Biochem. J. (1967) **105**, 473
Printed in Great Britain

Further Properties and Possible Mechanism of Action of Adenosine 5′-Triphosphate–D-Glucose 6-Phosphotransferase from Rat Liver

By M. J. PARRY and D. G. WALKER
Department of Biochemistry, University of Birmingham

(*Received* 17 *February* 1967)

1. Magnesium ions are the most effective bivalent ions in the glucokinase reaction. 2. The molecular weight of rat hepatic glucokinase is 48000–49000 as assessed by gel filtration on Sephadex G-100. 3. Anomalous kinetic behaviour at low glucose concentrations appears to be due to the formation during the purification procedure of fragments possessing modified catalytic properties, but is unlikely to be of physiological significance. 4. Extension of previous studies (Parry & Walker, 1966) suggests that glucokinase catalyses a reaction of the random Bi Bi type similar to that of yeast hexokinase. 5. The inhibitory effects of various thiol reagents suggest that a thiol group may be involved at or near the binding site of the acceptor molecule.

A previous paper (Parry & Walker, 1966) described a procedure for an 870-fold purification of glucokinase (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2) from rat liver. The enzyme preparation had a specific activity of over 8 μmoles of glucose phosphorylated/min./mg. of protein, and certain properties such as its stability, substrate specificity and inhibition by the products of the reaction were given (Parry & Walker, 1966).

The present paper describes a number of other properties of this preparation of glucokinase, including the effect of thiol inhibitors, and presents steady-state kinetic data that suggest that the reaction catalysed by glucokinase occurs by a random Bi Bi rapid-equilibrium mechanism.

MATERIALS AND METHODS

Chemicals and enzymes. The sources of most of the materials were given previously (Parry & Walker, 1966). In addition, Sephadex G-25 and G-100 and Blue Dextran were obtained from Pharmacia (G.B.) Ltd., London, W. 13. Bovine plasma albumin was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., ribonuclease (ox pancreas) was from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and ovalbumin, soya-bean trypsin inhibitor and horse-heart cytochrome *c* were from Sigma (London) Ltd., London, S.W. 6. All other chemicals were A.R. or best quality reagent grade.

Preparation and assay of glucokinase. The enzyme was purified as described previously (Parry & Walker, 1966). For many experiments the final concentrated preparation (stage 8) was diluted as necessary, but the solution of enzyme at the end of stage 7 (i.e. before the final concentration step) was also sometimes used, after storage at 0°, within a few days of preparation. All specimens of enzyme used in these studies had a specific activity of not less than 7 μmoles/min./mg. of protein.

Glucokinase was assayed either by method 1 (which measures glucose 6-phosphate formation by coupling directly to the reduction of $NADP^+$) or by method 3 (a discontinuous procedure for measuring glucose 6-phosphate formation) as previously described (Parry & Walker, 1966). Method 1 had to be modified in the study on the effect of thiol compounds on glucokinase activity (see below).

'Tris buffer' consisted of 20mM-tris–HCl buffer, pH 7·0, containing $MgSO_4$ (4mM), EDTA (4mM) and *N*-acetylcysteine (4mM).

Effect of metal ions. The low concentrations of Mg^{2+} ions and EDTA present in the standard 'tris buffer' solution in which glucokinase was stored were removed for this study by passing the enzyme solution through a Sephadex G-25 column previously equilibrated with 20mM-tris–HCl buffer, pH 7·0, containing *N*-acetylcysteine (4mM). Other details are given along with the results.

Gel filtration on Sephadex G-100. The method used was based on that of Andrews (1965). A column (40cm. × 2·5 cm.) was packed under a low hydrostatic pressure. Proteins were eluted with 'tris buffer' containing KCl (0·4M) at a flow rate of 6–8ml./hr. and 3ml. fractions were collected. The column was calibrated with the following markers, which were located and estimated by the procedures given in parentheses: blue dextran, mol.wt. 2×10^6 (absorption at 625mμ); glucose 6-phosphate dehydrogenase, mol.wt. 110000 (reduction of $NADP^+$); bovine serum albumin, mol. wt. 67000 (absorption at 280mμ); ovalbumin, mol.wt. 44000–46000 (absorption at 280mμ); soya-bean trypsin inhibitor, mol.wt. 21500 (absorption at 280mμ); ribonuclease, mol.wt. 13700 (method of McDonald, 1955); horse-heart cytochrome *c*, mol.wt. 12400 (absorption at 412mμ).

Centrifugation studies. Preparative ultracentrifugation was performed in a 3×5ml. swing-out rotor in an MSE Super-Speed 40 Centrifuge at 37000rev./min., corresponding to $136000g_{av}$ in the 5ml. of enzyme solution in 'tris buffer'. The tubes were pierced at the end of the run and fractions collected as given with the results. In some experiments 0·75ml. of enzyme solution was layered over 4·25ml. of 'tris buffer' containing sucrose (2·22%, w/v).

Sucrose-density-gradient centrifugation was performed by layering 0·5ml. of the enzyme sample in 'tris buffer' over a linear gradient (total vol. 4·0ml.) of 5–20% (w/v) sucrose prepared in an aqueous medium containing $MgSO_4$ (4mM), EDTA (4mM), *N*-acetylcysteine (4mM) and KCl (0·4M). After centrifugation for various times (4–20hr.) the tubes were pierced and fractions (0·25ml.) collected manually.

Effect of thiol compounds. The *N*-acetylcysteine used as stabilizer during the purification procedure had first to be removed by passing the enzyme through a Sephadex G-25 column (as above) previously equilibrated with 20mM-tris–HCl buffer, pH7·0, containing $MgSO_4$ (4mM), EDTA (4mM), and KCl (0·4M). The glucokinase thus eluted free of *N*-acetylcysteine was used for kinetic studies within 2hr. of preparation.

