The Kinetics of Rabbit Muscle Pyruvate Kinase

INITIAL-VELOCITY, SUBSTRATE- AND PRODUCT-INHIBITION AND ISOTOPIC-EXCHANGE STUDIES OF THE REVERSE REACTION

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1. An assay, based on the transfer of label from [γ - ^{32}P]ATP to [^{32}P]phosphoenolpyruvate, suitable for a steady-state kinetic analysis of pyruvate kinase in the reverse direction (i.e. phosphoenolpyruvate synthesis), is described. 2. This assay was used in a kinetic investigation of the rabbit muscle enzyme, including initial-rate and product-inhibition experiments, at a pH of 7.4 and constant concentrations of total K^+ and free Mg^{2+} . 3. These studies indicate that there is a random release of ADP and phosphoenolpyruvate from the enzyme and that there is a competitive substrate inhibition by ATP. Some of the results were suggestive that the rapid-equilibrium assumption, generally used for this enzyme was not valid. 4. Techniques were developed to measure the rate of isotopic exchange between all the substrate-product pairs. 5. By using these techniques the rates of isotopic exchange at chemical equilibrium were measured. The results indicate that this enzyme does not catalyse a truly rapid-equilibrium random mechanism, although in the forward reaction all initial-rate data obtained to date are consistent with this assumption.

Pyruvate kinase (EC 2.7.1.40) from rabbit muscle catalyses a reaction between ADP and phosphoenolpyruvate that gives steady-state kinetics in the forward direction (i.e. ATP synthesis)

that can be interpreted by using the Michaelis-Menten rapid-equilibrium assumption (Reynard *et al.*, 1961; Ainsworth & MacFarlane, 1973). The enzyme has an obligatory requirement for a bivalent and a univalent cation, the roles of which have not been clearly established.

The bivalent metal ion (usually Mn^{2+} and Mg^{2+}) can bind directly to the enzyme (Mildvan & Cohn, 1965) and it has been suggested that it is a true substrate for the enzyme in the forward reaction (Ainsworth & MacFarlane, 1973). Other workers, however, have suggested that the true substrates for the reaction are the Mg-chelate forms of the nucleotides (Melchior, 1965; Cleland, 1967a).

For a complete kinetic analysis of an enzyme the reaction should be studied in both directions, as only in this way can sufficient information about the enzyme complexes, and their rates of formation, be obtained. Several kinetic studies of the forward reaction of rabbit muscle pyruvate kinase have been undertaken (Reynard *et al.*, 1961; Mildvan & Cohn, 1966; Ainsworth & MacFarlane, 1973), but no systematic steady-state studies of the reverse reaction have been reported. As a result of the unfavourable

equilibrium constant of the reaction (McQuate & Utter, 1959; Krimskey, 1959), only a very small proportion of the substrates are converted into products at equilibrium. Such kinetic studies of the reverse reaction that have been made were limited to conditions of high concentrations of both substrates (McQuate & Utter, 1959; Krimskey, 1959) such that sufficient amounts of products were formed to allow measurement by usual enzymic means. Consequently, an assay more sensitive than those already available was required to measure the reverse reaction of this enzyme and permit a full kinetic analysis.

In the present paper a sensitive assay for the reverse reaction of pyruvate kinase, based on the transfer of ^{32}P from [γ - ^{32}P]ATP to [^{32}P]phosphoenolpyruvate, is described. A study of the steady-state kinetics of the enzyme by using this assay in the presence and absence of products is reported. The initial rates of isotopic exchange at chemical equilibrium are also reported. All reactions took place at pH 7.4 and 25°C; K^+ was present at all times at a saturating concentration (100 mM) and the free Mg^{2+} concentration was held constant at 0.5 mM.

Theory

In the present study the enzyme reaction in the reverse direction was followed by measuring the initial rate of isotope flux from ATP into phosphoenolpyruvate. In the absence of products this flux is the same as that of the overall chemical reaction.

However, since this enzyme has a particularly unfavourable equilibrium constant it was found that the reaction could be close to chemical equilibrium,

formation of two dead-end complexes (Scheme 1), the initial rate of isotopic exchange from ATP into phosphoenolpyruvate† is

$$v^* = \frac{V[MgATP][Pyr]}{[MgATP][Pyr] + K_m^{Pyr}[MgATP] \left(1 + \frac{[MgATP]}{K_1^{MgATP}}\right) + K_m^{MgATP}[Pyr] \left(1 + \frac{[MgADP]}{K_1^{MgADP}}\right) + K_s^{MgATP}K_m^{Pyr} \left(1 + \frac{[MgADP]}{K_s^{MgADP}} + \frac{[Pyr-P]}{K_s^{Pyr-P}} + \frac{[MgADP][Pyr-P]}{K_s^{MgADP}K_m^{Pyr-P}}\right)} \quad (1)$$

yet far from isotopic equilibrium. One consequence of this is that it is very difficult to measure the initial rate of the chemical reaction, yet it is relatively easy to measure the initial rate of isotopic exchange. In order to utilize this information, equations describing the initial rate of isotopic exchange need to be derived.

In a rapid-equilibrium random mechanism the initial velocity of isotopic exchange from a labelled substrate, A*, into a labelled product, Q*, v^* , may be written as

$$v^* = k_{+1}[EA^*B]$$

where k_{+1} is the rate-limiting first-order rate constant

$$\frac{1}{v^*} = \frac{1}{V} \left(1 + \frac{K_m^{Pyr}}{[Pyr]}\right) + \frac{K_m^{MgATP}}{V} \left\{1 + \frac{K_s^{Pyr}}{[Pyr]} \left(1 + \frac{[MgADP]}{K_s^{MgADP}} + \frac{[Pyr-P]}{K_s^{Pyr-P}} + \frac{[MgADP][Pyr-P]}{K_s^{MgADP}K_m^{Pyr-P}}\right) + \frac{[MgADP]}{K_1^{MgADP}} + \frac{K_m^{Pyr}[MgATP]^2}{K_1^{MgATP}K_m^{MgATP}[Pyr]}\right\} \cdot \frac{1}{[MgATP]} \quad (2)$$

