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A Kinetic Study of Rabbit Muscle Pyruvate Kinase

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The paper reports a study of the kinetics of the reaction between phosphoenolpyruvate, ADP and Mg^{2+} catalysed by rabbit muscle pyruvate kinase. The experimental results indicate that the reaction mechanism is equilibrium random-order in type, that the substrates and products are phosphoenolpyruvate, ADP, Mg^{2+}, pyruvate and MgATP, and that dead-end complexes, between pyruvate, ADP and Mg^{2+}, form randomly and exist in equilibrium with themselves and other substrate complexes. Values were determined for the Michaelis, dissociation and inhibition constants of the reaction and are compared with values ascertained by previous workers.

Pyruvate kinase (EC 2.7.1.40) from rabbit muscle catalyses a reaction between ADP and phosphoenolpyruvate, which shows relationships between initial velocity and substrate concentrations that have been interpreted by using the Michaelis–Menten assumptions (Reynard *et al.*, 1961). The enzyme has an obligatory requirement for a bivalent cation, such as Mg^{2+} or Mn^{2+}, and a univalent cation, preferably K^+ (Kachmar & Boyer, 1953).

The mechanism of the reaction has not been clearly established. Reynard *et al.* (1961) concluded that the substrates bind randomly to the enzyme and that the enzyme–substrate complexes are in equilibrium with one another (equilibrium random-order mechanism). McQuate & Utter (1959), Siebert *et al.* (1965) and Pon & Bondar (1970) reported that the (presumably apparent) Michaelis constant of phosphoenolpyruvate varies with ADP concentration: Pon & Bondar (1970) argued that this finding is inconsistent with random-order kinetics, whereas Siebert *et al.* (1965) concluded that the mechanism is ordered, with ADP binding to the enzyme first and phosphoenolpyruvate second. However, Mildvan & Cohn (1966) supported the random-order mechanism and suggested that the quaternary complex may be formed either by the separate addition of Mn^{2+} and ADP, or by addition of the Mn^{2+}-bound nucleotide. Mildvan *et al.* (1971) subsequently reported that the same mechanism applies when Ni^{2+} is present as the obligatory cation.

Cleland (1967*a*) has questioned whether the bivalent metal cation and ADP can bind separately to the enzyme and has stated that nucleoside di- and tri-phosphates always react in the form of their bivalent metal-ion complexes. This does not seem certain, however, for Macfarlane & Ainsworth (1972) have shown that free ADP alone is the nucleotide substrate of yeast pyruvate kinase.

To date, there has been no complete product-inhibition study of muscle pyruvate kinase: it has been found, however, that ATP competes with phosphoenolpyruvate and ADP and that pyruvate competes with phosphoenolpyruvate (Reynard *et al.*, 1961; Mildvan & Cohn, 1966).

The present paper reports a study of the kinetics of the reaction between phosphoenolpyruvate and ADP catalysed by muscle pyruvate kinase, in the presence and absence of single products. The reactions took place at pH 6.2 and 25°C; K^+ was present at all times at a saturating concentration (Macfarlane & Ainsworth, 1972).

Theory

Substrate-level equilibria

The possibility that the substrates of muscle pyruvate kinase may react in their free or Mg^{2+} complexed forms, and that Mg^{2+} itself may be regarded as a substrate, makes the definition of the different species a first requirement to a kinetic study of the enzyme-catalysed reaction. The concentrations of the different species involved have been determined from the known equilibrium constants for their inter-conversions by using the method described by Macfarlane & Ainsworth (1972). This examination showed that five ADP and four phosphoenolpyruvate species are present in significant proportions at pH 6.2: their identity and contributions to the sum of the free and Mg^{2+}-bound forms are indicated below:

$$[\text{Pyr-}P]_{\text{total}} \equiv A_t = \begin{cases} \Sigma[\text{Pyr-}P] = [\text{Pyr-}P^{3-}] + [\text{HPyr-}P^{2-}] + [\text{KPyr-}P^{2-}] \equiv A \\ \Sigma[\text{MgPyr-}P] = [\text{MgPyr-}P^{-}] \equiv \alpha \end{cases}$$

$$[\text{ADP}]_{\text{total}} \equiv B_t = \begin{cases} \Sigma[\text{ADP}] = [\text{ADP}^{3-}] + [\text{HADP}^{2-}] + [\text{KADP}^{2-}] \equiv B \\ \Sigma[\text{MgADP}] = [\text{MgADP}^{-}] + [\text{MgHADP}] \equiv \beta \end{cases}$$

$$\text{where } [Mg^{2+}]_{\text{total}} \equiv C_t = C + \alpha + \beta$$

The total Mg^{2+} concentration contains an addition for Mg^{2+}-bound ATP when this product is present as an inhibitor. Under the conditions of these experiments, 91% of the Mg^{2+}-complexed nucleotide in solution is present as $MgATP^{2-}$.

Identification of substrates

We propose to determine whether the free or Mg^{2+}-complexed forms of ADP and phosphoenolpyruvate are the true substrates of muscle pyruvate kinase, by using the test devised by Macfarlane & Ainsworth (1972). The basis of the test is to measure the reciprocal velocity as a function of Mg^{2+} concentration under conditions where the concentrations of different combinations of possible substrates are kept constant, and to estimate thereby the powers of Mg^{2+} concentration which appear in the empirical equation representing each situation. Comparison of the estimated powers with the values given in Table 1 identifies the true substrates and, in so doing, limits the possible mechanisms that need be considered.

Reaction mechanism

The experimental results indicate that the reaction mechanism is of the Random Tri Bi type (Cleland, 1963*a*) in which phosphoenolpyruvate, ADP and Mg^{2+} bind randomly to the enzyme, forming complexes in equilibrium with one another. Phosphoryl transfer takes place, by a rate-limiting step, in the quaternary complex of the substrates, with the random dissociation of pyruvate and MgATP. The results also suggest that pyruvate can form dead-end complexes with all the substrates other than phosphoenolpyruvate. The mechanism and the pyruvate interactions are illustrated in Schemes 1 and 2, where A, B, C, P, Q and E represent phosphoenolpyruvate, ADP, Mg^{2+}, pyruvate, MgATP and the enzyme–K^+ complex respectively. The theoretical aspects of this mechanism will now be described.

The inverted initial-velocity equation for pyruvate formation is given in eqn. (1). In this equation, Dalziel's (1969) nomenclature for the kinetic constants has been modified to indicate which terms are affected by products added singly to the reaction solutions. The composition of the kinetic constants in terms of the equilibrium constants defined by Schemes 1 and 2 are given in Table 2.

$$\frac{E_0}{v}=\left[\left(\frac{\phi_{ABC}^{PQ}}{C}+\phi_{AB}^{P}\right)\frac{1}{B}+\frac{\phi_{AC}^{P}}{C}+\phi_{A}^{P}\right]\frac{1}{A}+\left[\left(\frac{\phi_{BC}}{C}+\phi_{B}\right)\frac{1}{B}+\frac{\phi_{C}}{C}+\phi_{0}\right] \quad (1)$$

Eqn. (1) is cast in a form illustrating that the double-reciprocal plot is linear and also that the eight kinetic constants may be obtained by a stepwise procedure in which the slopes and intercepts of the primary plots are replotted against B^{-1} to obtain secondary line constants, which are then plotted against C^{-1} (Dalziel, 1969). Eqn. (1) also shows that, when A is the varied substrate, P and Q are competitive inhibitors.

