The Control of Rat-Heart Phosphofructokinase by Citrate and Other Regulators

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1. The effects of ATP, inorganic phosphate and citrate on the relationship between fructose 6-phosphate concentration and initial velocity of reaction has been investigated with a partially purified preparation of rat-heart phosphofructokinase. 2. At low concentrations of ATP ($< 80 \mu\text{M}$) rate curves for fructose 6-phosphate approximated to Michaelis-Menten kinetics. At higher ATP concentrations rate curves were sigmoid, the K_m for fructose 6-phosphate increased and the reaction appeared to be first-order with respect to fructose 6-phosphate at concentrations above its K_m and of a higher order at concentrations below its K_m . Inorganic phosphate lowered the K_m for fructose 6-phosphate and the concentration at which the apparent kinetic order decreased. 3. At $40 \mu\text{M}$ -ATP, citrate was an activator at low concentration ($< 100 \mu\text{M}$) and an inhibitor at higher concentrations. At 0.5 mM -ATP, citrate was inhibitory at all concentrations tested. 4. A new method for phosphofructokinase assay using $[\text{U-}^{14}\text{C}]$ fructose 6-phosphate is described which allows measurements to be made of the velocity of the forward reaction at known concentrations of the products of the reaction. With this method confirmatory evidence has been obtained that concentrations of ATP, AMP, phosphate and citrate may regulate phosphofructokinase in the perfused rat heart.

In the perfused rat heart the rate of glycolysis is increased by anoxia and diminished in alloxan-diabetes and by respiration of fatty acids, ketone bodies or pyruvate. Measurements of the concentrations of hexose phosphates suggest that these changes in glycolytic rate are mediated through alterations in the activity of PFK† (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11). This conclusion is valid for rat heart (though not necessarily for other muscles) because of the low or absent fructose diphosphatase activity in this muscle (Newsholme & Randle, 1961, 1962, 1964; Regen, Davis, Morgan & Park, 1964; Krebs & Woodford, 1965).

Passonneau & Lowry (1962) have proposed that the increased glycolytic flow in anoxia may be explained by the kinetic properties of PFK. They find that the enzyme from rabbit skeletal muscle is inhibited by ATP and activated by AMP, inorganic phosphate, fructose 6-phosphate and fructose 1,6-diphosphate at concentrations within the physiological range. The changes which anoxia induces in the concentration of these metabolites in rat heart are consistent with this proposal (Newsholme & Randle, 1964; Regen *et al.* 1964). This mechanism

does not, however, account for the inhibitory effect of alloxan-diabetes or of the respiration of fatty acids, ketone bodies or pyruvate. These agents do not induce consistent changes in the concentration of adenine nucleotides, hexose phosphates or inorganic phosphate in rat heart (Newsholme & Randle, 1964; Regen *et al.* 1964). An alternative mechanism has been proposed for the inhibition produced under these conditions, based upon the concomitant rise in intracellular citrate concentration (Garland & Randle, 1964). Experiments with rat-heart extracts have indicated that PFK may be inhibited by citrate at concentrations within the physiological range (Garland, Randle & Newsholme, 1963), and evidence derived from perfusion experiments with fluorocitrate have added weight to the view that citrate concentration is a major factor in controlling glycolysis *in vivo* (Bowman, 1964; Williamson, Jones & Azzone, 1964).

The regulatory significance of citrate in the control of rat-heart PFK has now been studied in greater detail with a partially purified preparation of the enzyme substantially free of contaminating activities. Kinetic studies of inhibition and activation of PFK are open to two difficulties, which, on the basis of published evidence, may not have been obviated in earlier work with enzyme preparations from other tissues. Sulphate is an

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† Abbreviation: PFK, phosphofructokinase.

activator of rat-heart PFK and is potent in overcoming citrate inhibition (Garland *et al.* 1963); it therefore became necessary to remove this ion from the enzyme and all reagents used in the assay of PFK activity. If kinetics are not to be complicated by fructose 1,6-diphosphate activation, it seems necessary to measure initial reaction velocities and not steady-state rates, which are only attained after an autocatalytic phase lasting 2–5 min. Assay methods for PFK activity have been devised with these difficulties in mind.

To confirm further that the observed variations in concentration of adenine nucleotides, phosphate and citrate are instrumental in effecting changes in PFK activity *in vivo*, experiments simulating conditions *in vivo* with appropriate concentrations of substrates, products and known PFK effectors have been designed with ^{14}C -labelled fructose 6-phosphate.

A preliminary report of some of these findings has been published (Randle & Pogson, 1965).

MATERIALS AND METHODS

Chemicals. ATP, ADP, AMP, D-glucose 6-phosphate (sodium salt), D-fructose 1,6-diphosphate (tetracyclohexylammonium salt), phosphoenolpyruvate (tricyclohexylammonium salt), 3-phosphoglycerate (sodium salt), NADH, NADP and triethanolamine hydrochloride were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Cyclic 3',5'-AMP was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Tris (as Trizma base or Sigma 121) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Bovine serum albumin fraction V was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and was used without further purification. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Thorn Electronics, Tolworth, Surbiton, Surrey. All other chemicals were of A.R. grade or were the purest available.

Radioactive chemicals. D-[U- ^{14}C]Glucose 6-phosphate (sodium salt), 99% pure, of specific activity 3.5 mc/m-mole, was obtained from The Radiochemical Centre, Amersham, Bucks. This was dissolved in distilled water and unlabelled glucose 6-phosphate and phosphoglucose isomerase (100 $\mu\text{g.}/\text{ml.}$, $(\text{NH}_4)_2\text{SO}_4$ -free as below) were added to give a final concentration of hexose monophosphates 6.0 mM with a specific activity 350 $\mu\text{C}/\text{m-mole}$. Three standards were prepared by spotting 10 $\mu\text{l.}$ portions of a 1:10 dilution of this solution in distilled water on to 2 cm. by 1 cm. rectangles of Whatman no. 3MM paper (W. and R. Balston Ltd.). The rectangles were placed in counting vials with 3 ml. of scintillation fluid [0.35% of 2,5-diphenyloxazole and 0.005% of 1,4-bis-(5-phenyloxazol-2-yl)benzene in sulphur-free toluene] and were counted with each experiment.

