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Purification and Properties of Adenosine 5'-Triphosphate–D-Glucose 6-Phosphotransferase from Rat Liver

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1. An 870-fold purification of glucokinase from rat liver is described which involves ammonium sulphate fractionation and the use of DEAE-Sephadex, DEAE-cellulose and polyacrylamide columns. 2. The preparation is free of any interfering enzymes and has a specific activity of 8 μmoles/min./mg. of protein. 3. Glucokinase catalyses the phosphorylation of glucose, mannose and 2-deoxyglucose. 4. The enzyme is inhibited by high concentrations of glucose 6-phosphate only; ADP is an inhibitor whose effect depends on the Mg^{2+} concentration. 5. The properties of glucokinase are compared briefly with those of other phosphotransferases.

The presence of a hexokinase in liver tissue having a high K_m for glucose was first reported by DiPietro, Sharma & Weinhouse (1962), Walker (1962) and Viñuela, Salas & Sols (1963). Studies on crude tissue extracts and simple ammonium sulphate fractions (Viñuela *et al.* 1963; Walker, 1963; Walker & Rao, 1963) indicated that this enzyme, designated glucokinase (adenosine 5'-triphosphate–D-glucose 6-phosphotransferase, EC 2.7.1.2), also phosphorylated mannose and 2-deoxyglucose but had a narrower specificity for the phosphoryl acceptor than other mammalian hexokinases, which have comparatively low K_m values.

This paper presents the procedure for an 870-fold purification of glucokinase from the supernatant fraction of rat-liver homogenate resulting in a preparation of specific activity of over 8 μmoles of glucose phosphorylated/min./mg. of protein. Some properties of this enzyme are described and compared with those of other mammalian hexokinases. A 200-fold-purified preparation of glucokinase from rabbit liver has been described by Salas, Salas, Viñuela & Sols (1965), with properties that are very similar to those of the rat-liver enzyme.

MATERIALS AND METHODS

Materials

Animals. Well-fed male rats of the Wistar strain and at least 6 months old were used as the source of liver tissue.

Chemicals and enzymes. ATP (disodium salt) and NADH were obtained from Sigma (London) Ltd.; $NADP^+$, ADP, phosphoenolpyruvate (sodium salt), glucose 6-phosphate (sodium salt), triethanolamine hydrochloride, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (140 units/mg.), pyruvate kinase (EC 2.7.1.40) (125 units/mg.), phosphoglucose isomerase (EC 5.3.1.9) (390 units/mg.) and lactate dehydrogenase from rabbit muscle (EC 1.1.1.27) (360 units/mg.) were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; 2-deoxyglucose, *N*-acetylglucosamine and D-lyxose were from Mann Research Laboratories Inc., New York, N.Y., U.S.A. All other chemicals were A.R. or best quality reagent grade. DEAE-Sephadex was purchased from Pharmacia, Uppsala, Sweden, and the Bio-Gels from Bio-Rad Laboratories, Richmond, Calif., U.S.A., through Kodak Ltd., Kirkby, Lancs.

Buffers. Buffer solutions and all other solutions were prepared in glass-distilled water and pH was measured at 4° on a Pye Universal pH-meter standardized against solutions prepared with standard buffer tablets.

Methods

Assay of glucokinase. Three different methods were employed, depending on the experimental requirements. The method used in every case is recorded in the Results section. In each method extinction changes were recorded on a Gilford model 2000 Multiple Sample Absorbance Recorder attached to a Unicam SP.500 Spectrophotometer light-source and monochromator.

Method 1. In this method the formation of glucose 6-phosphate was measured by coupling with excess of glucose 6-phosphate dehydrogenase to the reduction of $NADP^+$. The reaction mixture except where otherwise stated contained (final concentrations) in a total volume of 1·5 ml.: glycylglycine buffer, pH 7·5 (50 mM), $MgSO_4$ (7·5 mM), ATP (5 mM), $NADP^+$ (0·5 mM), KCl (100 mM), glucose (100 mM) and glucose 6-phosphate dehydrogenase (0·4 unit). The enzyme preparation was added last and the rate followed at 340 mμ and at 28° in a cuvette having a 1 cm. light-path. The initial rate was taken as the linear portion of the progress curve after the initial 2 min., during which time the steady state with the subsidiary enzyme was attained.

Method 2. The formation of ADP was measured by coupling in the presence of phosphoenolpyruvate with

excess of both pyruvate kinase and lactate dehydrogenase (muscle) to the oxidation of NADH. The reaction mixture unless otherwise stated contained (final concentrations) in a total volume of 2·0ml.: triethanolamine hydrochloride buffer, pH7·5 (100mM), $MgSO_4$ (7·5mM), ATP (5mM), KCl (100mM), phosphoenolpyruvate (0·75mM), NADH (0·25mM), glucose (100mM), pyruvate kinase (25μg.) and lactate dehydrogenase (50μg.). The glucokinase preparation was added last and the extinction change followed as above.

