Effectors of Rat-Heart Hexokinases and the Control of Rates of Glucose Phosphorylation in the Perfused Rat Heart

By P. J. ENGLAND AND P. J. RANDLE
Department of Biochemistry, University of Bristol

(Received 14 June 1967)

1. The kinetic properties of the soluble and particulate hexokinases from rat heart have been investigated. 2. For both forms of the enzyme, the K_m for glucose was $45\ \mu\text{M}$ and the K_m for ATP 0.5 mM . Glucose 6-phosphate was a non-competitive inhibitor with respect to glucose (K_i 0.16 mM for the soluble and 0.33 mM for the particulate enzyme) and a mixed inhibitor with respect to ATP (K_i $80\ \mu\text{M}$ for the soluble and $40\ \mu\text{M}$ for the particulate enzyme). ADP and AMP were competitive inhibitors with respect to ATP (K_i for ADP was 0.68 mM for the soluble and 0.60 mM for the particulate enzyme; K_i for AMP was 0.37 mM for the soluble and 0.16 mM for the particulate enzyme). P_i reversed glucose 6-phosphate inhibition with both forms at 10 mM but not at 2 mM , with glucose 6-phosphate concentrations of 0.3 mM or less for the soluble and 1 mM or less for the particulate enzyme. 3. The total activity of hexokinase in normal hearts and in hearts from alloxan-diabetic rats was $21.5\ \mu\text{moles}$ of glucose phosphorylated/min./g. dry wt. of ventricle at 25° . The temperature coefficient Q_{10} between 22° and 38.5° was 1.93 ; the ratio of the soluble to the particulate enzyme was $3:7$. 4. The kinetic data have been used to predict rates of glucose phosphorylation in the perfused heart at saturating concentrations of glucose from measured concentrations of ATP, glucose 6-phosphate, ADP and AMP. These have been compared with the rates of glucose phosphorylation measured with precision in a small-volume recirculation perfusion apparatus, which is described. The correlation between predicted and measured rates was highly significant and their ratio was 1.07 . 5. These findings are consistent with the control of glucose phosphorylation in the perfused heart by glucose 6-phosphate concentration, subject to certain assumptions that are discussed in detail.

A number of mechanisms have been suggested for the regulation of hexokinases (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1) in various animal tissues, quite apart from the possibility of control by the intracellular concentrations of glucose and ATP. These include: regulation by the concentrations of glucose 6-phosphate, ADP and AMP, which may act as inhibitors (Weil-Malherbe & Bone, 1951; Crane & Sols, 1955), and by the concentration of P_i , which may be an activator (Tiedemann & Born, 1959); regulation by the concentration of enzyme (DiPietro & Weinhouse, 1960; Niemeyer, Clark-Turri, Garcés & Vergara, 1962; Viñuela, Salas & Sols, 1963; Walker & Rao, 1964; Hernandez & Sols, 1963; Borrebaek, 1966; McLean, Brown, Greenslade & Brew, 1966); regulation by the proportions of soluble and particulate hexokinases (Hernandez & Crane, 1966); and regulation by the proportions of isoenzymes of hexokinases (Katzen, 1966; Grossbard & Schimke, 1966).

In the perfused rat heart the uptake of glucose is

believed to involve, in sequence, transport of the sugar across the cell membrane and then intracellular phosphorylation to glucose 6-phosphate, catalysed by hexokinases. Other possible methods of glucose phosphorylation, such as that proposed by Smith, Taylor & Whelan (1967) to account for the high rate of synthesis of glycogen from glucose in skeletal muscle, have not been excluded in the perfused heart, but glycogen is not the major end product of glucose metabolism in this tissue (Regen, Davis, Morgan & Park, 1964; Williamson, 1965). In the present paper it has been assumed that the major pathway of glucose phosphorylation in the perfused heart is through hexokinase. Some experimental support for this assumption is afforded by the observation that the glucose 6-phosphate pool in the heart equilibrates rapidly with [^{14}C]glucose added to the perfusion medium (within 4 min.; R. M. Denton, P. J. England & P. J. Randle, unpublished work).

In the perfused rat heart, the rate of intracellular

phosphorylation of glucose may control glucose uptake when the perfusion medium contains glucose at a concentration above approx. 6mM and insulin (Morgan, Henderson, Regen & Park, 1961b). Alterations in the rate of glucose phosphorylation that have been attributed to changes in hexokinase activity in the perfused heart are seen in alloxan-diabetes (Morgan, Cadenas, Regen & Park, 1961a), in starvation (Newsholme & Randle, 1961), during anaerobic perfusion (Morgan, Randle & Regen, 1959) and during perfusion with ketone bodies, fatty acids and pyruvate (Newsholme Randle & Manchester, 1962). The mechanism of these changes in glucose-phosphorylation rate is not established, though it has been suggested that inhibition or activation of hexokinase by alterations in the intracellular concentration of glucose 6-phosphate, as a consequence of activation or inhibition of phosphofructokinase, are involved.

In the present study the kinetics of soluble and particulate hexokinases from rat heart have been investigated, and a number of metabolites tested as inhibitors. The data have been used to predict rates of phosphorylation of glucose in the perfused heart, and these have been compared with rates measured in a small-volume recirculating perfusion apparatus developed for this purpose.

MATERIALS

Rats. Hearts were obtained from male albino Wistar rats (each rat weighed 250–400g.) fed on stock laboratory diet 41B (Short & Parkes, 1949). Unless otherwise stated, the animals were fed until the time of the experiment. Alloxan-diabetes was induced as described by Newsholme & Randle (1964).

Chemicals and enzymes. Glycolytic intermediates, adenine nucleotides, coenzymes, UDP-glucose, triethanolamine hydrochloride and all enzymes were from C. F. Boehringer and Sohne G.m.b.H., Mannheim, Germany. 3',5'-(Cyclic)-AMP and tris were from Sigma Chemical Co., St Louis, Mo., U.S.A. Sodium DL-3-hydroxybutyrate, sodium 6-phosphogluconate, D-glucose, malate, citrate, glucosamine 6-phosphate and 1,4-bis-(5-phenyloxazol-2-yl)-benzene were from British Drug Houses Ltd., Poole, Dorset. 2,5-Diphenyloxazole was from Thorn Electronics, Tolworth, Surbiton, Surrey. D-[U-¹⁴C]Sorbitol, [U-¹⁴C]sucrose, D-[U-¹⁴C]glucose and [³H]water were from The Radiochemical Centre, Amersham, Bucks. Dowex 1 (AG1-X4; Cl⁻ form; 200–400 mesh) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and was converted into the formate form, as described by Denton & Randle (1967). Alloxan was from Eastman-Kodak Co., Rochester, N.Y., U.S.A. Crystalline insulin was a gift from Boots Pure Drug Co. Ltd., Nottingham, Burroughs Wellcome and Co., Beckenham, Kent, or Novo Terapeutisk Laboratorium, Copenhagen, Denmark; the hormone was dissolved in 3mN-HCl to give a stock solution of 20 i.u./ml., which was stored at –20°. Acetyl-CoA, palmityl-CoA and acetyl-DL-carnitine were given by Mr D. Nicholls, and UDP-N-acetylglucosamine and N-acetylglucosamine 6-phosphate by Dr M. Luscombe.