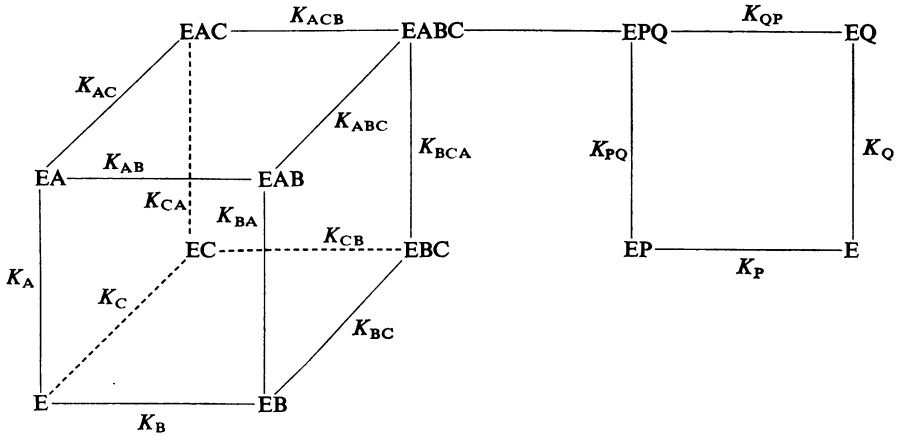
Eqn. (2) illustrates that the triple-plotting procedure may be undertaken in a different order and that, when B is the varied substrate, Q is a competitive inhibitor and P a non-competitive inhibitor.

$$\frac{E_0}{v}=\left[\left(\frac{\phi_{ABC}^{PQ}}{C}+\phi_{AB}^{P}\right)\frac{1}{A}+\frac{\phi_{BC}}{C}+\phi_{B}\right]\frac{1}{B}+\left[\left(\frac{\phi_{AC}^{P}}{C}+\phi_{A}^{P}\right)\frac{1}{A}+\frac{\phi_{C}}{C}+\phi_{0}\right] \quad (2)$$

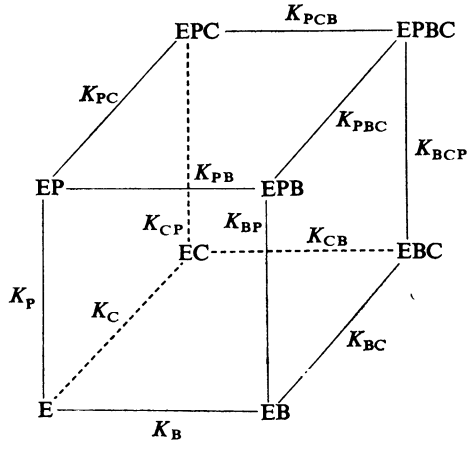
Table 1. *Relationship between Mg^{2+}-dependence in the rate equation and hypothetical true substrates*

Values of i for individual terms $f_x\,(C^t)_x$ appearing in the inverted rate equation when named substrates are kept constant are given. Values of i are obtained by representing the hypothetical true substrates (x) in the reaction in terms of the species kept constant by the use of an apparent Mg^{2+}-binding constant for each substrate.

X^{-1}	A and B constant	A and β constant	α and B constant	α and β constant	A_t and B_t constant
A	0	0	+1	+1	0, +1
B	0	+1	0	+1	0, +1
C	−1	−1	−1	−1	−1
α	−1	−1	0	0	−1, 0
β	−1	0	−1	0	−1, 0



Scheme 1. *Enzyme–substrate complexes formed by muscle pyruvate kinase*



Scheme 2. *Pyruvate dead-end complexes formed with muscle pyruvate kinase*

Michaelis constants and dissociation constants of individual substrates from the binary and ternary complexes are readily obtained by forming ratios of the kinetic constants. These are given in Table 3.

Values of K_Q may be obtained from data described by eqn. (1) or eqn. (2). Replots of the slopes of the respective double-reciprocal plots as a function of Q allows the direct calculation of K_Q from the resulting secondary slopes if ϕ_{ABC} is known.

Values of the pyruvate inhibition constants may be obtained from data described by eqn. (2). Replotting the slopes or intercepts of the double-reciprocal plot as a function of P provides secondary slopes, which, in both instances, contain two pyruvate inhibition constants. These may be separately evaluated, with simultaneous equations, by employing additional data obtained at different fixed concentrations of A and C (Figs. 8 and 9).

Table 2. *Composition of kinetic constants corresponding to the enzyme mechanism depicted in Schemes 1 and 2, in the presence or absence of a single product*

Kinetic coefficient	Kinetic constant $\times \left(\frac{K_A K_{AB} K_{ABC}}{V_f}\right)^{-1}$
ϕ_A^P	$\frac{1}{K_B K_{BC}}\left(1+\frac{P}{K_{BCP}}\right)$
ϕ_B	$\frac{1}{K_A K_{AC}}$
ϕ_C	$\frac{1}{K_A K_{AB}}$
ϕ_{AB}^P	$\frac{1}{K_C}\left(1+\frac{P}{K_{CP}}\right)$
ϕ_{AC}^P	$\frac{1}{K_B}\left(1+\frac{P}{K_{BP}}\right)$
ϕ_{BC}	$\frac{1}{K_A}$
ϕ_{ABC}^{PQ}	$1+\frac{P}{K_P}+\frac{Q}{K_Q}$
ϕ_o	$\frac{1}{K_A K_{AB} K_{ABC}}$

Experimental

Materials

ATP, ADP, NADH, pyruvate (all as sodium salts), phosphoenolpyruvate, 3-phosphoglycerate (cyclohexylammonium salts), rabbit muscle lactate

dehydrogenase (15371 ELAC), glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and rabbit muscle pyruvate kinase were products of Boehringer Corp. (London) Ltd., London W.5, U.K. Tetrapropylammonium hydroxide was supplied by Eastman-Kodak Co., Rochester, N.Y., U.S.A. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden. All other reagents were either AnalaR or reagent grade as supplied by British Drug Houses Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Enzyme preparation

Rabbit skeletal muscle pyruvate kinase was purchased from Boehringer Corp. (London) Ltd. and was used without further purification (Mildvan & Cohn, 1965, 1966). The enzyme had a specific activity of 162 units/mg at 25°C, measured by the assay described by Bergmeyer (1963). However, when NH_4^+ was removed from pyruvate kinase and lactate dehydrogenase, and Na^+ from ADP and phosphoenol pyruvate (see the next section) the enzyme was found to have a specific activity of 300 units/mg at 25°C. For this assay the reaction mixture contained, in a total volume of 1.0ml, tetrapropylammonium cacodylate (100μmol of cacodylate), pH6.2, KCl (100μmol), $MgCl_2$ (15μmol), ADP (5μmol), phosphoenolpyruvate (5μmol), NADH (0.15μmol) and 50μg (12 units) of lactate dehydrogenase. The buffer was prepared by adjusting the pH of cacodylic acid to pH6.2 with 10% (w/v) tetrapropylammonium hydroxide.

Removal of metal ions from substrates, products and enzymes

All substrates and products that were purchased as sodium salts (except NADH) were converted into the tetrapropylammonium salt by passing them through a column of Dowex 50W -X8 (tetrapropylammonium form) (Phillips *et al.*, 1963; Kayne & Suelter, 1965). Stock solutions of these compounds were adjusted to pH7.0 by addition of 10% (w/v) tetrapropylammonium hydroxide, and were stored at −15°C. ADP and ATP concentrations were determined spectrophotometrically at 259nm (Bock *et al.*, 1956). Phosphoenolpyruvate and pyruvate concentrations were determined at 230nm (Pon & Bondar, 1970).