When method 1 (Parry & Walker, 1966) was used for velocity measurements of glucokinase activity by the standard procedure, the velocity gradually decreased over a period of approx. 15min., after which time rates remained linear. Acceptable initial-velocity values were obtained by modifying the procedure so that all the reagents including the glucokinase and thiol compound, but excluding the ATP, $MgSO_4$ and glucose 6-phosphate dehydrogenase, were preincubated at 28° for 15min. Linear rates were then obtained within 1min. of adding these last three reagents. The concentrations of thiol compounds used in these experiments had no significant effect on the activity of glucose 6-phosphate dehydrogenase (cf. Salas, Salas, Viñuela & Sols, 1965).

RESULTS

As described previously (Parry & Walker, 1966) it has not been possible to date to obtain sufficient quantities of the enzyme for determination of its purity. Further studies with disk electrophoresis on polyacrylamide gels of samples at various stages of the purification procedure lead us to believe that the major band seen with the purest preparations does represent glucokinase.

Metal ion specificity

Two types of test were used. First, metal ions were removed from the preparation on a Sephadex G-25 column and the effects of a range of bivalent metal ions examined in the presence of K^+ ions and *N*-acetylcysteine as the thiol stabilizer. Secondly, the effects of adding a 5mM concentration of various bivalent metal ions in the presence of 5mM-Mg^{2+} ions under similar conditions were examined. The ATP concentration was 5mM and Table 1 shows that Mg^{2+} ions were the most effective activators, but that Mn^{2+} ions, and to a smaller extent Co^{2+}, Ca^{2+} and Cd^{2+} ions, had some activating potential. All the other bivalent ions inhibited the activity in the presence of Mg^{2+} ions, indicating that the latter are preferred.

Molecular weight

The gel-filtration method with Sephadex G-100 was employed and samples of the fractions collected were assayed by method 1 for glucokinase activity.

Table 1. *Specificity of rat-liver glucokinase for bivalent metal ions*

Activities were measured by the discontinuous assay method 3 (Parry & Walker, 1966). The activities in columns 2 and 3 represent those estimated in the absence of added Mg^{2+}, and those in columns 4 and 5 represent activities estimated in the presence of 5mM-Mg^{2+}. The concentration of bivalent metal ion added was 5mM in every case. Further details are given in the text.

Bivalent metal ion	Activity in the presence of 5mM-ATP (μmoles of glucose 6-phosphate formed/min./mg. of protein)	% of activity with Mg^{2+}	Activity in the presence of 5mM-ATP and 5mM-$MgSO_4$ (μmoles of glucose 6-phosphate formed/min./mg. of protein)	% of activity with Mg^{2+} only
Mg^{2+}	7·40*	100	6·12*	100
Mn^{2+}	4·00	54	3·10	51
Co^{2+}	1·90	25	2·00	33
Ca^{2+}	1·55	21	4·00	65
Cd^{2+}	0·97	13	1·94	32
Ni^{2+}	0·47	6·5	2·85	47
Zn^{2+}	0·00	0	0·15	2·5
Cu^{2+}	0·00	0	0·00	0
Fe^{2+}	0·00	0	0·00	0
Hg^{2+}	0·00	0	0·00	0

* Different enzyme preparations were used in the two experiments.

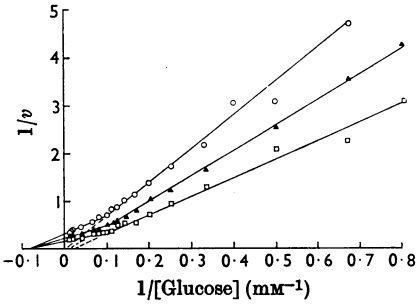

Fig. 1. Effect of glucose concentration on the velocity at several concentrations of glucokinase. The velocity, v, was measured by method 1 (Parry & Walker, 1966) and is expressed as μmoles of glucose 6-phosphate formed/min./mg. of protein. The reaction mixture included: ATP, 5mM; $MgSO_4$, 7·5mM; the temperature was 28°. ○, 0·023unit of glucokinase/ml.; ▲, 0·034unit of glucokinase/ml.; □, 0·045unit of glucokinase/ml.

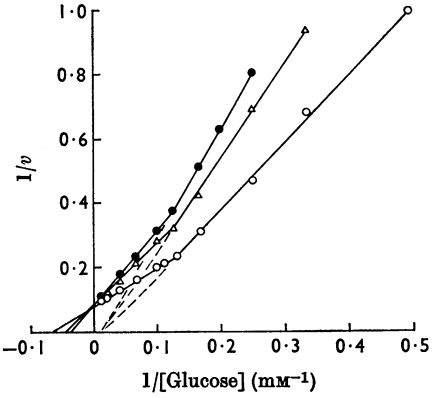

Fig. 2. Effect of mannose and *N*-acetylglucosamine on the velocity of glucose phosphorylation. The velocity, v, was measured by method 1 (Parry & Walker, 1966) and is expressed as μmoles of glucose 6-phosphate formed/min./mg. of protein. The reaction mixture included: ATP, 5mM; $MgSO_4$, 7·5mM; glucokinase, 0·07 unit/ml.; the temperature was 28°. ○, No inhibitor; △, 10mM-mannose; ●, 0·2mM-*N*-acetylglucosamine.

Examination of several preparations each of the crude supernatant preparation of liver homogenate, of glucokinase partially purified to stage 4 of the purification procedure (Parry & Walker, 1966) and of the final concentrated preparation (stage 8) all gave a molecular weight of 48000–49000.

