition	Concn. (mM)	Activity	Inhibition (%)
Control	—	5·4	—
Citrate	0·5	2·7	50
Succinate	2·5	2·9	46
Acetyl-CoA	0·5	4·9	9
Fumarate	2·5	5·1	5
α-Oxoglutarate	2·5	3·0	44
Malate	2·5	3·6	33
Acetoacetate	5·0	3·9	28
EDTA	1·0	5·4	0

by ammonium sulphate precipitation was stable for several days at 4°, but after removal of this salt the extract lost activity fairly rapidly (33% in 2·5hr.).

(2) The activity of PFK was increased by the addition of ammonium sulphate (Table 1). Evidence has been obtained that this effect was due to the relief of ATP inhibition. In Fig. 1 the presence of ammonium sulphate has a similar effect to AMP or FDP: the curve was displaced to the left and the $V_{max.}$ was reached at a much lower hexose monophosphate concentration. In Fig. 4 the effects of increasing the concentration of ATP in the presence of two concentrations of ammonium sulphate are shown. At a higher concentration of the salt the curve was displaced to the right, without increasing the maximum activity of the enzyme. The activity

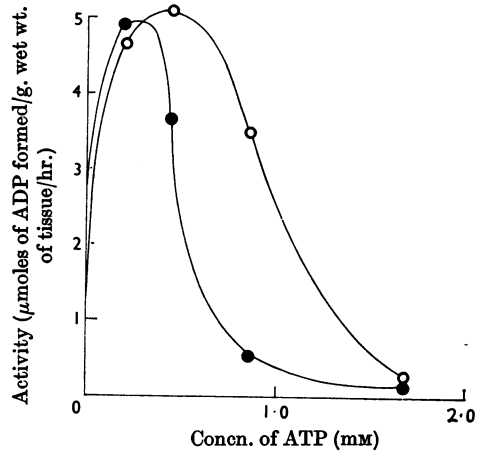

Fig. 4. Inhibition of partially purified liver PFK by ATP and the effect of ammonium sulphate. ●, 13mM-Ammonium sulphate; ○, 67mM-ammonium sulphate.

Table 4. *Effect of concentration of extract on the activity of partially purified liver PFK*

The volume of extract was increased in the assay cuvette from 25μl./3ml. of assay medium to 200μl./3ml. of assay medium. The activities are expressed as μmoles of ADP formed/g. wet wt. of tissue/hr. and are presented both as absolute activities and as ratios of activities at different extract concentrations. Experimental details are given in the text.

Vol. of extract (μl.)	Activity	Vol. used for ratio (μl.)	Ratio of activities
25	0·6	25/50	0·19
50	3·2	50/100	0·52
100	6·2	100/200	0·85
200	7·3		

Table 5. *Effect of AMP, ammonium sulphate and a high glucose 6-phosphate concentration on the activity of partially purified liver PFK at two extract concentrations*

The activities have been measured at two extract concentrations, by adding either 25 or 50 μl. of extract in 3 ml. of assay medium in the cuvette. The activities are expressed as μmoles of ADP formed/g. wet wt. of tissue/hr. at 25 and 50 μl. of extract, and also as a ratio of activities at these extract concentrations. Experimental details are given in text. The conditions of the assay in each experiment were as follows: Expt. 1, 0·01 mM-ATP; Expt. 2, 0·01 mM-ATP and 5 mM-hexose monophosphate; Expt. 3, 0·016 mM-ATP and 3·3 mM-hexose monophosphate.

Expt. no.	Addition	Concn. (mM)	Activity 25 μl. of extract	Activity 50 μl. of extract	Ratio of activities 25 μl./50 μl.
1	Glucose 6-phosphate	1·5	0·6	1·7	0·35
	Glucose 6-phosphate	·5	3·3	3·7	0·89
2	None (control)	—	3·1	6·6	0·47
	AMP	1·0	6·6	6·2	1·06
3	None (control)	—	8·1	14·5	0·56
	$(NH_4)_2SO_4$	33·3	22·6	21·7	1·04

was decreased to very low values by higher concentrations of ATP, even in the presence of a high concentration of ammonium sulphate.

Effect of extract concentration. At low hexose monophosphate concentrations the activity of PFK in the partially purified extract (expressed as μmoles/g. wet wt. of tissue/hr.) was dependent on the concentration of extract in the assay cuvette; at greater dilutions the activity of PFK was decreased (Table 4). This effect was not due to denaturation of the enzyme at low extract concentrations because preincubation of the extract with the assay medium, but lacking either ATP or glucose 6-phosphate, did not change the enzyme activity. At higher concentrations of hexose monophosphate, or in the presence of AMP or ammonium sulphate, the degree of dilution had little or no effect on the activity of PFK (Table 5). These three latter conditions are also those that relieved the inhibition of PFK by ATP. This suggested that the effect of increasing the extract concentrations was to protect the enzyme from inhibition by ATP. The effect of ATP at two extract concentrations was investigated and the results support the contention that ATP was less inhibitory at the higher extract concentration (Fig. 5). Thus conditions that removed ATP inhibition of PFK also prevented loss of activity on extract dilution. It is concluded that the decrease in activity of PFK on dilution of the extract can be explained by the concomitant increase in the degree of ATP inhibition.