Enzymes. Phosphoglucose isomerase, aldolase, triose phosphate isomerase, α -glycerophosphate dehydrogenase, 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, myokinase, pyruvate kinase and rabbit-muscle lactate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H.

Buffer solutions. Veronal buffer was prepared by appropriate mixture of equimolar solutions of 5,5'-diethylbarbituric acid and of the corresponding sodium salt. All other buffers were adjusted to the appropriate pH (glass electrode) with N-NaOH or N-acetic acid.

Special preparations. For kinetic studies, all assay enzymes were dialysed to remove $(\text{NH}_4)_2\text{SO}_4$. Lengths (12 in.) of $\frac{1}{4}$ in. Visking seamless cellulose dialysis tubing (Hudes Merchandising Corp. Ltd., London) were boiled with several changes of distilled water for 12–18 hr. The preparation was then washed twice with glass-distilled water and stored in glass-distilled water at 4°.

A mixture of 15 mg. of aldolase, 3.75 mg. of triose phosphate isomerase and 3.75 mg. of α -glycerophosphate dehydrogenase was dialysed against six changes of imidazole buffer (20 mM-imidazole–0.15 M-potassium acetate–5 mM- MgCl_2 –0.01% bovine serum albumin, pH 7.4) over 24 hr. The enzyme solution was then adjusted to a final volume of 10 ml. by addition of imidazole buffer, and 1.0 ml. fractions of this solution were stored frozen at -15° . No SO_4^{2-} ion was detectable (by BaCl_2 –HCl) in the final preparation.

Phosphoglucose isomerase (5 mg.) was dialysed as above, and the resulting solution was diluted to 2.0 ml.

Glucose 6-phosphate solutions were assayed and standardized enzymically by the method of Hohorst (1963), with glucose 6-phosphate dehydrogenase. The concentration of hexose monophosphates was finally adjusted to 18 mM, pH 7.4, dialysed phosphoglucose isomerase being added to a final concentration of 12.5 $\mu\text{g.}/\text{ml.}$ Fractions (1.0 ml.) of this glucose 6-phosphate–fructose 6-phosphate equilibrated mixture were stored at -15° .

Fructose 1,6-diphosphate was assayed with aldolase, triose phosphate isomerase and α -glycerophosphate dehydrogenase, essentially the same system as that described below for assay of total PFK activity being used. A solution of 2.0 mM-fructose 1,6-diphosphate thus standardized was stored at -15° .

ATP solutions (pH 7.4) were assayed and standardized enzymically with 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. For kinetic experiments, solutions (3 mM, 30 mM and 300 mM) were stored in 1.0 ml. fractions. For 'simulated *in vivo*' experiments ATP solutions were also assayed for contamination by ADP and AMP.

ADP and AMP solutions (pH 7.4) were assayed and standardized enzymically with pyruvate kinase, lactate dehydrogenase and myokinase. ADP solutions were also assayed for AMP content, due allowance being made for this in subsequent experiments. Full details of assay procedures are given in Newsholme & Randle (1964). All adenine nucleotides, both solid and solutions, were kept at -15° . Solutions of ATP and ADP were never kept for more than 2 weeks, to avoid experimental errors due to increased amounts of AMP and P_i .

Since stored solutions of NADH may contain appreciable amounts of AMP, fresh solutions in imidazole buffer, pH 7.4, were made up each day immediately before use. The AMP content of fresh solutions of NADH was negligible ($<0.5\%$ of NADH).

Preparation of DEAE-cellulose. DEAE-cellulose (Whatman DE 50, now known as DE 11; W. and R. Balston Ltd.) was stirred with 40 vol. of 50 mM-NaOH for 1 hr. Sodium hydroxide was removed by exhaustive washing with distilled water and the larger particles were freed from fines and smaller particles by decantation, so as to allow

subsequent fast flow rates. The DEAE-cellulose was finally resuspended in 50 mM-tris-2 mM-EDTA, pH 8.0.

A 2.2 cm. diameter column with a coarse glass sinter (porosity 1) was filled with DEAE-cellulose to a depth of 4.5 cm. without packing. The column was equilibrated with 250 ml. of 50 mM-tris-2 mM-EDTA, pH 8.0.

Assay of total PFK activity. Activity in extracts was measured enzymically by converting fructose 1,6-diphosphate into α -glycerophosphate, the resulting disappearance of NADH being followed at 340 m μ in a 1 cm. light-path in a Beckman DU spectrophotometer at room temperature (22°). Fructose 6-phosphate was generated *in situ* with glucose 6-phosphate and phosphoglucose isomerase. The reaction mixture contained: 50 mM-tris; 1 mM-MgCl₂; 2 mM-ATP; 1 mM-glucose 6-phosphate; 0.1 mM-NADH; 10 mM-cysteine; 0.01% bovine serum albumin; 20 μ g. of aldolase/ml.; 20 μ g. of phosphoglucose isomerase/ml.; 7.5 μ g. of triose phosphate isomerase/ml.; 7.5 μ g. of α -glycerophosphate dehydrogenase/ml.; final volume was 1.0 ml. All enzymes used were the commercially available suspensions in (NH₄)₂SO₄. The reaction was initiated by addition of a suitable dilution of the PFK preparation (20 μ l.); blank determinations without glucose 6-phosphate were carried out simultaneously. After 1 min., allowed for equilibration, readings were taken every minute for 4 min.; the rate always remained linear over this 4 min. period. One unit of PFK is defined as that amount of enzyme which catalyses the formation of 1.0 μ mole of fructose 1,6-diphosphate/min. at 22°.