DEAE-cellulose. DEAE-Cellulose (Whatman DE-11; W. and R. Balston Ltd., Maidstone, Kent) was washed five times with 5 vol. of water, the fines being sucked off each time, and then washed twice with 5 vol. of each of the following: 5mN-NaOH; 5mN-HCl; and water. It was then suspended in 50mM-tris, adjusted by a glass electrode to approx. pH 7 with conc. HCl, and stored at 4°.

METHODS AND PROCEDURE

Preparation of rat heart hexokinase. Rats (usually in batches of five) were killed by decapitation, and the ventricle muscle was excised, pressed between filter paper to remove blood and weighed. All subsequent operations were carried out at 0–4°. The ventricles were cut into small pieces in 25ml. of buffer, the buffers used being: for the soluble enzyme, 50mM-tris-chloride, pH 8.0, containing MgCl₂ (10mM), glucose (10mM) and EDTA (2mM); for the particulate enzyme, 20mM-tris-chloride, pH 7.5, containing KCl (0.115M), MgCl₂ (10mM) and EDTA (2mM). The wash liquid was decanted and the pieces of ventricle were homogenized for 1min. in buffer (3ml./g.) in a Sorvall Omni-Mixer at full speed.

To separate particulate hexokinase, the homogenate was centrifuged for 5min. at 500g (MSE 18 centrifuge; 500g was attained in 15sec.), and the supernatant was centrifuged at 20000g for 20min. The pellet (containing particulate hexokinase) was washed twice in the same buffer by resuspending and centrifuging, and finally resuspended in 15ml. of 0.1M-triethanolamine-chloride, pH 7.7, containing MgSO₄ (10mM).

To separate soluble hexokinase, the homogenate was centrifuged at 40000g for 30min. The soluble hexokinase was further purified by layering the supernatant on to a fast-flowing (5–8ml./min.) column of DEAE-cellulose (8cm. × 2.5cm.), previously equilibrated with the buffer used for extracting the enzyme. The column was rapidly washed with 100ml. of the same buffer, and hexokinase was then eluted at a rate of 5–8ml./min. with 0.1M-tris-chloride, pH 8.0, containing MgCl₂ (10mM), glucose (10mM) and EDTA (2mM). Most of the hexokinase eluted was collected in two 10ml. fractions between elution volumes of 40ml. and 60ml. Further purification was achieved by fractionation with (NH₄)₂SO₄, the enzyme being precipitated between 40 and 60% saturation. The enzyme was redissolved either in 50mM-tris-chloride, pH 8.0, containing MgCl₂ (10mM), glucose (10mM) and EDTA (2mM), or in 0.1M-triethanolamine-chloride, pH 7.7, containing MgSO₄ (10mM).

Activity and stability. Details of a typical partial purification of soluble hexokinase are given in Table 1. The final product had a specific activity of 0.4 unit/mg. of protein (for definition of the enzyme unit, see below) and was essentially free of adenosine triphosphatase, adenylate kinase, phosphoglucomutase, phosphohexoisomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. The particulate enzyme contained considerable adenosine triphosphatase activity (twice that of hexokinase), but was free of the other enzymes listed above. The particulate enzyme could be stored at 0–4°, the loss of activity after 1 week being 20%. No solubilization was detected during this time. The soluble enzyme was stable at 0° in the presence of glucose, but lost 80% of its activity in 24hr. when stored without glucose.

Table 1. *A typical purification of soluble hexokinase from 5g. of rat heart*

For further details see the Methods and Procedure section.

Purification step	Volume (ml.)	Protein (mg.)	Enzyme activities (units at 25°)				Purification factor	Yield (%)
			Hexokinase	Adenosine triphosphatase	Adenylate kinase	Phosphohexoisomerase		
Original homogenate	20	750	20	150	—	—	1	100
40000g supernatant	15	150	6	120	—	—	1.5	30
DEAE-cellulose eluent	20	12	1.5	0.1	5	2	6	7.5
40–60% -satd. (NH ₄) ₂ SO ₄ fraction	2	3	1.2	0.01	0	0	15	6

Because of its influence on stability, glucose was included in the buffers used for DEAE-cellulose chromatography, where it had the additional effect of increasing the concentration of tris-chloride needed to elute the enzyme from less than 50mm to 0.1M.

Protein estimations. These were made by the method of Oyama & Eagle (1956).

Assay of hexokinase activity

Spectrophotometric assays. These were carried out at 25° in a Hilger spectrophotometer fitted with a Gilford absorbance converter and recorder, at 340m μ , with a 1cm. light path and a reaction volume of 3ml. Unless otherwise stated the buffer was 0.1M-triethanolamine-chloride, pH 7.7, containing MgSO₄ (20mm). In the first assay system, the glucose 6-phosphate formed was oxidized to 6-phosphogluconate, with reduction of NADP. The reaction mixture contained, in 3ml., 0.6 unit of glucose 6-phosphate dehydrogenase and 0.3 μ mole of NADP. In the second assay, the ADP formed was converted into ATP with phosphoenolpyruvate and the pyruvate formed reduced to lactate, with concomitant oxidation of NADH₂. The reaction mixture contained 0.5 unit each of pyruvate kinase and lactate dehydrogenase, 6 μ moles of phosphoenolpyruvate and 0.2–0.3 μ mole of NADH₂. Corrections were applied for adenosine triphosphatase activity, and any ADP present in the ATP was converted into ATP immediately before the addition of the hexokinase preparations.

Assays of particulate and soluble hexokinase were made with samples containing 2.5×10^{-3} – 1×10^{-2} unit/cuvette, the reaction rate being proportional to the enzyme concentration. After an initial lag phase of 0.5 min., the rates were linear for at least 4 min. except at very low substrate concentrations (<0.05mm-glucose and <0.1mm-ATP).

Radioactivity assay. This assay was developed for use with the particulate enzyme, where the higher adenosine triphosphatase activity precluded the use of the spectrophotometric assay based on ADP production in studies of glucose 6-phosphate inhibition. The [U-¹⁴C]glucose 6-phosphate formed from [U-¹⁴C]glucose in the hexokinase reaction was separated from the glucose remaining, and assayed by liquid-scintillation spectrometry. The incubation medium was 0.1M-triethanolamine-chloride, pH 7.7, containing MgSO₄ (20mm), [U-¹⁴C]glucose (specific radioactivity 1 μ C/ μ mole), ATP, and phosphoenolpyruvate

(5mm) and 0.5 unit of pyruvate kinase to regenerate ATP (except when ADP was added to the incubation). The incubations, in a final volume of 1 ml., were made in 15 ml. centrifuge tubes at 25°, and the reaction was started by the addition of approx. 10^{-2} unit of hexokinase. The reaction was stopped after 2 min. by the addition of 0.07 ml. of 70% (w/v) HClO₄. After centrifugation, 0.9 ml. of supernatant was separated and neutralized with 0.25 ml. of saturated KHCO₃ solution and, after the mixture had been cooled to 0°, the KClO₄ was removed by centrifugation. Glucose 6-phosphate was separated from glucose by the following method. The supernatant (0.9 ml.) was applied to a small column (6 cm. $\times$ 0.3 cm.) of Dowex 1 (formate form), and glucose and other radioactive contaminants were eluted with 20 ml. of water and 20 ml. of N-formic acid. Glucose 6-phosphate was then eluted in a volume of 4 ml. with ammonium formate, pH 4.0, and this solution was assayed for radioactivity by addition of 1 ml. of it to 15 ml. of dioxan scintillator (Butler, 1961) containing 2 ml. of 6N-formic acid to prevent cloudiness. The samples were assayed in a Nuclear-Chicago liquid-scintillation spectrometer, at least 5000 counts being recorded. Quenching corrections were applied by using the channels-ratio method.

