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A Kinetic Study of Baker's-Yeast Pyruvate Kinase Activated by Fructose 1,6-Diphosphate

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The paper reports a study of the kinetics of the reaction between phosphoenolpyruvate, ADP and Mg^{2+} catalysed by yeast pyruvate kinase when activated by fructose 1,6-diphosphate and K^+. The experimental results indicate that the reaction mechanism is of the Ordered Tri Bi type with the substrates binding in the order phosphoenolpyruvate, ADP and Mg^{2+}. Direct phosphoryl transfer takes place in the quaternary complex, with pyruvate released before MgATP. A dead-end enzyme–pyruvate complex is also indicated. Values have been determined for the Michaelis, dissociation and inhibition constants of the reaction. Several of the rate constants involved have also been evaluated.

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) exists in several forms (Kachmar & Boyer, 1953; Tanaka *et al.*, 1967; Haekel *et al.*, 1968). Yeast pyruvate kinase is a typical member of the 'allosteric' variety, for it catalyses a reaction between ADP and phosphoenolpyruvate that shows a sigmoidal relationship of initial velocity to phosphoenolpyruvate concentration. With the further addition of the activator fructose 1,6-diphosphate, the relationship:

$$v = f([\text{Pyr-}P])$$

takes a non-inflected form and the affinity for phosphoenolpyruvate is enhanced. There is throughout a strict requirement for Mg^{2+} or Mn^{2+} and a univalent cation, preferably K^+ (Washio & Mano, 1960; Haekel *et al.*, 1968; Hunsley & Suelter, 1969*b*).

The present paper reports a study of the kinetics of the reaction between phosphoenolpyruvate and ADP catalysed by the fructose diphosphate-activated form of yeast pyruvate kinase. The reactions took place at pH 6.2 and at 25°C, and K^+ and Mg^{2+} were present throughout.

Complex-formation of this sort necessarily affects the concentration of free Mg^{2+} and hence, assuming that it is a substrate, the rate of reaction. But, in addition, it remains to be determined whether the other substrates act in their free or Mg^{2+}-complexed forms. The first requirement is therefore to determine the concentrations of the different possible substrates by using the known equilibrium constants for their interconversions. This may be achieved by fixing the free Mg^{2+}, K^+ and H^+ concentrations, together with the total substrate concentrations, and calculating from them the concentrations of free and complexed substrates present in solution. The total Mg^{2+} or K^+ to be added to the solution may then be calculated from the conservation equations. A detailed description of the method and the constants employed is given in the Appendix.

This examination shows 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:

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

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

where

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

Theory

Substrate-level equilibria

The strict requirement for a bivalent cation (in our experiments Mg^{2+}) indicates that the ion may be regarded as a substrate in the reaction. The other substrates in the reaction, ADP and phosphoenolpyruvate, can also form complexes with Mg^{2+}.

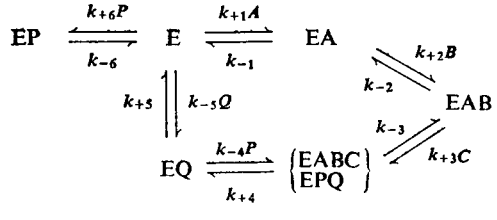
Identification of the substrates of pyruvate kinase

We now seek to determine whether the free or Mg^{2+}-complexed forms of ADP and phosphoenolpyruvate are the true substrates of yeast pyruvate kinase. The test we propose requires two assumptions, which will be justified below by the results. These are: (1) the reaction velocity may be represented by a first-degree equation in the true substrates; (2) the

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

Values of i for individual terms $f_x(C^i)_x$ appearing in the inverted rate equation when named substrates are held constant are given. Values of i are obtained by representing the hypothetical true substrates (x) in the reaction in terms of the species whose concentrations were held 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

Mg^{2+}-bound and free species of a given substrate are forms kinetically distinct from one another but indistinguishable within themselves.

The basis of the test is therefore as follows. The inverted form of the general first-degree rate equation may contain terms in A^{-1}, B^{-1}, C^{-1}, α^{-1} and β^{-1}, together with combinations of these determined by the possible complexes of substrates with the enzyme. Apparent Mg^{2+}-binding constants, valid at fixed pH and K^+ concentrations, are now calculated from the true constants. By using these constants, the inverted rate equation may be expressed throughout in terms of either the free or bound species of ADP and phosphoenolpyruvate; in addition, by their use the concentrations of the chosen species are held constant as C is increased. As a result, the measured reciprocal velocity is represented by a polynomial in C in which the powers of C that appear are determined by which species of ADP and phosphoenolpyruvate are held at constant concentration and by which species correspond to the true substrates of the reaction. Table 1 gives values of i for the relations $f_x(C^i)_x$ arising from terms in the individual substrates (x): the degree of the Mg^{2+}-dependence arising from a product of substrate concentrations is obtained by adding the values of i for the individual substrates involved.

Comparison of the observed Mg^{2+}-dependence of the reciprocal velocity with the values predicted by Table 1 identifies the true substrates of yeast pyruvate kinase. In doing so, the comparison also limits the possible number of mechanisms that need be considered.