Anomalous behaviour at low glucose concentrations

This was noted previously (Parry & Walker, 1966) and is illustrated in detail in Fig. 1. At glucose concentrations below about 8mM activities with the purified enzyme were lower than those expected by extrapolation of the double-reciprocal plots (Lineweaver & Burk, 1934). Lines drawn through the points obtained with these low glucose concentrations and with several enzyme concentrations appeared to converge near the abscissa to the right of the ordinate. Similar types of plots were obtained with one enzyme concentration and adding a competitive inhibitor that acts at the phosphoryl-acceptor site (Fig. 2). This anomalous behaviour was found to occur with all glucokinase preparations purified to a point from stage 3 onwards, but was not seen with the initial supernatant fraction (stage 1) or immediately after the ammonium sulphate stage.

Numerous modifications of the assay medium were made, including addition of several different concentrations of bovine serum albumin, potassium chloride and *N*-acetylcysteine. Potassium chloride, *N*-acetylcysteine or Mg^{2+}–EDTA were removed from the purified enzyme preparations either individually or in combinations by use of Sephadex G-25 columns. None of these procedures had any influence on the behaviour illustrated in Figs. 1 and 2.

The results in Fig. 1 were redrawn in several different ways and did not suggest that the effect of substrate concentration was due to substrate activation. In particular, the break in the line in Fig. 1 (and similar plots) is a rather sharp one. Further studies therefore revolved around the possibility that the anomalous behaviour was due to the existence of more than one form of glucokinase. Schachman (1960) reported the dissociation of yeast hexokinase by certain hexoses, and Kenkare & Colowick (1965) have presented evidence for the existence of active half-units of yeast hexokinase under certain conditions.

Solutions of preparations of the enzyme were centrifuged for long periods and the distribution of glucokinase was then determined. Typical results are shown in Fig. 3. Whereas the activity of the crude supernatant fraction sedimented in a normal manner, the preparation from stage 4 showed unusual behaviour in that a portion of the activity sedimented rapidly to the bottom of the tube while some remained distributed throughout almost the length of the tube. All preparations from stage 3 of the preparative procedure onwards showed this evidence for more than one molecular form. The distribution of glucokinase activity after different time-periods supported this conclusion. Plot *A* in Fig. 3 is typical for a single protein species (Trautman & Breese, 1959) and corresponds (Schachman,

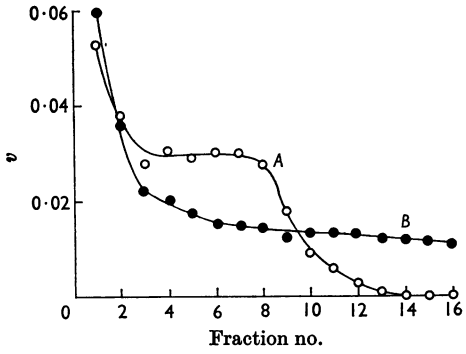

Fig. 3. Distribution of glucokinase activity after centrifugation of 5 ml. of crude and partially purified glucokinase preparations. Centrifugation was at 136000*g* at 4°; further details are given in the text. The velocity, *v*, was measured by method 1 (Parry & Walker, 1966) and is expressed as ΔE_{340}/min./20 μl. of fraction. ○, 100000*g* Supernatant of a 33% (w/v) homogenate of liver centrifuged for 18 hr.; the total initial activity was 1·57 units. ●, Stage 4 eluate centrifuged for 18 hr.; the total initial activity was 1·42 units. Fraction 1 represents that at the bottom of the tube in each case.

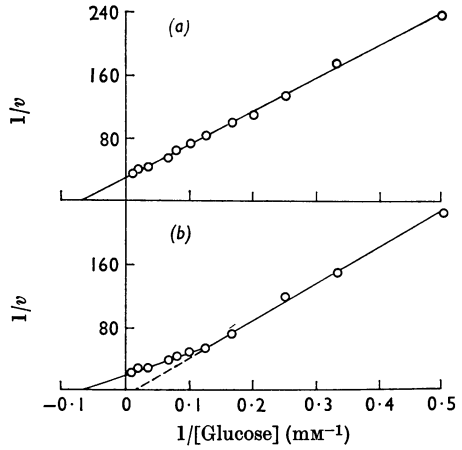

Fig. 4. Effect of glucose concentration on the glucose-phosphorylating activity in the combined 'heavy' and 'light' components separated as described in the text and illustrated in Fig. 3. The velocity, *v*, was measured by method 1 (Parry & Walker, 1966) and is expressed (*a*) as ΔE_{340}/min./100 μl. of 'heavy' fraction and (*b*) as ΔE_{340}/min./40 μl. of 'light' fraction. The points represent the means of several closely agreeing determinations. The reaction mixture included: ATP, 5 mM; $MgSO_4$, 7·5 mM; the temperature was 28°. (*a*) 'Heavy' component; (*b*) 'light' component.

1959) to an *S* value of 3–4 s. If the glucokinase molecule is spherical this indicates a molecular weight a little less than that of haemoglobin. The 'heavy' component also has a similar *S* value. The plot *B* for the stage 4 eluate (Fig. 3) was obtained after centrifugation for 18 hr. No significant difference in distribution pattern was observed when the centrifugation time was varied from 3 to 20 hr. This was in contrast with the changes in enzyme activity distribution, which occurred as predicted when the supernatant fraction (stage 1) was centrifuged for these various time-periods.

Portions of the glucokinase comprising fractions 1–2 and 7–13 of Fig. 3 (curve *B*) were combined and designated the 'heavy' and 'light' components respectively. The effect of glucose concentration on the activity of these two components was studied by using method 1 and examined in the form of double-reciprocal plots (Fig. 4). That for the 'heavy' component (Fig. 4*a*) gave straight-line relationships down to 2 mM-glucose and indicated the normal K_m of glucokinase for glucose, whereas the plot for the 'light' component (Fig. 4*b*) was biphasic, as in Fig. 1. Two forms of glucokinase, each individually responsible for the two portions of the biphasic plots, had therefore not been separated. The anomalous behaviour at low glucose concentrations appears to be associated with the presence of the 'light' component. It is possible that there could be partial conversion of the 'light' into the 'heavy' component when subjected to the conditions of activity assay.