Protein concentrations were determined from the extinction of solutions at 260 m μ and 280 m μ by the method of Warburg & Christian (1941) as given by Layne (1957).

PROCEDURE

Procedure in kinetic experiments with ATP, P_i and citrate

Preparation of PFK extract. PFK was purified as described below from rat-heart muscle about 15-fold (specific activity approx. 1 unit/mg. of protein) by centrifugation and DEAE-cellulose fractionation. This yielded a preparation virtually free of contaminating NADH oxidase (usually <0.1%) and adenosine triphosphatase (<1% of total PFK activity, as determined by ADP production). Since storage led to loss of activity, the enzyme was prepared freshly on each occasion.

Four male albino Wistar rats (300–400 g.) were decapitated and bled. The hearts were quickly removed, washed in ice-cold 0.9% NaCl for a few minutes, and the auricles and adhering connective tissue were removed. The ventricular muscle was then washed again in 0.9% NaCl before blotting and weighing.

All subsequent operations were carried out at 0–4°. The muscle was extracted with 4 vol. of 50 mM-tris-2 mM-EDTA, pH 8.0, in a Dounce homogenizer. The resulting homogenate was centrifuged in an MSE Highspeed 18 centrifuge at 14 000 *g* for 30 min. at 0°. The supernatant was added to the DEAE-cellulose column, which was allowed to run as fast as possible (usually approx. 5 ml./min.). The extract was washed in with 20 ml. of 50 mM-tris, pH 8.0, and the column was washed with 10 ml. of 0.2 M-tris, pH 8.0. Activity was then eluted with 0.3 M-tris, pH 8.0, 15 ml. of

eluate being collected immediately after addition of this buffer to the column.

Procedure for obtaining kinetic data. The reaction medium contained: 20 mM-imidazole; 0.15 M-potassium acetate; 5 mM-MgCl₂; 0.04 mM-NADH; 0.01% bovine serum albumin; 40 μ g. of aldolase (dialysed); 6 μ g. of triose phosphate isomerase (dialysed); 6 μ g. of α -glycerophosphate dehydrogenase (dialysed); ATP, sodium citrate and sodium phosphate (added at concentrations given in text, Tables or Figures); total volume 3.0 ml.; pH 7.4. The reaction was followed with a 4 cm. light-path, cell width 0.4 cm. The change in extinction at 366 m μ was followed on an Eppendorf spectrophotometer (Netheler und Hinz G.m.b.H., Hamburg, Germany; obtained through V. A. Howe Ltd., London) coupled to a Honeywell chart recorder (Honeywell Controls Ltd., London) fitted with a variable sensitivity control.

All non-protein solutions were maintained in a water bath at 26°, and all reactions were carried out at this temperature.

An incubation period of 2 min. was allowed to elapse after the addition of PFK extract. The reaction was then initiated by addition of equilibrated hexose phosphate mixture. The time-course of the reaction showed an initial autocatalytic phase, lasting for 2–5 min., followed by a linear steady-state period. To obtain initial velocities, rates were determined at various intervals during the autocatalytic phase; these values were plotted logarithmically against time so that initial velocities could be determined by extrapolation to zero time. Typical plots are shown in Fig. 1. (Rates with added fructose 1,6-diphosphate were in all cases at least 20 times faster than those obtained in experiments with added

Fig. 1. Typical plots observed in experiments for obtaining kinetic data. The time *t* taken for a given change in extinction at 366 m μ (0.005–0.0155), i.e. a measure of $1/v$, is plotted logarithmically against the total time after the addition of substrate to the reaction mixture. Plots were extrapolated to zero time to give values of $\log t$ from which initial rates were calculated. For any one experiment, plots thus obtained were parallel for concentrations of fructose 6-phosphate in the range 0.05–1.0 mM. O, 126 μ M-Fructose 6-phosphate; 36 m-units of PFK/ml.; given change in extinction at 366 m μ , 0.0104. ●, 180 μ M-Fructose 6-phosphate; 24 m-units of PFK/ml.; given change in extinction at 366 m μ , 0.0104. Δ, 216 μ M-Fructose 6-phosphate; 24 m-units of PFK/ml.; given change in extinction at 366 m μ , 0.0154. Concentrations of Mg²⁺, ATP and sodium citrate were 5 mM, 0.2 mM and 0.1 mM respectively throughout. Other conditions were as described in the text.

PFK.) Since specific activity varied somewhat from one preparation to another, reaction velocities are expressed in terms of total PFK activity in the extracts, i.e. as μ moles of fructose 1,6-diphosphate formed/min./unit of enzyme.

Procedure in experiments simulating conditions in vivo

This procedure was developed to measure the velocity of the forward reaction with PFK at known concentrations of products (fructose 1,6-diphosphate and ADP) in addition to known concentrations of other regulators. Both products are known to be activators of PFK from a variety of sources (e.g. Passonneau & Lowry, 1962).

Preparation of PFK extract. PFK was purified 20–25-fold by centrifugation and precipitation with ethanol to give a preparation entirely free of aldolase activity. Enzyme was prepared freshly each day from two to four rat hearts removed, washed and weighed as previously described. The ventricular muscle was extracted with 4 vol. of 50 mM-veronal–50 mM-magnesium acetate–0.1 M-sucrose, pH 8.0, at 0°. The resulting homogenate was centrifuged in an MSE Highspeed 18 centrifuge at 14 000 *g* for 30 min. at 0°. The supernatant was cooled to –2° in ice-salt mixture. Ethanol (96%, cooled in acetone–solid CO₂) (0.123 vol.) was added dropwise with rapid stirring. After addition was complete, stirring was continued for a further 30 min. and the precipitate was then removed by centrifugation at 11 000 *g* for 20 min. at –2°. To the supernatant a further 0.082 vol. of 96% ethanol was added as before. After centrifuging, the supernatant was discarded, and the inside of the centrifuge tube was dried quickly with absorbent tissue to remove residual ethanol. The pellet was then dissolved in 2 ml. of 50 mM-tris, pH 8.0. Total PFK activity was assayed as previously described.