Reaction mechanism

The experimental results indicate that the reaction mechanism is of the Ordered Tri Bi type (Cleland, 1963*a*) with the substrates binding in the order phosphoenolpyruvate, ADP and Mg^{2+}. Direct phosphoryl transfer takes place in the quaternary complex, with pyruvate released before MgATP. A dead-end enzyme–pyruvate complex is also indicated. This mechanism is illustrated in Scheme 1, where A, B, C, P, Q and E represent the concentrations of A (phosphoenolpyruvate), B (ADP), C (Mg^{2+}), P (pyruvate), Q (MgATP) and E (enzyme) respectively. (For the present study, E may be regarded as an enzyme–Fru-1,6-di-*P*–K^+ complex.) The theoretical aspects of this mechanism are now described.

The inverted initial-velocity equation for pyruvate formation is given in eqn. (1):

$$\frac{E_0}{v} = \left[\left(\frac{\phi^{DPQ}_{ABC}}{C} + \phi^{DQ}_{AB}\right)\frac{1}{B} + \phi^{DQ}_{A}\right]\frac{1}{A} + \left[\left(\frac{\phi^{P}_{BC}}{C} + \phi_B\right)\frac{1}{B} + \frac{\phi^{P}_{C}}{C} + \phi^{P}_{0}\right] \quad (1)$$

In this equation Dalziel's (1969) nomenclature for the kinetic constants has been modified to indicate which terms are affected by the inhibition of products added singly to the reaction solutions; the presence of dead-end inhibition by pyruvate is also indicated by

Table 2. *Composition of the kinetic constants corresponding to the enzyme mechanism for pyruvate kinase depicted in Scheme 1, in the presence and in the absence of a single product*

$K_{IQ} = k_{+5}/k_{-5}$, $K_{DP} = k_{-6}/k_{+6}$, $K_{IP} = k_{+5}(k_{-3}+k_{+4})/k_{-3}k_{-4}$, $K_{IIP} = (k_{+4}+k_{+5})/k_{-4}$. It should be noted that $\phi_0 = 1/V_f = (k_{+4}+k_{+5})/k_{+4}k_{+5}$, where V_f is the maximum velocity for unit concentration of enzyme.

Constant	$k_{+1}k_{+2}k_{+3}k_{+4}k_{+5}\phi_j^i$
ϕ_{ABC}^{DPQ}	$k_{-1}k_{-2}k_{+5}(k_{-3}+k_{+4})\left[\left(1+\frac{P}{K_{DP}}\right)\left(1+\frac{P}{K_{IP}}\right)+\frac{Q}{K_{IQ}}\right]$
ϕ_{BC}^{P}	$k_{+1}k_{-2}k_{+5}(k_{-3}+k_{+4})\left(1+\frac{P}{K_{IP}}\right)$
ϕ_{AB}^{DQ}	$k_{-1}k_{+3}k_{+4}k_{+5}\left(1+\frac{P}{K_{DP}}+\frac{Q}{K_{IQ}}\right)$
ϕ_{A}^{DQ}	$k_{+2}k_{+3}k_{+4}k_{+5}\left(1+\frac{P}{K_{DP}}+\frac{Q}{K_{IQ}}\right)$
ϕ_{C}^{P}	$k_{+1}k_{+2}k_{+5}(k_{-3}+k_{+4})\left(1+\frac{P}{K_{IP}}\right)$
ϕ_{B}	$k_{+1}k_{+3}k_{+4}k_{+5}$
ϕ_{0}^{P}	$k_{+1}k_{+2}k_{+3}(k_{+4}+k_{+5})\left(1+\frac{P}{K_{IIP}}\right)$

the superscript D. The composition of the kinetic constants in terms of the rate constants defined by Scheme 1 is given in Table 2. Eqn. (1) is cast in a form which illustrates that the double-reciprocal plot is linear and also that the seven kinetic constants may be obtained by a stepwise procedure in which the slopes and intercepts of the primary plots are re-plotted against B^{-1} to obtain secondary line constants, which are then plotted against C^{-1} (Dalziel, 1969). Eqn. (1) also shows that A is competitively inhibited by Q and non-competitively inhibited by P.

Eqn. (2) illustrates that the triple-plotting procedure may be undertaken in a different order and that B is non-competitively inhibited by both P and Q:

$$\frac{E_0}{v} = \left[\left(\frac{\phi_{ABC}^{DPQ}}{C}+\phi_{AB}^{DQ}\right)\frac{1}{A}+\frac{\phi_{BC}^{P}}{C}+\phi_B\right]\frac{1}{B}+\left[\frac{\phi_A^{DQ}}{A}+\frac{\phi_C^{P}}{C}+\phi_0^{P}\right] \quad (2$$

The identification of B and C with ADP and Mg^{2+} respectively required by Scheme 1 can be confirmed by initial-rate experiments conducted at saturating concentrations of one of the two substrates (Cleland, 1970).

At infinite B eqn. 2 reduces to:

$$\frac{E_0}{v} = \frac{\phi_A}{A}+\frac{\phi_C}{C}+\phi_0 \quad (3)$$

Thus, whichever substrate has its concentration varied, a double-reciprocal plot is a straight line showing only an intercept variation with change in concentration of the fixed substrate. In contrast, if C is infinite, both the slope and intercept of the primary plot vary with the concentration of the fixed substrate.

