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Kinetics of Rat Liver Glucokinase

CO-OPERATIVE INTERACTIONS WITH GLUCOSE AT PHYSIOLOGICALLY SIGNIFICANT CONCENTRATIONS

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The kinetics of glucokinase from rat liver were studied over wide ranges of glucose and $MgATP^{2-}$ concentrations. The initial rate shows a co-operative dependence on the glucose concentration, with Hill coefficients in the range 1.2–1.5. The degree of glucose co-operativity increases with the $MgATP^{2-}$ concentration, but no co-operativity was detected for the dependence of the rate on the $MgATP^{2-}$ concentration. The effects observed occur at physiologically reasonable concentrations of glucose and $MgATP^{2-}$ and are consistent with the presumed function of glucokinase in maintaining a constant concentration of glucose in the blood.

It has long been recognized that a major function of the mammalian liver is to regulate the concentration of glucose in the blood, and that this is achieved by synthesis and storage of glycogen when the blood-glucose concentration rises, and by breakdown of glycogen when it falls (Bernard, 1848, 1855). In accordance with the usual principles of feedback control (Stadtman, 1970), one might expect the conversion of glucose into glycogen to be controlled at the first step, i.e. the phosphorylation of glucose by ATP. So the enzyme responsible for this reaction ought to be sensitive to the glucose concentration. In fact, the isoenzymes of hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1) found in most tissues, i.e. hexokinase types I, II and III, are barely affected by changes in the blood-glucose concentration, because they are all largely saturated throughout the physiological range (Hanson & Fromm, 1965; Copley & Fromm, 1967; Bachelard *et al.*, 1971), and they are strongly and allosterically inhibited by glucose 6-phosphate at low concentrations. This means that they are well adapted to supply tissues with glucose at a rate that depends on the need (indicated by the concentration of glucose 6-phosphate) and not significantly on the supply. By the same token, however, they are poorly adapted to regulate the supply.

The observation that liver slices can phosphorylate glucose at a rate that varies with the glucose concentration (Cahill *et al.*, 1958) suggested that a hexokinase with a much higher Michaelis constant should exist in the liver, and led to the discovery of glucokinase (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2, sometimes also referred to as hexokinase type IV) (Walker, 1962; DiPietro *et al.*, 1962; Viñuela *et al.*, 1963), which is half-saturated in the physiological range of glucose concentrations. Further, it is inhibited only weakly and non-allosterically by glucose 6-phosphate (Viñuela *et al.*, 1963; Parry & Walker, 1966), so the ability of the liver to phosphorylate glucose at high concentrations of glucose 6-phosphate is readily explainable. Glucokinase has a greater specificity for glucose than the low-K_m hexokinases, though the specificity is incomplete (Parry & Walker, 1966).

To shed light on the physiological role of glucokinase, and as a first step in elucidating its mechanism of action, we have studied the kinetics of glucokinase from rat liver over a wide range of concentrations of glucose and $MgATP^{2-}$. The results are reported in this paper.

Materials and Methods

Glucokinase

Glucokinase was purified from the livers of well-fed Wistar rats by the method described by Holroyde *et al.* (1976), with the use of materials as given there. For most of the kinetic studies the last stages of the purification (ion exchange on DEAE-Sephadex and gel filtration on Sephadex G-200) were omitted, and the enzyme used had a specific activity of about 20 units/mg of protein rather than the 150 units/mg that was obtained for the homogeneous enzyme. Tests for contaminating enzymes

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showed that no hexokinase, glucose dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, phosphomannose isomerase, glucose 6-phosphatase, adenylate kinase or adenosine triphosphatase activity was present in the preparation. Glucose 6-phosphate dehydrogenase was detected, but there was no point in removing it as it was a constituent of the coupled assay normally used (see below). Comparison between samples of the homogeneous and partially purified preparations of glucokinase revealed no differences in initial-rate behaviour, apart from the difference in specific activity. The partially purified enzyme was preferred because it could be obtained more quickly and in higher yield, and it lost activity more slowly when stored, and far more slowly under assay conditions.