Procedure for incubations. Reactions were carried out in a water bath at 37° with a final volume of 0.5 ml. in 2 in. $\times$ $\frac{1}{2}$ in. flat-bottomed tubes. Reaction systems were made up previously by the addition of appropriate volumes of the following solutions: 100 mM-ATP; 20 mM-ADP; 4 mM-AMP; 10 μ M-cyclic 3'5'-AMP; 2 mM-fructose 1,6-diphosphate; 50 mM-Na₂SO₄; 50 mM-sodium phosphate (pH 7.4); 10 mM-trisodium citrate; 6 mM-[U-¹⁴C]hexose monophosphate (equilibrated mixture) to a medium with final concentrations 10 mM-tris, 5 mM-magnesium acetate and 125 mM-KCl, pH 7.4. These reaction systems were kept at –15° until use, and were then preincubated at 37° for 3 min., during which stirring was effected with a small magnetic 'flea' at a sufficient rate to ensure thorough mixing.

Reactions were initiated by addition of PFK (25–50 μ l.) and were stopped after 10, 20 or 30 sec. periods by addition of 0.8 ml. of 10% pyridine in absolute ethanol containing 1.0 mM-*p*-chloromercuribenzoic acid, a reagent found to produce immediate cessation of enzyme activity without resulting in marked change of pH.

The tubes were allowed to stand for 1 hr. at room temperature, and the contents were then transferred to conical centrifuge tubes with two washings of a 1:1 dilution of the pyridine–ethanol–*p*-chloromercuribenzoic acid reagent with distilled water. The samples were then frozen and dried *in vacuo* over conc. H₂SO₄ overnight. The residue was dissolved in 0.4 ml. of distilled water; this was facilitated by warming for a few minutes in a water bath at 60°.

Electrophoresis. Separation of phosphate esters was

achieved by electrophoresis, by a modification of the method of Vanderheiden (1964). Sheets of Whatman no. 3MM paper (46 cm. $\times$ 19 cm.) were washed successively with three changes of 20 l. of N-HCl, five changes of 20 l. of distilled water and finally with three changes of 20 l. of 0.02% EDTA. They were then allowed to dry in a current of air at room temperature.

The origin was 7 cm. from the cathode. Fractions (40 μ l.) were applied in 1 cm. lengths, 3.5 cm. apart. In this way, three samples and a marker solution were run on each paper. The marker solution was of similar composition to the test samples but contained added fructose 1,6-diphosphate.

Electrophoresis was carried out with pyridine–acetic acid–water (5:16:229, by vol.) buffer, pH 3.9, at 1050 v for 2 $\frac{1}{2}$ hr. in a tank filled with white spirit (Esso Petroleum Co. Ltd., Cambridge), which was cooled by water in a glass coil. Phosphate esters were detected on marker strips, with an acid molybdate dip followed by exposure to H₂S, as described by Burrows, Grylls & Harrison (1952). Fructose 1,6-diphosphate appeared as a fast-moving spot well separated from both hexose monophosphates (after 2 $\frac{1}{2}$ hr. phosphate had moved approx. 28 cm., fructose 1,6-diphosphate 27 cm., ATP 20 cm. and glucose and fructose 6-phosphates 16 cm.).

For each sample, an area 28 cm.², corresponding to the position of the fructose 1,6-diphosphate spot on the marker strip, was cut out as eight 3.5 cm. $\times$ 1 cm. rectangles. Each rectangle was put into a counting vial, covered with scintillator fluid and counted for 10 min. in a liquid scintillation counter (Nuclear–Chicago series 720; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The total number of counts above background recorded in a 10 min. period were 300–3000. Appropriate reagent and paper blanks were similarly prepared and counted.

Fructose 1,6-diphosphate recovery. A fraction (0.2 ml., 0.75 unit) of a PFK preparation (aldolase-free as above) was added to a mixture containing 2.0 ml. of 50 mM-tris, 10 mM-MgSO₄, 2.5 mM-ATP, pH 8.0, 0.2 ml. of 6 mM-[U-¹⁴C]hexose monophosphate equilibrated mixture and 20 μ g. of phosphoglucose isomerase. A blank without PFK was similarly prepared. The mixtures were incubated at 37° for 10 min., and reaction was stopped by placing in a boiling-water bath for 10 min. After denatured protein had been removed by centrifugation, the fructose 1,6-diphosphate content was assayed enzymically, and 50 μ l. fractions were added to 'typical' reaction systems prepared as described above. After addition of pyridine–ethanol–*p*-chloromercuribenzoic acid, the mixtures were freeze-dried and subjected to electrophoresis as usual. Recoveries for three determinations were 81.2%, 84.1% and 81.0%. An average recovery of 82% was taken as a basis for calculation of experimental results.

RESULTS

Effect of enzyme concentration. As shown in Table 1, reaction velocity and enzyme concentration were directly proportional at a number of different ATP and citrate concentrations. It was necessary to establish that this was so because some variation in enzyme concentration was required in kinetic experiments to achieve measurable rates. The concentration of Mg²⁺ in these and following

Table 1. *Effect of phosphofructokinase concentration on reaction velocity*

Rates of PFK reaction were measured as described in the Procedure section for obtaining kinetic data.

10 ⁻³ × Concn. of enzyme (unit)	Concn. (mm)			Rate (μmole of fructose 1,6-diphosphate formed/min./unit of enzyme)
	ATP	Fructose 6-phosphate	Citrate	
11	0.5	2.0	0.4	0.112
22				0.112
33				0.118
9.5	0.05	3.0	1.0	0.229
19.0				0.239
28.5				0.208

experiments was fixed at 5mm, which was greatly in excess of the highest ATP concentration used (0.5mm); qualitative experiments indicate that excess of Mg²⁺ has no inhibitory action on PFK activity.