Values of some rate constants may be obtained from initial-rate data (Dalziel, 1969). Thus ϕ_A and ϕ_B are the reciprocals of k_{+1} and k_{+2} respectively. We also have:

$$k_{-1}/k_{+1} = \phi_{ABC}/\phi_{BC} = \phi_{AB}/\phi_B$$
$$k_{-1}/k_{+2} = \phi_{AB}/\phi_A$$

and

$$k_{-2} = \phi_{ABC}/\phi_{AB}\phi_C = \phi_{BC}/\phi_B\phi_C$$

In both the product inhibitions by Q, a linear relationship between inhibitor concentration and slope or slope and intercept of the primary plot is predicted, although it is clear that the inhibitor constant can be obtained directly only in the experiment involving competitive inhibition. When P is the product inhibitor, a linear relationship between inhibitor concentration and intercept is required, but a non-linear relationship with slope. In these experiments the apparent inhibitor constants determined are functions both of the true constants and other

kinetic constants together with the concentrations of fixed substrates.

Experimental

Materials

ATP, ADP, NADH, pyruvate (all as sodium salts), fructose 1,6-diphosphate, phosphoenolpyruvate, 3-phosphoglycerate (all as cyclohexylammonium salts), lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase were obtained from the 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 BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Preparation of pyruvate kinase

Pyruvate kinase was isolated from fresh yeast (Distillers Co. Ltd.), obtained locally, according to the procedure of Hunsley & Suelter (1969*a*). The kinetic study employed a single preparation of the enzyme with a specific activity of 150 units (μmol of NADH oxidized/min)/mg of enzyme at 25°C. It was stored as a suspension in 90%-saturated $(NH_4)_2SO_4$ at 4°C, and showed no loss of specific activity over a period of 9 months. The purified enzyme sedimented as a single symmetrical peak during ultracentrifugation at 56100 rev./min at 20°C in the Beckman model E analytical ultracentrifuge (AN-D rotor) yielding $s_{20,w}$ 8.22S at a protein concentration of 2mg/ml in 0.1M-tris–HCl buffer, pH7.5, and $s_{20,w}$ 8.57S at a protein concentration of 2mg/ml in 0.1M-tetramethylammonium cacodylate buffer, pH6.2, containing KCl (0.1M), $MgCl_2$ (26mM) and fructose diphosphate (1mM) (Kuczenski & Suelter, 1970*b*). The enzyme was also examined by polyacrylamide-gel electrophoresis by using the method described by Hunsley & Suelter (1969*a*): the protein migrated as two bands with all the enzymic activity associated with the major staining band (Hunsley & Suelter, 1969*a*).

Removal of metal ions from substrates and enzymes

All substrates that were purchased as sodium salts (except NADH) were converted into the tetrapropylammonium salts by passage through a column of Dowex 50W (X8) in the tetrapropylammonium form (Phillips *et al.*, 1963). Stock solutions of these compounds were adjusted to pH7.0 with 10% (w/v) tetrapropylammonium hydroxide and 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).

Pyruvate kinase was chromatographed on Sephadex G-25 equilibrated with 0.1M-tris–HCl buffer, pH7.5 (Kuczenski & Suelter, 1970*a*,*b*), and left for 3h at room temperature before use. Pyruvate kinase concentrations were determined spectrophotometrically at 280nm ($E_{1cm}^{0.1\%} = 0.653$) (Hunsley & Suelter, 1969*a*). Lactate dehydrogenase, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase were dialysed at 4°C against 0.2M-tris–HCl buffer, pH7.0. Samples of all treated enzymes were tested with $BaCl_2$ to confirm the absence of NH_4^+ ions (Hunsley & Suelter, 1969*b*).

Enzyme assays

Preliminary experiments were performed to establish the concentrations of KCl and fructose diphosphate necessary to saturate the enzyme over the concentration range of substrates to be employed: these concentrations of activators were then employed throughout the kinetic experiments.

Reaction mixtures contained (in a total volume of 1ml) tetrapropylammonium cacodylate buffer, pH6.2 (0.1M in cacodylate), NADH (0.15μmol), fructose diphosphate (2.0μmol) and KCl (100μmol), with substrates and single products at the concentrations indicated in the figures.

Initial-velocity and ATP-inhibition experiments employed the lactate dehydrogenase coupled assay method described by Hunsley & Suelter (1969*a*): it was confirmed, in trial experiments, that the linked assay method provided the same initial-velocity data as the direct spectrophotometric assay method of Pon & Bondar (1970).

In the pyruvate-inhibition experiments a linked assay method was employed by using phosphoglycerate kinase (50μg), glyceraldehyde 3-phosphate dehydrogenase (150μg) and 3-phosphoglycerate (20μmol). Control experiments showed that 3-phosphoglycerate did not inhibit pyruvate kinase and that the pyruvate, phosphoenolpyruvate and ADP concentrations used in the kinetic experiments did not inhibit the coupling enzymes. It was also shown that there was no difference between the reaction velocities recorded by the two coupled assay systems when pyruvate was absent. In the presence of pyruvate, however, a small decrease in absorbance with time occurred before the addition of pyruvate kinase, probably because of a small contamination of the assay enzymes by lactate dehydrogenase: the resulting rate was subtracted from the initial rate observed in the presence of pyruvate kinase.