Because the behaviour of the 'heavy' component on centrifugation appeared to be similar to that of the glucokinase activity in the crude supernatant preparation and there was no evidence of anomalous behaviour with these crude preparations, it seemed possible that the 'light' component was formed from the natural form of glucokinase, with which the 'heavy' component is identical with respect to apparent molecular weight. Several attempts were made both to prevent appearance of the 'light' fraction during purification of glucokinase and to bring about its disappearance as indicated by behaviour on centrifugation; these included addition of bovine serum albumin (up to 4 mg./ml.) to the incubation medium and variation in the concentrations of various reactants. When the 'light' component was passed through a Sephadex G-100 column it behaved exactly as did the 'heavy' component and other glucokinase preparations at various stages of purification, i.e. corresponding to mol.wt. 48000–49000, and gave no indication of a lower-molecular-weight component as might be expected from the sedimentation rate. When the 'light' component was centrifuged under similar conditions to those used in Fig. 3, but with the addition of 4 mg. of sperm-whale myoglobin, it sedimented in a similar manner to that in Fig. 3 (curve *B*) and as though its molecular weight was no higher than that of the myoglobin.

Further centrifugation studies

Several other studies failed to provide any further evidence as to the mechanism of or reason for the appearance of the 'light' component. Briefly, therefore, when purified glucokinase was layered over 2·22% (w/v) sucrose and centrifuged at 136000*g* for 20hr. it sedimented at a slightly lower rate than that of bovine serum albumin (mol.wt. 67000) but behaved as one component only. Addition of 5mM- or 100mM-glucose with or without 5mM-ATP–Mg^{2+} had no effect on the rate of sedimentation. The 'light' component separated by centrifugation as described above and in Fig. 3 also sedimented at the same rate as the 'heavy' component and purified glucokinase under the same conditions. Purified glucokinase sedimented as one component when layered over a 5–20% (w/v) sucrose density gradient and centrifuged at 136000*g* for periods ranging from 4 to 20hr. Addition of glucose or ATP–Mg^{2+} or both as above did not effect the sedimentation rate. Full details of these experiments have been given (Parry, 1966). The principal technical difficulty in all these experiments was that they have to be performed in the presence of a thiol reagent, otherwise activity is lost too rapidly (see below) for such periods of centrifugation. Thus it has not proved possible to study the influence, if any, of these compounds on the phenomena reported above.

Mechanism of action

Some basic characteristics, including product inhibition, of glucokinase activity have been described previously. Mannose, which is phosphorylated by glucokinase with apparent K_m 50mM and apparent K_i 14mM with respect to glucose (Parry & Walker, 1966), acts as a non-competitive inhibitor with respect to $MgATP^{2-}$ as the concentration of the latter is varied at a constant glucose concentration (100mM); the apparent K_i for this inhibition was 150mM (Fig. 5).

Effect of variation of the concentration of one substrate in the presence of several fixed concentrations of the other substrate. The results are given in Figs. 6 and 7. The fixed concentrations were chosen over a wide range on both sides of the apparent K_m values. The concentration of ATP–Mg^{2+} had no effect on the apparent K_m for glucose (16·7mM in this experiment) and the concentration of glucose had no effect on the K_m for ATP (1·0mM in this experiment). Secondary plots of $1/V_{max.}$ against 1/[S] in each case (Florini & Vestling, 1957) gave straight-line relationships and intercepts on the abscissae corresponding to identical apparent K_m values (Fig. 8). Other secondary plots (not shown) derived from the data in Figs. 6 and 7 for the slopes

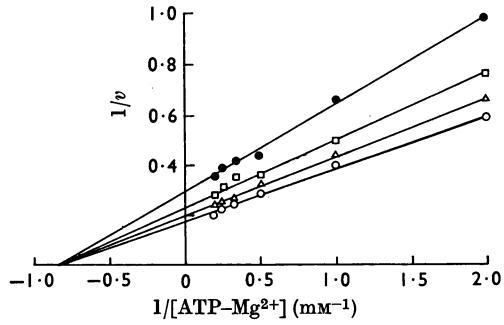

Fig. 5. Effect of mannose on the velocity as the ATP–Mg^{2+} concentration is varied and the glucose concentration remains constant. The Mg^{2+} and ATP concentrations were kept at a constant 1:1 molar ratio. The velocity, *v*, was measured by method 1 (Parry & Walker, 1966) and is expressed as μmoles of glucose 6-phosphate/min./mg. of protein. The reaction mixture included: glucose, 100mM; enzyme, 0·019unit/ml.; the temperature was 28°. ○, No inhibitor; △, 20mM-mannose; □, 50mM-mannose; ●, 100mM-mannose.

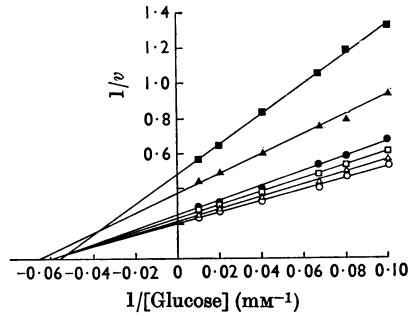

Fig. 6. Effect of several concentrations of ATP–Mg^{2+} on the velocity as the glucose concentration is varied. The velocity, *v*, was measured by method 1 (Parry & Walker, 1966) and is expressed as μmoles of glucose 6-phosphate/min./mg. of protein. The Mg^{2+} and ATP concentrations were kept at a constant 1:1 molar ratio. The reaction mixture included: enzyme, 0·021unit/ml.; the temperature was 28°. ○, 5mM-ATP; △, 4mM-ATP; □, 3mM-ATP; ●, 2mM-ATP; ▲, 1mM-ATP; ■, 0·5mM-ATP.

of the lines versus the corresponding 1/[S] values also gave straight lines converging on the abscissa at points corresponding to identical apparent K_m values.