Method 3. This was a discontinuous method in which the rate of glucose 6-phosphate formation was determined by measuring enzymically with glucose 6-phosphate dehydrogenase the amount of glucose 6-phosphate in samples taken from a reaction mixture at definite time-intervals. The reaction mixture for glucokinase activity contained except where stated otherwise (final concentrations) in a total volume of 1·5ml.: triethanolamine hydrochloride buffer (100mM), $MgSO_4$ (7·5mM), ATP (5mM), KCl (100mM) and glucose (100mM). The glucokinase preparation was added last. Samples (0·4ml.) were taken at 0, 3 and 6min. and added to 1ml. of ice-cold 6% (w/v) $HClO_4$ containing methyl orange indicator. The solution was neutralized with 6M-K_2CO_3. The precipitate was removed by centrifugation and a 1ml. sample of the supernatant assayed for glucose 6-phosphate by adding (to make final concentrations) triethanolamine hydrochloride buffer, pH7·5 (80mM), $MgSO_4$ (3mM), $NADP^+$ (0·25mM) and glucose 6-phosphate dehydrogenase (0·4 unit) in a final volume of 1·5ml. The reaction was followed to completion at room temperature and the change in extinction at 340mμ measured.

One unit of glucokinase activity is defined as that which catalyses the formation of 1μmole of glucose 6-phosphate/min. Specific activity is defined as units of enzyme activity/mg. of protein (except where otherwise stated in Figs. 1 and 2).

Protein estimations. Protein was determined in crude preparations by a biuret method (Gornall, Bardawill & David, 1946) and in more purified preparations by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein in the fractions from columns was monitored by the ultraviolet method of Warburg & Christian (1941).

Purification of glucokinase

Preamble. Viñuela *et al.* (1963) reported that the glucokinase occurred in the protein fraction precipitated in the 0·60–0·75-saturated $(NH_4)_2SO_4$ fraction and could thus be separated from hexokinase (EC 2.7.1.1), which was also present in the liver supernatant preparation and which precipitated in the 0·25–0·50-saturated $(NH_4)_2SO_4$ fraction. In our experience glucokinase is precipitated over a wider range of $(NH_4)_2SO_4$ concentrations and complete fractionation is not possible by this means. Maximum recovery of glucokinase assessed against protein fractionation was obtained by using the 0·45–0·65 saturation range. Repeated or further subfractionations with $(NH_4)_2SO_4$ resulted in large losses of glucokinase activity and were abandoned.

The importance of having both K^+ and a thiol compound present throughout the preparation became apparent at an early stage. As described in the Results section, K^+ stabilizes the enzyme; the concentration of K^+ was never permitted to fall below 0·1M during the preparation and 0·4M-KCl was present during storage. 2-Mercaptoethanol was used in many early experiments but was subsequently replaced by *N*-acetylcysteine.

Dialysed portions of 0·45–0·65-saturated $(NH_4)_2SO_4$ fractions from liver supernatant preparations were used to investigate other fractionation procedures. Glucokinase activity was irreversibly destroyed at pH5. The enzyme adsorbed on calcium phosphate gel (Keilin & Hartree, 1938) but only small percentages of that activity could be eluted. Three types of columns—Sephadex G-200, DEAE-cellulose and CM-cellulose—were tested as convenient fractionation media but in all cases glucokinase activity was eluted off the columns with the main protein peak. DEAE-cellulose was found to be useful after preliminary fractionation on DEAE-Sephadex (see below). Gel filtration was also suitable on more purified preparations and is used in the last step described below.

Stage 1: preparation of extract. Ten normal well-fed adult male rats of the Wistar strain were killed by cervical dislocation, exsanguinated by cutting the jugular veins and the livers were removed and chilled for several minutes in ice-cold homogenizing medium (see below). All subsequent operations were performed in a cold room at 0–4°. Homogenates (33%, w/v) of each liver were prepared in a Potter–Elvehjem-type homogenizer (glass tube and Teflon pestle) and pooled. The homogenizing medium contained (final concentrations): KCl, 0·15M; $MgSO_4$, 4mM; EDTA, 4mM; *N*-acetylcysteine, 4mM; adjusted to pH7·0 with 1·0N-KOH. The combined homogenate was centrifuged at 38000*g* for 10min. (MSE High Speed 18 Centrifuge; 8×50ml. rotor). The supernatant was further centrifuged at 105000*g* for 1hr. (MSE Super Speed 40 Centrifuge; 8×50ml. rotor); this second supernatant was collected with a pipette, avoiding contamination with the fatty layer at the top of the centrifuge tube.

Stage 2: ammonium sulphate fractionation. This latter supernatant was brought to 0·45 saturation with respect to $(NH_4)_2SO_4$ by the addition, per 100ml. of extract, of 82ml. of saturated (at 4°) $(NH_4)_2SO_4$ solution prepared in 4mM-$MgSO_4$–4mM-EDTA–4mM-*N*-acetylcysteine, previously adjusted to pH7·0 with 1·0N-KOH. After equilibration for 20min. the precipitate was removed by centrifugation at 40000*g* for 10min. and discarded. The clear 0·45-saturated $(NH_4)_2SO_4$ supernatant solution was brought to 0·65 saturation by the addition of 57ml. of the same saturated $(NH_4)_2SO_4$ solution/100 ml. of this supernatant solution. After equilibration the precipitate was collected by centrifugation at 40000*g* for 10min. The $(NH_4)_2SO_4$ paste remaining after removal of the supernatant could be stored at −15° for several days without appreciable loss of glucokinase activity.