Effects of concentrations of fructose 6-phosphate and ATP. To define conditions for investigating effects of citrate on PFK activity the effects of varying fructose 6-phosphate and ATP concentrations on reaction velocity were investigated. Earlier studies with PFK from a number of tissues have shown that ATP is an inhibitor of the enzyme in addition to being a substrate (Lardy & Parks, 1956; Passonneau & Lowry, 1962) and preliminary evidence indicated that this was true for PFK from rat heart (Garland *et al.* 1963).

As shown in Fig. 2, the relationship between fructose 6-phosphate concentration and reaction velocity is dependent upon ATP concentration. The sigmoid character of these curves varies with ATP concentration, being most marked at 0.5mm and least so at 0.1mm. The inhibitory effect of ATP was most evident at low concentrations of fructose 6-phosphate. At high concentrations of fructose 6-phosphate the effect of increasing ATP concentrations upon *V*_{max} predominated and reaction velocity increased with ATP concentration.

At ATP concentrations below 80 μM, rate curves with fructose 6-phosphate concentrations in the range used (0.18–0.9mm) conformed with Michaelis-Menten kinetics. In Fig. 3 is shown a plot of 1/*V*_{max} against 1/*s* obtained under these conditions (Florini & Vestling, 1957). The apparent *K*_m values were 0.3mm for ATP and 0.15mm for fructose 6-phosphate. These values are higher than those reported for guinea-pig-heart and mouse-brain PFK (Mansour, 1963; Lowry & Passonneau, 1964). However, the assay systems employed in those studies may have contained sufficient SO₄²⁻ ion to ensure at least partial activation of the enzyme.

In Fig. 4 the relationship between fructose 6-phosphate concentration and reaction velocity is expressed in the form log(*v*/*V*_{max} - *v*) against

Fig. 2. Interrelation between the inhibition of rat-heart PFK by ATP and the concentration of fructose 6-phosphate. ○, 0.1 mm-ATP; ●, 0.2 mm-ATP; △ 0.5 mm-ATP. Experimental details were as given in the text.

Fig. 3. A Florini-Vestling plot of 1/*V*_{max} against 1/*s* for fructose 6-phosphate and ATP at non-inhibitory concentrations. ○, Values of 1/*V*_{max} at varying concentrations of fructose 6-phosphate; ●, the values with varying ATP. *K*_m for fructose 6-phosphate and ATP are 0.15mm and 0.3mm respectively.

Fig. 4. Plots of $\log(v/V_{\max} - v)$ against $\log[\text{fructose 6-phosphate}]$ at two ATP concentrations, one inhibitory (0.5 mM), the other virtually non-inhibitory (50 μM). ○, 50 μM-ATP; ●, 0.5 mM-ATP. $[\text{Mg}^{2+}]$ was constant at 5 mM; other conditions were as described in the text.

$\log[\text{fructose 6-phosphate}]$. Under conditions where the relationship conforms to Michaelis-Menten kinetics $\log(v/V_{\max} - v) = \log s - \log K_m$. At a low concentration of ATP (50 μM) the relationship was linear with a slope of 1.2 at concentrations of fructose 6-phosphate above 0.2 mM. The relationship is thus close to the predicted line of unit slope for Michaelis-Menten kinetics. At a higher concentration of ATP (0.5 mM) the slope was greater, being approx. 3.3 at low fructose 6-phosphate concentrations and 1.8 at high fructose 6-phosphate concentrations. The apparent K_m for fructose 6-phosphate was increased about threefold on raising the ATP concentration from 50 μM to 0.5 mM [calculated from $\log K_m = \log s$ when $\log(v/V_{\max} - v)$ is 0].

The effects of fructose 6-phosphate concentration on reaction velocity with inhibitory concentrations of ATP in the absence and presence of an activator of the enzyme (inorganic phosphate) are shown in Figs. 5 and 6. In the plot of reaction velocity against fructose 6-phosphate concentration at ATP concentration 0.5 mM the sigmoid character of the curve is most marked in the absence of phosphate and least marked at 2.5 mM-phosphate. In plots of $\log(v/V_{\max} - v)$ against $\log[\text{fructose 6-phosphate}]$ conformity with Michaelis-Menten kinetics was closest at high concentrations of fructose 6-phosphate and of inorganic phosphate and the lower concentration of ATP. At the higher concentration of ATP and low concentrations of fructose 6-

Fig. 5. Activation of rat-heart PFK by inorganic phosphate in the presence of ATP at an inhibitory concentration (0.5 mM). ○, No P_i; ●, 1.0 mM-P_i; ▽, 2.5 mM-P_i. $[\text{Mg}^{2+}]$ was constant at 5 mM; other conditions were as described in the text.

Fig. 6. Plots of $\log(v/V_{\max} - v)$ against $\log[\text{fructose 6-phosphate}]$ showing the activating effect of inorganic phosphate at two ATP concentrations: (a) 0.1 mM-ATP; (b) 0.5 mM-ATP. ○, No P_i; ●, 0.5 mM-P_i; △, 1.0 mM-P_i; ▲, 2.5 mM-P_i. $[\text{Mg}^{2+}]$ was constant at 5 mM; other conditions were as described in the text.

phosphate and inorganic phosphate the slope was considerably in excess of unity (between 7 and 10).

Apparent K_m values for fructose 6-phosphate with varying ATP and inorganic phosphate concentrations calculated from plots of $1/v$ against $1/s$ or $\log(v/V_{\max} - v)$ against $\log s$ are given in Table 2. Increasing ATP concentrations increased K_m whereas increasing inorganic phosphate concentrations lowered it.

