# Kinetic Studies with Skeletal-Muscle Hexokinase

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Rat skeletal-muscle hexokinase was partially purified by ammonium sulphate fractionation and gel filtration. The mechanism of the skeletal-muscle hexokinase was studied kinetically by initial-velocity analysis and product inhibition. Glucose 6-phosphate was a non-competitive inhibitor of glucose and ATP. ADP was a non-competitive inhibitor of glucose and a competitive inhibitor of ATP. The data on product inhibition and initial-velocity analysis of skeletal-muscle hexokinase support an ordered sequential mechanism (ordered Bi Bi) where the addition of substrates and release of products is in the order: ATP, glucose, glucose 6-phosphate and ADP.

Hexokinase (ATP–D-hexose 6-phosphotransferase; EC 2.7.1.1) catalyses the reaction:

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose 6-phosphate} + \text{ADP} \quad (1)$$

Though this reaction is the same for hexokinase whatever its source, different hexokinases may show marked differences in a number of properties. Animal hexokinases generally are markedly inhibited by low concentrations of glucose 6-phosphate (Crane & Sols, 1954; Fromm & Zewe, 1962*a*) whereas yeast hexokinase (Colowick & Kalckar, 1943; Weil-Malherbe & Bone, 1951) and hepatic glucokinase (Salas, Salas, Viñuela & Sols, 1965) are not. Likewise, different hexokinases are inhibited to different degrees by ADP. With brain hexokinase ADP is a non-competitive inhibitor with respect to ATP but uncompetitive with respect to glucose (Fromm & Zewe, 1962*a*). Liver glucokinase (ATP–D-glucose 6-phosphotransferase; EC 2.7.1.2) is inhibited non-competitively by ADP with respect to glucose and ATP (Salas *et al.* 1965). Differences in the mechanisms of the reactions of hexokinase of different origins would account for these variable inhibitory effects.

The mechanism of the hexokinase reaction has been recently studied in yeast and in brain. On the basis of kinetic studies and isotope-exchange rates at equilibrium, yeast hexokinase was postulated to have a rapid-equilibrium random type mechanism (Fromm & Zewe, 1962*b*; Fromm, Silverstein & Boyer, 1964). However, from kinetic studies and an examination of product inhibition, Hammes & Kochavi (1962*a*,*b*) postulated an ordered sequential type mechanism for the yeast enzyme. From kinetic studies and product-inhibition data Fromm & Zewe (1962*a*) postulated an ordered mechanism for brain hexokinase, in which the first product leaves the enzyme before the addition of the second substrate.

Some work on muscle hexokinase has appeared (Kerly & Leaback, 1957; Slein, Cori & Cori, 1950; Liebecq, 1953), but no definitive study has been undertaken to determine the mechanism of the hexokinase reaction in muscle. The present paper reports a study of the kinetic properties of skeletal-muscle hexokinase. Analysis of initial velocity and studies of product inhibition have been made. The data presented for skeletal-muscle hexokinase support an ordered sequential type mechanism.

## EXPERIMENTAL

*Materials.* D-Glucose was obtained from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A. Tris was obtained from British Drug Houses Ltd., Poole, Dorset. ATP, ADP, $NADP^+$, NADH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type V), lactate dehydrogenase (type II), pyruvate kinase (type I) and phosphoenolpyruvate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

*Preparation of skeletal-muscle hexokinase.* Skeletal muscle was dissected from the back and hind legs of adult male hooded rats (Quebec Breeding Farms, St Eustache, Quebec, Canada) immediately after decapitation. The muscle was immediately placed in 10 vol. of cold water (3°) and homogenized in a Waring Blendor for 3 min. The homogenate was centrifuged for 60 min. at 100000*g* (Spinco model L centrifuge, rotor no. 30). The supernatant was 40% saturated with $(NH_4)_2SO_4$ at pH 7·8. The precipitate was removed by centrifugation and resuspended in a small volume of 0·1 M-EDTA, pH 7·8. This fraction, which contained most of the hexokinase and some myokinase (ATP–AMP phosphotransferase; EC 2.7.4.3), but virtually no adenosine triphosphatase (ATP phosphohydrolase; EC 3.6.1.4), was used for all initial-velocity experiments. For experiments on product inhibition by ADP and glucose

6-phosphate the fraction was further purified by gel filtration: Sephadex G-200 was equilibrated with 0·1M-$Na_2HPO_4$, pH7·8, for 24hr. at 3°. A portion (5ml.) of the resuspended 40%-saturated $(NH_4)_2SO_4$ sediment was placed on the column and eluted with 0·1M-$Na_2HPO_4$, pH7·8. Hexokinase, free of myokinase, was eluted just after the void volume.