Immediately before proceeding to the next stage the paste was dissolved in a minimum volume of 20mM-tris–HCl buffer, pH7·0, containing $MgSO_4$ (4mM), EDTA (4mM) and *N*-acetylcysteine (4mM) (referred to below as 'tris buffer') to which KCl had been added to give 0·1M concentration. The solution was dialysed in a cellophan bag with gentle agitation against 20–25 vol. of 0·1M-KCl in 'tris buffer' for 1hr., thus removing most of the $(NH_4)_2SO_4$.

Stage 3: batch chromatography on DEAE-Sephadex. Preliminary tests had shown that glucokinase is adsorbed on DEAE-Sephadex when the concentration of KCl is less than about 0·2M and is eluted by 0·3M-KCl. Direct application of the dialysed solution to a column of this adsorbent resulted in only small fractions of the glucokinase activity

being retained on the column. It was found preferable to adsorb the glucokinase on small portions of the DEAE-Sephadex A-50 (medium grade). This was freed from fines, washed with water and equilibrated at 4° with 0·1 M-KCl in 'tris buffer'. About 40 ml. (containing approx. 3 g. of dry adsorbent) of a suspension of DEAE-Sephadex in 0·1 M-KCl in 'tris buffer' was added to the dialysed solution (35–40 ml.) containing the glucokinase. After 15 min. the mixture was centrifuged at 40000*g* for 10 min. The supernatant was removed and a small portion assayed for glucokinase activity. This adsorption process was repeated two or three times with further lots of adsorbent until less than 5% of the original glucokinase activity remained in the final supernatant. The several lots of adsorbent were combined and transferred to a glass column (5 cm. diam.), small volumes of the 0·1 M-KCl in 'tris buffer' being used to assist the process. The height of material in the column was now 6–8 cm. The column was washed with 2 bed-volumes of 0·1 M-KCl in 'tris buffer'. Glucokinase activity was eluted at a rate of 100 ml./hr. with 0·3 M-KCl in 'tris buffer'. Fractions (8 ml.) were collected and those containing the bulk of the activity combined.

Stage 4: gradient chromatography on DEAE-Sephadex. The pooled fractions were diluted with an equal volume of 'tris buffer' to bring the concentrations of KCl to about 0·15 M. This diluted solution was applied to a column (3 cm. × 17 cm.) of DEAE-Sephadex A-50 (medium grade) previously washed and equilibrated with 0·15 M-KCl in 'tris buffer'. Protein not adsorbed to the column was washed through with 1 bed-volume of this same solution. Glucokinase activity was eluted overnight at a flow rate of 10 ml./hr. by a linear gradient of 0·15–0·6 M-KCl in 'tris buffer', total volume 500 ml. Fractions (3–4 ml.) were collected; activity was eluted between approx. 0·25 M- and 0·35 M-KCl. The active fractions were pooled. Fig. 1 shows an elution diagram.

Stage 5: gradient chromatography on DEAE-cellulose. The pooled fractions were diluted with 3 vol. of 'tris buffer' to give a final KCl concentration of approx. 0·1 M. The diluted solution was applied to a column (3 cm. × 31 cm.) of DEAE-cellulose that had previously been freed from fines, washed with water and equilibrated with 0·1 M-KCl in 'tris buffer'. Activity was eluted overnight at a flow rate of 20 ml./hr. by a linear gradient of 0·1–0·6 M-KCl in 'tris buffer', total volume 500 ml. Fractions (3–4 ml.) were collected; activity was again eluted between 0·25 M- and 0·35 M-KCl. Fig. 2 shows an elution diagram. The most active fractions were pooled.

Stage 6: concentration step. The volume containing glucokinase activity obtained from the DEAE-cellulose column was too large (60–70 ml.) to apply directly to the next (gel-filtration) column. The pooled fractions were diluted with an equal volume of 'tris buffer' to give a final KCl concentration just below 0·2 M. The diluted solution was applied to a very small column (1·0 cm. × 1·5 cm.) of DEAE-Sephadex A-50 (medium grade) that had been previously equilibrated with 0·2 M-KCl in 'tris buffer'. Activity was eluted with 0·4 M-KCl in 'tris buffer' at a flow rate of 0·25 ml./min. Small fractions (about 6 drops each) were collected manually. Almost all the activity could thus be concentrated into less than 3 ml. and further purification was achieved (see the summary in Table 1).

Stage 7: gel filtration on Bio-Gel P.225. Preliminary trials indicated that but little fractionation could be achieved by the use of Bio-Gel P.150. On the other hand, use of Bio-Gel P.300 was impracticable because of the extremely low flow rate obtained. A Bio-Gel P.225 preparation was made by first mixing equal volumes of Bio-Gels P.150 and P.300, each having previously been allowed to swell in water for 3 days. The concentrated solution containing glucokinase activity from the previous step was applied to a column (3·5 cm. × 30 cm.) of Bio-Gel P.225. Activity was eluted with 0·4 M-KCl in 'tris buffer' at a flow rate of 3–4 ml./hr. Fractions (3–4 ml.) were collected and those containing activity pooled. Fig. 3 shows an elution diagram.