Effect of thiol reagents. To permit a study of the effect of several thiol reagents on glucokinase it was necessary to remove the *N*-acetylcysteine used as stabilizer (see the Materials and Methods section). Glucokinase is highly unstable under such conditions, its activity decreasing approx. 50% in 1hr.

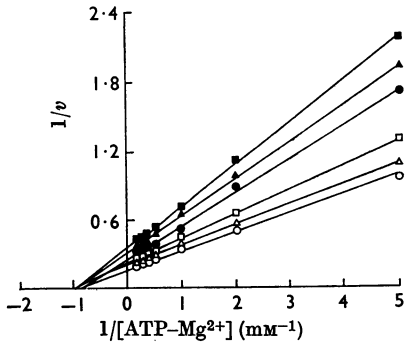

Fig. 7. Effect of several concentrations of glucose on the velocity as the ATP–Mg^{2+} concentration is varied. The velocity, v, was measured by method 1 (Parry & Walker, 1966) and is expressed as μmoles of glucose 6-phosphate/min./mg. of protein. The Mg^{2+} and ATP concentrations were kept at a constant 1:1 molar ratio. The reaction mixture included: enzyme, 0·021 unit/ml.; the temperature was 28°. ○, 100mM-Glucose; △, 50mM-glucose; □, 25mM-glucose; ●, 15mM-glucose; ▲, 12·5mM-glucose; ■, 10mM-glucose.

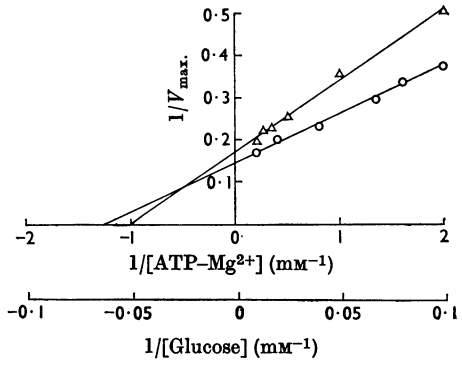

Fig. 8. Plot of the reciprocal of the maximum reaction velocity, $V_{max.}$ (obtained from the intercepts on the ordinate in Figs. 6 and 7), expressed as μmoles of glucose 6-phosphate/min./mg. of protein, versus reciprocals of the molar concentrations of either glucose or ATP. The intercepts on the abscissa give the reciprocals of the corresponding K_m values. ○, Glucose from Fig. 7; △, ATP from Fig. 6.

It proved perfectly feasible to perform the following experiments and obtain linear plots if all results were obtained within about 90min. of removal of *N*-acetylcysteine and were corrected for the percentage loss of activity in control incubations, with identical assay conditions, of enzyme that had not been treated with thiol inhibitor. Fig. 9 shows the effect of increasing concentrations of phenylmercuric acetate and *p*-chloromercuribenzoate.

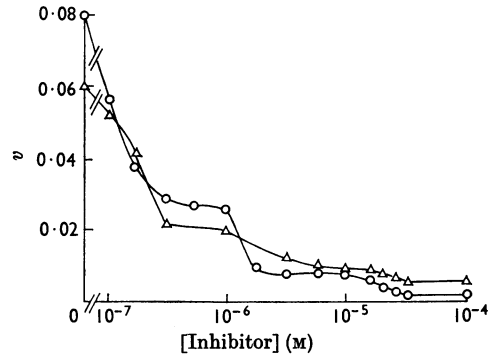

Fig. 9. Effect of increasing concentrations of phenylmercuric acetate and *p*-chloromercuribenzoate on the activity of glucokinase in the absence of *N*-acetylcysteine. The procedure for the removal of *N*-acetylcysteine from the glucokinase preparation is described in the Materials and Methods section. The velocity, v, was measured by a modification of method 1 (Parry & Walker, 1966) and was corrected for the loss of activity of control samples of the enzyme kept under identical conditions but in the absence of inhibitor; these procedures are described in the text. The velocity is expressed as ΔE_{340}/min./100 μl. Glucose was present during the period of incubation in the presence of the inhibitor and ATP was added to begin the assay of activity. ○, Phenylmercuric acetate; △, *p*-chloromercuribenzoate.

There appear to be three levels of inhibition at 0–0·1 μM, 1–2 μM and 20–50 μM. Only about 25% of the original activity was restored when 4mM-*N*-acetylcysteine was added after *N*-acetylcysteine-free glucokinase had been incubated for 10min. in the presence of 0·1mM-phenylmercuric acetate.

The inhibitory effect of several thiol compounds was studied as the concentration of one substrate was varied in the presence of a fixed saturating concentration of the other substrate. It proved possible to obtain satisfactory linear rate plots only when glucose was present during the period of incubation of the enzyme with the thiol reagent and ATP was added to commence the activity assay at the end of the 15min. incubation period. Under these conditions apparent K_i values for the various inhibitors could be calculated. Fig. 10 shows that glucose protects the enzyme against inhibition by thiol reagents in a competitive manner. If at the end of the period of incubation in the presence of glucose and inhibitor different concentrations of $MgATP^{2-}$ are added and the activity measured, the inhibition is seen to be non-competitive with respect to ATP (Fig. 11). Table 2 summarizes the apparent K_i values obtained by using at least two different concentrations of the inhibitor in each case. Only small changes in the apparent K_i values for phenylmercuric acetate were noted when a non-saturating