In all experiments enough $Mg^{2+}$ was added to bind all the EDTA remaining in the enzyme preparation and to maintain an optimum $Mg^{2+}$/ATP ratio for the hexokinase reaction. When insufficient $Mg^{2+}$ was present non-linear double-reciprocal plots were obtained, presumably due to the presence of insufficient $Mg^{2+}$ owing to its binding by EDTA. The double-reciprocal plots were linear (except Fig. 5) when the hexokinase was prepared without EDTA or when an optimum $Mg^{2+}$/ATP ratio was used.

*Methods.* Hexokinase activity was measured spectrophotometrically by coupling glucose 6-phosphate formation to a glucose 6-phosphate dehydrogenase system and measuring $NADP^+$ reduction or by coupling ADP production to a pyruvate kinase and lactate dehydrogenase system and measuring the oxidation of NADH by pyruvic acid produced from phosphoenolpyruvate. Kinetic measurements were made at 340m$\mu$ in cuvettes of 1cm. light-path. Continuous readings were made with a Unicam SP.800 spectrophotometer equipped with a variable-speed 'slave' recorder with a full scale of 0–0·1$E$ unit. Four reactions were studied simultaneously with the Unicam automatic cell-changer. All reagents except enzyme were maintained at 29° in the same water bath that circulated water through the cuvette holder in the spectrophotometer. The reaction was started by the addition of a small volume of a diluted enzyme solution maintained at 0°. The amount of hexokinase used was adjusted to permit measurement of linear initial reaction velocities for a period of at least 5min. The control cuvettes contained buffer and all reactants except glucose.

In the glucose 6-phosphate-dehydrogenase-coupled system all reaction vessels contained, in a total volume of 3ml., tris–HCl buffer, pH8 (0·08M), $NADP^+$ (0·13mM) and excess of glucose 6-phosphate dehydrogenase; $MgCl_2$, ATP, D-glucose and ADP were added at the times shown in the Results section.

In the experiments on glucose 6-phosphate inhibition all reaction vessels contained, in a total volume of 3ml., lactate dehydrogenase and pyruvate kinase both in excess, NADH (0·084mM), phosphoenolpyruvic acid (5mM) and tris–HCl buffer, pH8 (0·08M); $MgCl_2$, ATP, D-glucose and glucose 6-phosphate were added at the times shown in the Results section. Initial velocity is expressed as change in $E_{340m\mu}$/min.

Many of the experiments were done in triplicate. The standard errors of the mean are indicated by vertical bars in the graphs.

## RESULTS

*Initial-velocity analysis.* In Figs. 1 and 2 are shown the results for the kinetics of the hexokinase reaction. In the experiment shown in Fig. 1 the concentration of glucose was varied at different fixed concentrations of ATP. In the experiment shown in Fig. 2 the concentration of ATP was varied at different fixed concentrations of glucose. The graphs show that the reciprocal plots of both Figs. 1 and 2 intersect to the left of the vertical axis. This indicates that both substrates, ATP and glucose, add sequentially to the enzyme before any product is released. The replots of slopes and intercepts from Figs. 1 and 2 versus the reciprocal of the changing concentration of the fixed substrate are linear (Figs. 3 and 4), indicating that the mechanism does not include alternative reaction sequences and that no substrate reacts with more than one enzyme form. The steady-state rate equation describing these data can be derived by Cleland's (1963*a*) method in the form (2):

$$v = \frac{V_1[A][B]}{K_i^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B]} \quad (2)$$

where $V_1$ is the maximum velocity in the forward direction and where A is ATP, B is glucose, $K_m^A$

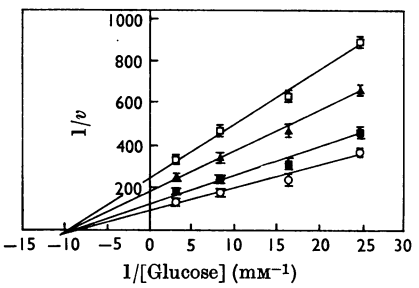



Fig. 1. Plot of the reciprocal of initial velocity against the reciprocal of the concentration of D-glucose. The $MgCl_2$ concentration was held constant at 6mM. The ATP concentration was held constant at 0·3mM (□), 0·45mM (▲), 0·9mM (■) and 3·0mM (○). Other details are given in the Experimental section.