Before use pyruvate kinase was passed through Sephadex G-25 (medium grade) equilibrated with 0.1M-Tris–HCl, pH7.5 (Kuczenski & Suelter, 1970) at 4°C. Pyruvate kinase concentrations were established from the absorbance at 280nm ($E_{1cm}^{0.1\%} = 0.54$) (Bücher & Pfleiderer, 1955*b*). Lactate dehydrogenase, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase were dialysed at 4°C against 0.2M-Tris–HCl, pH7.0. Samples of all treated enzymes were tested with $BaCl_2$ (1.8%, w/v) to confirm the absence of SO_4^{2-} and hence of NH_4^+ (Hunsley & Suelter, 1969).

Purity of substrates

ADP decomposes by approx. 1–3% per month (Phillips *et al.*, 1963) and was therefore used within 2 weeks of arrival. The purity of each sample was checked by paper-chromatographic analysis (Krebs & Hems, 1953) with development in isobutyric acid–aq. NH_3 (sp.gr. 0.88)–water (66:1:33, by vol.). Examination of the chromatograms in u.v. light did not reveal any impurities. Stock solutions, stored at −15°C, were discarded after 1 week, as longer storage produced sufficient 5'-AMP to be detectable by the chromatographic procedure described above. The concentration of each fresh stock solution of phosphoenolpyruvate and ADP was checked enzymically. The reaction mixture contained, in a total volume of 1ml, tetrapropylammonium cacodylate (0.1M in cacodylate), pH6.2, KCl (100μmol), $MgCl_2$ (15μmol), NADH (0.15μmol) and 50μg (12 units) of lactate dehydrogenase. For the determination of ADP, excess of phosphoenolpyruvate (2μmol) was added, and for the determination of phosphoenolpyruvate excess of ADP (2μmol) was added. Then 0.1μmol of the other substrate was added and the reaction was initiated by the addition of 5 units of pyruvate kinase. The reaction was then allowed to proceed for 10min and the change in NADH concentration determined spectrophotometrically. The blank cuvettes contained identical assay components, except that pyruvate kinase was omitted. The concentrations of substrates determined by direct absorption measurements and enzymic analysis were in agreement to within ±2%.

Enzyme assays

Reaction mixtures contained, in a total volume of 1ml, tetrapropylammonium cacodylate buffer (0.1M in cacodylate), pH6.2, KCl (100μmol), NADH (0.15μmol), with substrates and sometimes a single product at the concentrations indicated in the Figures. Initial-velocity and ATP-product-inhibition studies were performed by using lactate dehydrogenase as a coupling enzyme (Bücher & Pfleiderer, 1955*a*, *b*). Pyruvate-inhibition studies were performed by using the assay procedure described by Macfarlane & Ainsworth (1972).

After addition of the necessary components, the reaction mixtures were incubated for 5min before addition of NADH and the coupling enzymes. The reactions were initiated by the addition of 10μl or less of pyruvate kinase, and the initial velocities estimated

Table 3. *Constants for the uninhibited reaction catalysed by muscle pyruvate kinase*

Kinetic constants were calculated by assuming that pyruvate kinase has a molecular weight of 237000 (Warner, 1958) and the enzyme has two active sites per molecule (Mildvan & Cohn, 1965; Reynard *et al.*, 1961).

Kinetic constants		Michaelis and dissociation constants, and maximum velocity
ϕ_A	$4.64\ (\pm1.18)\times10^{-10}$ M·min	$K_{BCA} = K_{CBA} = \phi_A/\phi_O = 3.01\ (\pm0.78)\times10^{-5}$ M
ϕ_B	$-2.53\ (\pm1.18)\times10^{-9}$ M·min	$K_{CAB} = K_{ACB} = \phi_B/\phi_O = 4.25\ (\pm0.51)\times10^{-5}$ M
	$6.54\ (\pm0.72)\times10^{-10}$ M·min (Fig. 1)	$K_{ABC} = K_{BAC} = \phi_C/\phi_O = 1.20\ (\pm0.069)\times10^{-3}$ M
ϕ_C	$1.85\ (\pm0.05)\times10^{-8}$ M·min	$K_A = \phi_{ABC}/\phi_{BC} = 7.97\ (\pm1.0)\times10^{-5}$ M
ϕ_{AB}	$-1.01\ (\pm1.3)\times10^{-13}$ M²·min	$K_B = \phi_{ABC}/\phi_{AC} = 8.09\ (\pm1.4)\times10^{-4}$ M
	$3.58\ (\pm0.31)\times10^{-14}$ M²·min (Fig. 1)	$K_C = \phi_{ABC}/\phi_{AB} = 5.92\ (\pm0.86)\times10^{-2}$ M
ϕ_{AC}	$2.62\ (\pm0.33)\times10^{-12}$ M²·min	$K_{AB} = \phi_{BC}/\phi_C = 1.44\ (\pm0.08)\times10^{-3}$ M
ϕ_{BC}	$2.66\ (\pm0.13)\times10^{-11}$ M²·min	$K_{BA} = \phi_{AC}/\phi_C = 1.42\ (\pm0.18)\times10^{-4}$ M
ϕ_{ABC}	$2.12\ (\pm0.25)\times10^{-15}$ M³·min	$K_{AC} = \phi_{BC}/\phi_B = 4.07\ (\pm0.49)\times10^{-2}$ M
ϕ_O	$1.54\ (\pm0.07)\times10^{-5}$ min	$K_{CA} = \phi_{AB}/\phi_B = 5.47\ (\pm0.76)\times10^{-5}$ M
		$K_{BC} = \phi_{AC}/\phi_A = 5.64\ (\pm1.6)\times10^{-3}$ M
		$K_{CB} = \phi_{AB}/\phi_A = 7.71\ (\pm2.0)\times10^{-5}$ M
		$V_f = 1/\phi_O = 6.49\ (\pm0.3)\times10^5\ \text{min}^{-1}$
		$= 1.08\ (\pm0.05)\times10^4\ \text{s}^{-1}$ (turnover number)

Table 4. *Inhibition constants arising from the inhibition of muscle pyruvate kinase by MgATP*

Inhibition constants are obtained from the slope of the secondary replot against MgATP concentration. Apparent inhibition constants obtained directly from the COMP program of Cleland (1963*b*) are given as (secondary intercept)/(secondary slope) and values of 1.54 ± 0.1 mM and 1.42 ± 0.12 mM were obtained with A and B as varied substrate respectively.

Product inhibitor	Varied substrate	Fixed substrate (mM)	Replot	Inhibition constant (K_Q) (mM)
Q	A	B (0.028)	Slope	2.07 ± 0.28
Q	B	A (0.066)	Slope	2.09 ± 0.34

Table 5. *Inhibition constants arising from the inhibition of muscle pyruvate kinase by pyruvate*

Inhibition constants are obtained by replotting the slope and intercepts of $v^{-1} = f(\Sigma\text{ADP})^{-1}$ (eqn. 2) as a function of pyruvate concentration. The resulting slopes are each a function of two pyruvate inhibition constants, which may be separately evaluated by employing additional data for different fixed concentrations of phosphoenolpyruvate and Mg^{2+}.

Kinetic constants	(mM)
K_P	12.6 ± 4.25
K_{CP}	1.61 ± 0.82
$K_{PC} = K_C K_{CP}/K_P$	7.56 ± 4.6
$K_{PB} = K_B K_{BP}/K_P$	0.21 ± 0.08
K_{BP}	3.31 ± 0.56
$K_{BCP} = K_{CBP}$	5.15 ± 2.48
$K_{BPC} = K_{PBC} = K_{BC} K_{BCP}/K_{BP}$	8.77 ± 5.11
$K_{PCB} = K_{CPB} = K_{CB} K_{BCP}/K_{CP}$	0.24 ± 0.18

from the linear slopes of progress curves recorded by an Optica spectrophotometer at 340nm.