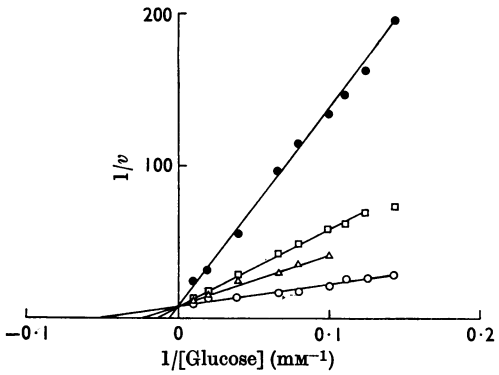

Fig. 10. Effect of thiol inhibitors on the velocity as the glucose concentration is varied at a constant ATP–Mg^{2+} concentration. The Mg^{2+} and ATP concentrations were kept at a constant 1:1 molar ratio. The methods used are given in the legend to Fig. 9 and in the text. The velocity, v, is expressed as ΔE_{340}/min./100 μl. The reaction mixture included ATP–Mg^{2+}, 5 mM; enzyme, a constant volume; the temperature was 28°. ○, No inhibitor; △, 0·1 μM-$HgCl_2$; □, 0·5 μM-phenylmercuric acetate; ●, 0·5 μM-*p*-chloromercuribenzoate.

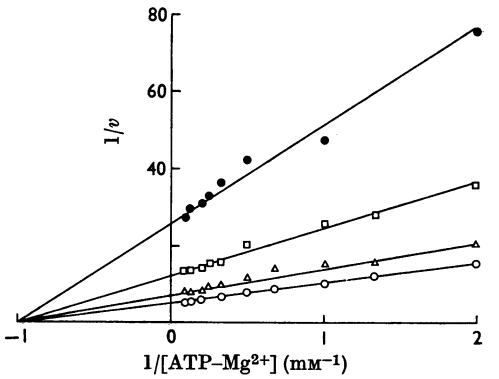

Fig. 11. Effect of thiol inhibitors on the velocity as the ATP–Mg^{2+} concentration is varied at a constant glucose concentration. The Mg^{2+} and ATP concentrations were kept at a constant 1:1 molar ratio. The methods used are given in the legend to Fig. 9 and the text. The velocity, v, is expressed as μmoles of glucose 6-phosphate formed/min./mg. of protein. The reaction mixture included: glucose, 100 mM; enzyme, a constant volume; the temperature was 28°. ○, No inhibitor; △, 0·02 μM-$HgCl_2$; □, 0·1 μM-phenylmercuric acetate; ●, 0·1 μM-*p*-chloromercuribenzoate.

concentration of the fixed-concentration substrate was employed. Sodium benzoate is not a thiol reagent and the apparent K_i values for its inhibitory effect were very much higher than those for the other reagents possessing a phenyl group; this group is therefore not likely to contribute significantly to the values for those reagents.

Table 2. *Inhibition of rat hepatic glucokinase by thiol inhibitors: K_i values*

Results were calculated from data such as those in Figs. 10 and 11. The K_i^A values were obtained when the glucose concentration was varied in the presence of a fixed concentration (5 mM) of $MgATP^{2-}$; the K_i^B values were obtained when the $MgATP^{2-}$ concentration was varied in the presence of a fixed concentration (100 mM) of glucose. Activities were measured by a modification of assay method 1 (Parry & Walker, 1966) as described in the text. The K_i values represent the means of values obtained with at least two concentrations of inhibitor and selected to be both above and below the K_i values; the inhibitors were adjusted to pH 7·5. The K_m values for glucose and ATP–Mg^{2+} for the preparation of glucokinase used in this study were 20 mM and 1·0 mM respectively.

Reagent	K_i^A (M)	K_i^B (M)
$HgCl_2$	$6·7\times10^{-8}$	$4·0\times10^{-8}$
p-Chloromercuribenzoate	$5·6\times10^{-8}$	$2·5\times10^{-7}$
Phenylmercuric acetate	$2·0\times10^{-7}$	$8·0\times10^{-8}$
Sodium iodoacetate	$4·0\times10^{-5}$	$1·7\times10^{-6}$
N-Ethylmaleimide	$9·0\times10^{-5}$	$9·0\times10^{-5}$
Sodium benzoate	$1·5\times10^{-4}$	$2·3\times10^{-5}$
Phenylmercuric acetate	$8·5\times10^{-8}$*	$1·2\times10^{-7}$†

* K_i^A value when the fixed ATP–Mg^{2+} concentration is 0·75 mM.

† K_i^B value when the fixed glucose concentration is 10 mM.

DISCUSSION

The present studies extend our previous observations (Parry & Walker, 1966) and permit a tentative analysis of the mechanism of glucokinase action. They also raise a number of new problems that are difficult to study because of the lability of the enzyme under all but a fairly narrow range of experimental conditions, the lack of certainty about the purity of the preparation and the very small amount of enzyme that can conveniently be prepared.

The anomalous kinetic behaviour of glucokinase at low glucose concentrations (Fig. 1) was not seen in early studies (Walker, 1963; Walker & Rao, 1963) with crude liver-supernatant preparations, and would in any case be at least partially masked by the presence of hexokinases in those extracts. The anomalous behaviour was of interest because it seemed possible that it was of physiological importance but, on the present evidence, it would be wrong to draw this inference.

The marked difference in sedimentation behaviour between the crude and purified preparations (Fig. 3) indicates that the 'light' component arises during an early purification step and suggests that during the preparation some of the naturally occurring form of the enzyme is degraded by unknown means, yielding one or more fragments that

retain enzymic activity of a modified nature. These fragments might be stabilized by the presence of reagents such as the thiol compound *N*-acetylcysteine; this point cannot be checked because all the glucokinase activity is soon lost in the absence of the stabilizer. No means of preventing formation of the 'light' component or reconverting it into the original form have been found and no explanation is apparent for differences in its behaviour during sedimentation and on a Sephadex G-100 column. We tend to the view that there is more than one form of glucokinase, but we have not been able to effect interconversion. Substrate activation of the enzyme cannot be excluded, however, on the basis of these experiments. Several forms of yeast hexokinase (Kenkare, Schultze, Gazith & Colowick, 1964) are believed to arise by proteolytic action during purification.