PFK and FDPase activities in crude extracts and cell fractions. Liver and kidney-cortex tissue contain both PFK and FDPase, and in crude extracts the FDPase/PFK activity ratio was 8·8 and 2·9 for liver and kidney cortex respectively (Table 6). The fractionation of liver homogenate showed that the two enzymes were present mainly in one fraction, namely the soluble supernatant (Table 6).

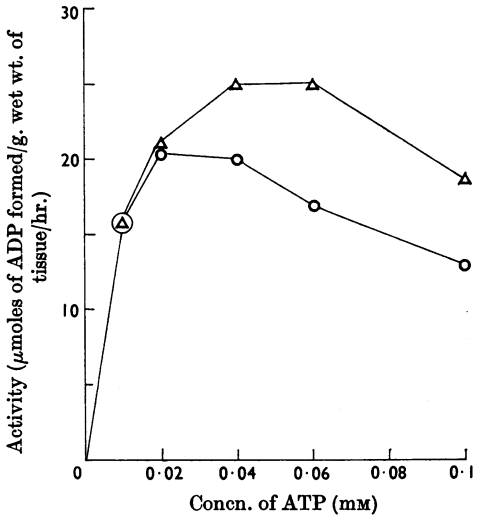

Fig. 5. Inhibition of partially purified liver PFK by ATP at two extract concentrations. Ammonium sulphate present in the extract was removed by Sephadex treatment. ○, 0·025 ml. of extract in 2 ml. of assay medium in the cuvette; △, 0·05 ml. of extract in 2·0 ml. of assay medium in the cuvette.

DISCUSSION

The presence of a very active FDPase in liver creates a problem for the assay of PFK activity in extracts of this tissue. FDPase has a very low K_m for FDP (approx. 2·0 μM; see Taketa & Pogell, 1963; Underwood & Newsholme, 1965), and in

Table 6. *Activities of PFK and FDPase in crude extracts of liver and kidney cortex, and in cell fractions of liver*

Activities are expressed as μmoles of FDP formed/g. wetwt. of tissue/hr. Experimental details are given in the text.

Expt. no.	Tissue	Cell fractions	PFK activity		FDPase activity	
			Actual	As % of whole homogenate	Actual	As % of whole homogenate
1	Liver	Whole homogenate	20·4	—	533	—
		Cell debris	1·6	8	39	7
		Mitchondria	0·0	0	0	0
		Microsomes	4·3	21	16	3
		Soluble fraction	16·3	80	494	93
2	Liver	Whole homogenate	77	—	678	—
	Kidney cortex	Whole homogenate	174	—	512	—

crude extracts is several times more active than PFK. This enzyme could therefore interfere in an assay system for PFK based on the formation of FDP. Moreover, FDPase is inhibited by low concentrations of AMP, partially inhibited by FDP and, in crude extracts of liver, ATP partially reverses this inhibition by AMP (Underwood & Newsholme, 1965). In this assay system any effects of these nucleotides could be interpreted as changes in the activity of either PFK or FDPase. Passonneau & Lowry (1964) have investigated liver PFK activity by using an assay system based on FDP formation, without removing FDPase activity; for the reasons given above some of the properties they report are open to several interpretations. In the present study hepatic PFK activity has been assayed by following the formation of ADP, after partial purification of the liver extract to decrease, to a very low level, the activities of enzymes that could interfere in the assay system (e.g. adenylate kinase, α-glycerophosphate dehydrogenase).

The present results show that some properties of liver PFK are very similar to those of the same enzyme that is present in skeletal muscle, brain and cardiac muscle (Passonneau & Lowry, 1962, 1963; Mansour, 1963). ATP inhibited the activity of the enzyme by decreasing the affinity for the other substrate, fructose 6-phosphate. The inhibition can therefore be removed by increasing the concentration of fructose 6-phosphate. This inhibition was also reversed by low concentrations of AMP or FDP, which appeared to compete with the inhibitory function of ATP, but not with its action as a substrate for PFK. AMP appeared to be more effective than FDP in this respect. The enzyme activity was also inhibited by citrate, which became more inhibitory as the ATP concentration was increased; this suggests that citrate potentiates the inhibition by ATP. The decrease of citrate inhibition by AMP or FDP, or conversely the ability of citrate to decrease the effects of AMP or FDP on the inhibition by ATP, support the above suggestion. Other intermediates of the tricarboxylic acid cycle also inhibited PFK activity, but they were much less effective than citrate. This action of citrate could not be a simple chelation effect, because EDTA at a similar concentration to citrate was not inhibitory. The activity was also increased by the presence of ammonium sulphate, which relieved the inhibition by ATP in a similar manner to AMP or FDP. The response of liver PFK to physiological concentrations of these nucleotides and intermediates, and the similarity with PFK from other tissues, suggest that these properties play an important role in the regulation of PFK activity in liver.