Computer analysis of data

The results of Figs. 2–9 were analysed by using the SEQUEN, COMP and NON-COMP programs of Cleland (1963*b*), and the weighted linear regression of Wilkinson (1961) (Macfarlane & Ainsworth, 1972).

Equilibrium constants were obtained from the resulting slope and intercept data as described above and the resulting values are recorded in Tables 3–5. The straight lines appearing on the double-reciprocal plots represent computer fits to the data of the individual figures.

Results

Identification of the substrates

Measurements of the initial velocity of the pyruvate kinase-catalysed reaction were made as a function of

the free Mg^{2+} concentration with either the free, Mg^{2+}-bound, or total ADP and phosphoenolpyruvate kept constant. The results (Fig. 1) demonstrate that the reciprocal velocity is represented by a relationship:

$$v^{-1} = \Sigma_x f_x C_x^i$$

in which $i = 0, -1$ only when A and B are kept constant; in all other cases inhibition is observed. Further replotting of the data shows that the limiting value of i is +1 when either α and B or A and β are kept constant and +2 when either α and β or A_t and B_t are fixed. These observations reproduce exactly the behaviour of yeast pyruvate kinase reported by Macfarlane & Ainsworth (1972), and therefore support identical conclusions. These may be summarized as follows.

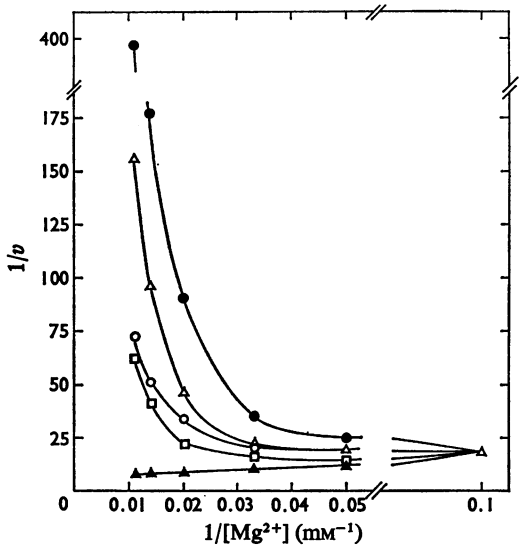


Fig. 1. *Dependence of the reaction velocity on increasing concentration of* Mg^{2+}

Reaction mixtures contained tetrapropylammonium cacodylate (100μmol of cacodylate), pH6.2, KCl (100μmol), NADH (0.15μmol) and 50μg of lactate dehydrogenase, in a final volume of 1.0ml. Phosphoenolpyruvate and ADP concentrations were: ●, [MgPyr-*P*] (0.025mM), [ΣMgADP] (0.086mM); △, total [Pyr-*P*] (0.075mM), total [ADP] (0.1mM); ○, [ΣPyr-*P*] (0.05mM), [ΣMgADP] (0.086mM); □, [MgPyr-*P*] (0.025mM), [ΣADP] (0.014mM); ▲, [ΣPyr-*P*] (0.05mM), [ΣADP] (0.014mM). △, is the coincident point for all five experiments at a reciprocal Mg^{2+} concentration of 0.1mM^{-1} and for △ and ○ at 0.05mM^{-1}. v is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

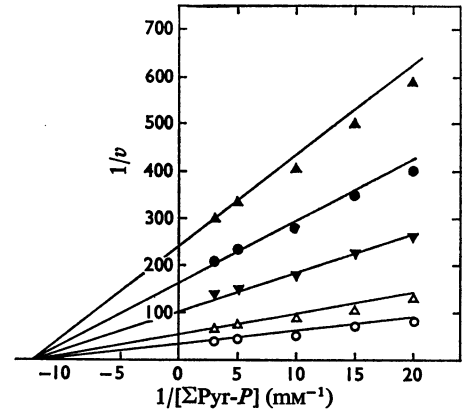


Fig. 2. *Effect of* [Σ*ADP*] *on the initial velocity of the forward reaction with* Σ*Pyr-P as the varied substrate*

The free Mg^{2+} concentration was 0.4mM. Assay conditions were as described in Fig. 1. The concentrations of ΣADP were: ○, 0.28mM; △, 0.17mM; ▼, 0.085mM; ●, 0.051mM; ▲, 0.034mM. v is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase. The lines corresponding to different concentrations of ΣADP intersect at values of v^{-1} and $[\Sigma\text{Pyr-}P]^{-1}$ of −3.4 and -12.7×10^3 respectively.

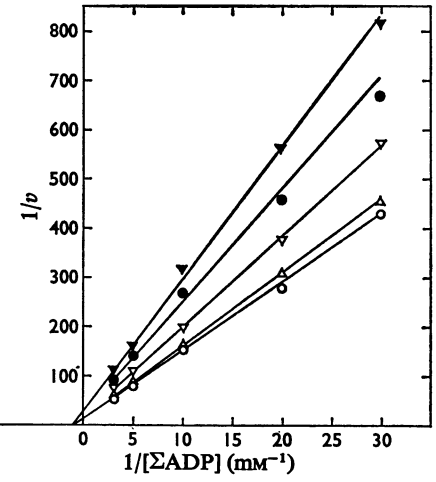


Fig. 3. *Effect of* [Σ*Pyr-P*] *on the initial velocity of the forward reaction with* Σ*ADP as the varied substrate*

The free Mg^{2+} concentration was 0.3mM. Assay conditions were as described in Fig. 1. The concentrations of ΣPyr-*P* were: ○, 0.33mM; △, 0.2mM; ▽, 0.1mM; ●, 0.067mM; ▼, 0.05mM. v is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase. The lines corresponding to different concentrations of ΣPyr-*P* intersect at values of v^{-1} and $[\Sigma\text{ADP}]^{-1}$ of −4.51 and -1.29×10^{-3} respectively.