Effects of citrate. In confirmation of earlier experiments with aqueous extracts of rat-heart

muscle (Garland *et al.* 1963) citrate was found to be a potent inhibitor of rat-heart PFK. Fig. 7 shows the inhibitory effect of various concentrations of citrate (0.1, 0.4 and 1.0mm) on PFK activity at two concentrations of ATP, one inhibitory (0.5mm), the other apparently non-inhibitory (50µm). At both concentrations of ATP, citrate increased K_m for fructose 6-phosphate without altering the V_{max} . (calculated from Lineweaver-Burk plots not shown). The inhibitory effect of citrate was greatest at the higher ATP concentration, indicating the synergism between ATP and citrate.

In Fig. 8 the effects of varying citrate concentration (0.01–1.2mm) on PFK activity with a constant concentration of ATP (40µm) and fructose 6-phosphate (0.36mm) are given. The concentration

of ATP used in this experiment was within the range of concentrations at which an inhibitory effect could not be detected. At low concentrations (below 0.1mm) addition of citrate led unexpectedly to an increase in reaction rate. This activating effect was reproducible and has been observed with all preparations of rat-heart PFK. The effect could not be reproduced with EDTA at concentrations up to 2mm and it is thus unlikely to be due to any known chelating action of citrate. At higher concentrations of citrate (above 0.1mm) the more characteristic inhibitory effect of citrate was seen.

The effect of citrate at two ATP concentrations (50µm and 0.5mm) on the relationship between $\log(v/V_{max} - v)$ and $\log s$ are shown in Fig. 9. With

Table 2. Effect of ATP and inorganic phosphate on K_m for fructose 6-phosphate of rat-heart phospho-fructokinase

K_m values were calculated from plots of $1/v$ against $1/s$ or of $\log(v/V_{max} - v)$ against $\log s$ with the assay procedure described for kinetic data.

Concn. of inorganic phosphate (mm)	K_m for fructose 6-phosphate (µm)		
	0.1mm-ATP	0.2mm-ATP	0.5mm-ATP
0	305	700	1300
0.5	47	176	370
1.0	34	110	190
1.5	28	49	83
2.0	22	22	69

Fig. 8. Activation and inhibition of rat-heart PFK by citrate. Concentrations of fructose 6-phosphate and ATP were 0.36 mm and 40µm respectively. Other conditions were as described in the text.

Fig. 7. Effect of citrate concentration at two ATP concentrations: (a) 50µm-ATP; (b) 0.5mm-ATP. O, No citrate; ●, 0.1mm-citrate; △, 0.4mm-citrate; ▲, 1.0mm-citrate. $[Mg^{2+}]$ was constant at 5mm; other conditions were as described in the text.

Fig. 9. Plots of $\log(v/V_{\max} - v)$ against $\log[\text{fructose 6-phosphate}]$ demonstrating inhibition by citrate at two ATP concentrations: (a) $50 \mu\text{M-ATP}$; (b) 0.5 mM-ATP . $\circ$, No citrate; $\bullet$, 0.1 mM-citrate ; $\triangle$, 0.4 mM-citrate ; $\blacktriangle$, 1.0 mM-citrate .

$50 \mu\text{M-ATP}$, citrate at 0.1 mM concentration increased the slope somewhat (from 1.2 to 1.5), but the slope returned to near unity with mM-citrate . With 0.5 mM-ATP no increase in slope was seen with 0.1 mM- and 0.4 mM-citrate . At both ATP concentrations citrate increased K_m for fructose 6-phosphate.

Effect of changing concentrations of key metabolites to simulate conditions in vivo on PFK activity. The effects of anoxia and alloxan-diabetes on the overall cell concentration of metabolites which may regulate PFK in the perfused heart has been investigated by Newsholme & Randle (1964) and Regen *et al.* (1964). The effects of respiration of fatty acids and ketone bodies have also been given by Newsholme & Randle. The two investigations have yielded similar results except that lower concentrations of inorganic phosphate were obtained by Regen *et al.* (1964) with an improved procedure employing extraction at a lower temperature. In the experiments to be described the activity of partially purified rat-heart PFK has been measured in reaction systems which simulate *in vivo* concentrations of ATP, ADP, AMP, fructose 6-phosphate, fructose 1,6-diphosphate, citrate and inorganic phosphate. The concentration of cyclic $3',5'$ -AMP was fixed at μM (as given for rat skeletal muscle by Posner, Hammermeister, Bratvold & Krebs, 1964); the effects of anoxia, diabetes or respiration of fatty acids and ketone bodies on the concentration of this nucleotide in rat heart are not known. No data are available for the concentrations of free Mg^{2+} and SO_4^{2-} in rat heart or for the effects of anoxia, diabetes, fatty acids or ketone bodies on them. The concentration of Mg^{2+}

Fig. 10. Linearity of reaction velocity with enzyme concentration in the simulated *in vivo* assay system. The reaction mixture contained: 10 mM-tris ; 125 mM-KCl ; $5 \text{ mM-magnesium acetate}$; 10 mM-ATP ; 2 mM-ADP ; 0.4 mM-AMP ; $1 \mu\text{M-cyclic } 3',5'\text{-AMP}$; $2.5 \text{ mM-sodium phosphate}$; $1.0 \text{ mM-Na}_2\text{SO}_4$; $0.4 \text{ mM-sodium citrate}$; $0.12 \text{ mM-fructose 1,6-diphosphate}$; 0.6 mM equilibrated $[\text{U-}^{14}\text{C}]\text{hexose monophosphate}$, $\text{pH } 7.4$. Other conditions were as given in the text.

was arbitrarily fixed at 5 mM . The SO_4^{2-} concentrations chosen (0.6 mM and 1 mM) are within the range of values for a number of other rat tissues given by Dietzler (1963). At these concentrations SO_4^{2-} is an activator of rat-heart PFK (see Table 3, Expt. 4).