$$\frac{1}{v^*} = \frac{1}{V} \left\{1 + \frac{K_m^{MgATP}}{[MgATP]} \left(1 + \frac{[MgADP]}{K_1^{MgADP}}\right)\right\} + \frac{K_m^{Pyr}}{V} \left\{1 + \frac{K_s^{MgATP}}{[MgATP]} \left(1 + \frac{[MgADP]}{K_s^{MgADP}} + \frac{[Pyr-P]}{K_s^{Pyr-P}} + \frac{[MgADP][Pyr-P]}{K_s^{MgADP}K_m^{Pyr-P}}\right) + \frac{[MgATP]}{K_1^{MgATP}}\right\} \cdot \frac{1}{[Pyr]} \quad (3)$$

for the conversion of the ternary complex EA^*B into EPQ^* , irrespective of whether the system is at chemical equilibrium. The concentration of the ternary complex, $[EA^*B]$, can be expressed in terms of the concentrations of the various enzyme forms by using the relevant equilibrium constants and the conservation equation for enzyme. For comparison the initial velocity of the chemical reaction, v , is defined as

$$v = k_{+1}[EAB] - k_{-1}[EPQ]$$

where k_{+1} and k_{-1} are the rate-limiting first-order rate constants for the interconversion of the central complexes in the forward and reverse directions respectively, and $[EAB]$ and $[EPQ]$ are the concentrations of these central complexes. For any reaction therefore the ratio between v and v^* depends on both k_{-1} and the amount of EPQ formed.

If muscle pyruvate kinase catalyses a rapid-equilibrium random Bi-Bi mechanism with the

where V is the maximal velocity of the chemical reaction in the reverse direction, and the kinetic constants are as defined in Scheme 1.

In the absence of products the rate equation (1) simplifies to that derived for the chemical reaction. Eqns. (2) and (3) are the inverted forms of eqn. (1). They show that in all cases replots of the slopes of the asymptotes of the double-reciprocal plots as a function of product concentration should be linear in the presence of either only one product, or one product at variable concentration and one product at constant concentration. The asymptotic condition is equivalent to the case where no substrate inhibition occurs.

If the rate equations are derived by assuming that the reaction involves three substrates in one direction (Mg^{2+} , ADP and phosphoenolpyruvate) and two in the other ($MgATP$ and pyruvate) a linear replot of slopes against product concentration, irrespective of the presence of the second product is again predicted for the initial rate of both the chemical and isotopic-exchange reactions. The difference between the equations derived to explain the initial rate of isotopic exchange in a Bi-Bi and a Ter-Bi rapid-equilibrium random mechanism resides in the make up of the constant denominator term. As the equations have the same overall form, product-inhibition experiments of the isotopic-exchange rate can be used to investigate the kinetic mechanism (Cleland, 1963b). Calculation of the kinetic constants, however, is dependent on a knowledge of the correct rate equation to use.

† Abbreviations: Pyr, pyruvate; Pyr-P, phosphoenolpyruvate.

Scheme 1. Reaction scheme for the rapid-equilibrium random Bi-Bi mechanism of the rabbit muscle pyruvate kinase reaction if the true substrates are MgADP, phosphoenolpyruvate, MgATP and pyruvate

Two dead-end complexes form, namely E_{Pyr}^{MgADP} and $E_{(ATP)}^{MgATP}$. The nature of the second ATP species to bind is unknown and is indicated as (ATP). k_{+1} and k_{-1} are the first-order rate constants for the interconversion of the ternary complexes in the forward and reverse reactions respectively.

Materials and Methods

Rabbit muscle pyruvate kinase and pig heart lactate dehydrogenase were from Boehringer Corp., London W.5, U.K. Yeast hexokinase, sodium pyruvate, Tris base, EGTA [ethanedioxybis(ethylamine)-tetra-acetate] and Dowex resins were from Sigma Chemical Co., London S.W.6, U.K. [γ - ^{32}P]ATP, [2 - 3H]ATP, [2 - 3H]ADP and sodium [1 - ^{14}C]pyruvate were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Purification of ATP

Disodium ATP (800mg) was dissolved in approx. 50ml of water and the solution adjusted to pH 7 with $1M-NH_3$. This solution was placed on a Dowex 1 (Cl^- form, 200–400 mesh) column (2.5cm $\times$ 20.5cm) equilibrated with water. P_i , AMP and ADP were eluted by passing at least 10 litres of $10mM-HCl/40mM-KCl$ through the column over a 16–18 h period. At this stage the E_{259} of the eluate was the same as that of the eluting medium. The ATP was eluted

by passing $50mM-Tris/HCl/0.5M-KCl$, pH 7.4, and the peak fractions (approx. 100ml elution volume) were pooled. The ATP was immediately precipitated by adding an excess of saturated barium acetate solution. The insoluble barium salt was collected by low-speed centrifugation (5000g-min) and washed with $3 \times 50ml$ of acetone/water (1:1, v/v). The salt was dried by washing with acetone, acetone/diethyl ether (1:1, v/v) and finally diethyl ether. At all stages of the purification the temperature was never higher than $4^\circ C$. In this form the ATP could be stored *in vacuo* over silica gel at $-20^\circ C$ until required, although it was more stable in solution as its tetrapotassium salt.

The barium salt of ATP was converted into the K^+ salt by adding Dowex 50 (K^+ form) batchwise to a suspension of the salt in water. The concentration of ATP was determined by using the linked hexokinase–glucose 6-phosphate dehydrogenase reaction (Lamprecht & Trautschold, 1963). Any ADP present was detected by using the pyruvate kinase–lactate dehydrogenase linked reaction (Adam, 1963) and was usually 0.2% or less of the ATP concentration.

Purification of [γ - 32 P]ATP

The freeze-dried ammonium salt, with an initial specific radioactivity greater than 10 Ci/mmol, was dissolved in 50 mM-Tris/HCl, pH 7.4, and stored in liquid N₂. Immediately before use the radioisotope was purified by ion-exchange chromatography. Approx. 0.1 mCi was placed on a Dowex 1 (Cl⁻ form, 200–400 mesh) column (5 mm $\times$ 5 mm) equilibrated with water. After washing with 8 ml of 10 mM-HCl/40 mM-KCl to remove contaminants, the radioisotope was recovered in 2.2 ml of 20 mM-HCl/0.4 M-KCl and neutralized by adding 0.1 ml of 1 M-Tris base.

Potassium pyruvate

This was prepared from sodium pyruvate (4.5 mmol) by converting it into the free acid by passage over a Dowex 50 (H⁺ form) column (0.85 cm $\times$ 145 cm) and neutralization with KOH. The concentration of Na⁺ in the product was less than 1% of that of K⁺ as determined by flame photometry. Pyruvate concentration was determined by using the lactate dehydrogenase reaction (Bücher *et al.*, 1963).