The recovery of glucose 6-phosphate was 96–100%, and the separation from glucose was complete. Samples were taken at zero time of incubation and the radioactivity assayed was subtracted from the radioactivity assayed after incubation. The zero-time radioactivity varied from 2% to 30%, depending on the amount of glucose 6-phosphate formed. With these conditions of assay, the reaction rate was constant for 6 min. and the concentration of glucose 6-phosphate produced did not exceed 25 μ M after 2 min. of incubation. Each assay was carried out in duplicate.

Total hexokinase activity. The heart, freed of obvious fat, was minced and homogenized as described for the preparation of the particulate enzyme, but with 10 ml. of the same buffer/g. of muscle. In some experiments the buffer contained 2-mercaptoethanol (2mm). Hexokinase activity was assayed spectrophotometrically by glucose 6-phosphate dehydrogenase with ATP (10mm), glucose (3mm), Mg²⁺ (20mm) and a volume of extract equivalent to 2.5×10^{-3} – 10×10^{-3} unit of hexokinase. The rate of the reaction was directly proportional to the volume of homogenate used, and was constant for 4 min. Measurements of rate were made between 1 and 4 min.

Assays for contaminating enzymes

Adenosine triphosphatase activity was assayed spectrophotometrically, ADP production from ATP being measured as described for the assay of hexokinase but without glucose. Adenylate kinase was assayed by the rate of ATP formation from ADP with glucose (1 mM), ADP (2 mM) and glucose 6-phosphate dehydrogenase (0.5 unit), with the further addition of yeast hexokinase (0.5 unit). Phosphoglucosomerase and phosphoglucomutase were assayed by glucose 6-phosphate dehydrogenase with fructose 6-phosphate or glucose 1-phosphate as substrate. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed by the reduction of NADP, with glucose 6-phosphate (2 mM) and 6-phosphogluconate (5 mM).

Enzyme unit. One unit of enzyme activity is defined as the amount of enzyme catalysing the transformation of 1 μ mole of substrate/min. to products at the temperature stated.

Heart perfusions and analyses

For measurements of the rates of glucose phosphorylation in the intact heart, perfusions were carried out with the recirculating apparatus of Morgan *et al.* (1961*b*). Since precise estimates of the rate of glucose phosphorylation and of alterations in the rate were required, the apparatus was modified so that the disappearance of glucose from the perfusion medium could be measured at intervals of 1–3 min. The modifications to the bubble trap and the perfusion chamber shown in Fig. 1 enabled satisfactory perfusion to be made with as little as 5 ml. of medium. The hearts were attached to a cannula fitted with a two-way tap and pre-perfused by drip-through with medium containing glucose at the same concentrations as that used during the subsequent perfusion. They were then switched to recycling, and, as soon as the perfusion pressure had stabilized at 35–45 mm. Hg (0.5 min.), a sample of medium (50 μ l.) was taken for analysis. Further samples were then taken from the chamber at suitable intervals (1–3 min.). With such small volumes of perfusion medium, mixing is rapid, since the whole of the perfusion medium is passed through the heart three or four times each minute.

The hearts were removed for perfusion as described by Newsholme & Randle (1964) and the medium used was the bicarbonate-buffered medium of Krebs & Henseleit (1932), gassed with O₂+CO₂ (95:5) or N₂+CO₂ (95:5) and containing calcium EDTA (0.2 mM). [U-¹⁴C]Sorbitol was added to the perfusion medium to enable the total volume of perfusion medium to be calculated from the dilution of radioactivity (some medium is carried over in the heart from preperfusion).

At the end of the perfusion the hearts were trimmed free of fat, blotted and weighed, or, if required for analysis, frozen with the tissue clamp of Wollenberger, Ristau & Schoffa (1960) and powdered and extracted with HClO₄ as described by Denton & Randle (1967). Glucose 6-phosphate was assayed spectrophotometrically in muscle extracts as described by Newsholme & Randle (1964), but with a 4 cm. light-path cuvette and 0.3–0.5 ml. of extract. Glucose was assayed in the muscle extracts and in the perfusion medium as glucose 6-phosphate with 3 mM-ATP and 1 unit of yeast hexokinase. ATP was assayed in muscle extracts as glucose 6-phosphate with 2 mM-glucose and

Fig. 1. Diagram of the small-volume recirculating perfusion apparatus. A, Sintered-glass disk, porosity 1; B, gas supply; C, perfusion chamber; D, roller pump; E, bubble trap; F, manometer; G, water jackets, water temp. 37°.

1 unit of yeast hexokinase. ADP and AMP were assayed as described by Newsholme & Randle (1964). [U-¹⁴C]-Sorbitol was assayed in dioxan scintillator. Total water, glucose and sorbitol spaces were calculated as described by Morgan *et al.* (1959).

Mitochondrial space determinations

Mitochondria were prepared from approx. 6 g. of rat ventricles by the method of Chance & Hagihara (1961), and the mitochondrial pellet was suspended in 12 ml. of 20 mM-tris-chloride-40 mM-methylamine-chloride (as penetrant cation), pH 7.4, containing KCl (120 mM). Conical centrifuge tubes (15 ml.) were coated with a layer of Repelcote (Hopkin and Williams Ltd., Chadwell Heath, Essex), and set up containing three layers. The bottom layer was 1 ml. of 30% (w/v) glycerol containing 7% (w/v) HClO₄ (sp.gr. 1.09), the middle layer was 1.5 ml. of silicone oil (sp.gr. 1.03), and the top layer was 2 ml. of mitochondria suspension containing [³H]water (1.0 μ C/ml.), [U-¹⁴C]-sucrose (2 mM, 0.2 μ C/ml.) and glucose 6-phosphate (2 mM). After the requisite time at 25°, the tubes were centrifuged

for 5 min. at 2000g and the mitochondria, substantially free of extramitochondrial water, passed to the bottom layer (Werkheiser & Bartley, 1957). The top layer was deproteinized with 0.1 ml. of 70% (w/v) HClO_4 , and the top and bottom layers were brought to pH 6.8–7.4 with saturated KHCO_3 solution (narrow-range indicator papers, pH 5.4–7.0 and pH 7.0–8.5). After separation of KClO_4 , glucose 6-phosphate was assayed spectrophotometrically, and $[^3\text{H}]$ water and $[\text{U-}^{14}\text{C}]$ sucrose were assayed in dioxan scintillator. Quenching corrections were applied with an external standard and the proportions of the two isotopes were calculated as described by Hendler (1963).