After purification, glucokinase was stored in 0.3M-KCl, 50mM-glucose, 0.5mM-dithiothreitol and 20% glycerol at pH7.2 and 0–2°C. Under these conditions it was usable for several months, losing activity at a rate of about 5% per month. The composition of the storage mixture was established by thorough testing and all of the components contributed to the stability. Several other possible stabilizing materials were tested, of which some, such as ascorbate, accelerated inactivation, and others, such as $MgATP^{2-}$ (instead of glucose) and 10mM-mercaptoethanol, were less effective than the components used. The high concentration of glucose in the storage mixture necessitated special precautions in some of the kinetic experiments. When very low concentrations were required the excess was removed by absorbing the enzyme on DEAE-cellulose and eluting it with glucose-free storage mixture. Residual glucose was determined with a glucose assay kit (blood sugar GOD–Perid method) from Boehringer, Mannheim, W. Germany. Glucose added with the enzyme was always allowed for when calculating the glucose concentrations in reaction mixtures. (Neglect of this correction would decrease or eliminate the apparent sigmoidicity of the kinetics and might give a spurious suggestion of hexokinase contamination.) The glucose in the storage mixture was not the cause of the kinetic behaviour described below, as glucokinase that had been stored in the absence of glucose behaved in the same way.

Other materials

ATP (disodium salt) was obtained from Kyowa Hakko Kogyo Co., Tokyo, Japan. Dithiothreitol, NADH, $NADP^+$, phosphoenolpyruvate, glucose 6-phosphate dehydrogenase (type VII, from baker's yeast), pyruvate kinase (type III, from rabbit muscle), lactate dehydrogenase (type XI, from rabbit muscle), and Dowex 50 (X8) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tetramethylammonium hydroxide, tetramethylammonium chloride, Zerolit DM-F and 1M stock solutions of $MgCl_2$ were obtained from BDH Chemicals, Poole, Dorset, U.K. All other reagents were AnalaR or best quality reagent grade from Fisons Scientific Apparatus, Loughborough, Leics., U.K., or from BDH Chemicals.

Solutions of reagents were made up in glass-distilled water and the pH was adjusted when necessary by adding 1M-HCl or 1M-KOH.

Preparation of standard solutions

ATP and $NADP^+$ were obtained as sodium salts, but were converted into tetramethylammonium salts before use. Dowex 50 (X8) was washed with 1M-NaOH, 1M-HCl and glass-distilled water, and was then converted into its tetramethylammonium form by washing with an excess of tetramethylammonium hydroxide, followed by water until the pH of the wash was neutral. Solutions of the sodium salts of ATP or $NADP^+$ were adjusted to pH7.0 and then stirred gently with the calculated equivalent of tetramethylammonium Dowex. The solution was filtered and the solute concentration determined, ATP by the spectrophotometric method of Bock *et al.* (1956), and $NADP^+$ by a similar method ($\epsilon_{260} = 15000\,\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$). Contamination of ATP with ADP was determined by coupling the conversion of ADP into ATP to the oxidation of NADH, by using pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate (see under 'Enzyme assays'). ADP was found to account for 1–2% of the total nucleotide content of fresh solutions, and increased only slightly after the frozen solution was stored for 2 weeks.

Glucose 6-phosphate dehydrogenase was obtained as a suspension in 3.2M-$(NH_4)_2SO_4$ and was dialysed overnight against 20mM-Tris/HCl buffer at pH7.5 before use. The activity after dialysis was about double that of the undialysed enzyme, because of relief of inhibition by sulphate. Pyruvate kinase and lactate dehydrogenase were obtained as salt-free freeze-dried powders and were dissolved in 20mM-Tris/HCl at pH7.5 before use.