Assay of pyruvate kinase activity in the forward direction (v_{+1})

(NH₄)₂SO₄ in commercial pyruvate kinase was removed by exhaustive dialysis against 25 mM-Tris/HCl (pH 7.4)/1 mM-EGTA. The dialysed enzyme was stored at 2°C. An assay modified from that by Bücher & Pfeleiderer (1955) was used to measure the activity of the enzyme in the forward direction. Pyruvate kinase (approx. 0.4 μ g of protein in 10 μ l) was added to the reaction mixture (final volume 3 ml), containing 25 mM-Tris/HCl, pH 7.4, 75 mM-KCl, 8 mM-MgSO₄, 1 mM-EGTA, 0.8 mM-phosphoenolpyruvate, 0.23 mM-ADP, 0.15 mM-NADH and 16 units of lactate dehydrogenase, equilibrated to 25°C. NADH oxidation was followed at 340 nm by using a Pye Unicam SP.1800 u.v. spectrophotometer and an AR 25 pen recorder. In this assay, the dialysed enzyme had a specific activity greater than 150 μ mol of pyruvate produced/min per mg of protein at 25°C. Protein was determined by the Biuret method (Gornall *et al.*, 1949).

Assay of pyruvate kinase in the reverse direction (v_{-1})

The reaction was measured at 25°C in a final volume of 1 ml, which contained 25 mM-Tris/HCl, pH 7.4, 1 mM-EGTA, 100 mM-K⁺ (total) and 0.5 mM-Mg²⁺ (free). The concentrations of ATP and pyruvate used are given in the text. A known amount of previously purified [γ - 32 P]ATP was added such that the specific radioactivity of the ATP was 1–5 μ Ci/ μ mol. The balance of K⁺ required over that present

in the substrates was added as KCl. Sufficient MgCl₂ was added to give the required free Mg²⁺ concentration calculated by using the dissociation constants of the Mg²⁺ chelates of ATP, ADP and phosphoenolpyruvate quoted by MacFarlane & Ainsworth (1972). After thermal equilibration the reaction was initiated by addition of a small volume of enzyme (generally 10 μ l).

After a suitable period, usually 5 min, the reaction was stopped by the addition of 0.2 ml of 5% (w/v) trichloroacetic acid, and the mixture kept in ice for 5 min. This solution was neutralized with 50 μ l of 1 M-Tris base before addition of 0.1 ml of 1 M-glucose and 0.5 μ mol of carrier phosphoenolpyruvate. After mixing, 4 units of hexokinase were added and the solution was incubated at 25°C for 20 min. The reaction mixture was diluted with 5 ml of water and placed on a Dowex 1 (Cl⁻ form, 200–400 mesh) column (0.8 cm $\times$ 2.5 cm) equilibrated with water. The bulk of the radioactivity, present as glucose 6-phosphate, was eluted in 10 ml of 10 mM-HCl/40 mM-KCl. Phosphoenolpyruvate was recovered by eluting with 20 mM-HCl/0.2 M-KCl. The eluate (10 ml) was neutralized with 1 M-Tris base and the phosphoenolpyruvate hydrolysed by the addition of 0.1 ml of 0.1 M-mercuric acetate. After incubation at room temperature (20°C) for 5 min, 0.5 ml of 3.75 M-H₂SO₄ and 0.2 ml of 6.6% (w/v) ammonium molybdate were added to the solution. The P_i, as its phosphomolybdate complex, was extracted into 5 ml of 2-methylpropan-1-ol. A 3 ml sample of the organic phase was washed with 5 ml of 0.5 M-H₂SO₄, and 1 ml of the washed organic layer was added to 12 ml of toluene/methanol (5:1, v/v) containing 5 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD)/litre and counted for radioactivity in a Beckman automatic liquid-scintillation counter. Under these conditions the counting efficiency for 32 P was better than 95%.

One of the major problems encountered in developing this assay was the high background count. This was substantially decreased by purification of the radioisotope immediately before use and converting the unchanged [γ - 32 P]ATP remaining in the assay into glucose 6-phosphate by using glucose and hexokinase as described above. In the absence of this step ATP and phosphoenolpyruvate could not be readily separated by chromatography on Dowex 1 (Cl⁻ form). In addition, the hydrolysis of ATP catalysed by ammonium molybdate (Lowry & Lopez, 1946) in the latter stages of the assay resulted in an unacceptable increase in the background count.

The background count also varied with the chemical concentration and the specific radioactivity of the ATP used. Control incubations, without enzyme, were always included in the procedure and their value was subtracted from the experimental

Fig. 1. Time-course of incorporation of label from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into $[\text{P}^{32}]\text{phosphoenolpyruvate}$ measured by using the assay for pyruvate kinase in the reverse direction

The assay conditions were as given in the Materials and Methods section with the following concentrations of substrates: ATP, 0.25 mM (containing $2.8\mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}/\mu\text{mol}$); pyruvate, 5 mM. Rabbit muscle pyruvate kinase was added at zero time to give a concentration in the assay medium of $0.054v_{+1}$ unit/ml.

assays. Since the specific radioactivity of the ATP was known, the amount of phosphoenolpyruvate formed could be calculated to give the rate of the reaction in the reverse direction. In order to normalize the rates measured in various experiments the rate in the reverse direction (v_{-1}) was expressed as a ratio, v , to that found in the forward direction (v_{+1}) when assayed in standard conditions.

Fig. 1 shows the formation of phosphoenolpyruvate with respect to time in the presence of low concentrations of substrates. The reaction was linear for at least 8 min under the conditions described; thus the standard incubation time of 5 min appears justified. The reaction rate was also linear with respect to enzyme concentration, to values of over twice that used in the conditions described in Fig. 1. In these conditions 1 ml of the zero-time acid-washed organic phase contained 105 d.p.m., whereas after 5 min incubation the same volume contained 2700 d.p.m. It was only at the highest concentration of ATP used (9.77 mM) that the background count exceeded 10% of the experimental count.

Recoveries of known amounts of phosphoenolpyruvate, up to $0.5\mu\text{mol}$, taken through the procedure showed that 1 ml of the acid-washed 2-methylpropan-1-ol contained 37.4% of the phosphate added as phosphoenolpyruvate, when determined by the method of Barenblum & Chain (1938). The column eluate, containing the P_i after mercuric acetate hydrolysis, was extracted into 5 ml of 2-methylpropan-1-ol. After extraction, only 3.5 ml remained, as this organic solvent has an appreciable solubility in water. In the conditions described only 3 ml of this 3.5 ml was used in the acid-wash step,

after which 2.2–2.3 ml of the organic phase could be recovered. This is equivalent to 2.56–2.68 ml if all the 3.5 ml were used and hence leads to an overall recovery of 96–100% of the phosphate added as phosphoenolpyruvate.