RESULTS

Total hexokinase activity. The total hexokinase activities of hearts from normal and alloxan-diabetic rats were identical and were not influenced by the inclusion of 2-mercaptoethanol in the extraction fluid. The value measured at 25° was in each instance 21.5 ± 1.0 units/g. dry wt. of ventricle (mean $\pm$ S.E.M. for ten normal hearts and six hearts from diabetic rats). The temperature coefficient Q_{10} between 22° and 38.5° was 1.93, and over this range temperature and hexokinase activity were linearly related. No significant differences between normal and diabetic rats were detected in the proportion of soluble enzyme in hearts extracted either with 50 mM-tris-chloride, pH 8.0, containing glucose (10 mM), magnesium chloride (10 mM) and EDTA (2 mM), or with 20 mM-tris-chloride, pH 7.5, containing potassium chloride (0.115 M), magnesium chloride (10 mM) and EDTA (2 mM), with or without 2-mercaptoethanol (2 mM). The soluble enzyme accounted for $29 \pm 2\%$ of the total activity in the normal hearts and for $31 \pm 3\%$ in the hearts from diabetic rats (mean $\pm$ S.E.M. for ten normal hearts and six hearts from diabetic rats). Starch-gel electrophoresis of soluble hexokinase in the 40 000g supernatant of normal rat-heart extracts, under the conditions for electrophoresis described by Katzen, Soderman & Nitowsky (1965), showed two bands corresponding to type I and type II hexokinases

(Katzen & Schimke, 1965). The intensity of staining suggested an excess of type I. An identical pattern was seen after starch-gel electrophoresis of particulate enzyme solubilized with 0.2 M-magnesium chloride in 20 mM-tris-chloride, pH 7.7, containing potassium chloride (0.115 M) and EDTA (2 mM) and separated by centrifugation at 100 000g for 30 min.

Kinetic data for soluble and particulate hexokinases

The data described in this section are summarized in Table 2.

Effects of pH and substrate concentration. With buffers containing Mg^{2+} (20 mM), glucose (5 mM) and ATP (3 mM), and with the spectrophotometric assay by glucose 6-phosphate dehydrogenase in excess, the pH optimum of both forms of hexokinase was broad, and lay between pH 7.2 and pH 8.8. (The buffers used were: 0.1 M-citrate-potassium hydroxide, pH 5.0; 0.1 M-potassium phosphate, pH 6.0 and pH 6.5; 0.1 M-triethanolamine-chloride, pH 7.0 and pH 7.5; 0.1 M-tris-chloride, pH 8.0 and pH 8.5; 0.1 M-glycine-potassium hydroxide, pH 9.0 and pH 10.0). Both forms of the enzyme required the addition of Mg^{2+} for activity, and with 2 mM- or 4 mM-ATP and 2 mM-glucose maximum rates were attained with Mg^{2+} concentrations of 3 mM or greater. There was no change in activity when the Mg^{2+} concentration was raised to 6–20 mM.

Both forms of the enzyme had K_m values of 45 μM for glucose and 0.5 mM for ATP. These were obtained from reciprocal plots (Lineweaver & Burk, 1934) with 5 mM-ATP and various glucose concentrations (range 0.025–1.0 mM) and 2 mM-glucose with various ATP concentrations (range 0.1–5.0 mM). These K_m values were obtained with a fixed Mg^{2+} concentration of 20 mM; identical values were obtained with $[\text{ATP}]/[\text{Mg}^{2+}]$ ratios of 1:1.

Effects of ADP, AMP and P_i . These were investigated spectrophotometrically with excess of glucose 6-phosphate dehydrogenase activity. ADP

Table 2. *Kinetic data for soluble and particulate rat heart hexokinases*

Details of the assays and calculations are given in the text.

Enzyme	Activity at 25° (units/ g. dry wt.)	K_m (glucose) (mM)	K_m (ATP) (mM)	K_i (ADP) versus ATP (mM)	K_i (AMP) versus ATP (mM)	K_i (glucose 6- phosphate) versus ATP (mM)	K_i (glucose 6- phosphate) versus glucose (mM)
Soluble	6.5	0.045	0.5	0.68	0.37	0.08†	0.16†
Particulate	15.0	0.045	0.5	0.60	0.16	0.04†	0.33†
Type I*		0.045	0.42	0.62		0.026	0.21
Type II*		0.25	0.75	0.22		0.021	0.16

* Data of Grossbard & Schimke (1966) given for comparison.

† Mixed inhibition.

‡ Non-competitive inhibition.

and AMP were competitive inhibitors with respect to ATP for both forms of the enzyme. The values of K_i were 0.68 mM (ADP) and 0.37 mM (AMP) for the soluble enzyme, and 0.6 mM (ADP) and 0.16 mM (AMP) for the particulate enzyme. These values of K_i were obtained from the intersection of straight lines relating $1/v$ and inhibitor concentration at two ATP concentrations (0.5 mM and 2 mM), as described by Dixon (1953). The concentration of glucose was 2 mM and the concentration ranges for ADP and AMP were 0–2 mM. With the particulate enzyme (but not the soluble enzyme), the K_m value for glucose was decreased by AMP concentrations of 0.2 mM and above, from 45 μ M in the absence of AMP to 25 μ M with AMP. The concentration of ATP was 5 mM and the range of glucose concentration was 0.025–1.0 mM. P_i (7 mM or 15 mM) had no effect on the activities of soluble or particulate hexokinase in the presence or absence of AMP.

These K_i values for ADP and AMP were obtained with a fixed Mg^{2+} concentration of 20 mM; identical values for ADP inhibition were obtained with $[ATP + ADP]/[Mg^{2+}]$ ratios of 1:1.

Inhibition of soluble hexokinase by glucose 6-phosphate. This was investigated spectrophotometrically by measuring ADP production. The range of glucose 6-phosphate concentrations (up to 1.5 mM) and ATP concentrations (5 mM or 10 mM) encompass those found in the perfused heart under a wide variety of conditions (Newsholme & Randle, 1964).

Fig. 2. Reciprocal plot of reaction velocity and ATP concentration at different glucose 6-phosphate concentrations for soluble hexokinase from rat heart. The reaction velocity was measured spectrophotometrically as ADP production with 2 mM-glucose and 20 mM- Mg^{2+} at various ATP concentrations as shown. ▲, No glucose 6-phosphate; ●, 0.3 mM-glucose 6-phosphate; ○, 0.7 mM-glucose 6-phosphate. The lines were fitted by the method of Wilkinson (1961).

Fig. 3. Derivation of K_i for glucose 6-phosphate (with respect to ATP) from a plot of the reciprocal of reaction velocity and the concentration of glucose 6-phosphate at different ATP concentrations. Reaction velocity was measured spectrophotometrically as ADP production with 2 mM-glucose and 20 mM- Mg^{2+} at various glucose 6-phosphate concentrations as shown. Δ, 1 mM-ATP; ●, 2 mM-ATP; □, 5 mM-ATP; ○, 10 mM-ATP.

Glucose 6-phosphate was a non-competitive inhibitor with respect to glucose, the K_i value being 0.16 mM [the K_i value was calculated from the change in the V_{max} value induced by 0.3 mM-glucose 6-phosphate containing ATP (5 mM) and glucose concentrations in the range 0.02–0.20 mM]. Glucose 6-phosphate was a mixed inhibitor with respect to ATP (see Fig. 2); the K_i value was 80 μ M (calculated from the intersection of straight lines relating $1/v$ and inhibitor concentration; see Fig. 3).