$MgATP^{2-}$ concentrations

The concentrations of $MgATP^{2-}$ in reaction mixtures were calculated from the total concentrations of Mg^{2+} and ATP as described by Storer & Cornish-Bowden (1976), by using the thermodynamic data of Phillips *et al.* (1966). In all reaction mixtures, the total Mg^{2+} concentration exceeded the total ATP concentration by 1.0mM. This ensured that the $MgATP^{2-}$ concentration would be a high and nearly constant proportion ($86\pm2\%$) of the total ATP concentration, that the ATP^{4-} concentration would be small and proportional to the $MgATP^{2-}$ concentration, and that the Mg^{2+}

concentration would be small (about 0.4mM) and nearly constant (Storer & Cornish-Bowden, 1976).

Enzyme assays

One unit of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1 μmol of substrate in 1 min at 30°C, in the complete assay mixture. The method used in most of the kinetic experiments was a coupled assay based on that of DiPietro & Weinhouse (1960), with glucose 6-phosphate dehydrogenase as coupling enzyme, under the detailed conditions (apart from glucose and $MgATP^{2-}$ concentrations) given previously (Storer & Cornish-Bowden, 1974). The reaction was followed by measuring the change in E_{340} in a Gilford recording spectrophotometer, model 2400-S, with digital print-out.

Another coupled assay was also used, in which the formation of ADP was coupled to the oxidation of NADH (0.25mM) by addition of pyruvate kinase (10 units), lactate dehydrogenase (10 units) and excess of phosphoenolpyruvate (1 mM). The reaction was carried out at 30°C in a total volume of 0.75 ml of tetramethylammonium glycylglycinate buffer (50mM), pH 8.0, containing 100mM-KCl, 0.25mM-NADH, glucose, ATP and $MgCl_2$ (1 mM excess over ATP). The mixture was preincubated for 10 min at 30°C before addition of glucokinase, to convert any ADP present into ATP.

The phosphorylation of glucose by ATP is accompanied by the production of an additional ionizable group. The reaction can therefore be followed in a pH-stat (cf. Gutfreund & Hammond, 1963), and this was done in some experiments, primarily to check that effects observed with the other assays were not artifacts of the coupled enzyme systems. The equipment used was obtained from Radiometer, Copenhagen, Denmark, and consisted of a TTT2 titrator with SBR3 titrigraph, ABU12 autoburette and TTA31 titration assembly. The pH was maintained at 8.0 by addition of 10mM-KOH and the reaction mixture contained 100mM-KCl, glucose, ATP and $MgCl_2$ (1 mM excess over ATP), in a total volume of 2.0 ml. All solutions were made up in glass-distilled water that had been treated with Zerolit DM-F to remove dissolved CO_2, and the reaction was carried out under a stream of CO_2-free N_2.

With all three assays, whenever the concentration of an ion was varied during a series of experiments, the ionic strength was kept constant by addition of tetramethylammonium chloride.

Analysis of results

Initial rates were obtained from the recorder traces with a ruler and pencil. No correction for curvature was necessary because the traces were essentially straight during the first 3 min of each assay.

To minimize any effects due to enzyme inactivation during a series of experiments, substrate concentrations were varied in a convoluted sequence, e.g. if concentrations in the range 2.5–100mM were required a sequence such as 100, 50, 20, 10, 2.5, 5, 15, 25, 75mM would be used. Duplicates (when measured) were obtained by repeating the sequence. In the event, no enzyme inactivation during a series of experiments was detected.

Kinetic constants given in this paper were obtained by eye from plots of s/v against s, where s is the concentration of the varied substrate and v is the measured rate. In all cases the experimental error was small enough for non-linear regression analysis (cf. Wharton *et al.*, 1974) to make no appreciable difference to the results.