Stage 8: final concentration of the preparation. The preparation thus obtained was dilute but of a convenient activity for the kinetic studies and the activity only decreased by about 20% per month when stored at 0°. A more stable concentrated solution could be obtained by diluting

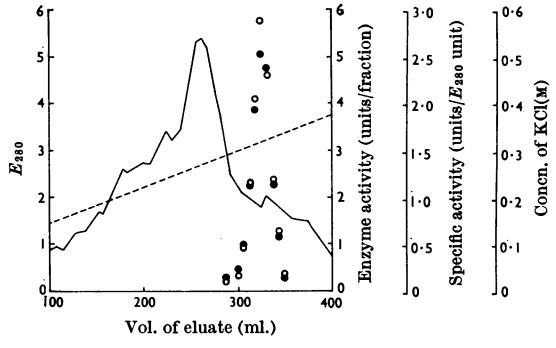

Fig. 1. Purification (stage 4) of rat hepatic glucokinase on a DEAE-Sephadex column. The experiment was carried out as described in the text. Fractions (3–4 ml.) were collected and samples (10 μl.) of those containing activity were assayed by method 1. ——, E_{280}; – – – –, concn. of KCl in effluent; ●, total activity (units/fraction); ○, specific activity (units/E_{280} unit).

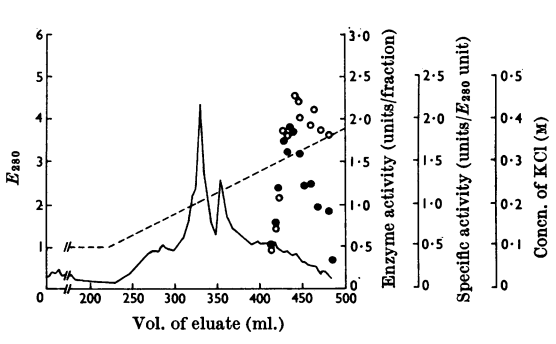

Fig. 2. Purification (stage 5) of rat hepatic glucokinase on a DEAE-cellulose column. The experiment was carried out as described in the text. Fractions (3–4 ml.) were collected and samples (10 μl.) of those containing activity were assayed by method 1. ——, E_{280}; – – – –, concn. of KCl in effluent; ●, total activity (units/fraction); ○, specific activity (units/E_{280} unit).

this solution with an equal volume of 'tris buffer' and then repeating the concentration step (stage 6).

The purification and recovery obtained by this procedure are shown in Table 1. The low amount of hexokinase activity precipitated with the glucokinase in the $(NH_4)_2SO_4$ fraction was removed during the subsequent batch chromatography on DEAE - Sephadex. 6-Phosphogluconate-dehydrogenase (EC 1.1.1.44) activity was also removed completely during that stage. Figs. 1 and 3 show that glucokinase activity was eluted only a little separated from the main protein peak in those column fractionations and indicate the need for a detailed survey of both glucokinase activity and protein concentration in each of the fractions from columns to decide which of them to combine and pass to the next stage.

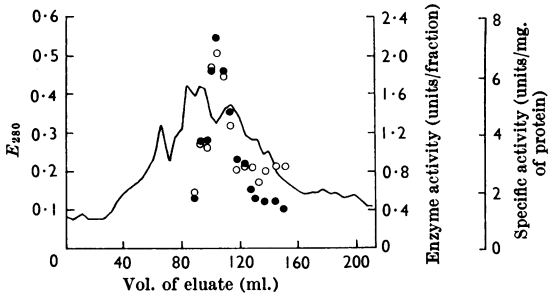

Fig. 3. Purification (stage 7) of rat hepatic glucokinase by gel filtration on Bio-Gel P.225. The experiment was carried out as described in the text. Fractions (3–4ml.) were collected and samples (10μl.) of those containing activity were assayed by method 1. ——, E_{280}; ●, total activity (units/fraction); ○, specific activity (units/mg. of protein; protein estimated by the u.v.-absorption method).

RESULTS

The small amount of enzyme prepared in the procedure described above limits the possibility of examining its purity. The effects of the various purifications steps could be assessed qualitatively by disk electrophoresis on a 7·5% (w/v) polyacrylamide gel (4cm. long) in tris–hydrochloric acid buffer, pH 7·9, at 150v and 5mA/cm.^{2}. The final preparation showed one major band and two minor bands. It has not proved possible to demonstrate that the major band (detected by staining for protein with Naphthalene Black) is that of glucokinase because enzymic activity is apparently lost during the procedures involved. It could not be detected by the histochemical staining procedure for glucokinase activity used by Moore, Chandler & Tettenhorst (1964).

Enzymic tests, using spectrophotometric methods when possible, revealed that the final preparation contained less than 0·2% of the glucokinase activity as hexokinase (determined with 0·5mM-glucose as

Table 1. *Purification of glucokinase*

Summary of a typical preparation from 120g. of rat liver, as described in the text. Enzyme activity units and specific activity are defined under 'Assay of glucokinase' in the Methods section.

Stage	Description	Protein (mg.)	Volume (ml.)	Total activity (units)	Specific activity (units/mg.)	Recovery (%)
1	Supernatant fraction of liver homogenate	9570	186	96*	0·01	100
2	Dialysed solution of $(NH_4)_2SO_4$ paste	2700	37	—†		
3	Combined fractions from first DEAE-Sephadex column	170	103	50	0·29	52
4	Combined fractions from second DEAE-Sephadex column	31	18	43	1·4	45
5	Combined fractions from DEAE-cellulose column	12	63	32	2·8	33
6	Concentrated preparation	3·0	2·0	14·6	4·9‡	15
7	Combined fractions from Bio-Gel P.225 column	1·8	22	14·3	7·9	15
8	Final concentrated preparation	1·1	2·2	9·6	8·7§	10

* This activity was calculated on the basis that 2 moles of $NADP^+$ were reduced/mole of glucose phosphorylated; all other activities were calculated on a 1:1 basis because 6-phosphogluconate-dehydrogenase activity had been removed (see the text).