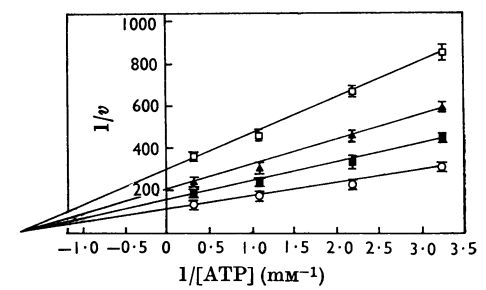



Fig. 2. Plot of the reciprocal of initial velocity against the reciprocal of the concentration of ATP. The $MgCl_2$ concentration was held constant at 6mM. The D-glucose concentration was held constant at 0·04mM (□), 0·06mM (▲), 0·12mM (■) and 0·3mM (○). Other details are given in the Experimental section.

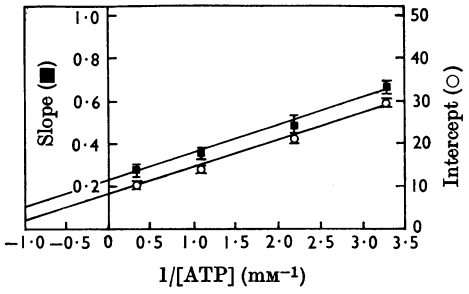



Fig. 3. Replots of slopes (■) and intercepts (○) from Fig. 1 against the reciprocal of the concentration of the fixed substrate, ATP.

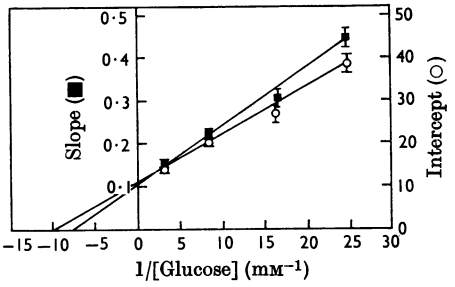



Fig. 4. Replots of slopes (■) and intercepts (○) from Fig. 2 against the reciprocal of the concentration of the fixed substrate, D-glucose.

and $K_m^B$ are the $K_m$ values for A and B respectively and $K_i^A$ is $k_2/k_1$ (eqn. 3). The $K_m$ values for the reactants calculated from the replots of intercepts in Figs. 3 and 4 are: $K_m^{Glc}$, 0·11 mM; $K_m^{ATP}$, 0·8 mM.

Eqn. (2) predicts a parabolic double-reciprocal plot of initial velocity versus reactant concentration when the concentrations of ATP and glucose are varied together at a constant ratio. The result of such an experiment is shown in Fig. 5. The solid curves are parabolas with the equation

$$y = 50x^2 + 50x + 80$$

for the lower curve and

$$y = 82x^2 + 60x + 80$$

for the upper curve, where $y = 1/v$ and $x = 1/[ATP]$. It is apparent that the experimental points fit the parabolic curves. The results in Fig. 5 confirm the presence of the constant term $K_i^A K_m^B$ in eqn. (2), thus ruling out any ordered mechanism for skeletal-muscle hexokinase in which the first product dissociates from the enzyme before the addition of the second substrate.