Results

Dependence of the rate on the glucose concentration

The glucokinase-catalysed rate of glucose phosphorylation, v, was measured at several fixed $MgATP^{2-}$ concentrations, with the glucose concentration varied in the range 0.1–100mM. For glucose concentrations above 10mM (Fig. 1), the Michaelis–Menten equation was obeyed accurately, with a Michaelis constant for glucose of 5mM, independent of the $MgATP^{2-}$ concentration. But at lower glucose concentrations plots of [glucose]/v against [glucose] were strongly curved (Fig. 2). The curvature agrees with the deviations from Michaelis–Menten kinetics observed previously (Parry & Walker, 1967; González *et al.*, 1967; Niemeyer *et al.*, 1975), but is more striking because results are shown at lower glucose concentrations, so that more of the curve is experimentally attested. Moreover, the form of plot used here gives a clearer indication of the behaviour at low concentrations than the double-reciprocal plot used by earlier workers. Niemeyer *et al.* (1975) comment that their results are different in character from those of Parry & Walker (1967), because they consider it unreasonable to draw pairs of straight lines through their experimental points. Actually this difference is more apparent than real, and results partly from the different ranges of glucose concentrations used in the two laboratories and partly from the subjective nature of lines drawn through experimental points (see Cornish-Bowden & Koshland, 1975). If the experimental points of Parry & Walker (1967) and Niemeyer *et al.* (1975) are compared, with the lines omitted and over the same range of glucose concentrations (2–50mM), the differences are slight.

The curvature in Fig. 2 indicates a sigmoid or co-operative dependence of the velocity on the glucose concentration, as may be seen more clearly by plotting v against [glucose] (Fig. 6, below). The

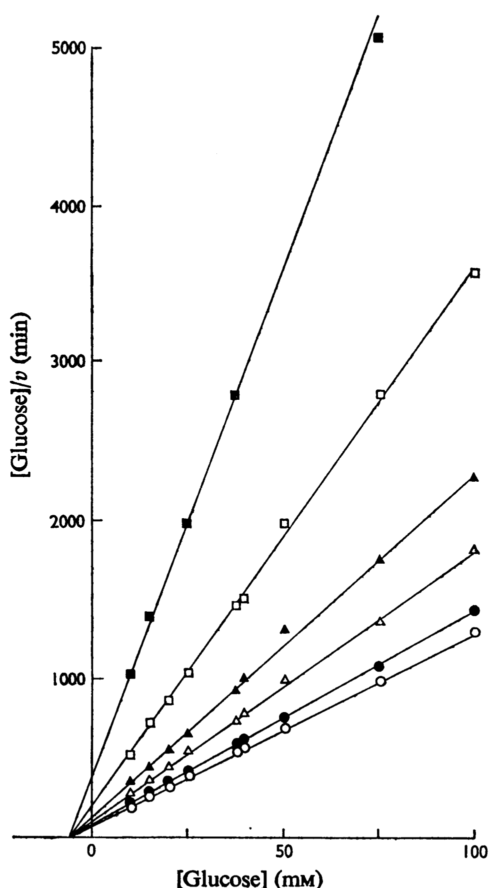

Fig. 1. *Dependence of the glucokinase-catalysed rate on the glucose concentration at high glucose concentrations*

Initial rates, v, were measured at 30°C in 50mM-tetramethylammonium glycylglycinate buffer at pH 8.0, containing 1 mM-$NADP^+$, 100mM-KCl, ATP (total concentrations in the range 0.125–5mM), $MgCl_2$ (total concentrations 1 mM in excess of total ATP concentrations), glucose (10–100mM, as indicated), glucokinase (0.077 unit/ml) and glucose 6-phosphate dehydrogenase (0.8 unit/ml). Concentrations of $MgATP^{2-}$ were calculated (Storer & Cornish-Bowden, 1976) to be 4.3mM (○), 2.15mM (●), 0.86mM (△), 0.43mM (▲), 0.215mM (□) and 0.108mM (■).

sigmoidicity can be expressed by the maximum slope h of a Hill plot {of $\log[v/(V_{app.}-v)]$ against log[glucose], where $V_{app.}$ is the apparent maximum velocity at each concentration of $MgATP^{2-}$}, and varies with the $MgATP^{2-}$ concentration as shown in Fig. 3. The curve has the appearance of a rectangular hyperbola through the point (0,1) (though there is presumably no reason why it should be accurately hyperbolic) and a plot of $[MgATP^{2-}]/(h-1)$ against $[MgATP^{2-}]$ is nearly straight (Fig. 3, inset). This secondary plot indicates a lower limit of $h\rightarrow1.0$ as $[MgATP^{2-}]\rightarrow0$ and an upper limit of $h\rightarrow1.47$ as $[MgATP^{2-}]\rightarrow\infty$.