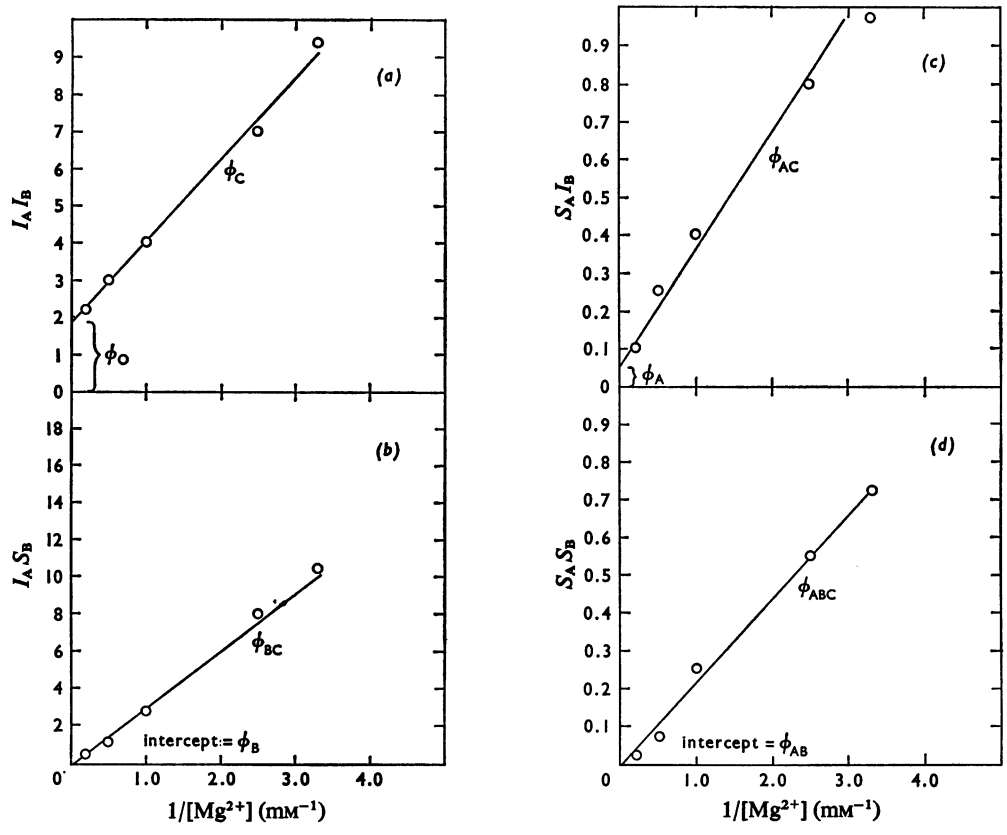


Fig. 4. *Plots of the relationships (a)–(d) in which the right-hand sides of the equalities were estimated by the SEQUEN program applied to data of the type shown in Figs. 2 and 3 at five free Mg^{2+} concentrations*

(*a*) $\phi_C/[Mg^{2+}]+\phi_O = I_A I_B$; (*b*) $\phi_{BC}/[Mg^{2+}]+\phi_B = I_A S_B$; (*c*) $\phi_{AC}/[Mg^{2+}]+\phi_A = S_A I_B$; (*d*) $\phi_{ABC}/[Mg^{2+}]+\phi_{AB} = S_A S_B$.

(1) From comparison of the values of i noted above with the predicted values given in Table 1, it is evident that phosphoenolpyruvate, ADP and Mg^{2+} are the true substrates of the enzyme-catalysed reaction.

(2) The linearity of the double-reciprocal plot, observed when A and B are kept constant, indicates that C is not a product inhibitor and that it must dissociate as part of the nucleotide complex, MgATP. It also demonstrates that α and β (the concentrations of which increase in proportion with C) are not inhibitors of the reaction.

(3) The limiting value of $i = +2$, obtained when α and β or A_t and B_t are kept constant, proves the reality of ϕ_{AB} and allows its evaluation. Similarly, the limiting value of $i = +1$ obtained when A and β are kept constant, allows ϕ_B to be calculated if ϕ_{AB} is known. Values of ϕ_{AB} and ϕ_B found in this way are given in Table 3.

Initial-rate studies

Figs. 2 and 3 show the fit to eqns. (1) and (2) of the initial velocities in the absence of product; together with the linear relation in Fig. 1, they demonstrate that the mechanism is linear in the true substrates and sequential in type. Fig. 4 shows four tertiary replots against C^{-1} in which the data points represent the values of slopes and intercepts of secondary plots against B^{-1} obtained by computation.

The kinetic constants obtained by computer analysis of the data are given in Table 3. The presence of ϕ_C demonstrates that the mechanism cannot require the addition of Mg^{2+} to the active site in a substrate-bound form, thus confirming the conclusion reached in the previous section. Fig. 4 shows that reliable estimates of ϕ_{AB} and ϕ_B cannot be obtained from the intercepts on the tertiary replots; the reality of these constants, however, has been demonstrated

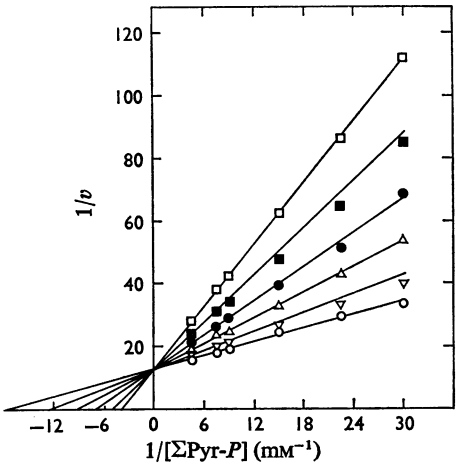


Fig. 5. *Inhibition of the forward reaction by MgATP with ΣPyr-P as the varied substrate*

The concentrations of free Mg^{2+} and ΣADP were kept constant at 10.0 and 0.028mM respectively. Assay conditions were as described in Fig. 1. The concentrations of MgATP were: ○, 0.0mM; ▽, 0.6mM; △, 1.25mM; ●, 2.5mM; ■, 4.0mM; □, 6.0mM. *v* is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

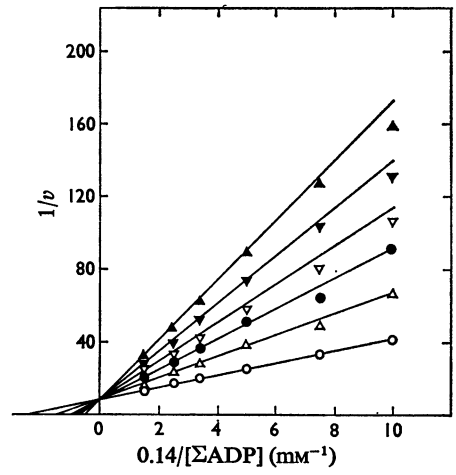


Fig. 6. *Inhibition of the forward reaction by MgATP with ΣADP as the varied substrate*

The concentrations of free Mg^{2+} and ΣPyr-*P* were kept constant at 10.0 and 0.066mM. Assay conditions were as described in Fig. 1. The concentrations of MgATP were: ○, 0.0mM; △, 1.0mM; ●, 2.0mM; ▽, 3.0mM; ▼, 4.0mM; ▲, 5.0mM. *v* is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

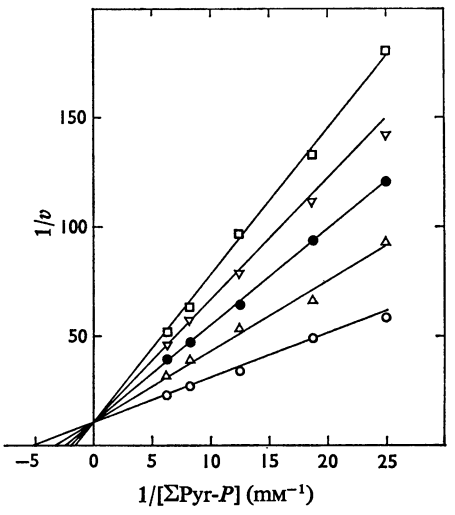


Fig. 7. *Inhibition of the forward reaction by pyruvate with ΣPyr-P as the varied substrate*

The concentrations of free Mg^{2+} and ΣADP were kept constant at 2.0 and 0.1 mM respectively. Reaction mixtures contained tetrapropylammonium cacodylate (100μmol), pH6.2, KCl (100μmol), NADH (0.15μmol), 3-phosphoglycerate (20μmol), 50μg of phosphoglycerate kinase and 150μg of glyceraldehyde 3-phosphate dehydrogenase, in a final volume of 1.0ml. The concentrations of pyruvate were: ○, 0.0 mM; △, 5.0mM; ●, 10.0mM; ▽, 15.0mM; □, 20.0mM. *v* is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

by Fig. 1. (Note: if ϕ_{AB} and ϕ_B had values that were large enough to allow their estimation from triple replots, then inhibition of reactions studied at constant total substrate concentrations would occur at much lower Mg^{2+} concentrations than is observed.)

The necessity to employ eight kinetic constants to describe the initial-rate data therefore establishes that the mechanism cannot require the ordered addition of A, B and C (Frieden, 1959; Cleland, 1963*a*; Dalziel, 1969) and suggests that an equilibrium random-order mechanism is involved.