*Product-inhibition studies.* With various con-

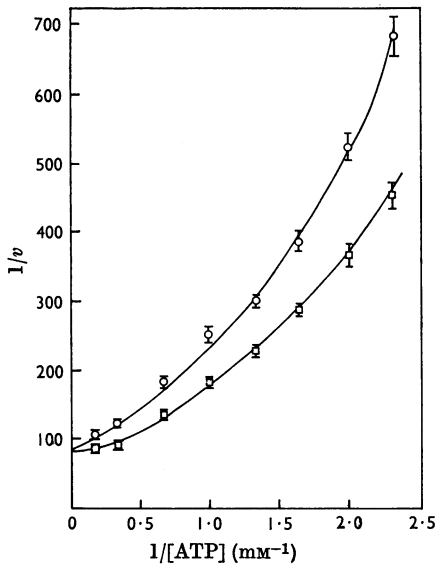



Fig. 5. Plot of the reciprocal of initial velocity against the reciprocal of the concentration of ATP and D-glucose. The ATP/glucose molar ratio was held constant at 7·5 (○) and 15 (□). $MgCl_2$ was present at a $Mg^{2+}$/ATP ratio 2. Other details are given in the Experimental section.

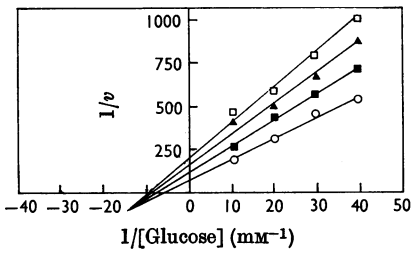



Fig. 6. Product inhibition of skeletal-muscle hexokinase by glucose 6-phosphate with D-glucose as the variable substrate. The ATP concentration was held constant at 3·0 mM. $MgCl_2$ was present at a $Mg^{2+}$/ATP molar ratio 2. The glucose 6-phosphate concentration was held constant at 0 μM (○), 16·7 μM (■), 33·4 μM (▲) and 50 μM (□). Other details are given in the Experimental section.

centrations of glucose as substrate, glucose 6-phosphate is a non-competitive inhibitor (Fig. 6). Replots of slopes and intercepts from Fig. 6 against glucose 6-phosphate concentration show that the increase in the degree of inhibition with increasing glucose 6-phosphate concentration is linear (Fig. 8). When studies were made in which the concentration of ATP was varied, inhibition by glucose 6-phosphate was shown to be non-competitive with respect to ATP (Fig. 7), and a replot of slopes and intercepts likewise is linear (Fig. 9).

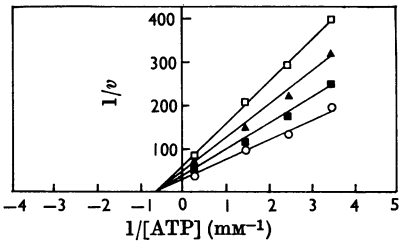



Fig. 7. Product inhibition of skeletal-muscle hexokinase by glucose 6-phosphate with ATP as the variable substrate. The D-glucose concentration was held constant at 13·3mM. $MgCl_2$ was present at a $Mg^{2+}$/ATP molar ratio 2. The glucose 6-phosphate concentration was held constant at 0μM (○), 16·7μM (■), 33·4μM (▲) and 50μM (□). Other details are given in the Experimental section.

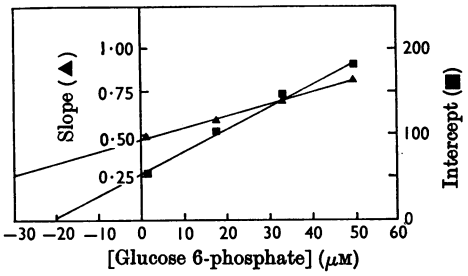



Fig. 8. Replot of slopes (▲) and intercepts (■) from Fig. 6 against the glucose 6-phosphate concentration.

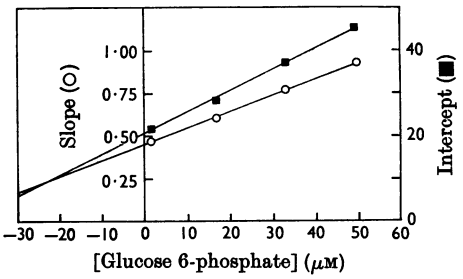



Fig. 9. Replot of slopes (○) and intercepts (■) from Fig. 7 against the glucose 6-phosphate concentration.