The measurements of PFK activity were made with $[\text{U-}^{14}\text{C}]\text{glucose 6-phosphate}$ as described in the Procedure section during 10, 20 and 30 sec. periods of incubation at 37° . Rates of reaction were linear and, as shown in Fig. 10, they were also directly proportional to enzyme concentration. Although enzyme preparations were standardized by assay as described, variation in rates under given conditions were observed with different preparations of rat-heart PFK. However, with single preparations (which formed the basis for comparisons of PFK activities with different concentrations of metabolites) reaction rates were reproducible.

The results of these experiments are shown in Table 3. In Expts. 1–3 as shown system A contained metabolites at concentrations found in hearts from normal rats perfused aerobically; in system B the concentrations were changed to those found in

Table 3. *Effect of metabolite concentrations on activity of rat-heart phosphofructokinase*

PFK activity was assayed by the rate of formation of [U-¹⁴C]fructose 1,6-diphosphate during 10, 20 and 30 sec. incubation at 37° (see the Materials and Methods section). All reaction tubes contained 10 mm-tris, 5 mm-magnesium acetate, 125 mm-KCl, 2 mm-ADP, μ M-cyclic 3',5'-adenosine monophosphate, pH 7.4, together with additions as detailed.

		Concn. in reaction mixture (mm)							PFK activity (μ moles of fructose 1,6-diphosphate formed/hr./unit of enzyme)	
Expt.	System	Phosphate	Sulphate	ATP	AMP	Citrate	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate	
1	A	7.5	1	10	0.4	0.4	0.42	0.18	0.12	49
	B	15	1	6	0.8	0.4	0.21	0.09	0.20	39
	C	7.5	1	10	0.4	1.6	0.57	0.23	0.06	29
2	A	2.5	1	10	0.4	0.4	0.42	0.18	0.12	3.5
	B	6.0	1	6	0.8	0.4	0.21	0.09	0.20	19.9
	C	2.5	1	10	0.4	1.6	0.57	0.23	0.06	2.9
3	A	2.5	0.6	10	0.4	0.4	0.42	0.18	0.12	3.6
	B	6.0	0.6	6	0.8	0.4	0.21	0.09	0.20	18.9
	C	2.5	0.6	10	0.4	1.6	0.57	0.23	0.06	0.9
4		3.0	0.5	10	0.4	0.4	0.42	0.18	0.12	2.6
		3.0	1.0							6.2
		5.0	0.5							9.4
		5.0	1.0							12.7

anaerobic perfusion; in system C concentrations corresponded to those seen in hearts perfused with fatty acids or ketone bodies or with hearts from alloxan-diabetic rats. In Expt. 1 the phosphate concentrations correspond to those found by Newsholme & Randle (1964) and in Expts. 2 and 3 lower phosphate concentrations similar to those found by Regen *et al.* (1964) were employed. In Expt. 3 the sulphate concentration was reduced as compared with Expts. 1 and 2.

In Expt. 1, system A, the rate of the PFK reaction was equivalent to a glycolytic flow of > 600 μ moles of glucose/g. of wet heart/hr. (assays have shown that 1 g. of heart yields 12–15 units of PFK by extraction). This value is considerably in excess of the calculated flow in the perfused heart under aerobic conditions (60–84 μ moles of glucose/g. of wet heart/hr.). When the concentrations of ATP, AMP, phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were manipulated to simulate anaerobic conditions the expected increase in the rate of the PFK reaction was not seen (system B). However, changing the concentrations of citrate, fructose 6-phosphate and fructose 1,6-diphosphate to simulate conditions observed in diabetes or with hearts perfused with fatty acids or ketone bodies diminished the rate of the PFK reaction by about 40%.

In Expt. 2, system A, the rate of the PFK reaction was equivalent to a glycolytic flow of > 42 μ moles of glucose/g. of wet heart/hr. This rate is of the same order of magnitude as that seen in the perfused heart under aerobic conditions (60–

84 μ moles of glucose/g. of wet heart/hr.). In system B changes in metabolite concentrations corresponding to those seen in anaerobic perfusion led to a five- to six-fold increase in rate. In system C changes in metabolite concentrations corresponding to those seen in the perfused heart with fatty acids, ketone bodies or diabetes led to a 25% reduction in rate. In Expt. 3, employing a lower sulphate concentration, similar manipulations in the concentrations of other metabolites led to a five- to six-fold increase in rate in system B and to a 75% reduction in rate in system C.

In Expts. 2B and 3B, simulating effects of anoxia in the perfused heart, lowering ATP and increasing AMP, phosphate and fructose 1,6-diphosphate concentrations increased PFK activity in spite of the lowered fructose 6-phosphate concentration. In Expts. 2C and 3C, simulating effects of diabetes, fatty acids and ketone bodies, PFK activity was lowered by raising citrate and lowering fructose 1,6-diphosphate concentrations in spite of the increased fructose 6-phosphate concentration.

DISCUSSION

Assay of phosphofructokinase. A number of procedures have been described for the assay of PFK; these fall basically into two groups, namely those measuring pH changes manometrically (Totton & Lardy, 1949) or potentiometrically (Dyson & Noltmann, 1965) and those measuring one or other of the products of the reaction with enzymes (e.g. Racker, 1947; Passonneau & Lowry,

1962; Mansour, 1963). Published methods based on pH changes appear to be less sensitive than those based on assay of products. In this study we have utilized the more sensitive spectrophotometric method in which fructose 1,6-diphosphate is converted into α -glycerophosphate with the concomitant oxidation of two equivalents of NADH. We have also developed a new method based on the conversion of $[U-^{14}C]$ fructose 6-phosphate into fructose 1,6-diphosphate.