The results shown in Figs. 1–3 were obtained with the coupled assay with glucose 6-phosphate dehydrogenase as coupling enzyme. To rule out any possibility that the deviations from simple kinetics were an artifact of this assay, the experiments were repeated on a more limited scale by using either the pH-stat assay or the coupled assay with pyruvate kinase and lactate dehydrogenase as coupling enzymes. These assays gave similar results, with the same sigmoid dependence of the rate on the glucose concentration.

Dependence of the rate on the $MgATP^{2-}$ concentration

When the rate was measured as a function of the

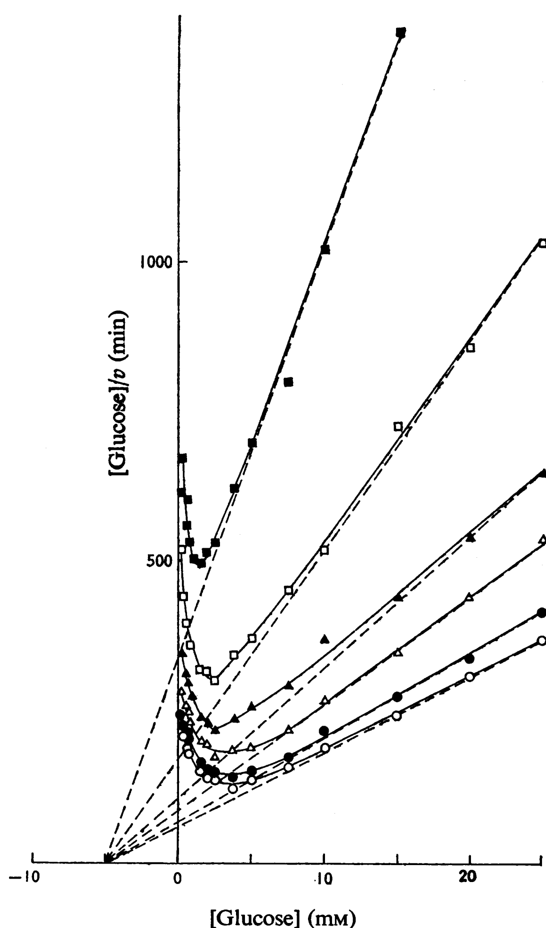

Fig. 2. *Dependence of the glucokinase-catalysed rate on the glucose concentration at low glucose concentrations*

Initial rates were measured at glucose concentrations in the range 0.1–25mM, as indicated. Other conditions were as for Fig. 1.

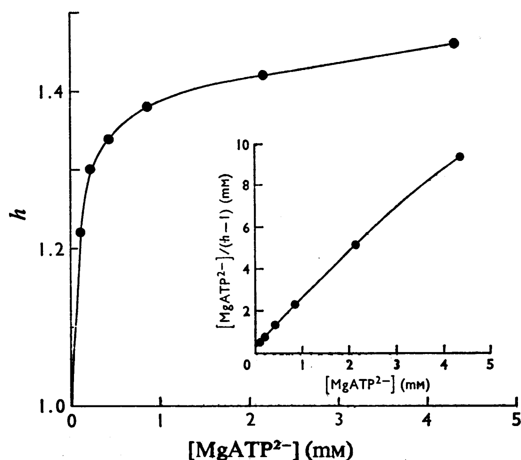

Fig. 3. *Dependence of the Hill coefficient on the* $MgATP^{2-}$ *concentration*

The Hill coefficient, h, was determined at each $MgATP^{2-}$ concentration as the maximum slope of a plot of $\log[v/(V_{app.}-v)]$ against log[glucose], where $V_{app.}$ is the apparent maximum velocity at the particular $MgATP^{2-}$ concentration. Inset: plot of $[MgATP^{2-}]/(h-1)$ against $[MgATP^{2-}]$. This plot would be exactly straight if the curve in the main part of the Figure were a rectangular hyperbola with a value $h = 1$ at $[MgATP^{2-}] = 0$.