The molecular weight of 48000–49000 determined by gel filtration is half the value of 96000 found for yeast hexokinase (Kunitz & McDonald, 1946; Kenkare & Colowick, 1965) and for the three types of mammalian hexokinase (Grossbard & Schimke, 1966). This raises the possibility (Pilkis & Krahl, 1966) that hepatic glucokinase is related to the other hexokinase molecules. Kenkare & Colowick (1965) have indeed demonstrated that yeast hexokinase can be dissociated into four inactive sub-units, which can be reaggregated to form the active enzyme; the dimeric form is active. The existence of a control system *in vivo* for altering the relative proportions of hexokinase and glucokinase in liver tissue has been suggested by McLean & Brown (1966). No evidence for this type of transformation *in vitro* has been found in the present work, and the peculiar properties of what appears to be a lower-molecular-weight derivative are not those of a low-K_m hexokinase. It must be noted that the first purification step (stage 2) used involves collecting the protein precipitated between 0·45 and 0·65 saturation with ammonium sulphate, yet glucokinase activity could be precipitated over a wider range of concentrations (Parry & Walker, 1966). It is possible that part of the enzyme having glucokinase activity but excluded from our preparation might have modified properties.

Mechanism of action

The characteristics of the phosphoryltransferase activity of hepatic glucokinase are in many respects those of an 'ideal' phosphokinase (Crane, 1964; Parry & Walker, 1966). The competitive inhibition with respect to both glucose and $MgATP^{2-}$ by glucose 6-phosphate, the competitive inhibition by $MgADP^-$ with respect to $MgATP^{2-}$ at higher concentrations of the latter and the non-competitive inhibition by $MgADP^-$ with respect to glucose suggest the existence of separate binding sites for the donor and acceptor molecules. This is supported first by the data in Figs. 6 and 7, which indicate that the affinity of the enzyme for either substrate is unaffected by the concentration of the other substrate, and secondly by the inhibitory effects of an acceptor analogue such as mannose, which is competitive with respect to glucose (Parry & Walker, 1966) but non-competitive with respect to $MgATP^{2-}$ (Fig. 5).

The results shown in Figs. 6 and 7 are not compatible with a two-stage Ping Pong mechanism (Cleland, 1963), in which the product of the first reaction leaves the enzyme before the attachment of the second substrate; such a mechanism would require parallel plots in those Figures (Cleland, 1963), as were obtained, for example, by Fromm & Zewe (1962*a*) for brain hexokinase. Plots converging at a point to the left of the ordinate, as for hepatic glucokinase, have been obtained for skeletal-muscle hexokinase (Fromm, Hanson & Bietz, 1966; Toews, 1966; cf. Hanson & Fromm, 1965) and are consistent with an ordered or random addition of the two substrates and removal of the products (Alberty, 1953) and a direct transfer of the terminal phosphoryl group from the donor to the acceptor without the formation of an enzyme–phosphate complex. Many other phosphokinases show similar behaviour, e.g. pyruvate kinase (Reynard, Haas, Jacobsen & Boyer, 1961; Haas, Boyer & Reynard, 1961), yeast hexokinase (Haas *et al.* 1961; Fromm & Zewe, 1962*b*; Hammes & Kochavi, 1962*a*,*b*; Fromm, Silverstein & Boyer, 1964; Kaji & Colowick, 1965), adenylate kinase (Callaghan & Weber, 1959), phosphoglycerate kinase (Bücher, 1955; Reynard *et al.* 1961), skeletal-muscle hexokinase (Fromm *et al.* 1966; Toews, 1966) and creatine kinase (Kuby & Noltmann, 1962; Morrison & James, 1965; Morrison & Cleland, 1966).

The nature of the inhibition by the products in such two-substrate two-product reactions may be treated theoretically (Cleland, 1963) and is affected by the formation of ternary complexes such as, for hexokinases, enzyme–ADP–glucose and enzyme–ATP–glucose 6-phosphate. The formation of such abortive complexes probably accounts for the curious biphasic inhibitory effect of $MgADP^-$ (Fig. 9 of Parry & Walker, 1966) and for the non-competitive inhibition recorded in certain other cases. An alternative explanation for the inhibitory effect of $MgADP^-$ could be the effect of the large difference in the binding constants for the formation of $MgADP^-$ and $MgATP^{2-}$ operating under the conditions where the ATP concentration is very much less than that of ADP.

The kinetic results obtained for hepatic glucokinase are similar in many respects to those obtained for yeast hexokinase (Fromm & Zewe, 1962*b*), and

for which additional observations on exchange rates at equilibrium (Fromm *et al.* 1964) indicated that the reaction proceeds by a random rapid-equilibrium Bi Bi mechanism (Cleland, 1963) in which the interconversion of the central ternary complexes is rate-limiting. This needs to be verified for hepatic glucokinase.