With ATP as the variable substrate, ADP is a competitive inhibitor (Fig. 12). The graph obtained by replots of slopes versus ADP concentration is linear (Fig. 13). The $K_{i\,(slope)}$ (Cleland, 1963*b*) for ADP calculated from Fig. 10 is 0·8mM. When glucose was the variable substrate and the concentration of ATP was held constant at 4mM, ADP was not inhibitory even at a concentration of 1·5mM (Fig. 10). However, when the concentration

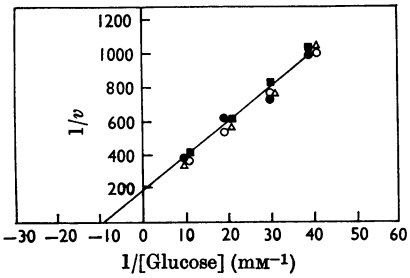



Fig. 10. Product inhibition of skeletal-muscle hexokinase by ADP with D-glucose as the variable substrate. The ATP concentration was held constant at 4mM. $MgCl_2$ was added with ATP at a molar $Mg^{2+}$/ATP ratio 2 and with ADP at a molar $Mg^{2+}$/ADP ratio 1. The ADP concentration was held constant at 0mM (●), 0·5mM (○), 1·0mM (△) and 1·5mM (■). Other details are given in the Experimental section.

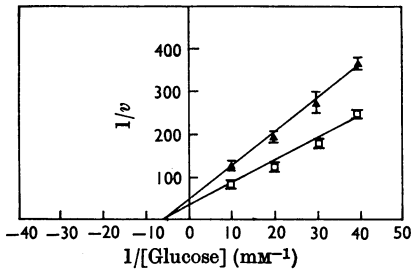



Fig. 11. Product inhibition of skeletal-muscle hexokinase by ADP with D-glucose as the variable substrate at non-saturating concentrations of ATP. The ATP concentration was held constant at 1mM. The $MgCl_2$ concentration was held constant at 6mM. ADP was held constant at 0mM (□) and 2mM (▲). Other details are given in the Experimental section.

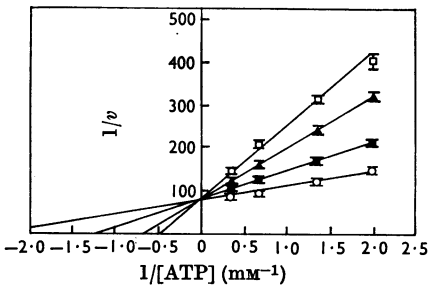



Fig. 12. Product inhibition of skeletal-muscle hexokinase by ADP with ATP as the variable substrate. The glucose concentration was held constant at 10mM. $MgCl_2$ was added with ATP at a molar $Mg^{2+}$/ATP ratio 2 and with ADP at a $Mg^{2+}$/ADP ratio 1. The ADP concentration was held constant at 0mM (○), 2·0mM (■), 4·0mM (▲) and 5·0mM (□). Other details are given in the Experimental section.

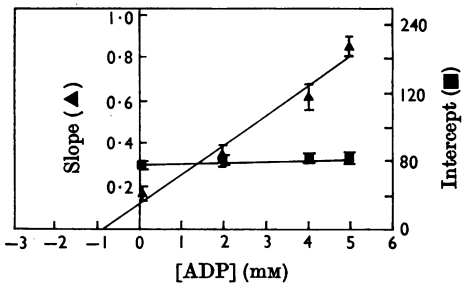
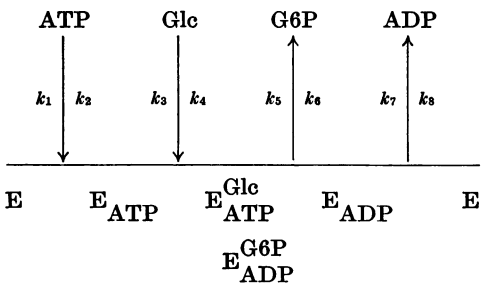



Fig. 13. Replot of slopes (▲) and intercepts (■) from Fig. 12 against the ADP concentration.

of the fixed substrate, ATP, is lowered to 1 mM then ADP is a non-competitive inhibitor with respect to glucose (Fig. 11).

## DISCUSSION

The simplest mechanism compatible with the initial-velocity analysis and the data on product inhibition is an ordered Bi Bi mechanism (Cleland, 1963*a*) as shown by:




(3)

where G6P refers to glucose 6-phosphate.

