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Purification and Properties of Sheep Liver Phosphofructokinase

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1. The activity of phosphofructokinase in sheep liver was found to be dependent on the composition and molarity of the buffer used in extraction. Under optimum conditions a value of 4–7 μmoles/min./g. wet wt. of tissue was obtained. 2. The enzyme was purified 480-fold by a combination of ammonium sulphate fractionation, heat treatment in the presence of ethanol, DEAE-cellulose chromatography and Sephadex G-200 gel filtration. The final specific activity was 18·5 μmoles/min./mg. of protein. 3. The purified enzyme was inhibited by ATP and citrate, the degree of inhibition depending on the concentration of fructose 6-phosphate, magnesium chloride and ammonium sulphate, as well as on the pH. ATP and citrate inhibition was overcome by AMP and fructose 1,6-diphosphate. 4. The enzyme was also inhibited by NADH and NADPH in a manner largely independent of other components of the assay medium. AMP and fructose 1,6-diphosphate were not able to overcome this type of inhibition. 5. Octanoate was not an inhibitor of phosphofructokinase. 6. Differences between these results and those of other workers are discussed.

PFK† has been recognized as one of the rate-limiting enzymes of glycolysis in a number of mammalian tissues. These include muscle (Karpatkin, Helmreich & Cori, 1964), heart (Park *et al.* 1961), brain (Rolleston & Newsholme, 1967), diaphragm (Newsholme & Randle, 1961), kidney (Underwood & Newsholme, 1967*b*) and liver (Aisenberg & Potter, 1957). PFK has been purified from rabbit skeletal muscle (Ling, Marcus & Lardy, 1965) and sheep heart (Mansour, Wahid & Sprouse, 1966), and its kinetic properties have been studied (Paetkau & Lardy, 1967; Mansour & Ahlfors, 1968). A complex pattern of activity-enhancing, inhibitory and deinhibitory effects has emerged, which has supported the postulated role of the enzyme as one of the regulators of glycolytic flux. However, in tissues capable of gluconeogenesis, e.g. liver and kidney, there has been little success in the purification of PFK, and its properties have been studied in relatively crude extracts (Passonneau & Lowry, 1964; Weber, Convery, Lea & Stamm, 1966; Underwood & Newsholme, 1965, 1967*a*). The presence in these extracts of high activities of fructose 1,6-diphosphatase, which catalyses the reverse reaction, has greatly complicated kinetic analysis. Disagreement between Passonneau & Lowry (1964) and Underwood & Newsholme (1965) over the properties of liver PFK was unlikely to be resolved while this contaminant was present. Accordingly, an extensive purification of sheep liver PFK was undertaken; this, together with an examination of the properties of the purified enzyme, forms the basis of this paper.

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† Abbreviations: PFK, phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate.

MATERIALS AND METHODS

Chemicals and enzymes. F6P (barium salt), FDP (trisodium salt), phosphoenolpyruvate (monosodium salt), ADP, GSH, aldolase, α-glycerophosphate dehydrogenase and triose phosphate isomerase mixture, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were obtained from the Boehringer Corp. (London) Ltd., London W.5. AMP, ATP, NAD^+, NADH, $NADP^+$, NADPH, bovine albumin (crystallized), Nitro Blue Tetrazolium and phenazine methosulphate were bought from Sigma Chemical Co., London S.W.6. Dithiothreitol was from Calbiochem Ltd., London W.1, and acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethyldiaminoethane were from B.D.H. Chemicals Ltd., Poole, Dorset. Tris–phosphate buffers were prepared by adjusting 0·1 M-, 0·5 M- and 0·7 M-tris to pH 8·0 with M-H_3PO_4.

Tissues. Livers were obtained when sheep from this Organisation's farms were sent to a local slaughterhouse. The animals were killed by electrical stunning followed by severance of the jugular vein; livers were removed within 20 min. of death, placed in polythene bags and cooled in ice. Enzyme extractions were carried out either immediately

or on tissue stored at −42°. There was no decrease in the amount of extractable PFK in livers stored frozen for up to 3 months.