Role of thiol groups. The lack of information about the purity of the glucokinase preparation rules out the estimation of the number of thiol groups/mol. and makes interpretation of the inhibition curves (Fig. 9) uncertain. The conventional linear double-reciprocal plots in Figs. 10 and 11, which are typical of competitive and non-competitive inhibitors respectively, were obtained with reagents many of which will form covalent linkages with thiol groups on the enzyme surface. These observations are not to be interpreted necessarily in terms of Michaelis–Menten kinetics, however. The competitive relationship between glucose and various inhibitors (Fig. 10) could be due to an effect of the substrate on the rate at which an enzyme thiol group undergoes an essentially irreversible reaction to form a covalently bonded complex with the inhibitor. On this interpretation the K_i values are presumably dependent on time of incubation of enzyme with inhibitor and cannot be given any absolute significance. The same conditions of incubation were used for the results in Fig. 11; under these circumstances of high glucose concentration only a little thiol reagent is likely to have reacted with a site protected by glucose. The non-competitive type of inhibition illustrated in Fig. 11 may be due to inhibition at this or another site. This type of interpretation of the data is subject to many limitations (Webb, 1966), but is in keeping with the ideas of Watts & Rabin (1962), who, as a result of studies on creatine kinase (EC 2.7.3.2), suggested that the hexokinase reaction could involve hydrogen bonding between a thiol group on the enzyme and a hydroxyl group of the acceptor molecule; a similar mechanism for lobster-muscle arginine kinase (EC 2.7.3.3) has been suggested by Virden & Watts (1966). Kaji (1966), on the other hand, has presented evidence that seems to confirm an earlier report (Fasella & Hammes, 1963) that the thiol groups on yeast hexokinase are not essential for enzyme action.

The protective effects of glucose in these studies *in vitro* may be an indication of how glucose lowers the rate of degradative processes *in vivo* and account for the increased hepatic glucokinase activities recorded in animals on a high-glucose diet (e.g. Walker, Khan & Eaton, 1966).

We thank the Medical Research Council for a grant towards the expenses and for a Training Studentship to M. J. P., and the Royal Society for a grant to purchase the Gilford Recording Spectrophotometer.

REFERENCES

Alberty, R. A. (1953). *J. Amer. chem. Soc.* **75**, 1928.
Andrews, P. (1965). *Biochem. J.* **96**, 595.
Bücher, T. (1955). In *Methods in Enzymology*, vol. 1, p. 415. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Callaghan, O. H. & Weber, G. (1959). *Biochem. J.* **73**, 473.
Cleland, W. W. (1963). *Biochim. biophys. Acta*, **67**, 104.
Crane, R. K. (1964). In *Comprehensive Biochemistry*, vol. 15, p. 226. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
Fasella, P. & Hammes, G. C. (1963). *Arch. Biochem. Biophys.* **100**, 295.
Florini, J. R. & Vestling, C. S. (1957). *Biochim. biophys. Acta*, **25**, 575.
Fromm, H. J., Hanson, T. L. & Bietz, J. A. (1966). *Fed. Proc.* **25**, 749.
Fromm, H. J., Silverstein, E. & Boyer, P. D. (1964). *J. biol. Chem.* **239**, 3645.
Fromm, H. J. & Zewe, V. (1962*a*). *J. biol. Chem.* **237**, 1661.
Fromm, H. J. & Zewe, V. (1962*b*). *J. biol. Chem.* **237**, 3027.
Grossbard, L. & Schimke, R. T. (1966). *J. biol. Chem.* **241**, 3546.
Haas, L. F., Boyer, P. D. & Reynard, A. M. (1961). *J. biol. Chem.* **236**, 2284.
Hammes, G. C. & Kochavi, D. (1962*a*). *J. Amer. chem. Soc.* **84**, 2069.
Hammes, G. C. & Kochavi, D. (1962*b*). *J. Amer. chem. Soc.* **84**, 2073.
Hanson, T. L. & Fromm, H. J. (1965). *J. biol. Chem.* **240**, 4133.
Kaji, A. (1966). *Biochim. biophys. Acta*, **122**, 43.
Kaji, A. & Colowick, S. P. (1965). *J. biol. Chem.* **240**, 4454.
Kenkare, U. & Colowick, S. P. (1965). *J. biol. Chem.* **240**, 4570.
Kenkare, U., Schultze, I. T., Gazith, J. & Colowick, S. P. (1964). *Abstr. 6th int. Congr. Biochem., New York*, p. 477.
Kuby, S. A. & Noltmann, E. A. (1962). In *The Enzymes*, vol. 6, p. 515. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Kunitz, M. & McDonald, M. R. (1946). *J. gen. Physiol.* **29**, 393.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
McDonald, M. R. (1955). In *Methods in Enzymology*, vol. 2, p. 427. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
McLean, P. & Brown, J. (1966). *Biochem. J.* **98**, 874.
Morrison, J. F. & Cleland, W. W. (1966). *J. biol. Chem.* **241**, 673.
Morrison, J. F. & James, E. (1965). *Biochem. J.* **97**, 37.
Parry, M. J. (1966). Ph.D. Thesis: University of Birmingham.
Parry, M. J. & Walker, D. G. (1966). *Biochem. J.* **99**, 266.
Pilkis, S. J. & Krahl, M. E. (1966). *Fed. Proc.* **25**, 523.
Reynard, A. M., Haas, L. F., Jacobsen, D. D. & Boyer, P. D. (1961). *J. biol. Chem.* **236**, 2777.
Salas, J., Salas, M., Viñuela, E. & Sols, A. (1965). *J. biol. Chem.* **240**, 1014.
Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 63. New York: Academic Press Inc.

Schachman, H. K. (1960). *Protein Structure and Function*, p. 49. New York: Brookhaven National Laboratory.
Toews, C. J. (1966). *Biochem. J.* **100**, 739.
Trautman, R. & Breese, S. S. (1959). *J. phys. Chem.* **63**, 1592.
Virden, R. & Watts, D. C. (1966). *Biochem. J.* **99**, 162.
Walker, D. G. (1963). *Biochim. biophys. Acta*, **77**, 209.
Walker, D. G., Khan, H. H. & Eaton, S. W. (1966). *Biol. Neonat.* **9**, 224.
Walker, D. G. & Rao, S. (1963). *Biochim. biophys. Acta*, **77**, 662.
Watts, D. C. & Rabin, B. R. (1962). *Biochem. J.* **85**, 507.
Webb, J. L. (1966). *Enzymes and Metabolic Inhibitors*, vol. 2, p. 647. New York: Academic Press Inc.