The reaction mixtures were incubated for 5min at 25°C before addition of NADH and the coupling enzymes. The reaction was initiated by adding 10μl

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

Kinetic constants were calculated assuming that pyruvate kinase has a molecular weight of 166000. One active site is assumed because the correct number of active sites/pyruvate kinase molecule is not known. Dissociation and rate constants are calculated on the basis of the mechanism given in Scheme 1.

Kinetic constants	Michaelis and dissociation constants and maximum velocity	Rate constants
ϕ_A 1.02 (±0.096) × 10^{-9} M·min	$K_A = \phi_A/\phi_0$ 3.76 (±0.41) × 10^{-5} M	$k_{+1} = 1/\phi_A$ 1.63 × 10^7 $M^{-1}\cdot s^{-1}$
ϕ_B − 0.445 (±1.4) × 10^{-9} M·min	$K_B = \phi_B/\phi_0$ 4.09 (±0.53) × 10^{-5} M	$k_{+2} = 1/\phi_B$ 1.5 × 10^7 $M^{-1}\cdot s^{-1}$
1.11 (±0.13) × 10^{-9} M·min (Fig. 1)		
ϕ_C 4.59 (±0.32) × 10^{-8} M·min	$K_C = \phi_C/\phi_0$ 1.69 (±0.15) × 10^{-3} M	$k_{-1} = \phi_{ABC}/\phi_{BC}\phi_A$ 1.54 × 10^3 s^{-1}
ϕ_{AB} − 0.48 (±1.5) × 10^{-12} M^2·min	$k_{-1}/k_{+1} = \phi_{ABC}/\phi_{BC}$ 9.5 (± 1.3) × 10^{-5} M	$k_{-2} = \phi_{BC}/\phi_B\phi_C$ 1.26 × 10^4 s^{-1}
0.97 (±0.15) × 10^{-13} M^2·min (Fig. 1)		
ϕ_{BC} 3.84 (±0.31) × 10^{-11} M^2·min	$k_{-1}/k_{+1} = \phi_{AB}/\phi_B$ 8.7 (± 1.7) × 10^{-5} M	
ϕ_{ABC} 3.67 (±0.43) × 10^{-15} M^3·min	$k_{-2}/k_{+2} = \phi_{BC}/\phi_C$ 8.4 (±0.89) × 10^{-4} M	
ϕ_0 2.71 (±0.15) × 10^{-5} min	$V_t = 1/\phi_0$ 3.68 × 10^4 min^{-1}	
	or 6.13 × 10^2 s^{-1} (catalytic constant)	

or less of pyruvate kinase and the resulting change in absorbance at 340nm recorded spectrophotometrically.

Analysis of initial-rate data

Analysis of data was done with the computer programs of Cleland (1963*b*) after a graphical check had been made that the double-reciprocal plots were linear. Initial-velocity data for uninhibited reactions at fixed free Mg^{2+} concentrations were analysed by the SEQUEN program. The secondary slopes and intercepts thus obtained, together with their standard errors, were then fitted as a linear function of the reciprocal free Mg^{2+} concentration by a weighted linear regression (Wilkinson, 1961). The tertiary slopes and intercepts that resulted are recorded, together with their standard errors, as the kinetic constants appearing in Table 3. The data from inhibited reactions were analysed according to the type of plot by means of the COMP or NONCOMP program. True inhibition constants were obtained algebraically from the apparent constants provided by the program (except where they are identical), the standard deviations of sums, products and quotients being calculated according to equations given by Margenau & Murphy (1956). Inhibition constants are given in Table 4. The straight lines appearing on double-reciprocal plots represent computer fits to the data on the individual figures appearing in this paper.

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 the free, the Mg^{2+}-bound or the total ADP and phosphoenolpyruvate concentrations held constant. The results, shown in 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 held 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 held constant and +2 when either α and β or A_t and B_t are fixed. These observations support the following conclusions.

(1) A branched mechanism, in which the substrates phosphoenolpyruvate and ADP can bind to the enzyme both in their free and Mg^{2+}-bound forms, has a rate equation that, when expressed in terms of A and B, is non-linear in C^{-1}. Fig. 1 shows that this relationship is linear and therefore indicates that the substrates bind in one form only.

Table 4. *Inhibition constants for the reaction catalysed by pyruvate kinase*

K_{IQ} and K_{DP} are dissociation constants for MgATP and pyruvate, respectively. For further details see the text.

Product inhibitor	Varied substrate	Fixed substrate (mM)	Replot	Inhibition constant (mM)	
Q	A	B: 0.14	Slope	K_{IQ}	1.64±0.29
		0.028	Slope	K_{IQ}	1.42±0.10
		0.014	Slope	K_{IQ}	1.25±0.10
Q	B	A: 0.48	Slope	K_{IQ}	1.22±0.27
			Intercept	K_{IQ}	1.34±0.26
		0.08	Slope	K_{IQ}	1.84±0.70
			Intercept	K_{IQ}	1.46±0.22
P	A	B: 0.014	Slope	K_{DP}	21.6 ±1.9
			(see the text)	K_{IP}	472±164
			(see the text)	K_{IIP}	12.4 ±0.40
		B: 0.028	Slope	K_{DP}	20.8 ±2.1
			(see the text)	K_{IP}	124±406
			(see the text)	K_{IIP}	12.5 ±0.5