Since both products of the PFK reaction are activators of the enzyme it is particularly important in kinetic studies to measure rates without significant accumulation of products. In the spectrophotometric method three enzyme-catalysed reactions are involved and precautions are necessary to ensure that product accumulation does not invalidate rate measurements (e.g. see Gutfreund, 1965). In an attempt to overcome this objection we have measured initial velocities by a process of extrapolation and the adequacy of rates in the following reactions has been verified by addition of fructose 1,6-diphosphate. With this procedure no anomalous effect of varying enzyme concentration (as reported for liver PFK by Underwood & Newsholme, 1965) was seen. This finding agrees with results reported for PFK from *Escherichia coli* by Atkinson & Walton (1965).

The new method employing $[U-^{14}C]$ fructose 6-phosphate is more time-consuming than the spectrophotometric method but it has the advantage that the rate of the forward reaction may be measured at known concentrations of products. With the short period of incubation used (up to 30 sec.) progress curves were linear and rates were proportional to enzyme concentration. As described the method can accurately detect rates of reaction which increase the concentration of products less than 5%.

Regulation of PFK in the perfused rat heart. The limitations of experiments in which PFK activity was measured at metabolite concentrations simulating those found in the perfused heart have not been overlooked. In particular the concentration of enzyme in the tissue may be different and the possibility of compartmentation of metabolites is recognized. Nevertheless the results show that changes in PFK activity which simulate those seen in the perfused heart with anoxia, with fatty acids or ketone bodies and in diabetes, may be induced with the isolated enzyme by manipulating concentrations of substrates, products, AMP, phosphate and citrate. The findings are consistent with the proposals that changes in the concentrations of adenine nucleotides and phosphate are responsible for the activation of PFK and glycolysis by anoxia (Passeau & Lowry, 1962) and that an increase in the concentration of citrate may mediate inhibitory

effects of fatty acids, ketone bodies and diabetes (Garland *et al.* 1963; Parmeggiani & Bowman, 1963).

The kinetic properties of PFK can also account for the increased glycolytic flow induced in rat heart by insulin. When ATP is present at inhibitory concentrations, increasing concentrations of fructose 6-phosphate may accelerate the PFK reaction both by reversal of ATP inhibition and through action at the substrate site. Insulin action leads to a marked increase in fructose 6-phosphate concentration in the heart presumably through acceleration of glucose transport (Newsholme & Randle, 1961, 1964). Other factors which may be important to effects of insulin on glycolytic flow are the increase in fructose 1,6-diphosphate concentration (Newsholme & Randle, 1961, 1964) and increased uptake of inorganic phosphate (Kaji & Park, 1961).

The possible importance of sulphate as a regulator of PFK requires further investigation. The present studies have confirmed the earlier finding (Garland *et al.* 1963) that sulphate is a potent activator of PFK but information concerning the concentration of free sulphate in rat heart under different conditions is not available.

Mechanism of PFK regulation. The sigmoid curves given by plots of reaction rate against fructose 6-phosphate concentration for rat-heart PFK are similar to those obtained with a number of regulatory enzymes including PFK from other sources (e.g. Changeux, 1964; Ling, Marcus & Lardy, 1965). Slopes in excess of unity in plots of $\log(v/V_{\max} - v)$ against $\log s$, which may provide a more sensitive method of detecting sigmoid rate curves (Atkinson, Hathaway & Smith, 1965), have similarly been described for regulatory enzymes. These kinetic findings have been variously interpreted in the light of hypotheses propounding induced fit (Koshland, 1958, 1963, 1964), allosteric transition (Monod, Changeux & Jacob, 1963) and, more recently, kinetic preference (Ferdinand, 1966). In the absence of direct evidence for specific regulatory sites or for changes in conformation few conclusions from kinetic data alone concerning regulatory mechanisms for rat-heart PFK seem justifiable.

At non-inhibitory concentrations of ATP, rate curves for fructose 6-phosphate [as $\log(v/V_{\max} - v)$ against $\log s$] suggest that reaction is first-order with respect to fructose 6-phosphate and therefore that co-operative effects are absent or minimal. With inhibitory concentrations of ATP the apparent order of reaction is considerably in excess of unity at concentrations of fructose 6-phosphate below its K_m ; but at concentrations of fructose 6-phosphate above its K_m first-order kinetics are approached. These findings suggest that fructose 6-phosphate is not itself an activator but that it antagonizes inhibitory effects of ATP. One explanation for

these findings is that there are one or more regulatory sites at which ATP is inhibitory and that fructose 6-phosphate interferes with the action of ATP at these sites. Citrate, which was also inhibitory, did not markedly increase the apparent kinetic order of reaction for fructose 6-phosphate and higher concentrations of citrate may have lowered it. One interpretation of these findings is that citrate may stabilize the enzyme in an ATP-inhibited form at a site or sites distinct from those at which ATP and fructose 6-phosphate exert regulatory effects.

Inorganic phosphate which activated PFK at inhibitory concentrations of ATP did not change the apparent kinetic order for fructose 6-phosphate. Its activating effect thus differed from that of fructose 6-phosphate which diminished the apparent kinetic order at concentrations above its K_m . The major effect of phosphate appeared to be to lower the concentration of fructose 6-phosphate required for first-order kinetics.

The kinetic properties of rat-heart PFK correlate well with the behaviour of the enzyme in the perfused heart with which this investigation has been particularly concerned. The interactions between ATP and fructose 6-phosphate concentrations provide for the controlling importance of the latter in the normal heart perfused with glucose under aerobic conditions and may contribute to the action of insulin on glycolytic flow as mentioned above. The effect of phosphate on the interaction between ATP and fructose 6-phosphate concentrations may be correlated with the increased glycolytic flow achieved in the perfused heart under anaerobic conditions at lower fructose 6-phosphate concentrations. Lastly the inhibitory effect of citrate which appears to prevent fructose 6-phosphate from reversing ATP inhibition may be correlated with its inhibitory effect in the diabetic heart or the heart perfused with ketone bodies or fatty acids under aerobic conditions where glycolytic flow is diminished at elevated fructose 6-phosphate concentrations.

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