Passonneau & Lowry (1964) have reported some properties of sheep-liver PFK that differ in three aspects from the properties reported in the present paper. They found that FDP was more effective than AMP in relieving ATP inhibition, that ATP inhibition was not relieved by ammonium sulphate and that ATP inhibition was not relieved by fructose 6-phosphate. This last finding would indicate that inhibition of liver PFK by ATP was not competitive with fructose 6-phosphate and therefore that the kinetics of this enzyme were distinct from those of PFK in other tissues. The reason for the differences in results is not known, but it may be related to the inadequacy of the assay system used by Passonneau & Lowry (1964) (see above).

The mechanism by which these nucleotides and metabolic intermediates are able to modify the catalytic action of the protein molecule is not known. Changes in the activity of PFK could be explained either by chemical or structural alterations in a single molecular species of PFK, or by affecting the rate of interconversion between two forms of PFK that differed in catalytic activity. Because the activity *in vitro* was dependent on the

interplay of many factors, including the concentrations of extract and of ammonium sulphate, it is not possible to extrapolate from the conditions *in vitro* to those that may exist inside the liver cell. However, the activity of the enzyme in the liver cell may be expected to respond to changes in the intracellular concentration of these factors in a similar manner to the enzyme *in vitro*. The parallelism between the properties of the liver enzyme and the enzyme from other tissues, and the likely metabolic significance of these properties in other tissues, suggest that they may play a role in the regulation of glucose metabolism in liver.

The reason for the relation between extract concentration and ATP inhibition is not known (Fig. 5). A simple explanation might be non-specific association of ATP and protein in the cuvette, so that more ATP would be bound at higher extract concentrations. Alternatively, if two forms of PFK exist in liver [see Mansour (1964) and Viñuela, Salas, Salas & Sols (1964) for evidence suggesting two forms of PFK in skeletal muscle and yeast respectively], then increasing the extract concentration may favour the conversion into a form that is less susceptible to inhibition by ATP.

Because of the rate-limiting nature of PFK and FDPase, the relative activities of these two enzymes are important in deciding whether the tissue synthesizes or degrades glucose. If the activity of one enzyme remained constant, changes in the activity of the second one could control both the rate and direction of the flow of residues through this reaction. Such a mechanism, however, would be energetically wasteful as ATP would be hydrolysed during phosphorylation of fructose 6-phosphate and subsequent dephosphorylation of FDP. This inefficiency would be overcome if the control mechanism were such that conditions that stimulate one reaction inhibited the other, or if the actual activity of one enzyme inhibited that of the other. The properties of PFK and FDPase of liver suggest that both these mechanisms may be operative.

The importance of AMP as an intracellular signal for the ATP concentration has been stressed by Krebs (1964); a change in the ATP concentration of the cell would produce, through the action of adenylate kinase, a larger but reciprocal change in the AMP concentration. Thus the dual function of AMP, as an inhibitor of FDPase and an activator of PFK, would mean that an increase in the ATP concentration of the cell would simultaneously diminish the activity of PFK and increase that of FDPase. A fall in the ATP concentration would produce the converse changes in activities. Similarly, the activities would respond in opposite directions to a change in concentration of FDP. The latter is the substrate for FDPase, which it inhibits, and the product of PFK, which it activates. Thus, for either enzyme, any change in activity would be increased by the resultant change in FDP concentration. Moreover, a change in activity of only one enzyme would result in a reciprocal change in the activity of the other enzyme, through the FDP concentration. For example, an increase in the concentration of citrate would inhibit PFK activity directly, which would lead to an increase in FDPase activity indirectly. Thus the FDP concentration, which is controlled by both enzyme activities, may constitute an important controlling system for the activities of these two enzymes.

The response of these two enzymes to concentration changes of the regulatory compounds could ensure that at any given time only one enzyme activity was dominant, and that the rate and direction of glucose metabolism could be controlled within this system. The regulatory relationship between these two enzymes may represent part of a complex overall control system for deciding the direction of carbohydrate metabolism in liver according to the nutritional and hormonal state of the animal.

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