$MgATP^{2-}$ concentration, at several fixed glucose concentrations, Michaelis–Menten kinetics were observed in all cases (Fig. 4). Secondary plots (Fig. 5) were in agreement with the results expected from the variable-glucose experiments, i.e. plots of both primary slopes and primary intercepts against [glucose] were linear at high glucose concentrations (above 10mM), but the plot of slopes curved upwards at low glucose concentrations. Just as the Michaelis constant for glucose at high glucose concentrations was independent of the $MgATP^{2-}$ concentration (Fig. 1), so also does the Michaelis constant for $MgATP^{2-}$ approach a glucose-independent value of 0.55mM at high glucose concentrations.

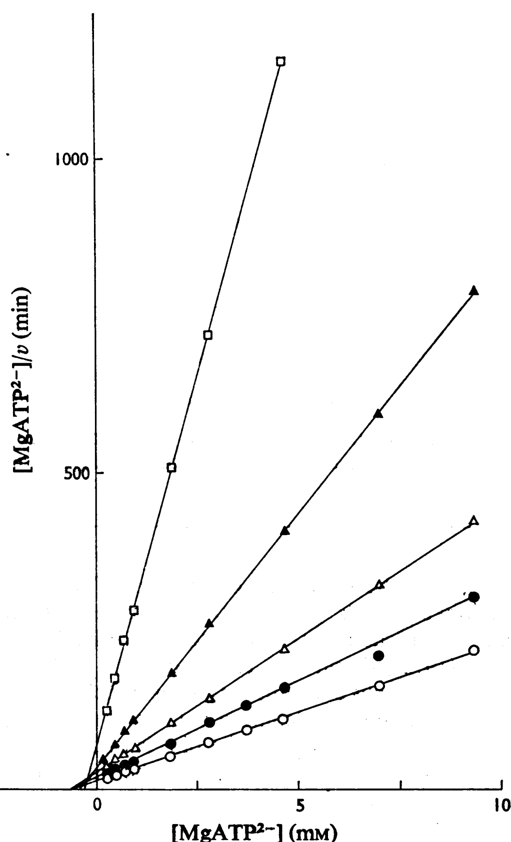

Fig. 4. *Dependence of the glucokinase-catalysed rate on the* $MgATP^{2-}$ *concentration*

Conditions were as for Fig. 1, apart from the following: $MgATP^{2-}$ concentrations were as indicated; glucose concentrations were 50mM (○), 10mM (●), 5mM (△), 2.5mM (▲) and 1mM (□); the glucokinase activity was 0.4unit/ml.

Discussion

The results show that the slight deviations from Michaelis–Menten kinetics observed by earlier workers (Parry & Walker, 1967; González *et al.*, 1967; Niemeyer *et al.*, 1975) are real and substantial, so that plots of rate against glucose concentration are sigmoid. The Hill coefficient ranges from 1.0 at low $MgATP^{2-}$ concentrations to about 1.5 at high $MgATP^{2-}$ concentrations. The smallness of this upper limit, by comparison with values observed for other co-operative enzymes, helps to explain why the deviations from simple kinetics observed previously were so slight. The suggestion of Parry & Walker (1967) that their results might be explained by a mixture of enzyme forms is untenable, because any deviations due to such a cause would be in the opposite sense (Childs & Bardsley, 1975).