Glucose 6-phosphate inhibition was partially released by P_i (15 mM), AMP (mM) and 3',5'-(cyclic)-AMP (50 μ M) at low concentrations of glucose 6-phosphate (<0.3 mM) that were below the physiological range. Thus, for example, with 0.1 mM-glucose 6-phosphate the inhibition was halved by 15 mM- P_i . At physiological concentrations of glucose 6-phosphate (0.7 mM and above) these compounds did not release inhibition by glucose 6-phosphate. The kinetics of glucose 6-phosphate inhibition were not altered by the use of high K^+ concentrations [20 mM-imidazole, pH 7.7, containing potassium acetate (0.15 M) and magnesium chloride (20 mM)]. A number of compounds whose concentrations change when hexokinase is activated or inhibited in the perfused heart were found to be without effect on the activity of soluble hexokinase or the inhibition by glucose 6-phosphate. The measurements were made with ATP (5 mM) and glucose (1 mM) with or without glucose 6-phosphate (0.3 mM). The compounds included citrate (1 mM), malate (0.5 mM), fructose 6-phosphate (0.4 mM), glucose 1-phosphate (0.1 mM), fructose 1,6-diphosphate (0.1 mM), 3-phosphoglycerate (0.1 mM), acetyl-CoA (40 μ M), CoA (0.14 mM), palmitoyl-CoA (50 μ M),

acetyl-DL-carnitine (0.5mM) and UDP-glucose (80 μ M).

Inhibition of particulate hexokinase by glucose 6-phosphate. This was investigated by the radioactivity assay, with the glucose 6-phosphate, ATP and glucose concentrations given for the soluble enzyme. Inhibition by glucose 6-phosphate was non-competitive with respect to glucose (K_i 0.33mM) and mixed with respect to ATP (K_i 40 μ M). The inhibition of particulate hexokinase by glucose 6-phosphate was partially released by 10mM- P_i (Fig. 7), but not by 2mM- P_i . The particulate enzyme was not affected by AMP, unlike the soluble enzyme. No effects on glucose 6-phosphate inhibition were seen with citrate (1mM) or with high- K^+ -concentration buffer [20mM-imidazole, pH 7.7, containing potassium acetate (0.15M) and magnesium chloride (20mM)].

These effects of glucose 6-phosphate were observed with a fixed Mg^{2+} concentration of 20mM; identical effects were seen with $[ATP]/[Mg^{2+}]$ ratios of 1:1.

Effects of other metabolites on particulate hexokinase. At the concentrations shown, the following compounds had no detectable effects on the K_m value for glucose or the V_{max} value of particulate hexokinase, with 5mM-ATP: citrate (1mM), malate (0.5mM), UDP-glucose (70 μ M), UDP-N-acetylglucosamine (0.6mM), glucosamine 6-phosphate (0.5mM), N-acetylglucosamine 6-phosphate (0.5mM), fructose 6-phosphate (0.4mM), fructose 1,6-diphosphate (0.1mM) and PP_i (0.2mM).

Correlation between rates of glucose phosphorylation measured in the perfused rat heart and predicted rates of glucose phosphorylation calculated from kinetic data for rat heart hexokinase

The results summarized in the preceding section appear to show that the factors that regulate hexokinases in rat heart are the concentrations of glucose, ATP, glucose 6-phosphate, ADP, AMP and P_i . (There was no evidence for any change in the total hexokinase activity or in the proportions of the particulate and soluble enzymes in diabetes, or for any effect of a number of other metabolites on the activity of the enzyme.) If the regulation of hexokinase by these metabolites determines the rate of glucose phosphorylation in the heart, then it should be possible to predict rates of glucose phosphorylation from the kinetic properties of the enzymes and the intracellular concentrations of the relevant metabolites. To simplify the calculation, and because of difficulties in the measurement of small intracellular glucose concentrations, conditions of perfusion associated with high intracellular glucose concentrations were selected.

Rates of glucose phosphorylation in the perfused

Fig. 4. Time-course of glucose disappearance from the perfusion medium with the isolated perfused rat heart. Hearts were preperfused for 5min. by drip-through and then switched to recirculation with the medium specified below, and samples were taken for glucose analysis at the times shown. (a): ▲, Normal heart, anaerobic perfusion, medium containing 14mM-glucose and 0.05 unit of insulin/ml.; ●, normal heart, aerobic perfusion, 33mM-glucose; ○, heart from diabetic rat, aerobic perfusion, 5.5mM-glucose, 0.05 unit of insulin/ml. (b): ■, Normal heart, aerobic perfusion, 33mM-glucose, insulin added after 12min., as shown by the arrow, to a final concn. of 0.05 unit/ml. (c): □, Normal heart, aerobic perfusion, 5.5mM-glucose, insulin added after 4min., as shown by the arrow, to a final concn. of 0.05 unit/ml.

heart. The rate of glucose phosphorylation in the perfused heart has been assumed to be the same as the rate of glucose uptake. This assumption appears justified, since in these experiments there was either no change in the muscle glucose concentration during the period of measurement of glucose uptake, or (in experiments with 3-hydroxybutyrate) the change was less than 5% of the overall glucose uptake.

Fig. 4 shows the results of typical experiments in which glucose uptake was measured in the small-volume recirculating perfusion apparatus. Linear rates of uptake were seen with normal hearts with

Fig. 5. Effect of DL-3-hydroxybutyrate on the time-courses of glucose disappearance from the perfusion medium and intracellular glucose 6-phosphate concentration in the perfused rat heart. Hearts were preperfused by drip-through and then recycled, and glucose was taken for analysis at the times shown, with medium containing 14mm-glucose and 0.05 unit of insulin/ml. DL-3-Hydroxybutyrate was added after 8 min., as shown by the arrow, to a final perfusate concn. of 5.5 mm. ○, Glucose disappearance; ●, intracellular glucose 6-phosphate concentration measured in hearts frozen at the times shown. The broken lines represent steady-state rates of glucose disappearance with and without 3-hydroxybutyrate. For further details of the analysis and the calculation of intracellular concentrations see the text.

or without insulin under aerobic or anaerobic conditions, and in hearts from diabetic rats. In some experiments with insulin the glucose uptake was measured in the absence of hormone in a control period of 12 min. of perfusion, during which time linear rates were recorded. Insulin was then added and a new linear rate was established within 3 min. (see Fig. 4b). In experiments with DL-3-hydroxybutyrate, which inhibits glucose phosphorylation, the ketone body (5.5 mm) was added after a control period of 8 min. of perfusion. There was a transient period of 12 min. after the addition of 3-hydroxybutyrate before a new steady-state

rate of glucose uptake was reached (Fig. 5). The rates of glucose phosphorylation measured in the perfused heart under various conditions are given in Table 4.

The intracellular glucose concentrations under the various conditions in which glucose uptake was measured are given in Table 3. They were calculated on the basis of an intracellular volume of distribution for glucose that is 0.75 times the volume of intracellular water (see Park, Reinwein, Henderson, Cadenas & Morgan, 1959). Under each of the conditions used, the intracellular glucose concentration was at least 70 times the K_m value for glucose of either the particulate or the soluble rat heart hexokinase.

Glucose 6-phosphate concentration. The concentrations of glucose 6-phosphate were measured in a further set of hearts perfused under conditions identical with those used for measurement of glucose uptake. The concentrations shown in Table 3 were those measured at the end of the period of perfusion used for the measurement of glucose uptake. Control experiments showed that, under conditions where glucose uptake rates are linear, glucose 6-phosphate concentrations do not vary significantly over the period of perfusion. In experiments with DL-3-hydroxybutyrate, the concentration of glucose 6-phosphate was measured at intervals during the transient period in which glucose uptake was changing, and after attainment of a new steady-state rate (see Fig. 5).