Polyacrylamide-gel electrophoresis. Acrylamide and *NN'*-methylenebisacrylamide were recrystallized as described by Loening (1967). Stock solutions for gel formation were: solution A, acrylamide (15g.) and *NN'*-methylenebisacrylamide (0·4g.) in 100ml. of water; solution B, tris (36·0g.), M-HCl (48·0ml.) and *NNN'N'*-tetramethyldiaminoethane (0·46ml.) in 100ml. of water; solution C, ammonium persulphate (1g.) in 10ml. of water, freshly prepared each day. The 3·75% gels were prepared by mixing 6ml. of solution A, 3ml. of solution B and 15ml. of water, and degassing *in vacuo* for 1min. Then 0·1ml. of solution C was added and the mixture pipetted into Perspex tubes (8·5cm. long × 6mm. internal diam.), fitted with rubber rings in the base to prevent the gels sliding out. Tops of the gels were overlaid with water, and polymerization allowed to proceed for 1hr.

The gels were set up in a Shandon disc-electrophoretic assembly, the lower (anodal) reservoir containing tris–glycine buffer, pH8·3 (6·0g. of tris base and 28·8g. of glycine/l.), and the cathodal reservoir containing the same buffer, supplemented with 0·1mM-ATP and 0·1mM-dithiothreitol. Samples (normally 0·05ml.) were applied in 0·25M-sucrose under the cathodal buffer, directly to the tops of the gels. Electrophoresis was carried out at a constant 3mA/gel for 3hr. in a cold-room.

At the end of the run gels were removed from the Perspex tubes by applying slight pressure with a rubber bulb, and washed briefly in water. For determination of protein bands, gels were stained by immersion in 1% Naphthalene Black in 7% acetic acid for 30min., and destained by exhaustive washing in 7% acetic acid. For location of areas of PFK activity, gels were soaked in a mixture (2·5ml.) of tris–HCl (50mM), $MgCl_2$ (6·7mM), KCl (100mM), F6P (2mM), ATP (1mM), sodium arsenate (5mM), NAD^+ (1mM), Nitro Blue Tetrazolium (2mg./ml,), phenazine methosulphate (0·025mg./ml.), aldolase (80μg.), triose phosphate isomerase (5μg.) and glyceraldehyde 3-phosphate dehydrogenase (5μg.), or for control purposes in the same mixture with F6P omitted. Staining was normally continued for 1–3hr. Gels were washed and preserved in 10% acetic acid.

Cellulose acetate electrophoresis. Cellulose acetate electrophoresis was carried out by using the Phoroslide system supplied by the Millipore Filter Corp., Bedford, Mass., U.S.A. The buffer used was 0·1M-tris–phosphate, pH8·0, containing 1mM-EDTA. Runs were carried out at 200v for 30min. at room temperature. Strips were stained with the same reaction mixtures as used for gels, but applied as a filter-paper overlay.

Assay of PFK activity. During purification the enzyme was assayed by following FDP formation in a coupled system containing aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase and NADH. AMP was present to inhibit fructose 1,6-diphosphatase and KCN to inhibit NADH oxidase (Underwood & Newsholme, 1965). Cuvettes contained, in 3·0ml., tris–HCl (50mM), $MgCl_2$ (6·7mM), KCl (100mM), KCN (0·3mM), F6P (2mM), ATP (2mM), AMP (2mM), dithiothreitol (1mM), NADH (0·1mM), aldolase (80μg.), α-glycerophosphate dehydrogenase (5μg.) and triose phosphate isomerase (5μg.), at pH8·0. After the second DEAE-cellulose column step in the purification, bovine serum albumin (1mg./ml.) was added to the assay medium. Measurements were made in a Unicam SP.800 double-beam recording spectrophotometer, fitted with a scale-expansion accessory, with the cuvette holder maintained at 25°. The reference cuvette contained the complete system except for F6P. After the first minute, rates were normally linear for 20–25min. Purified enzyme was assayed either by the above method, but with AMP and KCN omitted, or by following ADP formation in the presence of phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH. In the latter method cuvettes contained, in 3·0ml., tris–HCl (50mM), $MgCl_2$ (6·7mM), KCl (100mM), phosphoenolpyruvate (1mM), F6P (2mM), ATP (2mM), dithiothreitol (1mM), NADH (0·1mM), lactate dehydrogenase (5μg.) and pyruvate kinase (5μg.), at pH8·0. Alterations in $MgCl_2$, F6P, ATP and NADH concentrations are shown in the relevant figures and tables. For measurements at pH7·0, tris–HCl was replaced by 50mM-imidazole–HCl. When it was necessary to exclude $(NH_4)_2SO_4$ from the assay, the coupling enzymes were mixed in the correct ratio and passed through a short Sephadex G-25 column. Desalted coupling enzymes were unstable and were discarded at the end of their day of preparation. When coupling enzymes were not desalted, their addition to a cuvette made the assay medium 7mM with respect to $(NH_4)_2SO_4$.