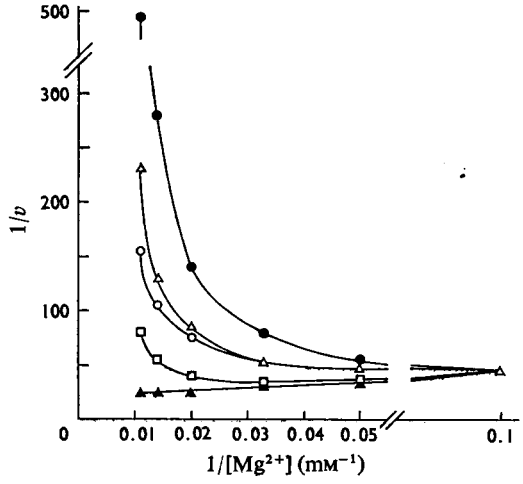


Fig. 1. *Dependence of the velocity of the reaction catalysed by pyruvate kinase on increasing Mg^{2+} concentration*

Reaction mixtures contained tetrapropylammonium cacodylate buffer, pH 6.2 (100 μmol), KCl (100 μmol), fructose diphosphate (2.0 μmol), NADH (0.15 μmol) and 50 μg of lactate dehydrogenase in a final volume of 1.0 ml. Phosphoenolpyruvate and ADP concentrations were: ●, [MgPyr-*P*] 0.1 mM and [ΣMgADP] 0.086 mM; △, total [Pyr-*P*] 0.3 mM and total [ADP] 0.1 mM; ○, [ΣPyr-*P*] 0.2 mM and [ΣMgADP] 0.086 mM; □, [MgPyr-*P*] 0.1 mM and [ΣADP] 0.014 mM; ▲, [ΣPyr-*P*] 0.2 mM and [ΣADP] 0.014 mM. Coincident point for all five experiments at a reciprocal Mg^{2+} concentration of 0.1 mM^{-1} and for △ and ○ at 0.05 mM^{-1} and 0.033 mM^{-1}. *v* is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

(2) On referring to Table 1, and to the values of *i* predicted for *A* and *B* constant, it will be observed that the corresponding data allow linear mechanisms in which A, B and C or α and B or A and β are substrates; i.e. the evident absence of $i = -2, -3$ excludes mechanisms in which more than one Mg^{2+} ion is incorporated in the active site.

(3) Reactions in which α and B or A and β are substrates that are not inhibited by increasing *C* when α and *B* or *A* and β respectively are held constant. Fig. 1 demonstrates that inhibition does occur under these conditions. It may be concluded therefore that the Mg^{2+}-free species of phosphoenolpyruvate and ADP are the true substrates of the enzyme-catalysed reaction and that the Mg^{2+}, essential for the catalytic process to take place, binds separately. Thus the inhibition observed when total or Mg^{2+}-bound phosphoenolpyruvate and ADP concentrations are held constant is apparent only because of the progressive removal of the kinetically important true substrates as the Mg^{2+} concentration increases.

(4) The evident absence of product inhibition by C when *A* and *B* are held constant indicates that the bound nucleotide dissociates from the enzyme as MgATP and not separately as Mg^{2+} and ATP.

(5) Increasing *C* causes a relative increase in α and β when *A* and *B* are held constant; therefore, because linearity is observed in the double-reciprocal plot of data obtained under such conditions, the species α and β are not dead-end inhibitors of the enzyme-catalysed reaction.

(6) The limiting value of $i = +2$, obtained when α and β or A_t and B_t are held constant, proves the reality of the constant ϕ_{AB} and allows its evaluation. This finding is important because ϕ_{AB} is too small to

demonstrate visually by the 'triple plotting' of initial-rate data obtained at lower Mg^{2+} concentrations. Similarly the limiting value of $i = +1$, obtained when A and β are held constant, allows ϕ_B to be calculated if ϕ_{AB} is known. Values of ϕ_{AB} and ϕ_B, based on these data, are recorded 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. Intercepts from computed fits to eqn. (2) are plotted in Fig. 5 as a function of A^{-1}, at several fixed values of C: this figure represents a test of eqn. (3) and indicates that ADP is bound to the enzyme as the second of three substrates.

The kinetic constants obtained by computer analysis of the data are given in Table 3. The absence of ϕ_{AC} indicates that the reaction is not a rapid random equilibrium; further, 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 preceding section. Fig. 4 demonstrates that reliable estimates of ϕ_{AB} and ϕ_B cannot be obtained from the intercepts on the tertiary plots: however, estimates of these constants may be obtained from the Mg^{2+}-inhibition data in Fig. 1 and are included in Table 3.