Under conditions where the Michaelis–Menten equation is obeyed, the Michaelis constant for glucose is 5mM, appreciably lower than the values of 10–20mM reported by earlier workers (Salas *et al.*, 1965; Parry & Walker, 1967; Pilkis, 1972; Grossman *et al.*, 1974). The discrepancy can be explained by the facts that previous results were analysed as double-reciprocal plots, which are liable to frustrate any attempts to obtain realistic estimates of kinetic constants (see Eisenthal & Cornish-Bowden,

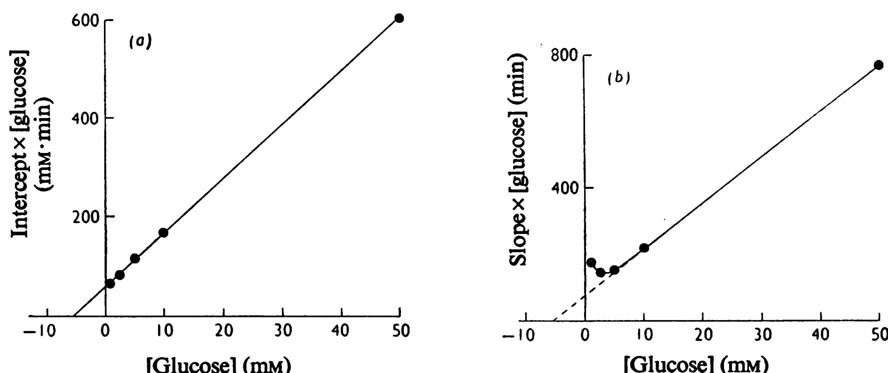

Fig. 5. *Dependence of apparent Michaelis–Menten parameters on the glucose concentration*

The intercept on the ordinate ($K_{m,app.}/V_{app.}$) and slope ($1/V_{app.}$) were estimated for each of the plots of [$MgATP^{2-}$]/v against [$MgATP^{2-}$] shown in Fig. 4. (*a*) Plot of $K_{m,app.}$[glucose]/$V_{app.}$ against [glucose]; (*b*) plot of [glucose]/$V_{app.}$ against [glucose].

1974), and the curvature of the 'linear' plots was overlooked or its importance was underestimated. Our results actually agree closely with those of Pilkis (1972), despite his reported value of 12.5mM for the Michaelis constant for glucose: the line shown on his plot actually defines a value of 8mM, and, if the manifest curvature of the plot is allowed for, a value of 4–5mM is reasonable for the limit at high glucose concentrations.

The sigmoidicity of plots of rate against glucose concentration is greatest at high $MgATP^{2-}$ concentrations (Fig. 3), but it is perceptible over the likely physiological range, and its disappearance at very low $MgATP^{2-}$ concentrations is unlikely therefore to be of physiological consequence. The effect of the sigmoidicity is that glucokinase is most sensitive to the glucose concentration at about 2.5mM-glucose, i.e. at a physiologically reasonable value, rather than at the origin, where a high sensitivity would be of no account. The value of dv/d[glucose] is about 140% higher at 2.5mM-glucose than the corresponding value for an enzyme that obeys the Michaelis–Menten equation with $K_m = 5$mM and gives the same rate at 2.5mM-glucose (Fig. 6). This advantage is somewhat less if the comparison is made with an enzyme with a very high K_m value, but glucokinase is 60% more sensitive than the most sensitive possible Michaelis–Menten enzyme and uses a far higher proportion of the catalytic potential at concentrations in the physiological range. Ideally one might expect the maximum sensitivity to occur at 5mM-glucose rather than 2.5mM. But this discrepancy is fairly small and in view of uncertainties about the detailed conditions in the cell it would be imprudent to conclude much from it.

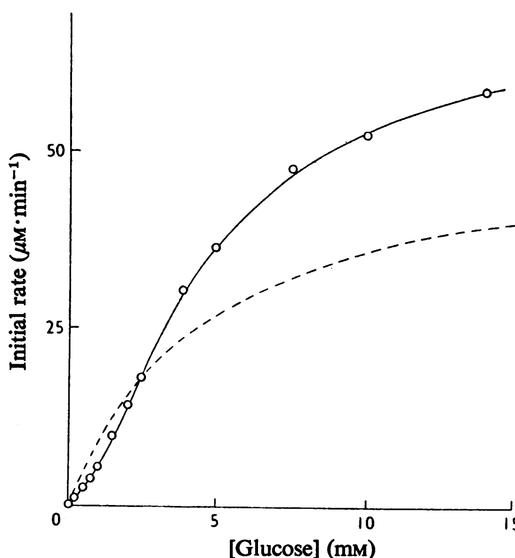

Fig. 6. *Sigmoid dependence of rate on glucose concentration*

The observed dependence (——) of the rate on the glucose concentration at 4.3mM-$MgATP^{2-}$ is compared with the dependence (----) expected for an enzyme that obeys the Michaelis–Menten equation with K_m = 5mM and gives the same rate at 2.5mM-glucose.