Preparation of phosphoenol[1- ^{14}C]pyruvate

Phosphoenol[1- ^{14}C]pyruvate was synthesized from [1- ^{14}C]pyruvate (specific radioactivity about $9\mu\text{Ci}/\mu\text{mol}$) by using phosphoenolpyruvate synthase (EC 2.7.9.2) partially purified to a specific activity of 1.92 units/mg of protein from lactate-grown *Escherichia coli* B (Cooper & Kornberg, 1967). Labelled phosphoenolpyruvate was prepared in a reaction mixture containing $400\mu\text{mol}$ of Tris/HCl, pH 8.4, $50\mu\text{mol}$ of MgCl_2 , $50\mu\text{mol}$ of sodium ATP, pH 8.4, $11.2\mu\text{mol}$ of sodium [1- ^{14}C]pyruvate ($100\mu\text{Ci}$ of carrier-free label) and 1.6 units of phosphoenolpyruvate synthase, in a final volume of 4 ml. The reaction was terminated after 30 min incubation at 30°C by addition of 1 ml of 5% (w/v) trichloroacetic acid and the mixture kept in ice for 5 min. The precipitated protein was removed by low-speed centrifugation (5000 g-min) and the supernatant neutralized with 1 M-Tris base. Unchanged ATP was converted into glucose 6-phosphate in a second incubation at 30°C in the presence of $600\mu\text{mol}$ of glucose and 32 units of hexokinase. This incubation mixture was diluted by addition of water (15 ml) and applied to a column (0.8 cm $\times$ 10 cm) of Dowex 1 (formate form, 200–400 mesh). AMP, ADP, glucose 6-phosphate and unchanged pyruvate were eluted at 4°C in 2.75 M-formic acid (160 ml) and the phosphoenolpyruvate was recovered by elution with 1 M-ammonium formate. The peak fractions were pooled, freeze-dried and the powder was taken up in water (0.5 ml). The yield of the purified radioisotope was 68–74% of the labelled pyruvate used. The final material contained less than 1% pyruvate.

Measurement of isotopic exchange rates

All the reactions were carried out at pH 7.4 and 25°C in the presence of 25 mM-Tris/HCl, 1 mM-EGTA, 100 mM- K^+ (total), 0.5 mM- Mg^{2+} (free) and approx. $0.05v_{+1}$ unit (as defined above) of enzyme/ml. In these conditions the equilibrium constant of the reaction was measured as 23.5×10^3 when expressed in terms of total nucleotide and phosphoenolpyruvate concentrations. This value for the equilibrium constant was used to determine equilibrium ratios of reactants.

In all the exchanges, except pyruvate-phosphoenolpyruvate, the equilibrium mixture of reactants (7.5 ml) was incubated for 5 min at 25°C , after which $75\mu\text{l}$ of enzyme (containing approx. $0.375v_{+1}$ unit) was added. After a further 10 min to ensure chemical equilibrium, the exchange reaction was started by

addition of 10–50 μ l of carrier-free labelled substrate. The maximal increase in the chemical concentration of the substrate on adding the label was for phosphoenolpyruvate, when the increase was 5%. Addition of [2- 3 H]ADP, [2- 3 H]ATP or [γ - 32 P]ATP displaced the equilibrium by less than 0.01%. Samples of the reaction mixture (1 ml) were taken at 1 min time-intervals over a 6 min time-course, added to 0.2 ml of 5% (w/v) trichloroacetic acid to stop the reaction and placed on ice for at least 5 min. In the pyruvate-phosphoenolpyruvate exchange the reaction was started by addition of enzyme to the mixture already containing radioisotope.

The reaction mixtures were resolved by chromatography on Dowex 1 (Cl^- form) as detailed below. The exchange rate, determined from the slope of the line in a plot of radioactivity (d.p.m.) appearing in the product against time, and the known specific radioactivity of the substrate, was expressed as nmol of product formed/min per v_{+1} unit of enzyme.

(a) *ATP-phosphoenolpyruvate exchange.* This was the same as the assay of enzymic activity in the reverse direction as described above.

(b) *ATP-ADP exchange.* Before use the [2- 3 H]-ATP supplied (specific radioactivity 18 Ci/mmol) was purified in the same way as the [γ - 32 P]ATP described above. This removed any endogenous labelled AMP and ADP present, which would otherwise increase the background count. Approx. 0.5 μ Ci was added per ml of incubation mixture to initiate the exchange. The acid-quenched sample after the exchange reaction was neutralized by adding 1 M-Tris base and diluted by addition of 15 ml of water. The solution was applied to a Dowex 1 (Cl^- form, 200–400 mesh) column (0.82 cm $\times$ 2.5 cm) equilibrated with water. A small volume of water (2 ml) was passed before the ADP was eluted in 10 mM-HCl/40 mM-KCl (35 ml). Samples of this (1 ml) were added to 10 ml of scintillant and counted for radioactivity as detailed below. Experiments indicated that more than 95% of the ADP was recovered in the 35 ml of eluate.

(c) *ADP-ATP exchange.* The exchange was initiated by addition of 1 μ Ci of label (specific radioactivity 18 Ci/mmol) per ml of reaction mixture. The acid-quenched sample after exchange was neutralized and applied to a Dowex 1 column as described above. The ADP was eluted as described for (b) above, and the ATP recovered by eluting with 20 mM-HCl/200 mM-KCl (20 ml). Portions (1 ml) of this were added to 10 ml of scintillant and counted for radioactivity as detailed below. Overall recovery was measured as being over 95%.

(d) *Pyruvate-phosphoenolpyruvate exchange.* The freeze-dried [1- 14 C]pyruvate supplied (specific radioactivity 8.9 μ Ci/mmol) was dissolved in a small volume of 10 mM-HCl to limit dimer formation and stored at -20°C . Immediately before use sufficient carrier-free radioisotope was neutralized with KOH

and purified by chromatography on a column (5 mm $\times$ 5 mm) of Dowex 1 (Cl^- form, 200–400 mesh). The pyruvate was eluted in 10 mM-HCl after a short water wash (1 ml); the peak fractions (volume 2 ml) were pooled and adjusted to pH 5 by addition of 1 M-Tris base.