† Activities rather lower than those recorded after stage 3 were obtained here; the contribution of 6-phosphogluconate-dehydrogenase activity at this stage was difficult to assess.

‡ The specific activity in the peak fraction was over 6 units/mg.

§ The specific activity in the peak fraction was over 11 units/mg.

substrate in method 1) and that the following enzyme activities were undetectable: glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase (EC 5.3.1.8), phosphoglucomutase (EC 2.7.5.1), glucose 6-phosphatase (EC 3.1.3.9), adenosine triphosphatase and adenylate kinase (EC 2.7.4.3).

Stability and storage. Glucokinase activity is soon lost unless it is stabilized by its substrates or high concentrations of K^+ (or both) and a thiol compound is present. For purification purposes the concentration of K^+ is never permitted to fall below 100mM and this concentration is also maintained in all enzyme assays. The ions Na^+, NH_4^+ and Li^+ are not very effective in these respects.

In many early preparations 2-mercaptoethanol was used as the thiol compound. Unless its concentration was maintained at a minimum of about 10mM in all solutions, large losses of activity occurred and these were not restored by the addition of more 2-mercaptoethanol. More recently *N*-acetylcysteine has been used instead and its properties have proved superior in that (*a*) lower concentrations (4mM) are needed to maintain enzymic activity, (*b*) it is far less volatile, (*c*) it is not so readily oxidized by air and (*d*) its molar extinctions at 260 and 280mμ are much lower. In a typical experiment, the *N*-acetylcysteine was removed from a glucokinase preparation by passage through a Sephadex G-25 column. The effluent from this column was assayed for activity 10min. after first applying the enzyme to the column and found to contain less than 10% of the initial activity. About 50–60% of this activity loss was restored on the addition of *N*-acetylcysteine to give a final concentration of 4mM.

Large losses of activity occurred when the purified enzyme was deep-frozen at $-20°$ although the ammonium sulphate paste (stage 2) could be stored at that temperature for several days without appreciable loss of activity. The final preparations were kept unfrozen at 0° and did not lose more than 10% of their activity in a month.

Effect of pH *on activity.* This was examined by using method 3 so that the effects measured could not be due to the influence of pH on the subsidiary enzyme system. Three different buffers were employed to cover the pH range. No evidence that the buffer concentration had any effect on the activity was found. The optimum pH was between pH 7·8 and 8·0 (Fig. 4). The marked decrease in activity at pH values below pH 7 was not due to a limitation in the active species of ATP because use of higher ATP–Mg^{2+} concentrations did not give greater rates.

Specificity for phosphoryl acceptor and effects of substrate concentrations. Figs. 5 and 6 illustrate

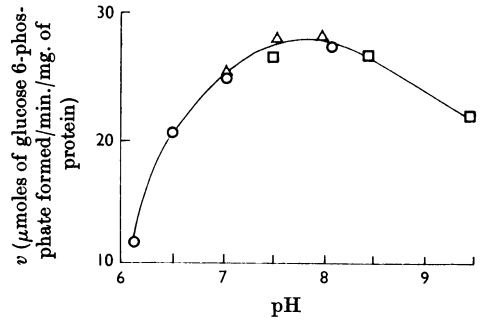

Fig. 4. Effect of pH on rat hepatic glucokinase activity at 30°. Activity was measured by method 3 as described in the text. The reaction mixture included: glucose, 100mM; $MgSO_4$, 7·5mM; ATP, 5mM; enzyme, 0·045 unit/ml. The buffers used (final concentrations) were: ○, 100mM-imidazole; △, 100mM-glycylglycine; □, 100mM-glycine. The pH of the remaining reaction medium was checked at the end of the incubations.

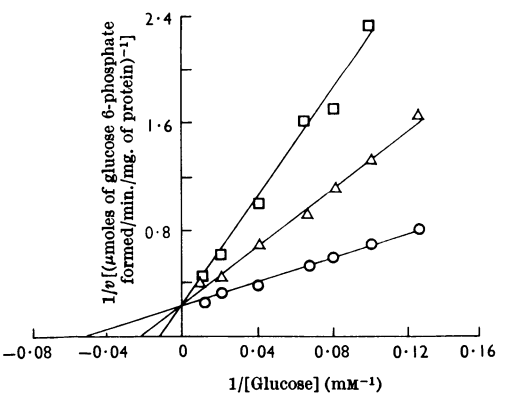

Fig. 5. Effect of the glucose concentration on the velocity and the effect of mannose on glucose phosphorylation. The velocity was measured by determining glucose 6-phosphate formation (method 1). Further details are given in the text. The reaction mixture included: ATP, 5mM; $MgSO_4$, 10mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, Glucose only; △, glucose+20mM-mannose; □, glucose+40mM-mannose.