In experiments in which inhibition was observed, controls were run to ensure that the coupling enzymes were not being interfered with. In the controls the rate of oxidation of NADH due to the addition of FDP (0·3mM) or ADP (0·3mM) was always several-fold greater than the maximum activity of PFK.

One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1μmole of product/min. at 25°. Since one assay measured FDP formation and the other ADP formation, specific activities are expressed in the figures and tables as FDP units/mg. of protein or ADP units/mg. of protein. With the purified enzyme no difference was found in the specific activity when measured by the two different methods; the designation FDP units or ADP units merely indicates the method of assay.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with albumin as standard.

EXPERIMENTAL

Extraction of PFK

Initially extractions of PFK were carried out with the buffer system (20mM-tris–5mM-$MgCl_2$–0·1mM-EDTA, pH8·0) described by Wallace & Newsholme (1967) and similar to that of Mansour *et al.* (1966). It was apparent from the pH of the extracts (6·2–6·5) that low-molarity buffers were inadequate to cope with the strong acidity of sheep liver. In view of the known susceptibility of PFK to acid inactivation, a study was made of the effect of buffer composition and concentration on extraction of the enzyme. This is shown in Table 1, which also contains a column indicating the effect of different buffers on the stability of crude extracts. High-molarity phosphate buffers were found to be the most effective in terms of both extracting the enzyme and conserving its stability, and were used as a routine thereafter.

The results recorded in Table 1 refer to extractions made

Table 1. *Effect of different extraction media on PFK values in liver and stability of extracts*

Portions (3–5g.) of frozen liver were thawed in 3 vol. of extraction medium, cut in pieces with scissors and homogenized in a Potter–Elvehjem-type tissue grinder (glass vessel and machine-driven Teflon pestle) at 0° until an even suspension resulted. The homogenate was centrifuged at 38000*g* for 20min. at 0°, and the supernatant filtered through a piece of calico. PFK was assayed both immediately and after 24hr. at 4°, as described in the Materials and Methods section.

Extraction medium	Activity (units/g. wet wt.) 0hr.	24hr.
Tris–HCl (20mM), $MgCl_2$ (5mM), EDTA (0·1mM), pH8·0	1·5	0·9
Tris–HCl (50mM), $MgCl_2$ (5mM), EDTA (0·1mM), pH8·0	2·9	2·0
Tris–HCl (50mM), pH8·0	2·9	2·1
Tris–HCl (100mM), pH8·0	3·1	2·4
Tris–phosphate (100mM), pH8·0	3·5	3·0
Potassium phosphate (100mM), pH8·0	5·1	4·9
K_2HPO_4 (100mM)	5·5	5·2
Na_2HPO_4 (100mM)	5·4	5·2
$(NH_4)_2SO_4$ (100mM)	4·0	0·3
$MgCl_2$ (100mM)	1·2	0·1
Sucrose (250mM)	1·8	0·3
Sucrose (250mM), tris–HCl (10mM), pH8·0	2·0	0·5

on different portions of a single liver. Provided that sections of liver were carefully freed of connective tissue and fat, PFK values with the same extractant were uniform through the organ. There was, however, some variation between the livers of different sheep. Fourteen livers from four different breeds were analysed with 0·1M-K_2HPO_4 as homogenizing medium; PFK activity was in the range 4–7units/g. wet wt. of tissue. All the PFK was in a soluble form and readily extractable in a single homogenization; no additional activity was obtained by incubating cell residues with ATP and $MgCl_2$, as is possible with sheep heart muscle and diaphragm (Mansour *et al.* 1966).

Purification of the enzyme

All operations were carried out at 0–4°, unless otherwise stated.

Step 1: *preparation of homogenate and centrifugation.* A portion of frozen liver (100g.) was allowed to thaw in 600ml. of 0·1M-K_2HPO_4. It was cut in small pieces with scissors and sliced in an MSE homogenizer run at maximum speed for 3min. The resultant preparation was further homogenized in a Potter–Elvehjem-type tissue grinder (glass vessel and Teflon pestle) until an even suspension resulted. This was centrifuged at 11750*g* for 30min. and the sediment discarded.

Step 2: *first ammonium sulphate precipitation.* To the supernatant was added an equal volume of saturated $(NH_4)_2SO_4$ solution, adjusted to pH8·0 with aq. NH_3. After 45min. stirring, the material was centrifuged at 11750*g* for 30min. and the supernatant discarded. The precipitate was dissolved to 270ml. in 0·1M-K_2HPO_4 containing 1mM-ATP, 0·1mM-FDP and 1mM-dithiothreitol.