Product-inhibition studies

Figs. 5–9 show the results from product-inhibition studies in which single products were added to the reactant solutions. The competition between both products and phosphoenolpyruvate, shown in Figs. 5 and 7, clearly indicates that the products are released randomly; because of this, the linearity observed in the double-reciprocal plots further demonstrates that the enzyme and enzyme–product

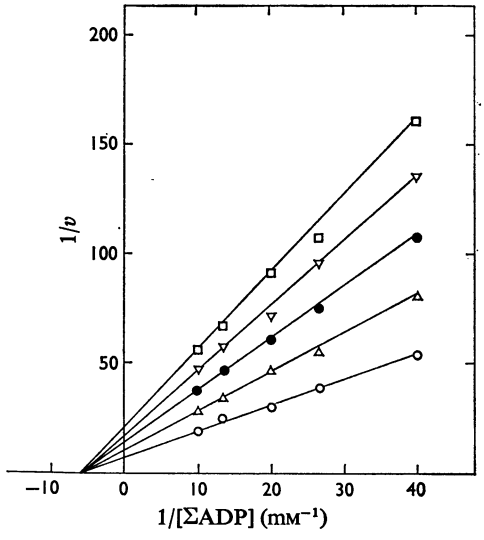


Fig. 8. *Inhibition of the forward reaction by pyruvate with ΣADP as the varied substrate*

The concentrations of free Mg^{2+} and ΣPyr-*P* were kept constant at 2.0 and 0.09mM respectively. Assay conditions were as described in Fig. 7. The concentrations of pyruvate were: ○, 0.0mM; △, 5.0mM; ●, 10.0mM; ▽, 15.0mM; □, 20.0mM. *v* is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

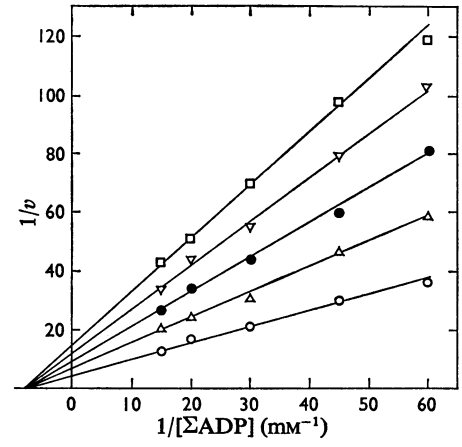


Fig. 9. *Inhibition of the forward reaction by pyruvate with ΣADP as the varied substrate*

The concentrations of free Mg^{2+} and ΣPyr-*P* were kept constant at 5.0 and 0.08mM respectively. Assay conditions were as described in Fig. 7. The concentrations of pyruvate were: ○, 0.0mM; △, 5.0mM; ●, 10.0mM; ▽, 15.0mM; □, 20.0mM. *v* is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase.

complexes are in equilibrium with one another. Fig. 6 shows that when ADP is the varied substrate, MgATP acts as a competitive inhibitor, in keeping with the suggested random-order mechanism. The random addition of substrates, and release of products, by muscle pyruvate kinase also requires that ADP and pyruvate should be competitive: instead, this pair are observed to be non-competitive with one another (Figs. 8 and 9). We therefore suggest that pyruvate can form dead-end complexes with all the substrates other than phosphoenolpyruvate. Eqns. (1) and (2) incorporate all these features and can be seen to represent the qualitative features of the results.

Values of the inhibition constants for MgATP and pyruvate, calculated in the manner described above, are given in Tables 4 and 5.

Discussion

The present study confirms the view of Reynard *et al.* (1961) that rabbit muscle pyruvate kinase has an equilibrium random-order mechanism. The study also suggests that the enzyme contains a site that will bind either phosphoenolpyruvate or pyruvate together with a second composite site that will bind either MgATP or Mg^{2+} and ADP. It further appears that the binding sites partially overlap because phosphoenolpyruvate and MgATP cannot bind simultaneously to the enzyme (Reynard *et al.*, 1961). The relationship of this study to previous work will now be briefly explored.

A substantial point of difference concerns the nature of the substrates, particularly ADP. Cleland (1967*a*) has suggested that nucleoside di- and triphosphates react only in the form of their bivalent metal-ion complexes. If this were true, one might imagine that the mechanism of pyruvate kinase would be bireactant in both directions, with phosphoenolpyruvate and MgADP as substrates and pyruvate and MgATP as products. Such a mechanism eliminates the dependence of initial velocity on Mg^{2+} concentration, provided that the nucleotides are expressed in terms of their Mg^{2+} complexes, and therefore does not explain the Mg^{2+} inhibition observed above and in other studies of the muscle and yeast enzymes (Melchior, 1965; Macfarlane & Ainsworth, 1972). Further, the mechanism requires fewer constants than those demonstrated by initial-velocity studies. Mildvan & Cohn (1966), in contrast, suggested that ADP reacts both in its free form and in complex, and cited, as evidence for the latter, the linearity of a plot of v^{-1} against $[MnADP]^{-1}$. Such an observation, however, is not inconsistent with ADP being the strict nucleotide substrate of pyruvate

kinase. For, assuming a simple equilibrium, we have $B = K\beta/C$ and $B = (B_t K)/(K+C)$, so that at constant free metal-ion concentration it is immaterial whether the reciprocal velocity is plotted against B^{-1}, β^{-1} or B_t^{-1}.

Reynard *et al.* (1961) have determined, in ultracentrifugal experiments, that the dissociation constant of phosphoenolpyruvate is 7.5×10^{-5}M, in the presence of 10mM-Mg^{2+} at 0°C and pH8.5. For comparison, the apparent constant $K_A' = K_A(1+C/K_C)/(1+C/K_{AC})$, based on our data, has a value of 7.47×10^{-5}M.

Comparisons of kinetically determined constants are more difficult because of lack of agreement on the true substrates of the reaction and also because previous workers (Reynard *et al.*, 1961; Mildvan & Cohn, 1965, 1966) have attempted to treat the reaction as equivalent to that of a two-substrate enzyme by keeping one of the substrates at saturating concentrations. Examination of the intercepts of eqns. (1) and (2), which represent this approach, shows that it is a valid stratagem provided that saturation by one substrate is achieved (Cleland, 1970) and maintained in the presence of different amounts of other substrates that chelate with it, and provided also that it is recognized (as it does not seem to have been) that the saturating substrate forms a complex with the enzyme taking the role of free enzyme in the normal two-substrate reaction.

Thus Reynard *et al.* (1961) have presented reciprocal velocity data, obtained in experiments where the Mg^{2+} concentration was kept throughout at 10mM, as functions of the reciprocal of either total [ADP] or total [phosphoenolpyruvate], with the non-varied substrate, phosphoenolpyruvate or ADP, kept at several fixed concentrations. The relationships are linear and intersect one another on the horizontal axis. Reynard *et al.* (1961) have taken the intersections to demonstrate the equality of the Michaelis and dissociation constants, which they evaluate as 3.2×10^{-5}M for phosphoenolpyruvate and 2.1×10^{-4}M for ADP.

The data of Mildvan & Cohn (1965, 1966) have been analysed again by assuming that the enzyme species that bound the varied substrate was itself an enzyme–substrate complex formed with the substrate (phosphoenolpyruvate or ADP) whose total concentration was kept at a high constant value throughout the experiment. The large variations that were obtained in the kinetic constants makes comparison with those recorded in Table 3 unwarranted, and suggests that saturation was not uniformly achieved. Cleland (1967*b*), moreover, has noted that a slope replot of the data in Fig. 6 of Mildvan & Cohn (1966) passes very near to the origin. The kinetic constant corresponding to the vertical intercept may be identified as ϕ_B. This is consistent with $K_{AC} = \phi_{BC}/\phi_B$ being large (as given in Table 3) but is not consistent with the value of K_D (i.e. K_{AC}), 7×10^{-5}M, given by Mildvan & Cohn (1966).