Table 3 also includes values of the rate constants that may be derived from the kinetic constants assuming the mechanism represented in Scheme 1. It may be

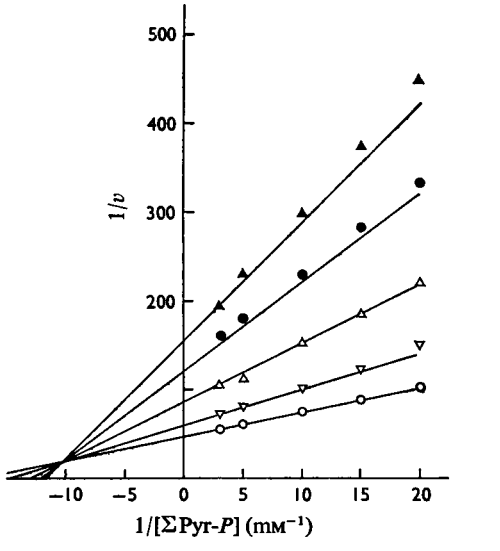


Fig. 2. *Effect of* [Σ*ADP*] *on the initial velocity of the forward reaction catalysed by pyruvate kinase with* Σ*Pyr-P as the variable substrate*

The free Mg^{2+} concentration was 0.67mM. Assay conditions were as described in Fig. 1. The concentrations of ΣADP were: ○, 0.35mM; ▽, 0.232mM; △, 0.140mM; ●, 0.095mM; ▲, 0.07mM. v is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

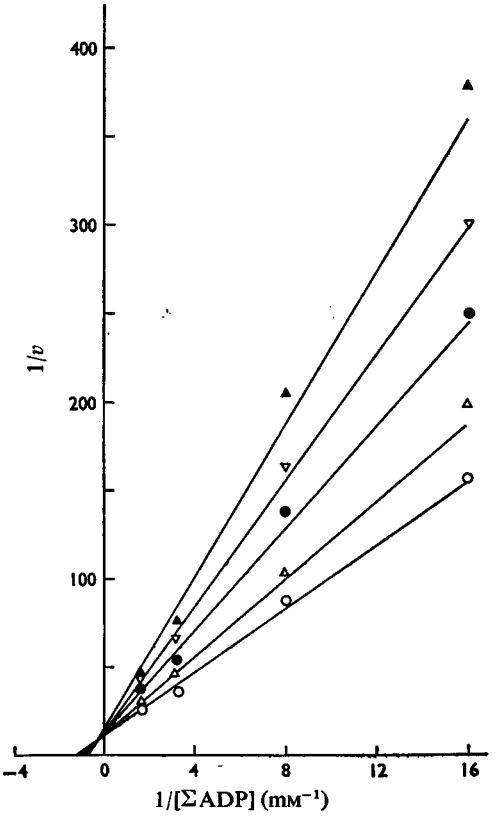


Fig. 3. *Effect of* [Σ*Pyr-P*] *on the initial velocity of the forward reaction catalysed by pyruvate kinase with* Σ*ADP as the varied substrate*

The free Mg^{2+} concentration was 1.0mM. Assay conditions were as described in Fig. 1. The concentrations of ΣPyr-*P* were: ○, 0.38mM; △, 0.192mM; ●, 0.096mM; ▽, 0.064mM; ▲, 0.048mM. v is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase. For each fixed value of [ΣPyr-*P*] an additional point at [ΣADP] (0.62mM) has been excluded from the graph to preserve clarity.