Step 3: *heat treatment.* Ethanol (13·5ml.) was added to the above solution and it was heated with constant stirring in a water bath maintained at 57°. As soon as the internal temperature reached 55°, the solution was cooled in ice–water and centrifuged at 38000*g* for 30min. The precipitate was discarded.

Step 4: *second ammonium sulphate precipitation.* To the supernatant was added an equal volume of saturated $(NH_4)_2SO_4$ adjusted to pH8·0. After 45min. the material was centrifuged at 38000*g*, the supernatant discarded and the precipitate dissolved to 104ml. in 0·1M-tris–phosphate buffer, pH8·0, containing 0·1mM-FDP and 1mM-dithiothreitol. It was dialysed overnight against 1l. of the same buffer.

Step 5: *first DEAE-cellulose column chromatography.* The dialysed material was run on to a DEAE-cellulose column (2cm.×22cm.) equilibrated with 0·1M-tris–phosphate buffer, pH8·0, containing 0·1mM-dithiothreitol. The column was washed with 250ml. of the same buffer, and the washings were discarded. The enzyme was eluted with 0·5M-tris–phosphate buffer, pH8·0, containing 0·1mM-dithiothreitol. Active fractions were combined and dialysed against two changes of 0·1M-tris–phosphate buffer, pH8·0, containing 0·1mM-ATP, 0·1mM-FDP and 1mM-dithiothreitol.

Step 6: *second DEAE-cellulose column chromatography.* The dialysed solution was adsorbed on a DEAE-cellulose column prepared exactly as in step 5. The column was washed with 100ml. of the equilibrating buffer, and the enzyme eluted in a linear gradient of 0·1M-tris–phosphate to 0·7M-tris–phosphate buffer, pH8·0, both buffers containing 0·1mM-dithiothreitol. Fractions (5ml.) were collected, those containing activity were combined and protein was precipitated by the addition of 2vol. of saturated $(NH_4)_2SO_4$, pH8·0. The suspension was centrifuged at 38000*g* and the precipitate dissolved to 4·8ml. in 0·1M-potassium phosphate buffer, pH8·0, containing 1mM-ATP and 1mM-dithiothreitol.

Step 7: *gel filtration on Sephadex G-200.* The redissolved precipitate was applied to a Sephadex G-200 column (1·5cm.×85cm.) packed in 0·1M-potassium phosphate

Table 2. *Purification of PFK*

Summary of a typical purification of enzyme from 100g. of frozen sheep liver. Details are given in the Results section.

Step no.	Fraction	Total protein (mg.)	Total activity (units)	Yield (%)	Sp. activity (units/mg. of protein)	Purification
1	11750*g* supernatant	12300	475	100	0·0386	1·0
2	First $(NH_4)_2SO_4$ precipitate	7750	425	90	0·055	1·4
3	Heat-treated	1580	350	74	0·11	5·7
4	Second $(NH_4)_2SO_4$ precipitate	970	350	74	0·35	9·1
5	First DEAE-cellulose column	144	338	71	2·34	60
6	Second DEAE-cellulose column	76	272	57	3·58	93
7	Sephadex G-200 gel filtration	5·7	106	22	18·5	480

buffer, pH 8·0, containing 1 mM-dithiothreitol. The same buffer was used for elution, 5 ml. fractions being collected. Maximum specific activity was found at the void volume, but there was considerable trailing of activity into later fractions. The first 5 ml. after the void volume was collected, made 5 mM with respect to dithiothreitol and conserved at 4° as the source of enzyme for kinetic studies.

A typical purification is shown in Table 2.

The purity of the enzyme after Sephadex G-200 filtration was examined by polyacrylamide-gel and cellulose acetate electrophoresis. On both media staining with Naphthalene Black revealed one intense anodically-migrating protein band, together with three other bands of much diminished intensity. Two portions of enzyme were then run on separate gels under identical conditions, and one gel was stained in Naphthalene Black and the other in the specific PFK mixture (see the Materials and Methods section). The major protein band corresponded to the area of PFK staining. It was not possible, however, to gain more than a rough idea of the degree of purification achieved. The preparation was free of the following enzymes, which might possibly have interfered with either of the assays: fructose 1,6-diphosphatase, aldolase, α-glycerophosphate dehydrogenase, adenylate kinase, NADH oxidases and enzymes hydrolysing ATP to ADP.