The trichloroacetic acid-treated sample after the exchange reaction was diluted by addition of 15 ml of water and adjusted to pH 3.8 by addition of 1 M-Tris base. The material was applied to a Dowex 1 (Cl^-) column as in (b) above. After a short water wash (2 ml) the pyruvate was eluted in 100 ml of 10 mM-HCl. The phosphoenolpyruvate was eluted in 10 ml of 20 mM-HCl/200 mM-KCl, of which 2 ml was added to 20 ml of scintillant and counted for radioactivity as detailed below. Overall recovery of phosphoenolpyruvate was better than 95%.

(e) *Phosphoenolpyruvate-pyruvate exchange.* The exchange reaction was initiated by addition of approx. 0.015 μ Ci of labelled phosphoenolpyruvate per ml of reaction mixture. The acid-quenched sample was diluted by addition of water (15 ml) and neutralized (pH 7) by addition of 1 M-Tris base. The anions in solution were bound to a Dowex 1 (Cl^- form) column as in (b) above. After passage of 2 ml of water the pyruvate was eluted in 75 ml of 10 mM-HCl. This eluate was neutralized by addition of 1 M-Tris base and applied to a second Dowex (Cl^- form) column of the same dimensions as the first. The pyruvate was collected in a small volume (5 ml) by eluting with 100 mM-HCl. Samples of this (2 ml) were added to 20 ml of scintillant and counted for radioactivity as detailed below. Overall recovery of pyruvate was measured as being over 95%.

Radiochemical purity

Radiochemical purity of the nucleotides and phosphoenolpyruvate was checked by using t.l.c. on polyethyleneimine-cellulose with 1 M-acetic acid/1 M-LiCl as the solvent. The purity of the pyruvate was determined by chromatography on Dowex 1 (Cl^- form). These results indicated that in the worst case no less than 98.5% of the total radioactivity was present as the labelled substrate. Hence little error was introduced by calculating the specific radioactivity of the substrates from a count of a known volume of the exchange incubation mixture.

Liquid-scintillation counting

Samples of either ^{14}C or ^3H were counted for radioactivity in a scintillation fluid containing 1 litre of toluene, 500 ml of Triton X-100, 4 g of 2,5-diphenyloxazole (PPO) and 0.5 g of 1,4-bis (4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP). This scintillation fluid maintains high efficiencies in the presence of appreciable quantities of aqueous

solutions. The prepared samples were pre-cooled to the counting temperature (10°C) and counted for radioactivity in a Philips liquid-scintillation counter. The counter corrected the observed c.p.m. for quenching by using external standardization, and by using the known efficiency for ^{14}C (96%) and ^3H (55%) calculated d.p.m. from the corrected c.p.m. In all experiments no less than 10000 counts were recorded for each experimental vial, giving a counting error of less than 1%.

Data analysis

The initial-rate data were fitted to the Michaelis-Menten equation (4) by the unweighted hyperbolic least-squares method of Wilkinson (1961) after a graphical check that the double-reciprocal plots (of reaction rate against substrate concentration) were linear. Slope and intercept secondary plots, including the standard errors of the variables, were constructed and used to identify the form of the rate equation. Finally all the data obtained in the absence of products were fitted to eqn. (5), the equation for linear competitive substrate inhibition, by using a program written in FOCAL-12 and run on a Digital Equipment Corporation PDP-12 computer and based on the method of Cleland (1967b).

$$v = \frac{V[S]}{K_m + [S]} \quad (4)$$

$$v = \frac{V[A][B]}{K_s^A K_m^B + (K_m^A + K_s^A K_m^B / K^B)[B] + K_m^B [A] + [A][B] + (K_m^A / K^B)[B]^2} \quad (5)$$

where A is the substrate that does not show substrate inhibition and B is the substrate that does show substrate inhibition.

Results

Initial-velocity patterns

Initial-rate studies with ATP as the variable substrate showed substrate inhibition (Giles *et al.*, 1975a). When pyruvate was the variable substrate, no substrate inhibition was observed at concentrations of pyruvate up to 90 mM (Fig. 2). A secondary plot of the ordinate intercepts from this primary plot is a linear function of the reciprocal of the ATP concentration (Fig. 3a), but the replot of slopes is non-linear (Fig. 3b). This is diagnostic of competitive substrate inhibition where the binding of a second inhibitory substrate molecule is in competition with the binding of the non-inhibitory substrate. By fitting the initial-rate data to eqn. (5), values for the kinetic constants can be obtained (Table 1). The maximal rate of the reverse reaction, V in Table 1, is expressed as a ratio of the enzyme activity in the

Fig. 2. Effect of ATP on the initial rate of the reverse pyruvate kinase reaction with pyruvate as the variable substrate

Conditions of the enzyme assay are given in the text, with total $K^+ = 100\text{ mM}$ and free $\text{Mg}^{2+} = 0.5\text{ mM}$. The total ATP concentrations used were: $\square$, 0.135 mM; $\blacksquare$, 0.27 mM; $\circ$, 0.54 mM; $\bullet$, 1.08 mM; $\triangle$, 9.77 mM. The points are the experimental data and the lines correspond to the least-squares fit to eqn. (5).

Fig. 3. Secondary plots of the initial-rate data in terms of the intercepts (a) and slopes (b) of the double-reciprocal plot (of enzyme activity against pyruvate concentration) as a function of the total concentration of the fixed substrate, ATP

The intercepts are a dimensionless ratio of activities and the slopes have units of mM. The error bars indicate the s.e.m. of the points obtained from the least-squares fit of each line in Fig. 2 to eqn. (4).

Table 1. Kinetic constants of the uninhibited reverse pyruvate kinase reaction

The constants were calculated by fitting the experimental data to eqn. (5), the rate equation for competitive substrate inhibition in a sequential Bi reaction. It was assumed that the binding constant of the inhibitory ATP molecule (K_1^{ATP}) for the enzyme is the same as that for the enzyme-substrate complex. If this assumption is not made no constants can be calculated. The constants involving ATP are quoted in terms of total nucleotide, and not in terms of the substrate species.