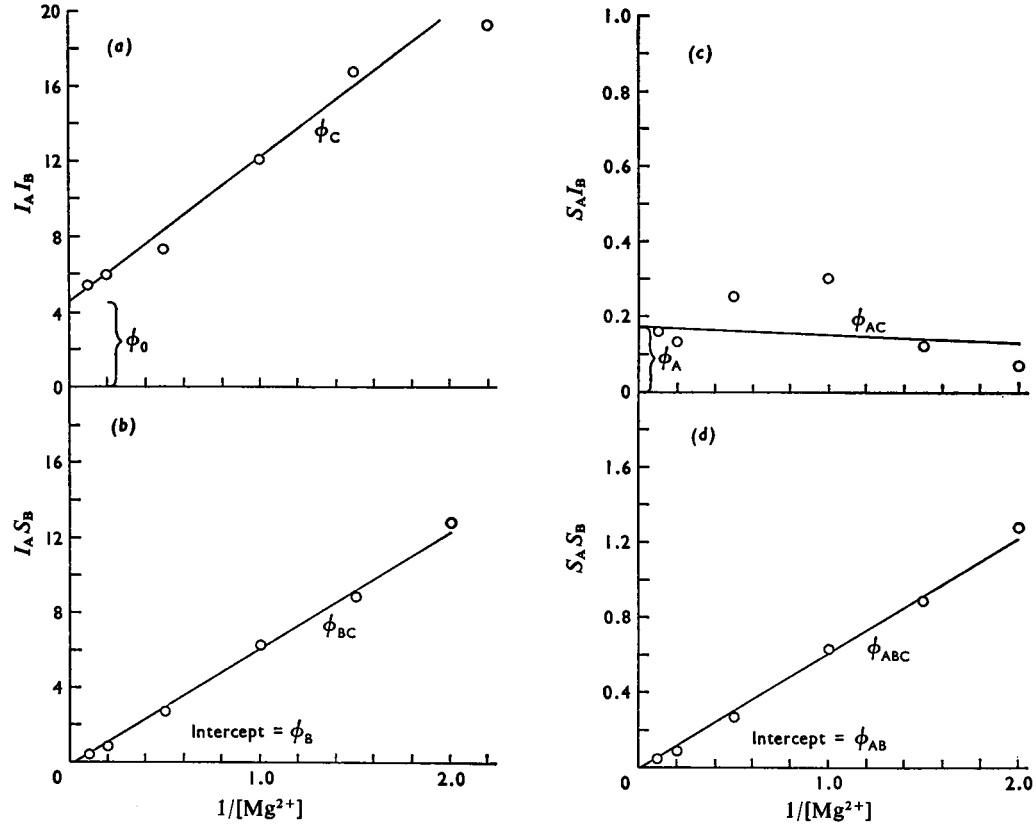


Fig. 4. *Plots of the relationships:*

(*a*) $\phi_C/[Mg^{2+}]+\phi_0 = I_A I_B$; (*b*) $\phi_{BC}/[Mg^{2+}]+\phi_B = I_A S_B$;
(*c*) $\phi_A = S_A I_B$; (*d*) $\phi_{ABC}/[Mg^{2+}]+\phi_{AB} = S_A S_B$

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 six* Mg^{2+} *concentrations*

noted that the values of k_{-1}/k_{+1} obtained from the ratios ϕ_{ABC}/ϕ_{BC} and ϕ_{AB}/ϕ_B are very similar, indicating that the estimate of the latter ratio from inhibition data is valid.

Product-inhibition studies

Figs. 6–9 show the fit to eqns. (1) and (2) of the initial velocities in the presence of a single product. All the inhibitions are non-competitive except for that in Fig. 6, which shows competition between phosphoenolpyruvate and MgATP. These data are consistent with a mechanism where phosphoenolpyruvate and MgATP alone bind to the free enzyme or with an Iso-Theorell–Chance mechanism in which the competing pair are interconverted directly by the reaction:

Phosphoenolpyruvate + E·MgADP ⇌ MgATP + E·pyruvate

Of the two alternatives the former is preferred because evidence exists to show that phosphoenolpyruvate binds to the free enzyme (Kuczenski & Suelter, 1971).

The product-inhibition pattern therefore, in conjunction with the evidence introduced in preceding sections, shows that the reaction mechanism is of the ordered Tri Bi type represented in Scheme 1 (Cleland, 1963*a*).

The possibility that an enzyme–pyruvate dead-end complex might exist follows from the observation that the slope variation with pyruvate in the inhibition of phosphoenolpyruvate (Fig. 8) is greater than the slope variation with pyruvate in the inhibition of

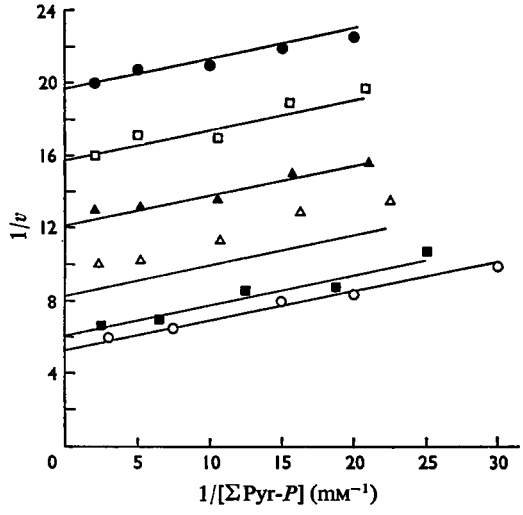


Fig. 5. *Effect of free Mg^{2+} on the initial velocity of the forward reaction catalysed by pyruvate kinase with ΣPyr-P as the varied substrate and [ΣADP] extrapolated to an infinite concentration*

The concentrations of free Mg^{2+} were: ●, 0.5mM; □, 0.67mM; ▲, 1.0mM; △, 2.0mM; ■, 5.0mM; ○, 10.0mM. v is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

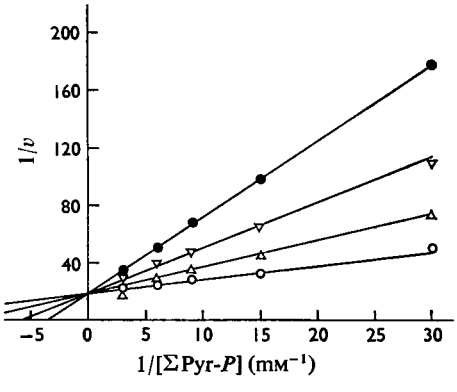


Fig. 6. *Inhibition by MgATP of the forward reaction catalysed by pyruvate kinase, with ΣPyr-P as the variable substrate*

The concentrations of free Mg^{2+} and ΣADP were held constant at 10.0 and 0.028mM respectively. Assay conditions were as described in Fig. 1. The concentrations of MgATP were: ○, 0.0mM; △, 1.0mM; ▽, 3.0mM; ●, 5.0mM. v is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

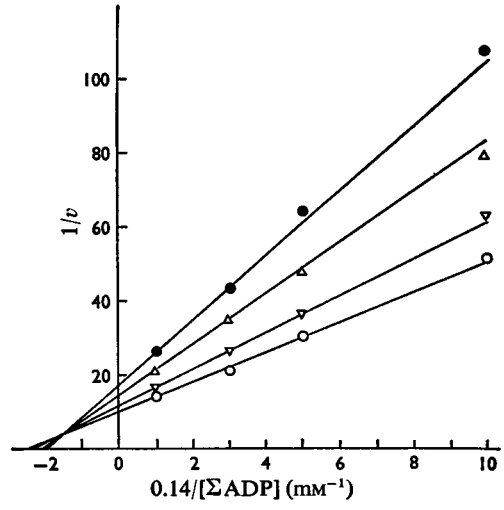


Fig. 7. *Inhibition by MgATP of the forward reaction catalysed by pyruvate kinase, with ΣADP as the varied substrate*