RESULTS

Properties of the purified enzyme

Stability. The enzyme was very labile at all stages of purification, and particularly so at high dilutions. At least two factors appeared to contribute to activity loss; the most important was susceptibility to oxidation. Mercaptoethanol (10 mM), GSH (1 mM) and dithiothreitol (1 mM) were tested at each of the last three stages of the purification; only dithiothreitol was effective in slowing the normal rapid disappearance of activity. In the absence of a thiol reducing agent the enzyme apparently dissociated, for when the Sephadex G-200 gel-filtration step was carried out without dithiothreitol no activity could be recovered at the void volume, although small amounts of activity emerged in later fractions. Even in the presence of 1 mM-dithiothreitol the purified enzyme lost 10% of its activity in 24 hr. at 4°; this could be largely but not completely restored by making the preparation 5 mM with respect to dithiothreitol and incubating it at pH 8·0 and 37° for 1 hr. Enzyme thus reactivated was reasonably stable over a period of 4 weeks at 4°; thereafter, unless protected by further addition of dithiothreitol, it lost activity very rapidly.

Table 3. *Effect of* pH *on activity of purified PFK*

$MgCl_2$ was 6·7 mM, ATP 0·5 mM, F6P 2 mM and NADH 0·1 mM. The $(NH_4)_2SO_4$ was not removed from the coupling enzymes. Activity is expressed in FDP units/mg. of protein.

Buffer	pH	Activity
Imidazole–HCl (50 mM)	6·5	4·4
Imidazole–HCl (50 mM)	7·0	12·1
Imidazole–HCl (50 mM)	7·5	14·4
Tris–HCl (50 mM)	7·5	14·0
Tris–HCl (50 mM)	8·0	16·9
Tris–HCl (50 mM)	8·5	16·4
Glycine–NaOH (50 mM)	8·5	15·1
Glycine–NaOH (50 mM)	9·0	15·9
Glycine–NaOH (50 mM)	9·5	14·6

Effect of pH. Imidazole–hydrochloric acid, tris–hydrochloric acid and glycine–sodium hydroxide buffers were used in the determination of optimum pH. None of these buffers appeared to inhibit PFK appreciably, since assays carried out at the same pH with different buffers gave similar values for enzyme activity. Maximum activity was found at pH 8·0, with a slow decline in the alkaline range and a considerably sharper decline in the acid range (Table 3).

Inhibition by ATP. The activity of PFK was inhibited by increasing concentrations of one of its substrates, ATP. The degree of inhibition by a fixed concentration of ATP, as well as the ATP concentration at which inhibition was first observed, was a function of pH (Figs. 1 and 2) and magnesium chloride (Fig. 1), ammonium sulphate (Fig. 2) and

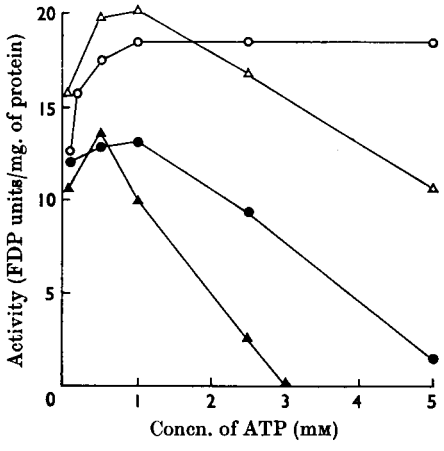

Fig. 1. Inhibition of purified PFK by ATP at different pH values and $MgCl_2$ concentrations. The assay was as described in the Materials and Methods section, with 2mM-F6P and 0·1mM-NADH. The $(NH_4)_2SO_4$ was not removed from the coupling enzymes. ○, pH8·0, 6·7mM-$MgCl_2$; △, pH8·0, 1mM-$MgCl_2$; ●, pH7·0, 6·7mM-$MgCl_2$; ▲, pH7·0, 1mM-$MgCl_2$.