The steady-state rate equation for this mechanism (Cleland, 1963*a*) can be written in the form:

$$v = \frac{V_1([A][B]-[P][Q]/K_{eq.})}{K_i^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B] + \frac{K_m^Q K_i^A K_m^B[P]}{K_m^P K_i^Q} + \frac{K_i^A K_m^B[Q]}{K_i^Q} + \frac{K_i^A K_m^B[P][Q]}{K_m^P K_i^Q} + \frac{K_m^Q K_m^B[A][P]}{K_m^P K_i^Q} + \frac{K_m^A[B][Q]}{K_i^Q} + \frac{[A][B][P]}{K_i^P} + \frac{K_i^A K_m^B[B][P][Q]}{K_i^B K_m^P K_i^Q}} \quad (4)$$

where A, B, P, Q and $V_1$ are ATP, glucose, glucose 6-phosphate, ADP, and the maximum velocity in the forward direction respectively; $K_m^A$, $K_m^B$, $K_m^P$ and $K_m^Q$ are the $K_m$ values for ATP, glucose, glucose 6-phosphate and ADP respectively; $K_{eq.}$ is the thermodynamic equilibrium constant and equals $k_1k_3k_5k_7/k_2k_4k_6k_8$; $K_i^A$ equals $k_2/k_1$, $K_i^B$ equals $(k_2+k_4)/k_3$, $K_i^P$ equals $(k_5+k_7)/k_6$ and $K_i^Q$ equals $k_7/k_8$. When eqn. (4) is simplified by making either [P] or [Q] equal to zero and rearranged, equations are obtained for the types of product inhibition reported in this paper:

$$\frac{1}{v} = \frac{1}{V_1}\left(1+\frac{K_m^A}{[A]}+\frac{K_m^A[Q]}{K_i^Q[A]}\right) + \frac{K_m^B}{V_1[B]}\left(1+\frac{K_i^A[Q]}{K_i^Q[A]}+\frac{K_i^A}{[A]}\right) \quad (5)$$

vary [B], inhibit with Q;

$$\frac{1}{v} = \frac{1}{V_1}\left(1+\frac{K_m^B}{[B]}\right) + \frac{K_m^A}{V_1[A]}\left(1+\frac{[Q]}{K_i^Q}+\frac{K_i^A K_m^B}{K_m^A[B]}+\frac{K_i^A K_m^B[Q]}{K_i^Q K_m^A[B]}\right) \quad (6)$$

vary [A], inhibit with Q;

$$\frac{1}{v} = \frac{1}{V_1}\left(1+\frac{K_m^A}{[A]}+\frac{[P]}{K_i^P}\right) + \frac{K_m^B}{V_1[B]}\left(1+\frac{K_m^Q[P]}{K_m^P K_i^Q}+\frac{K_i^A}{[A]}+\frac{K_m^Q K_i^A[P]}{K_m^P K_i^Q[A]}\right) \quad (7)$$

vary [B], inhibit with P;

$$\frac{1}{v} = \frac{1}{V_1}\left(1+\frac{K_m^B}{[B]}+\frac{K_m^Q K_m^B[P]}{K_m^P K_i^Q[B]}+\frac{[P]}{K_i^P}\right) + \frac{K_m^A}{V_1[A]}\left(1+\frac{K_m^Q K_i^A K_m^B[P]}{K_m^P K_i^Q K_m^A[B]}+\frac{K_i^A K_m^B}{K_m^A[B]}\right) \quad (8)$$

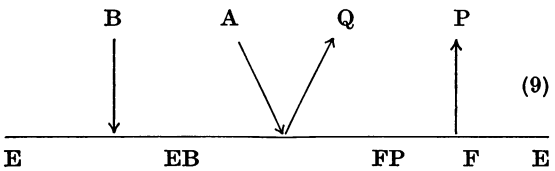
vary [A], inhibit with P.