results obtained by using methods 1 and 2 for the determination of K_m and K_i values. The range of substrate concentrations employed for K_m determinations was normally 8–100 mM. The two methods gave identical values for both K_m and $V_{max.}$ with glucose as substrate. Table 2 summarizes the results obtained. The K_m values were unaffected by the ATP concentration. Glucokinase catalyses the phosphorylation of mannose and 2-deoxyglucose in addition to glucose. Fructose is also phosphorylated; its effects on the velocity were examined over

a concentration range 100–1250mM. Extrapolation of the double-reciprocal plot suggested a K_m value for fructose approaching 2M with a $V_{max.}$ some 5 times that for glucose. The true K_m for the true substrate is likely to be considerably lower because the fructofuranose form of fructose which is phosphorylated (Slein, Cori & Cori, 1950) represents only about 20% of the total fructose concentration (Gottschalk, 1944). The other sugars tested were not phosphorylated in the presence of glucokinase. The K_i values for mannose, 2-deoxyglucose and fructose determined by method 1 with glucose as substrate were lower than their K_m values determined by method 2.

Phosphoryl donor. The true phosphoryl donor in phosphokinase reactions is considered to be an ATP–Mg^{2+} complex (Bock, 1960) and most hexokinases show maximal activity when the ATP/Mg^{2+} ratio is about unity. An excess of ATP over Mg^{2+} resulted in inhibition of glucokinase, but up to a tenfold excess of Mg^{2+} over the ATP concentration had no effect. The K_m for ATP (Fig. 7) was 0·5mM. ITP gave only a very low rate of phosphorylation.

Inhibition by the products. ADP is an inhibitor of glucokinase and the nature of the inhibition depends

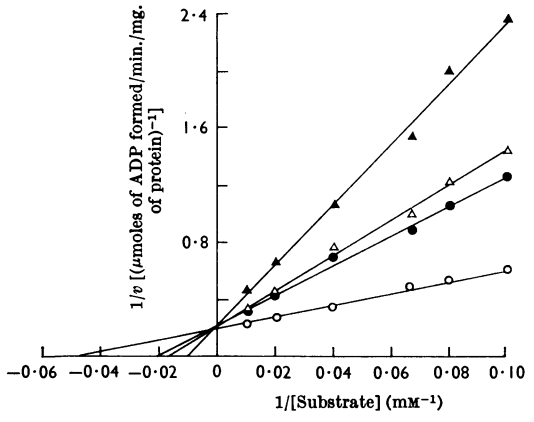

Fig. 6. Effect of glucose or mannose concentration on the velocity, and the inhibition of glucose and mannose phosphorylation by *N*-acetylglucosamine. The velocity was measured by determining ADP formation (method 2). Further details are given in the text. The reaction mixture included: ATP, 5mM; $MgSO_4$, 10mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, Glucose only; △, glucose+0·5mM-*N*-acetylglucosamine; ●, mannose only; ▲, mannose+0·5mM-*N*-acetylglucosamine.

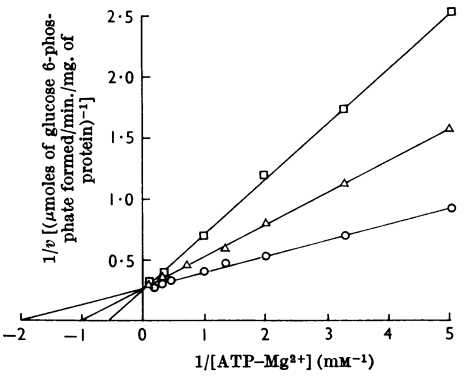

Fig. 7. Effect of the ATP–Mg^{2+} concentration on the velocity in the absence and presence of ADP. The velocity was measured by method 1. Further details are given in the text. The ATP and Mg^{2+} concentrations were kept constant at a 1:1 molar ratio. The reaction mixture included: glucose, 100mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, No inhibitor; △, 2mM-ADP; □, 4mM-ADP.

Table 2. *Substrate specificity of rat hepatic glucokinase: K_m and K_i values*

Results were obtained from Lineweaver–Burk plots of results such as those illustrated in Figs. 5 and 6. Further details are given in the text. The number (1 or 2) in parenthesis after each result refers to the method used as described in the Methods section. N.P., Not phosphorylated.

Substrate	Modified with respect to glucose at	Relative maximal rate	K_m (mM)	K_i (mM)
D-Glucose	—	1·0 (1, 2)	20 (1, 2)	—
D-Mannose	C-2	0·9 (2)	50 (2)	14 (1)
2-Deoxy-D-glucose	C-2	0·5 (2)	95 (2)	16 (1)
D-Fructose	C-1, C-2	*	Very high (1†, 2)	350 (1)
N-Acetyl-D-glucosamine	C-2	N.P.	—	0·5 (1, 2)
D-Glucosamine	C-2	N.P.	—	1·0 (1)
D-Galactose	C-4	N.P.	—	670 (1)
D-Lyxose	C-2, C-6	N.P.	—	83 (1)
D-Xylose	C-6	N.P.	—	120 (1)

* See comments in the text.