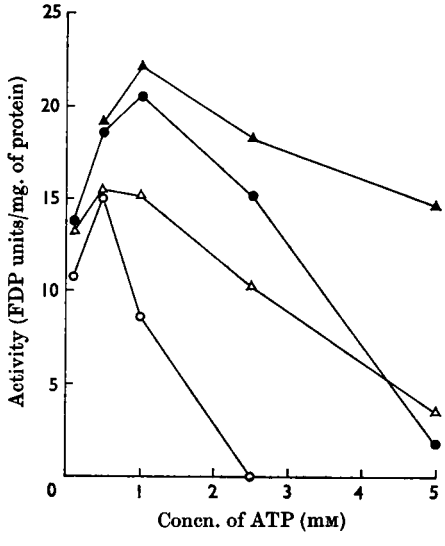

Fig. 2. Effect of $(NH_4)_2SO_4$ on ATP inhibition of purified PFK at pH7·0 and 8·0. $MgCl_2$ was 1mM, F6P was 2mM and NADH was 0·1mM. The $(NH_4)_2SO_4$ was removed from the coupling enzymes before use. ○, pH7·0, no $(NH_4)_2SO_4$; △, pH7·0, 20mM-$(NH_4)_2SO_4$; ●, pH8·0, no $(NH_4)_2SO_4$; ▲, pH8·0, 20mM-$(NH_4)_2SO_4$.

F6P concentrations (Fig. 3). Elevated pH, the presence of ammonium sulphate, or increased F6P concentration increased the activity of PFK at all concentrations of ATP, as well as delaying the onset of ATP inhibition. Changing from 1mM- to

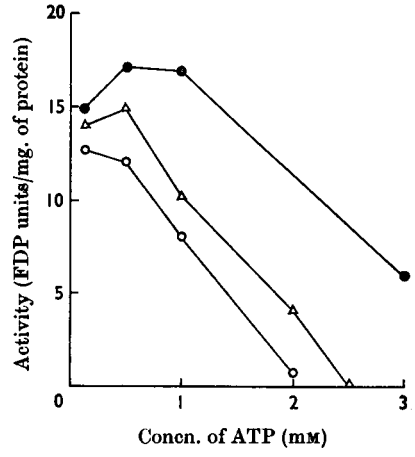

Fig. 3. Effect of F6P on ATP inhibition of purified PFK at pH7·0. $MgCl_2$ was 1mM and NADH was 0·1mM. The $(NH_4)_2SO_4$ was removed from the coupling enzymes before use. ○, 1mM-F6P; △, 2mM-F6P; ●, 5mM-F6P.

6·7mM-magnesium chloride, on the other hand, did not increase the activity of the enzyme, but afforded increased protection against ATP inhibition (Fig. 1). Fig. 1 shows that the conditions chosen for assay of the enzyme during purification (pH8·0, 6·7mM-magnesium chloride, 10mM-ammonium sulphate) were those where the activity was relatively independent of the ATP concentration.

Both FDP and AMP were able to relieve PFK inhibition (Table 4). Demonstration of the effect of FDP was complicated by the necessity of measuring PFK activity by following ADP formation with pyruvate kinase and lactate dehydrogenase. Pyruvate kinase required high concentrations of magnesium chloride, since its true substrate is $MgADP^-$ (Mildvan & Cohn, 1966), and high Mg^{2+} concentrations diminished ATP inhibition. Therefore the efficacy of FDP as an antagonist of ATP inhibition was not easily shown. AMP effects, on the other hand, could be demonstrated in the FDP-formation assay at low magnesium chloride concentration. In contrast with ammonium sulphate and F6P, AMP and FDP did not activate PFK, and exerted their effects only in the relief of ATP inhibition.

Effect of citrate. Citrate decreased the activity of PFK at all concentrations of ATP tested although the degree of inhibition became greater at high ATP concentrations (Table 5). Citrate inhibition was relieved by ammonium sulphate, AMP and phosphate and by increased concentrations of F6P and magnesium chloride. The relative effectiveness of these compounds in relieving both citrate and ATP inhibition is shown in Table 6.

Table 4. *Effect of AMP and FDP on activity of purified PFK at* pH 7·0

The F6P concentration was 2mM and NADH was 0·1mM. The $(NH_4)_2SO_4$ was removed from the coupling enzymes before use.

$MgCl_2$ (6·7mM) + ATP (3mM)				$MgCl_2$ (1mM) + ATP (2mM)	
Concn. of FDP (mM)	Activity (ADP units/mg. of protein)	Concn. of AMP (mM)	Activity (ADP units/mg. of protein)	Concn. of AMP (mM)	Activity (FDP units/mg. of protein)
0	4·5	0	4·5	0	1·2
0·17	7·9	0·17	6·2	0·1	4·5
0·33	10·3	0·33	8·9	0·5	10·3
0·67	8·5	0·67	11·1	1·0	10·7
1·0	8·5	1·0	11·0		

Table 5. *Effect of ATP on citrate inhibition of purified PFK*

Assays were carried out at pH 7·0 in the presence of 1mM-$MgCl_2$, 1mM-F6P and 0·1mM-NADH. Coupling enzymes were desalted before use. Citrate was added as the trisodium salt. Activity is expressed in FDP units/mg. of protein.