It is difficult to escape the inference that the complex kinetic behaviour of glucokinase is a useful phenomenon with relevance to its role in the liver. It is pertinent, therefore, to ask how our observations affect the analyses by Newsholme &

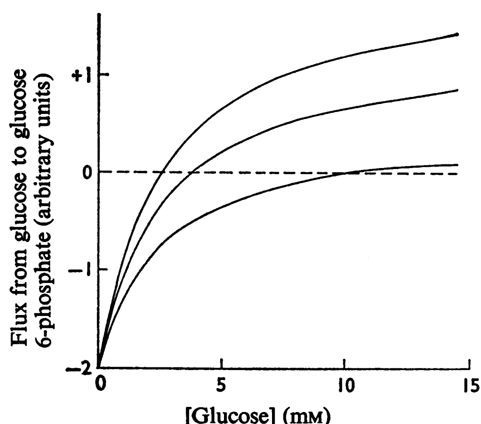

Fig. 7. *Effect of glucose and $MgATP^{2-}$ on cycling between glucose and glucose 6-phosphate in the liver*

The Figure shows a hypothetical dependence of the net flux from glucose to glucose 6-phosphate for a cycling system in which the rate of glucose phosphorylation varies with the glucose concentration according to the Michaelis–Menten equation and the rate of glucose 6-phosphate hydrolysis is constant. The three curves represent different $MgATP^{2-}$ concentrations and the broken line is drawn for zero flux.

Start (1973) and by Hue & Hers (1974) of cycling in the liver between glucose and glucose 6-phosphate. The two main effects not taken into account in those analyses were the dependence of the rate on the $MgATP^{2-}$ concentration and the sigmoidicity of its dependence on the glucose concentration. It is simplest to consider these separately, i.e. to discuss first the effect of [$MgATP^{2-}$] dependence as if glucokinase were not co-operative. The point of zero flux, at which the glucokinase and glucose 6-phosphatase reactions are exactly balanced, would then be highly dependent on the $MgATP^{2-}$ concentration (Fig. 7) so that, at lower $MgATP^{2-}$ concentrations, higher glucose concentrations would be required to achieve net flux towards glycogen. But if the main function of the cycling in the liver between glucose and glucose 6-phosphate is to regulate the concentration of glucose in the blood, a strong dependence on the $MgATP^{2-}$ concentration would be unhelpful, and perhaps damaging, even in the rather narrow range (1.4–2.1 mmol/kg wet wt.) that has been reported for the ATP content of the liver (Greenbaum *et al.*, 1971).

This analysis suggests that a possible function of co-operativity in glucokinase is to decrease the effect of $MgATP^{2-}$ on the cycle between glucose and glucose 6-phosphate. If the curves in Fig. 7 at higher $MgATP^{2-}$ concentration are made sigmoid, without altering their shapes at high glucose concentration, but the curves at lower $MgATP^{2-}$ concentration are changed less, the net effect is to bring the points of zero flux closer together, so that the direction of flux is made more dependent on the glucose concentration and less dependent on the $MgATP^{2-}$ concentration.

Although eventually it would be desirable to express these conclusions in more precise numerical terms, it would be unprofitable at the present state of knowledge, because not enough is known about the properties of glucose 6-phosphatase (see Arion *et al.*, 1975) or about the local concentrations of glucose, $MgATP^{2-}$ and glucose 6-phosphate, or indeed other metabolites that may affect the system. For the present, therefore, it is sufficient to conclude that the properties of glucokinase described in this paper are qualitatively in accordance with its presumed role in maintaining a constant concentration of glucose in the blood (Walker, 1966).

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