Studies in the perfused heart with the glucose analogue 3-O-methyl-D-glucose have suggested that glucose is confined to 70–80% of the intracellular water (Park *et al.* 1959). In calculating the intracellular glucose 6-phosphate concentrations given in Table 3, it has been assumed that glucose 6-phosphate is free and is distributed in an intracellular volume of 75% of the intracellular water. Some support for this assumption is provided by the data in Table 5, which appear to show that rat-heart mitochondria are impermeable to glucose 6-phosphate. Calculations based on data for mitochondrial protein (mg./g. of rat heart) given by Cleland & Slater (1953), and a value for mitochondrial water within the inner mitochondrial membrane of 2 μ l./mg. of mitochondrial protein, suggest that mitochondrial water may account for 25% of the volume of intracellular water (see also Williamson, 1965).

Concentration of ATP, ADP and AMP. The concentrations of ATP, ADP and AMP given in Table 3 are taken from Newsholme & Randle (1964), except for the anaerobic values, which were assayed in the extracts used for glucose 6-phosphate estimations. Control experiments showed that the concentrations of these nucleotides did not vary significantly during the period of perfusion used

Table 3. *Concentrations of effectors of rat heart hexokinase in perfused rat heart*

The concentrations of glucose and glucose 6-phosphate (given as means $\pm$ s.e.m.) in all perfusions and of ATP, ADP and AMP in anaerobic perfusions were measured in extracts of rat heart frozen with the tissue clamp of Wollenberger *et al.* (1960) after 5 min. of preperfusion followed by perfusion with bicarbonate-buffered medium (Krebs & Henseleit, 1932), containing [^{14}C]sorbitol (0.2 mg./ml., 0.02 $\mu\text{C}/\text{ml.}$) and other additions as shown. The minimum number of hearts perfused in each group was four. For further details see the text. The concentrations of ATP, ADP and AMP for the aerobic perfusions are taken from Newsholme & Randle (1964).

Condition	Insulin (0.05 unit/ml.)	Concn. of glucose in medium (mM)	Intracellular concn. (mM)				
			Glucose	Glucose 6-phosphate	ATP	ADP	AMP
Normal, aerobic	—	33	4.2 $\pm$ 0.5	0.85 $\pm$ 0.03	10	2.0	0.32
Normal, aerobic	+	33	17 $\pm$ 2.2	1.02 $\pm$ 0.05	10	2.0	0.32
Normal, anaerobic	+	14	3.7 $\pm$ 0.4	0.70 $\pm$ 0.03	8.2	2.2	1.0
Diabetic, aerobic	+	5.5	4.0 $\pm$ 0.7	1.82 $\pm$ 0.12	8.5	2.0	0.32
Normal, aerobic, with DL-3-hydroxybutyrate (5.5 mM) for:							
0 min.	+	14	4.0 $\pm$ 0.4	0.78 $\pm$ 0.02	10	2.0	0.32
5 min.	+	14		1.12 $\pm$ 0.04			
10 min.	+	14		1.24 $\pm$ 0.05			
15 min.	+	14	6.4 $\pm$ 1.3	1.26 $\pm$ 0.07	10	2.0	0.32

Table 4. *Measured and predicted rates of glucose phosphorylation in the perfused rat heart*

The conditions of perfusion were as described at the head of Table 3. Glucose phosphorylation rates were measured as glucose uptake in the heart and are given as means $\pm$ s.e.m. (for further details see the text and Fig. 4). For details of the calculation of predicted rates of glucose phosphorylation see the text.

Condition	Insulin (0.05 unit/ml.)	Concn. of glucose in medium (mM)	Rates of glucose phosphorylation (μ moles of glucose/min./g. dry wt. of muscle)				Ratio total predicted: total measured
			Measured in perfused heart	Predicted from kinetic data			
				Particulate hexokinase	Soluble hexokinase	Total	
Normal, aerobic	—	33	7.2 $\pm$ 0.1	5.7	2.3	8.0	1.11
Normal, aerobic	+	33	6.4 $\pm$ 0.2	5.1	1.5	6.6	1.03
Normal, anaerobic	+	14	9.2 $\pm$ 0.6	6.0	3.1	9.1	0.99
Diabetic, aerobic	+	5.5	2.1 $\pm$ 0.1	2.4	0	2.4	1.15
Normal, aerobic with DL-3-hydroxybutyrate (5.5 mM) for:							
0 min.	+	14	8.3 $\pm$ 0.1	6.0	2.7	8.7	1.05
5 min.	+	14	6.1 $\pm$ 0.1	4.7	1.1	5.8	0.95
10 min.	+	14	4.8 $\pm$ 0.1	4.5	0.8	5.3	1.11
15 min.	+	14	4.5 $\pm$ 0.1	4.4	0.8	5.2	1.17

for measurement of glucose uptake. For calculating intracellular concentrations it has been assumed that adenine nucleotides are free and uniformly distributed in intracellular water.

Correlation between rates of glucose phosphorylation and concentrations of glucose 6-phosphate in perfused rat heart. These results are shown in Tables 3 and 4 and Fig. 6. There was a negative correlation between rates of glucose phosphorylation and concentrations of glucose 6-phosphate. Two particular

observations call for further comment. Under aerobic conditions at a perfusate glucose concentration of 33 mM, insulin increased the concentration of glucose 6-phosphate but slightly decreased the rate of glucose phosphorylation. This unexpected finding was seen consistently in six separate perfusions (e.g. Fig. 4b). At lower perfusate concentrations of glucose (5.5 mM), where no intracellular glucose was detected in the absence of insulin, the hormone stimulated glucose uptake as

Table 5. $[^3\text{H}]$ Water, sucrose and glucose 6-phosphate spaces of rat heart mitochondria incubated for the times shown

Rat heart mitochondria prepared by the method of Chance & Hagihara (1961) were incubated for the time shown in 20 mM-tris-chloride-40 mM-methylamine-chloride, pH 7.4, containing KCl (120 mM), $[^3\text{H}]$ water (1 $\mu\text{C}/\text{ml.}$), $[\text{U-}^{14}\text{C}]$ sucrose (2 mM, 0.2 $\mu\text{C}/\text{ml.}$) and glucose 6-phosphate (2 mM), and isolated by centrifugation through silicone oil (Werkheiser & Bartley, 1957). Glucose 6-phosphate was assayed spectrophotometrically and $[^3\text{H}]$ water and $[\text{U-}^{14}\text{C}]$ -sucrose were measured by dual-isotope scintillation spectrometry. For further details see the text.

Time of incubation (min.)	Space ($\mu\text{l.}/\text{mitochondrial sample}$)		
	Water	Sucrose	Glucose 6-phosphate
5	62	30	29
10	57	34	38
15	67	38	39
25	71	35	31
35	65	29	30

Fig. 6. Relationship between intracellular glucose 6-phosphate concentration and rate of glucose phosphorylation in perfused rat heart. The data shown are taken from Tables 3 and 4.

expected (Fig. 4c). Perfusion with DL-3-hydroxybutyrate led to an increase in glucose 6-phosphate concentration, which only attained a new steady-state value after 12–15 min. of perfusion (Fig. 5). The decline in glucose phosphorylation paralleled the rise in glucose 6-phosphate concentration. The highest concentration of glucose 6-phosphate and the lowest rate of glucose phosphorylation were seen in the hearts from alloxan-diabetic rats (cf. Tables 3 and 4).