Concn. of ATP (mM)	Activity: No citrate	Activity: Citrate (0·25mM)	Inhibition by citrate (%)
0·1	7·5	5·8	23
0·25	8·0	5·4	33
0·5	6·0	3·1	48
1·0	1·5	0·7	55

Table 6. *Relief of ATP and citrate inhibition of purified PFK by magnesium chloride, ammonium sulphate, F6P, AMP, FDP and phosphate*

The basal system contained 1mM-$MgCl_2$, 1mM-F6P, 1mM-ATP and 0·1mM-NADH at pH 7·0. Where $MgCl_2$ or F6P was added to the basal system, the molarities in the first column are final concentrations in the cuvette. Coupling enzymes were desalted. Activity is expressed in FDP units/mg. of protein, and in the last row in ADP units/mg. of protein.

Addition	Activity: No citrate	Activity: Citrate (0·25mM)
None	1·6	0·7
$(NH_4)_2SO_4$ (20mM)	8·2	5·1
$MgCl_2$ (7·7mM)	6·6	3·1
F6P (5mM)	8·2	8·5
AMP (1mM)	4·9	1·8
AMP (5mM)	3·8	1·6
Potassium phosphate (20mM)	1·6	2·4
FDP (1mM)	6·1	2·5

Effect of NADH. PFK was inhibited by increasing concentrations of NADH. The onset of inhibition was sudden: 0·5mM-NADH gave near-maximum activity, whereas 1·0mM-NADH gave almost complete inhibition. Unlike citrate and ATP inhibition, NADH inhibition was not noticeably affected by pH, magnesium chloride concentration or F6P concentration (Fig. 4). The complete inhibition observed at 1·0mM-NADH was not relieved by 20mM-ammonium sulphate, 5mM-AMP or 5mM-FDP. NADPH produced effects identical with those of NADH; 1·0mM-NAD^+ and 1·0mM-$NADP^+$ were only slightly inhibitory. Similar results to those shown in Fig. 4 were obtained by using the ADP-formation assay, eliminating the possibility that NADH inhibition was operating at the level of the coupling enzymes.

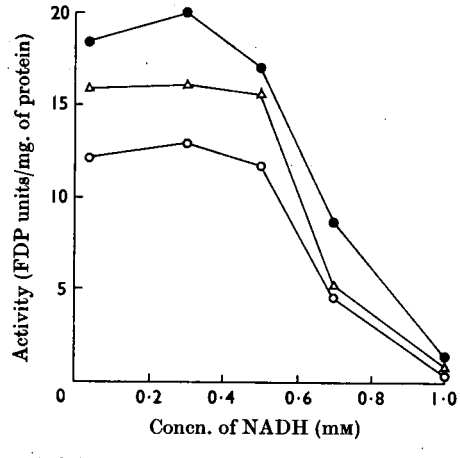

Fig. 4. Inhibition of purified PFK by NADH. ATP was 0·5mM. The $(NH_4)_2SO_4$ was not removed from the coupling enzymes before use. ○, pH 7·0, 1mM-$MgCl_2$, 1mM-F6P; △, pH 7·0, 1mM-$MgCl_2$, 5mM-F6P; ●, pH 8·0, 6·7mM-$MgCl_2$, 1mM-F6P.

Effect of octanoate. Octanoate was tested for inhibitory effects at concentrations between 0·1mM and 10mM. No inhibition was observed either under conditions where PFK was normally maximally

sensitive to effector molecules (pH 7·0, 1 mM-magnesium chloride, desalted coupling enzymes) or where it was least sensitive (pH 8·0, 6·7 mM-magnesium chloride, coupling enzymes not desalted).

DISCUSSION

When 0·1 M-dipotassium hydrogen orthophosphate was used to extract PFK from sheep liver, enzyme activity ranged from 4 to 7 units/g. wet wt. of tissue. This is much higher than the value of 0·48 unit/g. found by Mansour *et al.* (1966) and 0·5 unit/g. found by Wallace & Newsholme (1967). PFK was susceptible to inactivation below pH 7, and unless strong buffers were used the acidity of the sheep liver was great enough to destroy most of the enzyme during extraction. Thus when a low-molarity buffer was used in homogenization a value of only 1·5 units/g. was obtained. Wallace & Newsholme (1967), in a study of changes in activities of glycolytic enzymes during development, observed that there is a sharp decline in PFK activity (but not hexokinase or pyruvate kinase activity) in adult sheep livers compared with foetal livers. In the light of the above results it would be interesting to know whether this decline is real, or whether it reflects the difficulty in extracting the enzyme from an increasingly acid tissue.