K_m^{Pyr}	$4.9 \pm 1.5 \text{ mM}$
K_m^{ATP}	$0.184 \pm 0.034 \text{ mM}$
K_s^{Pyr}	$8.3 \pm 3.2 \text{ mM}$
K_1^{ATP}	$2.78 \pm 0.87 \text{ mM}$
V	0.0417 ± 0.0022

Fig. 4. Inhibition by ADP of the reverse reaction catalysed by rabbit muscle pyruvate kinase with ATP as the variable substrate

Assay conditions were as described in Fig. 2. The total concentrations of ATP were: $\circ$, $6 \mu\text{M}$; $\bullet$, $56 \mu\text{M}$; $\square$, $109 \mu\text{M}$; $\blacksquare$, $135 \mu\text{M}$; $\triangle$, $160 \mu\text{M}$. The concentration of pyruvate was 10 mM .

reverse reaction to that measured in a standard assay condition in the forward reaction (the v_{+1} unit described in the Materials and Methods section). This immediately gives the ratio between the maximal velocity in the reverse reaction and the enzyme activity in the v_{+1} assay. After allowance for the fact that only 46% of the maximal velocity of the forward reaction is measured in the v_{+1} assay the ratio of the maximal velocities in the forward and reverse reactions can be calculated as 54.5:1.

Product-inhibition studies

The results of the product-inhibition experiments, at a non-saturating concentration of the constant

Fig. 5. Inhibition by phosphoenolpyruvate of the reverse reaction catalysed by rabbit muscle pyruvate kinase with ATP as the variable substrate

Assay conditions were as described in Fig. 2. The total concentrations of phosphoenolpyruvate were: $\circ$, $0 \mu\text{M}$; $\bullet$, $17.4 \mu\text{M}$; $\square$, $34.8 \mu\text{M}$; $\blacksquare$, $52.5 \mu\text{M}$; $\triangle$, $69.6 \mu\text{M}$. The concentration of pyruvate was 10 mM .

Fig. 6. Inhibition by ADP of the reverse reaction catalysed by rabbit muscle pyruvate kinase with pyruvate as the variable substrate

Assay conditions were as described in Fig. 2. The total concentrations of ADP were: $\circ$, $<0.5 \mu\text{M}$; $\bullet$, $51.3 \mu\text{M}$; $\square$, $103 \mu\text{M}$; $\blacksquare$, $128 \mu\text{M}$; $\triangle$, $154 \mu\text{M}$. The total concentration of ATP was 0.251 mM .

substrate, are given in Figs. 4–6. The fourth pattern (phosphoenolpyruvate against pyruvate) is parabolic competitive inhibition (Giles *et al.*, 1975a). In these experiments the velocity measured was the initial rate of isotopic exchange, which is not necessarily the same as the initial rate of the chemical reaction (see the Theory section). This is because in some patterns substrates and both products were present, as it proved impossible to remove all the ADP from ATP. In no experiment did more than 20% of the total isotopic exchange occur in the 5 min incubation

Fig. 7. Secondary plots of the slopes of the double-reciprocal plots of the product-inhibition experiments as a function of inhibitor concentration

The primary plots from which the secondary plots were constructed are: (a) ADP versus ATP; (b) phosphoenolpyruvate versus ATP; (c) ADP versus pyruvate; (d) phosphoenolpyruvate versus pyruvate. The slopes all have units of mM. The error bars indicate the S.E.M. of the slopes obtained from the least-squares fit of each line individually to eqn. (4).

period and in most cases less than 10% of the isotope was transferred. Thus it is valid to derive the initial rate of isotopic exchange from this fixed-point assay. When ATP was the variable substrate only low concentrations were used. In these conditions little substrate inhibition occurred and the observed rates were close to the asymptote of the curve. These asymptotic rates describe the situation that occurs in the absence of substrate inhibition. No significant intercept effect was seen for any of the inhibition patterns, indicating that the inhibitions were all competitive. The competition between both products and both substrates implies that there must be a random combination with the enzyme, consistent with the rapid-equilibrium random mechanism proposed from the studies on the forward reaction. Analysis of the slope replots (Fig. 7) as a function of product concentration, however, is not consistent with this mechanism. Linear slope replots (Figs. 7a and 7b) were obtained with both products

from the data when ATP was the variable substrate, as predicted by the rapid-equilibrium model. When pyruvate was the variable substrate, however, non-linear slope replots were obtained (Figs. 7c and 7d). Such non-linear slope effects are normally the result of multiple combinations of the inhibitor with the enzyme. This possibility can be eliminated here, however, as the non-linearity is seen with both products against only one variable substrate and not both, as would be the case if a complex of the type enzyme-product-product occurred in a rapid-equilibrium random mechanism. Another possibility is that the rapid-equilibrium assumption is not valid.

No meaningful values for the true inhibition constants can be calculated from the data, owing to the non-linearity of the replots and the uncertainty in the identification of the substrates of the forward reaction. The apparent dissociation constants of ADP and phosphoenolpyruvate from their enzyme binary

Fig. 8. Linearity of the exchange of label from substrate into product as a function of time

The conditions of the assays for the various exchanges were as described in the text, with the following substrate and product concentrations: ATP, 1 mM; pyruvate, 12.9 mM; ADP, 25 μ M; phosphoenolpyruvate, 22.1 μ M. The exchanges measured were: ●, phosphoenolpyruvate-pyruvate; □, ATP-phosphoenolpyruvate; △, ADP-ATP; ▲, ADP-ATP in the absence of phosphoenolpyruvate and pyruvate.

complexes, calculated from the two linear competitive-inhibition plots, were 79.5 ± 11 and $29.4 \pm 5 \mu$ M respectively.

Isotopic exchange at equilibrium

The possibility that the reaction was not completely rapid-equilibrium was investigated by measuring the initial rates of isotopic exchange for five of the six possible exchange reactions at chemical equilibrium. In a truly rapid-equilibrium random reaction all the exchange rates should be identical.

Measuring the exchange between a reactant pair in both directions gave identical values, indicating that the system was at chemical equilibrium. This was confirmed by extending the preincubation of the equilibrium mixture of substrates with the enzyme for up to 1 h, when no change in the exchange rates

Table 2. Equilibrium exchange rates for the pyruvate kinase reaction

The exchange rates were measured at 25°C in a reaction mixture containing 25 mM-Tris/HCl (pH 7.4), 1 mM-EGTA, 100 mM-K⁺ (total) and 0.5 mM-Mg²⁺ (free). In condition (1) the total concentrations were: phosphoenolpyruvate, 22.1 μ M; ADP, 25 μ M; pyruvate, 12.9 mM; ATP, 1 mM. In condition (2) the total concentrations were: phosphoenolpyruvate, 50 μ M; ADP, 50 μ M; pyruvate, 7.65 mM; ATP, 7.65 mM. The exchange reaction was initiated by addition of a chemical concentration of labelled substrate not in excess of 5% of the total substrate concentration, and the exchange rate determined from a plot of d.p.m. appearing in the product as a function of time. Results are expressed as means $\pm$ S.E.M. (number of observations in parentheses).