Referring again to the experiments of Reynard *et al.* (1961), it is noteworthy that intersection on the horizontal axis may also be observed in the data shown in Figs. 2, 3, 8 and 9. Although calculation shows that the intersection, in each Figure, is not really on the axis but only appears to be so because of the scale of the drawing, it seems worthwhile to examine what factors determine such an appearance, the more so because Cleland (1967*b*) has warned against the practice of drawing lines to intersect on the axis.

The general condition for the intersection of lines on the axis is that the ratio of the intercept to the slope of a double-reciprocal plot should be independent of the concentration of the non-varied substrate. Thus, taking eqn. (1) as a basis for discussion:

$$-\frac{\text{Intercept}}{\text{Slope}} = \frac{1}{K_{app.}} = \frac{\left[\left(\frac{K_{AC}}{C}+1\right)\frac{K_{ACB}}{B}+\frac{K_{ABC}}{C}+1\right]}{K_{BCA}\left[\left(\frac{K_C}{C}+1\right)\frac{K_{CA}K_{ACB}}{BK_{BCA}}+\frac{K_{BC}}{C}+1\right]} \quad (3)$$

$1/K_{app.}$ is required to be independent of B. The necessary condition may be met in the following ways:

(1) $C \gg K_C, K_{AC}, K_{BC}, K_{ABC}; K_{CA} \equiv K_{BCA}$

This situation is the one postulated by Reynard *et al.* (1961).

(2) $K_{CA} \equiv K_{BCA}; K_{AC} \equiv K_C; K_{ABC} \equiv K_{BC}$

(3) $C \ll K_{AC}; C \ll K_C; C \geqq K_{ABC}; C \geqq K_{BC}$

If these inequalities hold, $1/K_{app.} \approx 1/K_A$

(4) $K_{ACB} \gg B; K_{CA}K_{ACB}/K_{BCA} \gg B$

With regard to (4), these inequalities indicate that the horizontal intercepts on a double-reciprocal plot are separated by decreasing intervals as B is diminished and, in the limit, may appear to coincide within the accuracy of the data. Further, the appearance of intersection may be exaggerated if the scale of the plot is arranged to accommodate preferentially the results for low values of B, for then the lines corresponding to high values of B have such small slopes that their point of intersection with the axis is difficult to determine.

Considering the data of Reynard *et al.* (1961), and assuming that the equilibrium constants given in Table 3 offer reasonable approximations to the values applicable to the conditions of their experiments, it is evident that the third and fourth conditions determine the appearance of intersection. In particular, the fourth condition gains its force when it is recognized that the free ADP is probably not more than 5% of the total ADP present, at pH8.5, when the concentration of Mg^{2+} is 10mM. The same conditions determine the intersection appearing in Fig. 2 of the present paper. This is reflected by the fact that the intersection gives an estimate of $K_A = 7.83 \times 10^{-5}$M, in very good agreement with the value obtained by computation.

functions of [MgATP]. The resulting estimates of $K_Q = 2$mM agree well with values reported by Macfarlane & Ainsworth (1972) for the yeast enzyme and with values for adipose-tissue pyruvate kinase provided by experiments of Pogson (1968), in which a correction for Mg^{2+} binding was also applied.

The binding of pyruvate to pyruvate kinase is obviously complex. Reynard *et al.* (1961) in equilibrium-dialysis experiments found that the muscle enzyme binds pyruvate at two to four sites with a dissociation constant around 0.5mM, and at a further 120 sites with a dissociation constant of 38mM; in these experiments, the solutions were at 0°C, pH8.5, and contained 10mM-$MgCl_2$. Representation of an apparent binding constant for pyruvate by eqn. (5),

$$\frac{1}{K_{app.}} = \frac{\left\{\left[\frac{K_{BC}}{C}\left(1+\frac{P}{K_{BP}}\right)+1+\frac{P}{K_{BCP}}\right]\frac{K_{BCA}}{A}+\frac{K_{BAC}}{C}+1\right\}}{K_{ACB}\left\{\left[\frac{K_C}{C}\left(1+\frac{P}{K_P}\right)+1+\frac{P}{K_{CP}}\right]\frac{K_{CB}K_{CBA}}{AK_{ACB}}+\frac{K_{AC}}{C}+1\right\}} \quad (4)$$

Eqn. (4) represents the horizontal intersections appearing in Figs. 3 and 8. Substitution of the estimated constants shows that intersection is not expected in either Figure. For Fig. 3 the calculated horizontal intercept varies, for the extreme values of A, from 0.94×10^3M^{-1} to 1.13×10^3M^{-1}. Nonetheless, the effect of scale is very marked in Fig. 3 and the apparent intersection gives an estimate of $K_B = 7.75 \times 10^{-4}$M, in good agreement with the computed value. The calculated range of intercept variation for the pyruvate inhibition is even larger, the extreme values being 1.70×10^3M^{-1} and 3.06×10^3M^{-1} (Fig. 8). The discrepancy between these values and those displayed on Fig. 8 probably arises from error in the original data and its propagation in the calculations: the apparent constant, however, is 1.72×10^{-4}M, a value very close to that of K_{PB}, the constant that would arise if the leading terms in P in eqn. (4) were dominant.

The inhibition of pyruvate kinase by ATP in the presence of Mg^{2+}, reported by Reynard *et al.* (1961), shows slopes of primary double-reciprocal plots (in which ADP or phosphoenolpyruvate is the varied substrate) increasing parabolically with ATP. A similar finding was reported by Mildvan & Cohn (1966) for the competition of phosphoenolpyruvate and ATP in the presence of Mn^{2+}. Mildvan & Cohn (1966) concluded that the additional inhibition may be due to removal of the bivalent activator by formation of metal–ATP complexes. This explanation is consistent with our observation that, when chelation of Mg^{2+} is allowed for, MgATP is competitive with both ADP and phosphoenolpyruvate and that the slopes of the primary plots are linear

$$K'_P = \frac{K_P(1 + C/K_C + B/K_B + BC/K_B K_{BC})}{(1 + C/K_{PC} + B/K_{PB} + BC/K_{PB} K_{PBC})} \quad (5)$$

and substituting the values given in Table 5, with B absent and $C = 10$mM, gives $K'_P = 6$mM. It therefore seems probable (with reservations arising from the different conditions of the experiments) that neither of the binding sites identified by Reynard *et al.* (1961) exerted important effects in our experiments with the muscle enzyme.

Mildvan & Cohn (1966) also found (in studies of the enhancement of the proton-relaxation rate of water) that pyruvate forms an enzyme–Mn^{2+}–pyruvate complex with a pyruvate dissociation constant of 0.6mM at pH7.5. They demonstrated that this complex was not kinetically important by showing that phosphoenolpyruvate did not compete for the pyruvate-binding site. In contrast, in the presence of saturating ADP, a second site was identified with a dissociation constant of 4mM, which agreed with the kinetically determined value of the inhibition constant. For comparison, calculations from our data with $B_t = 1$mM and $C = 5$ and 10mM provide values of K'_P equal to 5.5 and 6.0mM respectively.

These observations therefore do not conflict with our view that pyruvate occupies the phosphoenolpyruvate binding site and forms dead-end complexes, within the active site, by binding randomly in conjunction with Mg^{2+} and ADP. The mechanism of pyruvate inhibition is thus consistent with the overall nature of substrate binding, and the several constants that arise are all necessary to account quantitatively for the data.