Eqn. (5) predicts linear non-competitive inhibition by ADP, with glucose as the variable substrate. Because all terms in eqn. (5) in which [ADP] appears also have [ATP] in the denominator, namely $K_m^A[Q]/K_i^Q[A]$ and $K_i^A[Q]/K_i^Q[A]$, the observed $K_{i\,(slope)}$ and $K_{i\,(intercept)}$ would rise towards infinity as the concentration of the non-varied substrate, ATP, is raised. Therefore the non-competitive inhibition by ADP would be overcome by high fixed concentrations of ATP. In the data reported here, ADP is a non-competitive inhibitor when glucose is the variable substrate and the ATP concentration is held constant at 1 mM (Fig. 11). The inhibition by ADP is overcome when the concentration of the fixed substrate, ATP, is increased to 4 mM (Fig. 10). Eqn. (6) predicts linear competitive inhibition by ADP when ATP is the variable substrate. This is in agreement with the data for muscle hexokinase (Figs. 12 and 13). Eqn. (7) predicts linear non-competitive inhibition by glucose 6-phosphate when glucose is the variable substrate, which agrees with observed inhibition (Figs. 6 and 8). The observed linear non-competitive inhibition of hexokinase by glucose 6-phosphate

when ATP is the variable substrate (Figs. 7 and 9) is consistent with eqn. (8).

Eqn. (4) is useful because it can be used to distinguish the ordered Bi Bi mechanism from other sequential mechanisms. The presence of $K_i^A K_m^B$, [A][B][P] and [B][P][Q] terms in the denominator of eqn. (4) rules out any Ping Pong type mechanism (Cleland, 1963*a*). Likewise, the presence of [A][P], [B][Q], [A][B][P] and [B][P][Q] terms in the denominator of eqn. (4) rules out a rapid-equilibrium random Bi Bi mechanism. A simple Theorell–Chance mechanism is eliminated because of the presence of [A][B][P] and [B][P][Q] terms in the denominator of eqn. (4). An iso ordered Bi Bi mechanism (isomerization of the stable enzyme form E) is ruled out because the additional terms [A][P][Q], [A][B][Q] and [A][B][P][Q], predicted to be present in the denominator of the full rate equation for an iso ordered Bi Bi mechanism, are not present in eqn. (4). In a random Bi Bi mechanism additional terms appear in the numerator and denominator of eqn. (4) in such a way that normal hyperbolic kinetics with linear double-reciprocal plots do not occur. The observation that linear reciprocal plots were observed for muscle hexokinase (Figs. 1–4) as well as linear product inhibition by glucose 6-phosphate and ADP (Figs. 5–13) rules out a random Bi Bi mechanism.

Another mechanism that has a rate equation indistinguishable from eqn. (4) is an iso-Theorell–Chance mechanism (eqn. 9), where the order of addition of reactants is reversed and B and P react with different forms of free enzyme.




(9)

There is no way to distinguish this mechanism from an ordered Bi Bi mechanism by initial-velocity analysis or product inhibition. However, this mechanism is improbable because mechanisms without central complexes are unlikely to occur (Wratten & Cleland, 1963).

The most probable mechanism then for skeletal-muscle hexokinase is an ordered Bi Bi mechanism. Whether the complex $E^{Glc}_{ATP}$ isomerizes to $E^{G6P}_{ADP}$ before the release of products cannot be determined on the basis of the data reported above. However, this type of isomerization has been indicated in glutamate dehydrogenase (Frieden, 1959), lactate dehydrogenase (Novoa, Winer, Glaid & Schwert, 1959) and alcohol dehydrogenase (Wratten & Cleland, 1963).

Since the completion of this work another study of the kinetics of muscle hexokinase has been reported by Hanson & Fromm (1965). Though some of their results are qualitatively similar to those reported above, there are some major differences between their initial-velocity experiments and those in the present paper. Hanson & Fromm (1965) observed a parallel pattern in the double-reciprocal plots of the initial-velocity experiments, suggesting the absence of the $K_i^A K_m^B$ terms in eqn. (2). These findings are markedly different from the results described above (Figs. 1–5), which confirm the presence of the constant term $K_i^A K_m^B$ in eqn. (2). Although Hanson & Fromm (1965) have varied the ATP and glucose concentrations over a wide range in their initial-velocity experiments, these concentrations are well above their $K_m$ values so that the range of initial rates is quite small. If the substrate concentrations had been varied in a range near the $K_m$ resulting in a wider range of initial rates it is likely that an intersecting pattern would then have been observed. The subsequent interpretation of the kinetics would then be markedly different.

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