Exchange reaction	Exchange rate (nmol/min per v_{+1} unit of enzyme)	
	Condition (1)	Condition (2)
ATP-phosphoenolpyruvate	15.5 ± 2.0 (4)	12.7 ± 4.1 (2)
ATP-ADP	15.5 ± 1.7 (2)	—
ADP-ATP	13.8 ± 0.7 (6)	10.5 ± 2.1 (3)
Pyruvate-phosphoenolpyruvate	11.7 ± 0.5 (3)	—
Phosphoenolpyruvate-pyruvate	8.73 ± 1.5 (5)	5.8 ± 1.2 (4)

was seen. The rates of all the exchanges were linear with time (Fig. 8), even when the zero-time value was high relative to the slope of the line. Exchange from a substrate into a product was dependent on the presence of all reactants, giving direct evidence that a direct phosphate transfer occurs.

The initial rates for the various exchanges were determined in two sets of equilibrium conditions (Table 2). The exchange rate for any substrate-product pair was different in the two equilibrium conditions and the rates for the various exchanges in any one condition are different. In both equilibrium conditions the phosphoenolpyruvate-pyruvate exchange was the slowest.

Discussion

The present study confirms the view of Reynard *et al.* (1961) and Ainsworth & MacFarlane (1973) that rabbit muscle pyruvate kinase has a sequential mechanism, with the substrates and products binding randomly. The rapid-equilibrium assumption, which suffices to explain the data obtained for the forward reaction, does not hold for the reverse reaction. Direct evidence that it is an invalid assumption was obtained from isotopic-exchange studies at equilibrium. The results presented also demonstrate that the enzyme will form an enzyme-ATP-ATP dead-end complex, with the inhibitory

ATP molecule competing with pyruvate for the same enzyme form.

As far as we are aware this is the first rigorous kinetic analysis of the reverse reaction of pyruvate kinase. Considerable technical difficulties exist in assaying the enzyme in this direction. First there is a large unfavourable equilibrium constant, and secondly it does not appear possible to decrease the ADP content of ATP to less than 0.1%. This means that at times, a significant concentration of ADP with respect to its kinetic constants can be present. In our experience commercial supplies of ATP can contain up to 2% ADP, depending on the time after preparation and conditions of storage. Thus the presence of considerable concentrations of ADP, and the substrate inhibition by ATP, may explain the difference in the value of K_m^{ATP} quoted by Dyson *et al.* (1975) (0.9 mM) and the value obtained in the present study (0.184 mM). In comparison, studies of the forward reaction in the presence of ATP, allowing for the presence of ADP, as this was the variable substrate, gave a value of 0.14 mM for the dissociation constant of ATP from the enzyme (Boyer, 1969), in good agreement with the value of 0.315 mM that can be calculated from data in Table 1.

Dyson *et al.* (1975) noted that at high concentrations of ATP the enzyme activity fell, but their data were difficult to interpret, since they did not allow for the chelation of Mg^{2+} by ATP. In the light of our results it is possible that this is related to the substrate inhibition by ATP that we observed. It is not known at the moment which of the ATP species present is responsible for the inhibition.

The value for K_m^{Pyr} obtained in the present study agrees well with that reported by Rose (1960) from studies on the detritiation of pyruvate (5.3–6.6 mM), and by Dyson *et al.* (1975) using initial-rate kinetics (7.05 mM). The value for the dissociation constant, K_s^{Pyr} obtained in the present study (8.34 mM) also agrees with that calculated by Ainsworth & MacFarlane (1973) from pyruvate-inhibition studies of the forward reaction (6 mM) and with a value of about 4 mM for the binding constant obtained from direct physical studies (Mildvan & Cohn, 1966; Brevet *et al.*, 1975). The value for the ratio of the maximal velocities in the forward and reverse directions (54.5:1) is in excellent agreement with that reported by Dyson *et al.* (1975).

In the present investigations the free Mg^{2+} concentration was maintained constant at 0.5 mM with the consequence that the concentration of the Mg-chelate forms of the nucleotides and of phosphoenolpyruvate were always in a constant ratio to that of the Mg-free species. In the conditions of constant pH and metal-ion concentrations used in the present study, 84, 43 and 4% of the ATP, ADP and phosphoenolpyruvate respectively, were present as their Mg chelates. All the constants derived from the

data presented in this paper are quoted in terms of total chemical concentration. This approach was decided on because of the uncertainty that still exists as to the nature of the true substrates of the muscle enzyme. It is a simple matter to obtain the true kinetic constants once the true substrate species have been unambiguously identified, by multiplication by the appropriate constants. Similarly allowance will also be necessary if it transpires that Mg^{2+} is a true substrate of the enzyme in the forward direction, but not the reverse (as suggested by Ainsworth & MacFarlane, 1973), as it was always present and would be acting as a product inhibitor. In spite of these limitations to the quantitative nature of the results, the qualitative aspects, which are of more importance in the present study, are valid.

It has been demonstrated that rabbit muscle pyruvate kinase forms several dead-end complexes, enzyme-ADP-ADP (Janson & Cleland, 1974), enzyme-ADP-pyruvate (Ainsworth & MacFarlane, 1973; Brevet *et al.*, 1975) and enzyme-ATP-ATP (the present study). Further analysis of the results of inhibition of pyruvate kinase by ATP, reported by Reynard *et al.* (1961), shows non-linear replots of slope as a function of ATP concentration when either ADP or phosphoenolpyruvate was the variable substrate. It would be tempting therefore to suggest that these non-linear slope replots are solely due to formation of enzyme-ATP-ATP, but caution must be exercised, as Reynard *et al.* (1961) did not allow for chelation of Mg^{2+} by ATP. Nevertheless, at the concentrations of ATP used formation of the complex could contribute towards the observed non-linearity.