A comparison may now be drawn between the

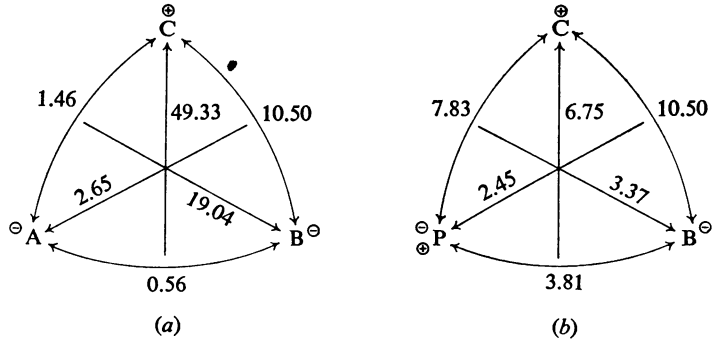
muscle and yeast enzymes (Macfarlane & Ainsworth, 1972). The mechanism of the latter (in its fructose 1,6-diphosphate-activated form) requires the addition of substrates and release of products in the ordered sequence A, B, C, P, Q. It is also found that an enzyme–pyruvate dead-end complex forms, in which pyruvate occupies the phosphoenolpyruvate site, the binding constant being insensitive to the concentration of ADP. Ordered mechanisms are common among the dehydrogenases (Wratten & Cleland, 1963; Heyde & Ainsworth, 1968), and it has been noted (Cleland, 1963*a*) for these enzymes that the simple ordered Bi Bi kinetics fail because ordering implies a conformational change, caused by the binding of the first substrate, which introduces additional enzyme species in the mechanism and corresponding terms in the rate equations.

In this connexion, the comparison K_P/K_A(yeast) = 234 and K_P/K_A(muscle) = 158 suggests that the phosphate group of phosphoenolpyruvate is responsible not only for the greater tightness of binding but also, in the yeast enzyme, for effecting an additional stabilization, which may reflect the conformational change that allows the binding of ADP. The observation that the dissociation constant of pyruvate, for yeast pyruvate kinase, is unaffected by ADP is therefore consistent with the view that pyruvate is incapable of inducing the conformational change that allows ADP to bind. It is probably for this reason, too, that in the reverse direction MgATP binds first, because it alone can effect the conformational change necessary for the reaction of pyruvate to take place. If so, the locus within the active site where conformational change is triggered lies in the region of overlap between the binding sites of phosphoenolpyruvate and MgATP.

In discussing the mechanism of yeast pyruvate kinase, Macfarlane & Ainsworth (1972) suggested that Mg^{2+} bridges the phosphate groups of phosphoenolpyruvate and ADP, assists the phosphorylation of the latter and is finally eliminated between the β and γ phosphate groups of ATP. The kinetic data introduced to identify the substrates suggest that the same basic mechanism applies to the muscle enzyme. We now wish to consider whether the dissociation constants for the individual equilibria are consistent with such a mechanism.

As a test of the required consistency, we shall compare the dissociation constant of a given substrate, say A, when it binds to the free enzyme with the constants that describe the binding of A to a binary or ternary complex of the enzyme with other substrates. The comparison is represented in Scheme 3 as a network of relationships, in which, for example, the line AC, associated with the value 1.46, corresponds to the ratios $K_A/K_{CA} = K_C/K_{AC} = 1.46$. (The value 1.46 indicates that the prior binding of A or C decreases the dissociation constant of C or A by a factor of 1.46; i.e. the binding of the second substrate is enhanced.) Again, the line originating between A and C and directed towards B, (AC)B = 19.04, corresponds to the ratio $K_B/K_{ACB} = 19.04$ and indicates that the prior binding of A and C decreases the dissociation constant of B by a factor of 19.04. It will be observed that the products of XY with (XY)Z are equal within the errors of measurement; this follows from the nature of the equilibrium box, depicted in Scheme 1, of which Scheme 3 is but an alternative representation.

Also shown in Scheme 3(*b*) is a second network, in which the relationships refer to binding of pyruvate rather than phosphoenolpyruvate.



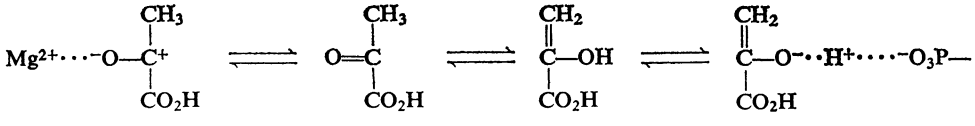
Scheme 3. *Interactions between substrates and pyruvate at the active site of muscle pyruvate kinase*

(*a*) Substrate interactions; (*b*) interactions in the presence of pyruvate. In (*a*) the line AC represents the ratio $K_A/K_{CA} = K_C/K_{AC}$, whereas (AC)B represents the ratio K_B/K_{ACB}. The other lines in (*a*) and (*b*) carry comparable meanings.

The disposition of the substrates in the two networks is intended to represent the suggested mechanism in which Mg^{2+} acts as a bridge. The fact that both phosphoenolpyruvate and pyruvate bind to the same enzyme site suggests that binding occurs mainly through their common structural feature, the carboxyl group. The negative charge associated with phosphoenolpyruvate therefore arises from its phosphate group. Similarly, the terminal phosphate group of ADP is represented by the negative sign associated with B.

Examination of the ABC network shows that AC and BC are both greater than 1, whereas AB is less. This suggests that the effect of the substrates on one another's binding arises from charge attraction and repulsion respectively. BC, however, is markedly greater than AC, indicating that the charge density on the phosphate residue of phosphoenolpyruvate is smaller than that on the terminal phosphate residue of ADP. Such a situation might arise if a negative charge on the phosphate group of phosphoenolpyruvate were responsible, in part, for its binding to the protein, a conclusion that is consistent with the observation that K_A is much smaller than K_P.

If the above conclusions are valid, the further observations that PB and PC are both greater than 1 can be rationalized only by supposing that pyruvate can bear either a positive or a negative charge, depending on the substrate with which it is interacting. As keto–enol tautomerization of pyruvate has already been suggested as a feature of the pyruvate kinase mechanism (Rose, 1960, 1970; Rose *et al.*, 1969), it is not unreasonable to extend the equilibria and allow positive or negative charges to be induced by the approach of a second substrate, the resulting interaction being of a charge-induced charge type, thus:



The observation that PC is greater than AC implies that the charge density on P is greater than on A: this conclusion seems reasonable if one compares the distribution of a negative charge over the single carbonyl oxygen atom of pyruvate with the distribution of one or two charges over the three oxygen atoms of the phosphate residue of phosphoenolpyruvate. Another factor involved, however, may be the loss of charge resulting from the interaction of the phosphate residue with the protein to which we referred above.

The relationships (XY)Z ($= \phi_{XYZ}\phi_O/\phi_{XY}\phi_Z$), although depending on different kinetic constants for their calculation than XY ($= \phi_{XYZ}\phi_Z/\phi_{XZ}\phi_{YZ}$), are nonetheless related to them through the closure of the equilibrium box. Therefore, because BC×(BC)A is approximately equal to BC×(BC)P, we may conclude that as PC>AC so (AC)B>(PC)B, and that as PB>AB so (AB)C>(PB)C. These inequalities are observed in the networks and are susceptible to the qualitative interpretations already advanced.

The detailed consideration we have given to the relative magnitudes of the substrate-equilibrium constants shows that they are consistent with the nature of the substrates and the enzyme mechanism that has been postulated.

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