† 0·8 unit of phosphoglucose-isomerase activity was added to the reaction mixture.

on the Mg^{2+} concentration. When the ATP/Mg^{2+} molar ratio is 1:1, ADP acts as a competitive inhibitor both with respect to ATP–Mg^{2+}, at a constant glucose concentration (Fig. 7), and to glucose, at a fixed ATP–Mg^{2+} concentration (Fig. 8). The K_i for ADP in both cases was 2mM. When the Mg^{2+} concentration is made equal to (or in small excess of) the total ATP plus ADP concentration, ADP–Mg^{2+} gives a complex inhibitory effect as the ATP–Mg^{2+} concentration is changed at a constant glucose concentration (Fig. 9). The K_i for the non-competitive component of the inhibition was about 2mM and that for the competitive component was 0·9mM. When the ATP–Mg^{2+} concentration is kept constant, ADP–Mg^{2+} is a non-competitive inhibitor with respect to glucose (Fig. 10) with a K_i of 2 mM.

Glucose 6-phosphate is a competitive inhibitor of glucokinase activity with respect to glucose at a constant ATP–Mg^{2+} concentration (Fig. 11) with a K_i of 65mM, but gives a mixed type of inhibition with respect to ATP–Mg^{2+} at a constant glucose concentration which is similar to that due to ADP–Mg^{2+} shown in Fig. 9.

Effect of temperature. An Arrhenius plot for glucokinase activity, measured by method 3 and with incubation times up to 6min., was a straight line over the temperature range 5–46·5°. Identical results over a slightly narrower range of temperatures were obtained by using method 1; the K_m value was independent of temperature over the range tested. The Q_{10} is 1·9 and the energy of activation is 12500cal./mole for the rate-determining step.

Anomalous behaviour at low glucose concentrations. None of the kinetic properties previously described were affected in any way by the concentration of thiol or K^+ present. However, in all the results so far given the final glucose concentration was not less than 8mM. We have consistently found, on examination of the effect of the glucose concentration on any preparation of glucokinase purified to

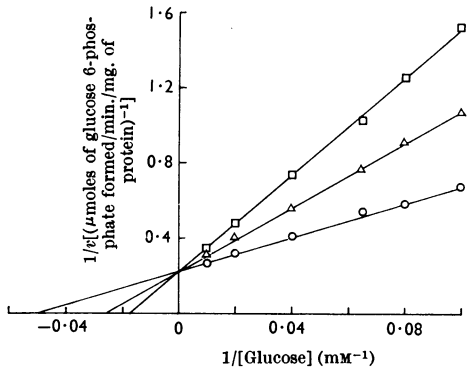

Fig. 8. Effect of ADP on the velocity as the glucose concentration is varied and the ATP–Mg^{2+} concentration remains constant. The velocity was measured by method 1. Further details are given in the text. The reaction mixture included: ATP–Mg^{2+}, 5mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, No inhibitor; △, 2mM-ADP; □, 4mM-ADP.

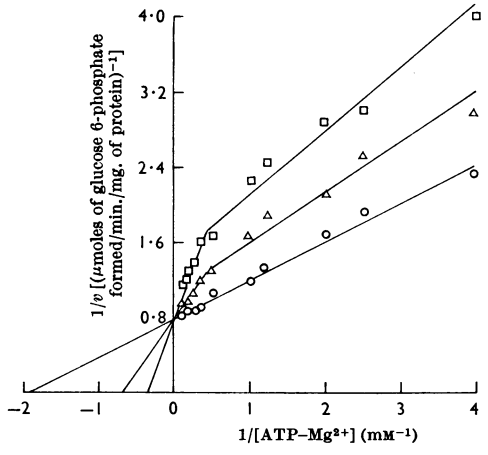

Fig. 9. Effect of ADP–Mg^{2+} on the velocity as the ATP–Mg^{2+} concentration is varied and the glucose concentration remains constant. The velocity was measured by method 1. Further details are given in the text. The ATP and Mg^{2+} concentrations were kept constant at a 1:1 molar ratio. The reaction mixture included: glucose, 100mM; enzyme, 0·015 unit/ml.; the temperature was 28°. ○, No inhibitor; △, 2mM-ADP–Mg^{2+}; □, 4mM-ADP–Mg^{2+}.

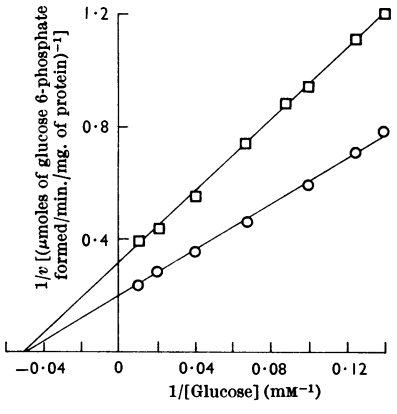

Fig. 10. Effect of ADP–Mg^{2+} on the velocity as the glucose concentration is varied and the ATP–Mg^{2+} concentration remains constant. The velocity was measured by method 1. Further details are given in the text. The reaction mixture included: ATP, 5mM; $MgSO_4$, 5mM; glucose, 100mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, No inhibitor; □, 3mM-ADP–Mg^{2+}.