Several of the purification steps used in the procedure described above were similar to those used with the enzyme from skeletal muscle (Ling *et al.* 1965). The liver enzyme was less stable under comparable conditions and it was necessary to maintain the pH close to 8 and to have dithiothreitol present throughout the purification. Even so, there was considerable activity loss at the last stage of purification, and this may to some extent account for the low final specific activity of 18·5 units/mg. of protein. Crystalline PFK from skeletal muscle has a specific activity of 180 units/mg. of protein (Ling, Paetkau, Marcus & Lardy, 1966), from sheep heart a specific activity of 120–157 units/mg. of protein (Mansour *et al.* 1966) and from yeast a specific activity of 116 units/mg. of protein (Lindell & Stellwagen, 1968). This suggests a specific activity for the fully purified liver enzyme of above 100 units/mg. of protein. Although electrophoresis indicated that the present preparation was still contaminated with other protein, it seems most unlikely that it was only 20% pure.

The kinetic properties of the purified liver enzyme were similar in many respects to those of the crystalline enzyme from muscle (Paetkau & Lardy, 1967), heart (Mansour & Ahlfors, 1968) and yeast (Lindell & Stellwagen, 1968), as well as the less pure preparations from kidney (Underwood & Newsholme, 1967*a*), liver (Underwood & Newsholme, 1965) and brain (Lowry & Passonneau, 1966). The present results show that PFK was inhibited by ATP and also by citrate, with citrate inhibition becoming more severe as the ATP concentration was raised. Both types of inhibition were relieved by high pH, by increased magnesium chloride or F6P concentrations, and by ammonium sulphate, FDP and AMP. Ammonium sulphate appeared to be a true activator, in increasing the activity of the enzyme at all concentrations of ATP, including non-inhibitory ones. F6P behaved in many ways like ammonium sulphate; its effects are probably more correctly described as those of a substrate with a variable Michaelis constant, dependent on ATP concentration. Magnesium chloride, FDP and AMP were all able to relieve ATP (or ATP and citrate) inhibition without having any primary activational effect on the enzyme.

The results discussed above differ in some respects from those of other workers. Passonneau & Lowry (1964), working with crude sheep liver extracts, have reported that both ammonium sulphate and F6P fail to reverse the inhibition due to ATP or ATP and citrate combined. This is disputed by Underwood & Newsholme (1965). The present results support the latter authors; in fact ammonium sulphate had to be rigorously excluded from the assay medium to enable citrate inhibition to be demonstrated at all. The dependence of ATP inhibition on the F6P concentration brings liver PFK into line with the enzyme from other tissues (Lowry & Passonneau, 1966; Mansour & Ahlfors, 1968; Passonneau & Lowry, 1962). Underwood & Newsholme (1965) further showed that FDP and AMP increased the activity of PFK several-fold at all concentrations of ATP. In the present study FDP and AMP effects were apparent only at inhibitory concentrations of ATP.

The observation that PFK is inhibited by NADH was first made by Weber, Lea, Convery & Stamm (1967), who showed that in crude rat liver extracts 2 mM-NADH decreased the activity of the enzyme by 30–40%. The present results show that both NADH and NADPH were potent inhibitors of the purified enzyme, and stopped the reaction completely at a concentration of 1 mM. The inhibition was of a different type from that observed with ATP and citrate, for it was independent of pH, magnesium chloride, ammonium sulphate, F6P, FDP and AMP. The effect of reduced nicotinamide nucleotides on the activity of PFK from other sources is worthy of examination, for it appears to be the most powerful inhibitor (on a molar basis) yet recognized. The implications of this kind of inhibition in the control of glycolysis have been discussed by Weber *et al.* (1967).

The inhibition of PFK by octanoate has been reported by Weber *et al.* (1967). This finding was not confirmed in the present study, even at

octanoate concentrations as high as 10mM. Since Weber *et al.* (1967) found it necessary to incubate crude liver extracts with octanoate for appreciable periods of time before assay in order to demonstrate inhibition, the possibility arises that the effects they observed were due to breakdown products of octanoate.

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