Predicted rates of glucose phosphorylation from kinetic data for rat heart hexokinase. The rates of glucose phosphorylation in the perfused heart have been measured under conditions where the intracellular concentration of glucose was at least 70 times the K_m for both forms of the enzyme. The concentration of glucose was therefore assumed to be saturating in calculating predicted rates. The predicted rate of glucose phosphorylation (R) in μmoles of glucose phosphorylated/g. dry wt. of muscle/min. at 37° has been calculated from:

$$R = R_p \cdot A_p \cdot F_p + R_s \cdot A_s \cdot F_s$$

where R_p and R_s are the specific activities of particulate and soluble hexokinases respectively, in units/g. dry wt. of muscle, measured at 37° with 10 mM-ATP-3 mM-glucose; A_p and A_s are the fractions of R_p and R_s operating at the concentrations of glucose 6-phosphate and ATP measured in the heart, and derived from experimental data given in Fig. 7; and F_p and F_s are corrections for the competitive inhibition by ADP and AMP with respect to ATP, calculated from similar equations (given for F_p only):

$$F_p = \frac{V_p^2}{R_p^2} \left[\frac{1}{1 + \frac{K_m}{[\text{ATP}]} \left(1 + \frac{[\text{ADP}]}{K_{\text{ADP}}} \right)} \cdot \frac{1}{1 + \frac{K_m}{[\text{ATP}]} \left(1 + \frac{[\text{AMP}]}{K_{\text{AMP}}} \right)} \right]$$

where V_p is $V_{\text{max.}}$ for particulate hexokinase, in units/g. dry wt. of muscle; K_m is K_m for ATP for particulate hexokinase; K_{ADP} is K_i for ADP (versus ATP) for particulate hexokinase; K_{AMP} is K_i for AMP (versus ATP) for particulate hexokinase; and $[\text{ATP}]$, $[\text{ADP}]$ and $[\text{AMP}]$ are the concentrations of these nucleotides measured in the heart.

Values of A_p , A_s , F_p and F_s for the perfusion conditions of Table 3 are given in Table 6.

This calculation assumes that the inhibition produced by glucose 6-phosphate is independent of that produced by ADP and AMP and vice versa. This has been tested experimentally by studying the effects of glucose 6-phosphate in the presence and absence of 2 mM-ADP (Fig. 7). The inhibition observed with both glucose 6-phosphate and ADP was similar to that calculated from the K_i value for ADP and the inhibition by glucose 6-phosphate alone. It has been assumed that AMP behaves similarly.

In calculating predicted rates of glucose phosphorylation, possible effects of P_i have been neglected. Under aerobic conditions of perfusion, the intracellular concentrations of P_i (2.5 mM;

Regen, *et al.*, 1964) are too low to influence hexokinase. Under anaerobic conditions of perfusion, the intracellular concentration of P_i in the heart

may rise to 9mm (Regen *et al.* 1964), and at this concentration P_i may reverse glucose 6-phosphate inhibition somewhat. At the anerobic concentration of glucose 6-phosphate (0.7mm), P_i (10mm) could increase the predicted rate of phosphorylation by not more than 8% through an effect on the particulate enzyme (Fig. 7).

Comparison of predicted and measured rates of glucose phosphorylation. This is shown in Table 4 and Fig. 8. The predicted and measured rates of glucose phosphorylation were correlated to a highly significant degree (r 0.94; P < 0.001). The ratio of predicted to measured rates of phosphorylation was 1.07 ± 0.03 (mean $\pm$ s.e.m.; P = 0.05 for the difference from unity).

Williamson (1965) has published values for rates of glucose uptake and intracellular concentrations

Fig. 7. Inhibition of soluble and particulate rat heart hexokinases by glucose 6-phosphate, and the effects of ATP concentration and of ADP and P_i . (a) Soluble hexokinase, spectrophotometric assay measuring ADP production with 1mm-glucose and 20mm- Mg^{2+} . $\bullet$, 5mm-ATP; Δ , 10mm-ATP. (b) and (c): Particulate hexokinase, radioactivity assay measuring $[^{14}C]$ glucose 6-phosphate production with 1mm-glucose and 20mm- Mg^{2+} . $\blacktriangle$, 10mm-ATP-10mm- P_i ; $\circ$, 10mm-ATP; $\blacksquare$, 5mm-ATP; $\blacktriangledown$, 10mm-ATP-2mm-ADP; $\square$, 5mm-ATP-2mm-ADP. Each point was the mean of duplicate determinations.

Fig. 8. Correlation between rate of glucose phosphorylation measured in the perfused heart and rate predicted from kinetic properties of particulate and soluble hexokinases. $\bullet$, Data from Table 4; $\circ$, calculated from the data of Williamson (1965). The line is drawn to a slope of 45° , and is not necessarily the best fit for the points.

Table 6. *Relative contributions of glucose 6-phosphate, ATP, ADP and AMP concentrations to inhibition of glucose phosphorylation predicted from kinetic data of rat heart hexokinases*

A_p and A_s are the fractions of the maximum activities of particulate and soluble hexokinases at the measured concentrations of glucose 6-phosphate and ATP. F_p and F_s are the fractions of maximum activities of particulate and soluble hexokinases at the measured concentrations of ATP, ADP and AMP. For further details of conditions of perfusion see Table 3. For further details of A_p , A_s , F_p and F_s see the text.

Condition	A_p	A_s	F_p	F_s
Normal, aerobic	0.23	0.20	0.79	0.85
Normal, aerobic, with insulin	0.20	0.13	0.79	0.85
Normal, anaerobic, with insulin	0.25	0.27	0.74	0.83
Diabetic, aerobic, with insulin	0.11	0	0.75	0.84
Normal, aerobic, with 3-hydroxybutyrate for: 0min.	0.24	0.24	0.79	0.85
5min.	0.19	0.10	0.79	0.85
10min.	0.18	0.07	0.79	0.85
15min.	0.17	0.06	0.79	0.85

of glucose, glucose 6-phosphate, ATP, ADP and AMP for normal hearts perfused under aerobic conditions with glucose (5mM) and insulin (0.002 unit/ml.), with or without acetate (10mM). In each instance, the intracellular glucose concentration was high (> 1 mM), so that it is possible to predict rates of glucose phosphorylation in Williamson's (1965) experiments from our kinetic data. This involves the assumption that the total activity and kinetic properties of rat-heart hexokinase were similar in the strains used in the present study and by Williamson (1965). The predicted rates of glucose phosphorylation were (in μ moles of glucose phosphorylated/min./g. dry wt. of muscle) 5.9 without acetate and 3.1 with acetate. The rates of glucose uptake observed by Williamson (1965) were 5.6 and 3.0 respectively.

DISCUSSION

Parameters of rat heart hexokinase. Detailed information is available about the properties of hexokinases in a number of rat tissues including skeletal muscle, but only a very small amount of data for rat-heart hexokinase has been published. The total activity of hexokinases in rat heart in the present study is comparable with that given by Mayer, Mayfield & Haas (1966), though the proportion of soluble enzyme (30%) was somewhat less than the value of 52% given by Mayer *et al.* (1966). The presence of type I and type II hexokinases in extracts of rat heart (Katzen, 1966) has been confirmed, and it has been shown in addition that both types are present in the soluble and in the particulate hexokinase, and that type I may predominate. The values of K_1 (for glucose 6-phosphate and ADP) for the rat-heart soluble and particulate enzymes are similar to those given for type I hexokinase from other rat tissues by Grossbard & Schimke (1966) (see Table 2). The K_m value for glucose is similar to that given for rat heart hexokinase by Mayer *et al.* (1966), but higher than the value of 8μ M reported by Crane & Sols (1955). The K_m value for ATP is higher than the value given for the particulate enzyme by Mayer *et al.* (1966), and the K_i value for glucose 6-phosphate (with respect to glucose) is higher than values reported by Mayer *et al.* (1966) and Crane & Sols (1955).