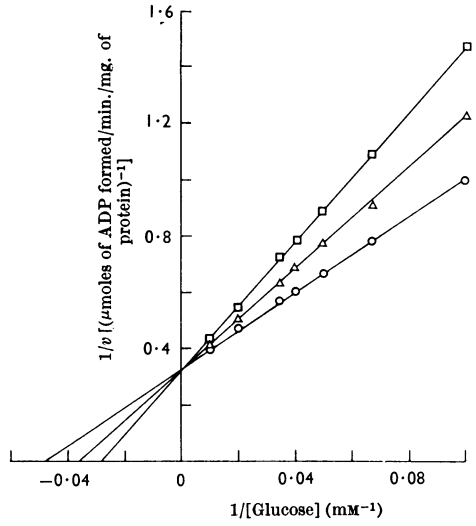

Fig. 11. Effect of glucose 6-phosphate on the velocity in the presence of different concentrations of glucose. The velocity was measured by method 2. Further details are given in the text. The reaction mixture included: ATP, 5mM; $MgSO_4$, 10mM; enzyme, 0·03 unit/ml.; the temperature was 28°. ○, No inhibitor; △, 22·5mM-glucose 6-phosphate; □, 45mM-glucose 6-phosphate.

stage 3 or beyond, that the activities with glucose concentrations of less than 8mM were lower than those predicted by extrapolation of the Lineweaver–Burk plot through the points obtained with higher glucose concentrations. This effect is not dependent on the presence or absence of K^+ or a thiol or both; it is complex and is the subject of further investigations.

DISCUSSION

The kinetic parameters for rat-liver glucokinase described in this paper have been obtained with a preparation that has a specific activity higher than that previously reported for any mammalian tissue hexokinase (although crystalline yeast hexokinase has a much higher specific activity of about 600 units/mg.; Darrow & Colowick, 1962). The substrate specificity recorded verifies the preliminary results with crude liver extracts (Walker, 1963; Walker & Rao, 1963) and is narrower than that of the widely distributed low-K_m animal hexokinases (Crane & Sols, 1955; Crane, 1962; Walker, 1966). Hepatic glucokinase is not absolutely specific for glucose, as is the glucokinase described in *Brevibacterium fuscum* (Saito, 1965), but the revised report on Enzyme Nomenclature (1965) covers the properties of the liver enzyme under the classification EC 2.7.1.2. The most striking feature of the specificity (Table 2) is the fact that mannose and 2-deoxyglucose are phosphorylated with high K_m values whereas glucosamine and *N*-acetylglucosamine, which are also modified with respect to glucose at C-2, are not phosphorylated but are competitive inhibitors with low K_i values. Other mammalian hexokinases catalyse the phosphorylation of glucosamine.

Under physiological conditions glucose is the only substrate likely to be phosphorylated by glucokinase. The parameters for fructose rule out the possibility of it being phosphorylated. The specificity explains why the ability of the liver to synthesize glycogen from both glucose and mannose is decreased in alloxan-diabetes (Wood, Leboeuf, Renold & Cahill, 1961), when glucokinase activities fall to very low values (Salas, Viñuela & Sols, 1963; Sharma, Manjeshwar & Weinhouse, 1963; Walker & Rao, 1964), and why conversion of glucose into glycogen in slices or normal liver is inhibited by glucosamine and *N*-acetylglucosamine (Spiro, 1958). The K_m value of 20mM for glucose is a little higher than that deduced with less pure preparations. We have noted on several occasions that the apparent affinity for glucose decreases after the purification step involving the use of DEAE-cellulose; before that step K_m values for glucose were 12–15mM.

The very low inhibitory effect of glucose 6-phosphate on glucokinase activity has been noted and the physiological significance discussed (Viñuela *et al.* 1963; Sols, Salas & Viñuela, 1964). Glucokinase does not possess the special allosteric site for inhibition by the product as do many mammalian hexokinases. Normal competitive inhibition at higher concentrations of glucose 6-phosphate is not surprising when one considers the probable mechanism of phosphoryl transfer (Crane, 1964). The inhibition by the other product, ADP, is complex; it depends on the concentrations of both Mg^{2+} and ATP–Mg^{2+}. Now Mg^{2+} forms complexes with the β- and γ-phosphate groups of ATP and with the α- and β-phosphate groups of ADP that may be designated $MgATP^{2-}$ and $MgADP^-$ respectively (Cohn & Hughes, 1962). Bock (1960) suggests that, in the physiological pH range and depending on the total Mg^{2+} concentration, the dominant species will be $MgATP^{2-}$ and either ADP^{3-} or $MgADP^-$; the latter form will occur to the extent permitted by the concentration excess of Mg^{2+} over ATP. On this basis, the conditions in Figs. 7 and 8 correspond to inhibitions by ADP^{3-} and in Figs. 9 and 10 to inhibition by $MgADP^-$. It is apparent that inhibition by ADP^{3-} is competitive whereas that due to $MgADP^-$ is more complex. Bock (1960) has also discussed the difficulties involved in creating precise conditions for obtaining exact kinetic information about the role of the nucleotides in phosphotransferase reactions. The conditions used in the present study do

not provide data on which the mechanism of action can be discussed. The possibility that ADP may regulate glucokinase activity *in vivo* is apparent and Mg^{2+} influences such effects. Hepatic glucokinase behaves as an 'ideal' phosphokinase (Crane, 1964) in that (i) there is competitive inhibition between glucose and glucose 6-phosphate, (ii) glucose 6-phosphate is competitive with $MgATP^{2-}$ and (iii) ADP^{3-} is competitive with $MgATP^{2-}$. Hence its phosphoryltransferase activity is similar to that of many other phosphokinases including yeast hexokinase. The basic and intriguing difference compared with other hexokinases is the comparatively low affinity for the acceptor molecule.

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