The results reported provide no evidence for the formation of the enzyme-ADP-ADP or enzyme-ADP-pyruvate complexes. But, because of the relatively low concentrations of ADP used, no significant formation of these complexes would be expected to occur. For example, formation of the enzyme-ADP-pyruvate complex by combination of ADP with enzyme-pyruvate complex, with a dissociation constant of $230 \mu M$, the same as that for K_s^{ADP} measured in the forward reaction (Janson & Cleland, 1974), would be expected to cause less than a 30% increase in the ordinate intercept in Fig. 6 on increasing the concentration of ADP from 0 to $160 \mu M$. A larger value for the dissociation constant will lead to a smaller intercept effect. The standard errors for each intercept, caused by experimental variation, were within the range 5–10%. It is obvious therefore that no significant intercept effect could be seen at the ± 2 s.e. (approx. 95%) confidence level.

It is interesting that two binding sites were observed by Stammers & Muirhead (1975) on diffusing high concentrations of ADP (10–100 mM) into crystals of cat muscle pyruvate kinase. The sites were revealed by constructing three-dimensional

Fourier difference maps from X-ray-crystallographic data. It is possible that one of these sites is the inhibitory nucleotide-binding site suggested from the results in the present paper. It is also possible that the rabbit muscle enzyme forms a mixed-nucleotide dead-end complex (enzyme-ATP-ADP) in a manner analogous to the pyruvate kinase from *Carcinus maenas* hepatopancreas (Giles *et al.*, 1975b).

The product-inhibition experiments are consistent with a random addition of reactants to the enzyme, but the analysis of the slope replots suggests that the rapid-equilibrium assumption is not valid. This was confirmed by the isotopic exchange rates at equilibrium. Unfortunately, because of the unfavourable equilibrium constant, and the problem of removing ADP from the ATP, the substrate:product ratios could only be studied over a limited range. From the data obtained it was found that the phosphoenolpyruvate-pyruvate exchange was always slower than the other exchanges, including the ATP-phosphoenolpyruvate exchange, which has phosphoenolpyruvate as a common partner. This is highly suggestive that the steps involving the binding and/or release of pyruvate contribute towards the rate-determining step(s) of the reaction. This result is consistent with that of Robinson & Rose (1972), who studied the detritiation of [3-³H]phosphoenolpyruvate in conditions of the net forward reaction. The results indicated that the release of pyruvate could not be rapid with respect to the reversal of the proton-transfer steps involved in the catalysis of the enolization reaction, steps presumably occurring in the interconversion of the central complexes. In the light of all the evidence it is theoretically invalid to use the rapid-equilibrium assumption to describe the pyruvate kinase reaction, although in the forward reaction all the steady-state kinetic data available are consistent with this assumption.

The rate equation for a random Bi Bi mechanism with no dead-end complexes is complicated and involves square terms in both the numerator and denominator of the rate equation. By using the method of King & Altman (1956) the rate equation for the mechanism in the presence of one product can be expressed as eqn. (6), where *a* to *z* represent functions of the rate constants for the individual steps.

$$v = \frac{a[A][B] + b[A][B][P] + c[A]^2[B]}{g + h[A] + i[B] + j[A][B] + k[A]^2 + l[B]^2 + m[A]^2[B] + n[A][B]^2 + o[A][P]^2 + p[A]^2[P] + q[B][P]^2 + r[B]^2[P] + s[A][B][P]^2 + t[A]^2[B][P] + u[A][B]^2[P] + v[A][P] + w[B][P] + x[A][B][P] + y[P]^2 + z[P]} \quad (6)$$

There are no kinetic constants equivalent to the Michaelis constants, although if certain terms become negligible compared with others the equation will simplify to the rate equation derived by using the rapid-equilibrium assumption. Computer-simulation studies conducted by assuming values for the various rate constants have shown that initial-rate and product-inhibition data can, in most cases, be adequately explained by rate equations derived by using the rapid-equilibrium assumption, even though this assumption is not theoretically valid (Gulbinsky & Cleland, 1968; I. G. Giles & P. C. Poat, unpublished work). With large differences in the values for the rate constants, however, it is possible to set up a preferred kinetic pathway, such that non-linearity is observed (Ferdinand, 1966).

It appears that the rate constants of the pyruvate kinase reaction are such that the forward reaction can be adequately described by the rapid-equilibrium assumption. In the presence of one product and a constant concentration of the second substrate, B, eqn. (6) can be simplified to,

$$v = \frac{a'[A] + b'[A][P] + c'[A]^2 + d'[A]^2[P]}{e' + f'[A] + g'[A]^2 + h'[A][P]^2 + i'[A]^2[P] + j'[A][P] + k'[P]^2 + l'[P]} \quad (7)$$

where *a'*–*l'* are new constants that are functions of the previous constants (*a*–*z*) and the concentration of B. From this equation, for example, one possible explanation for linear competitive inhibition with either product, and linear double-reciprocal plots, is that *f'* > *g'* and *a'* > *c'*. In addition, for the intercepts to be constant in the presence of the product (i.e. competitive inhibition) *f'* > *j'[P] + h'[P]^2* and *a'* > *b'[P]* over the concentration range of P used. For the inhibition to be a linear function of product concentration, two further conditions must be met, *b'* < *a'* and *e'* < *k'[P]*. Other inequalities can be derived to explain the non-linear slope replots and, since a systematic analysis of the inequalities was not undertaken, many more may well exist to explain the observed linear data. Unfortunately, because of the complexity of the system it has not been possible to reduce the relationships given to a form in which obvious relationships among the rate constants can be seen.

Previous work has shown that the rapid-equilibrium assumption in the thermodynamically favoured direction is valid, but the results in the present study suggest that it is theoretically invalid in the reverse direction. The large value of the equilibrium constant implies that there is a discrepancy between the ratio of the products of the first- and second-order rate constants involved in the forward and reverse reactions. At first sight this would appear to contradict the generalization that second-order rate constants for physiological substrates are more or

less invariant with values near the diffusion-controlled limit (Hammes & Schimmel, 1970). However, there is no evidence from the present study to suggest which rate constants are different in the forward and reverse reactions. Pre-steady-state techniques are required to measure the value of the individual rate constants in both directions.

The isotopic-exchange rates at equilibrium for several other kinases have been reported, and these include galactokinase (Gulbinsky & Cleland, 1968), hexokinase (Fromm *et al.*, 1964) and creatine kinase (Morrison & Cleland, 1966). Of these only the last was found to be truly rapid-equilibrium. The sugar-sugar phosphate exchange of the others was slower by a factor of 1.5–2.5:1. This is similar to rabbit muscle pyruvate kinase, where the analogous pyruvate-phosphoenolpyruvate exchange is slowest.

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