Regulation of glucose phosphorylation in the perfused rat heart. No change in the total activity of rat-heart hexokinase was detected in alloxan-diabetes in the present study. It would appear therefore that alterations in the concentration of hexokinase in the heart do not contribute to the defect in glucose phosphorylation in this tissue (Morgan *et al.* 1961a). This may be contrasted with the diminution of glucokinase activity in the liver

and of hexokinase in the adipose tissue in alloxan-diabetes (Viñuela *et al.* 1963; McLean *et al.* 1966). Katzen (1966) found that alloxan-diabetes in the rat was associated with loss of type II hexokinase from rat-heart muscle, and that this could be restored *in vitro* by incubation of hearts from diabetic rats with insulin or incubation of extracts with thiol. The total activity of extracts of hearts from diabetic rats in the present study was apparently the same as the normal, irrespective of the presence or absence of 2-mercaptoethanol in the extraction fluid. Since anaerobic perfusion increases the rate of glucose phosphorylation in hearts from diabetic rats to values that approach those seen in normal hearts it has been suggested by Morgan *et al.* (1961a) that hexokinase in the diabetic hearts may be inhibited and not decreased in concentration. The present findings are consistent with this explanation.

The present studies show that the concentrations of a number of effectors may control the rate of glucose phosphorylation in the intact heart under conditions where the intracellular glucose concentration is not rate-limiting. These are the concentration of glucose 6-phosphate, the concentration of ATP (which may modify the degree of inhibition by glucose 6-phosphate), the relative concentrations of ATP on the one hand and of ADP and AMP on the other (since ADP and AMP are competitive inhibitors with respect to ATP) and the concentration of P_i (which at high concentration may reverse inhibition by glucose 6-phosphate). The relative proportions of particulate and soluble hexokinases may also affect the rate of phosphorylation, since the soluble enzyme appears to be more sensitive to inhibition by glucose 6-phosphate at concentrations above 1 mM. It has been found that, when all these factors are taken into account, the rates of glucose phosphorylation that may be predicted from the kinetic properties of the hexokinases agree closely with rates measured in the perfused heart. The present findings are consistent therefore with the idea that alterations in the rate of glucose phosphorylation that may be induced in rat heart by alloxan-diabetes, by anaerobic perfusion or by perfusion with fatty acids, ketone bodies or pyruvate are due mainly to alterations in the intracellular concentration of glucose 6-phosphate. However, the calculation of predicted rates of glucose phosphorylation from kinetic data has involved a number of assumptions that do not appear to be open to direct investigation and that perhaps call for further comment.

Under all of the conditions of perfusion used in the present study the rate of glucose phosphorylation in the perfused heart, which ranged from 2.1 to 9.2μ moles/g. dry wt. of ventricle/min., was much less than the total activity of hexokinase extracted

from the muscle (46 units/g. at 37°). Since the concentration of glucose in intracellular water was at least 70 times the K_m value for glucose and that of ATP at least 16 times the K_m value for ATP, it would appear likely that the variations in the rate of glucose phosphorylation are due mainly to inhibition of the enzymes. Table 6 shows the extent to which the various factors may contribute to the inhibition observed. Under all conditions of perfusion the major factor contributing to the inhibition observed was the concentration of glucose 6-phosphate, which accounted for 92–98% of the inhibition (the extremes being the normal heart perfused anaerobically and the heart from the diabetic rat perfused aerobically). In the anaerobic perfusion, the fall in ATP concentration contributed to a small extent to the calculated inhibition by glucose 6-phosphate (approx. 5% of the calculated inhibition by glucose 6-phosphate). The relative concentrations of ATP on the one hand and of ADP and AMP on the other contributed 2–8% of the inhibition. The proportions of soluble and particulate enzyme were important factors in the calculation of predicted rates at high glucose 6-phosphate concentrations (i.e. in the heart from the diabetic rat and in the normal heart perfused with 3-hydroxybutyrate).

The major assumptions in the calculation of predicted rates of glucose phosphorylation appear to be the distribution of glucose 6-phosphate and the proportions of the soluble and particulate enzymes. In calculating the intracellular concentration of glucose 6-phosphate, a distribution volume of 75% of the volume of intracellular water has been used. This was based on the observation that analogues of glucose such as 3-O-methylglucose are distributed in 70–80% of the volume of intracellular water at equilibrium (Park *et al.* 1959), and the observation that mitochondria, which were shown to be impermeable to glucose 6-phosphate, may contain approx. 25% of the volume of intracellular water within the inner mitochondrial membrane. Some idea of the errors introduced by the assumption of this distribution volume may be obtained by considering the calculated phosphorylation rates if the distribution volume for glucose 6-phosphate was 70 or 80% of the intracellular water and not 75%. With anaerobic perfusion, for example, the calculated rate of phosphorylation would be increased by 7.5% if the distribution volume was 80% of the intracellular water and decreased by 3.5% if the distribution volume was 70%. In the hearts from diabetic rats the change would be 9% in both instances.

In calculating predicted rates of glucose phosphorylation, the proportions of soluble and particulate enzymes have been assumed to be 3:7 in all cases. The studies of Hernandez & Crane (1966)

have indicated that soluble and particulate hexokinases may be in equilibrium in pig heart and that the position of equilibrium may be changed in favour of the soluble form by increasing the ionic strength or the glucose 6-phosphate concentration. In some studies with the particulate enzyme from rat heart (not reported), it has been found that the proportion of particulate enzyme that is solubilized is increased from 19 to 30% by raising the glucose 6-phosphate concentration from 0.5 mM to 1.5 mM. The possibility exists therefore that the proportions of soluble and particulate hexokinases in the heart may vary with conditions of perfusion. Thus in Table 6, under conditions where A_p and A_s differ markedly, the predicted rates of glucose phosphorylation will be in error if the proportions of soluble and particulate hexokinases are very different from the assumed value. For example, if in the diabetic heart the proportion of soluble enzyme was 35% and not 30% as assumed, the predicted rate of glucose phosphorylation would be 86% of that calculated.

In calculating predicted rates of glucose phosphorylation it has also been assumed that $MgATP^{2-}$ and $MgADP^-$ are the predominant forms of ATP and ADP present in the cell. This assumption has been made for the following reasons. The apparent stability constants of $MgATP^{2-}$ and $MgADP^-$ are $10^4 M^{-1}$ and $1.4 \times 10^3 M^{-1}$ respectively (Sillen & Martell, 1964). The concentration of magnesium in rat heart (approx. $10 \mu\text{moles/g. wet wt.}$; Watchorn & McCance, 1937) is in excess of that of ATP and ADP (approx. $4.8 \mu\text{moles/g. wet wt.}$; Newsholme & Randle, 1964). If there is an appreciable concentration of ATP^{4-} in rat heart, the predicted rates of glucose phosphorylation may be in error because yeast and brain hexokinases are inhibited by ATP^{4-} (Gutfreund & Hammond, 1963; Newsholme, Rolleston & Taylor, 1967).

We thank Mrs I. M. Harris and Miss J. E. Saunders for skilled technical assistance, and the British Diabetic Association and the Medical Research Council for grants that contributed to the cost of these investigations. P. J. E. holds a Science Research Council studentship.

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