The concentrations of free Mg^{2+} and ΣPyr-P were held constant at 10.0 and 0.08mM respectively. Assay conditions were as described in Fig. 1. The concentrations of MgATP were: ○, 0.0mM; ▽, 1.0mM; △, 3.0mM; ●, 5.0mM. v is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

ADP (Fig. 9), and, also, because the intercept variation in the inhibition of phosphoenolpyruvate is smaller than the intercept variation in the inhibition of ADP. Examination of eqns. (1) and (2) shows that this is not expected if the effect of pyruvate is limited to product inhibition but is reasonable if an enzyme–pyruvate complex forms.

Values of K_{IQ}, obtained by computer analysis of both competitive and non-competitive experiments, are given in Table 4.

The corresponding values of the pyruvate-inhibition constants cannot be obtained so easily, and the procedure adopted only provides approximations to the constants. Fig. 10 shows that plots of the slope of eqn. (1) as a function of P, at two different values of B, are linear, the two lines nearly intersecting on the horizontal axis. The absence of curvature indicates that only one of the two pyruvate-inhibition constants concerned is important under the conditions of the experiment, and the insensitivity of the horizontal intersection to B shows that the important constant is K_{DP}. In these circumstances the value of the horizontal intercept provides a first approximation to K_{DP} (which is thus given directly by the NONCOMP program). With this value to hand and values for the

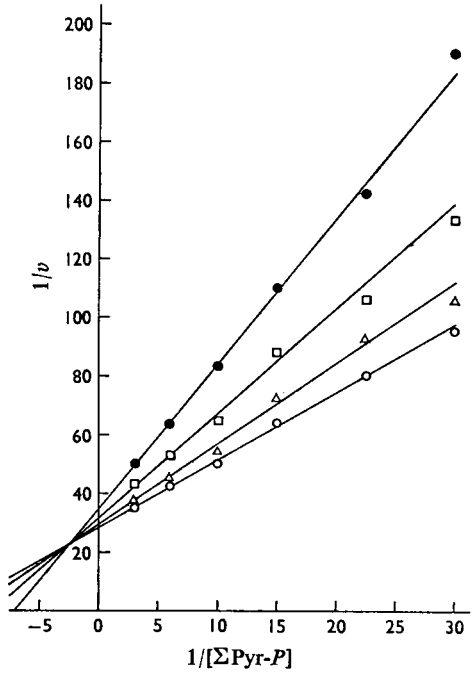


Fig. 8. *Inhibition by pyruvate of the forward reaction catalysed by pyruvate kinase, with ΣPyr-P as the varied substrate*

The concentrations of free Mg^{2+} and ΣADP were held constant at 10.0 and 0.028 mM respectively. Reaction mixtures contained tetrapropylammonium cacodylate buffer, pH 6.2 (100 μmol), KCl (100 μmol), fructose diphosphate (2.0 μmol), NADH (0.15 μmol), 3-phosphoglycerate (20 μmol), 50 μg of phosphoglycerate kinase and 150 μg of glyceraldehyde 3-phosphate dehydrogenase in a final volume of 1.0 ml. The concentrations of pyruvate were: ○, 0.0 mM; △, 5.0 mM; □, 10.0 mM; ●, 20.0 mM. *v* is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

intercepts of eqns. (1) and (2) applied to pyruvate inhibitions at constant *C*, the left-hand side of the equality:

$$\left(I_1 - I_2 + \frac{\phi_A^D}{A} - \frac{\phi_B}{B}\right) = \frac{\phi_{BC}}{BC}\left(1 + \frac{P}{K_{IP}}\right)$$

may be evaluated and fitted as a function of *P* by a weighted linear regression: the horizontal intercept of the resulting plot gives K_{IP}. Values of K_{IIP} are then calculated from I_2 by substituting the known constants and similarly fitting the results as a function of *P*. The estimates of K_{DP}, K_{IP} and K_{IIP} with their standard errors are given in Table 4.

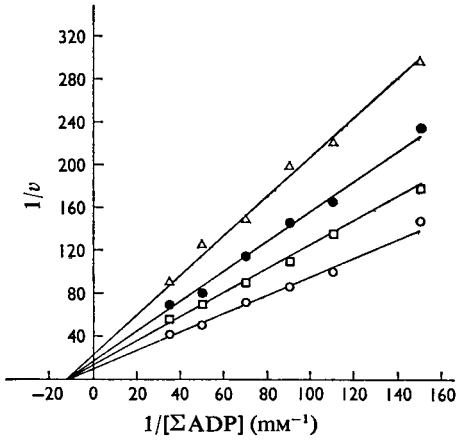


Fig. 9. *Inhibition by pyruvate of the forward reaction catalysed by pyruvate kinase, with ΣADP as the varied substrate*

The concentrations of free Mg^{2+} and ΣPyr-*P* were held constant at 10.0 and 0.05 mM respectively. Assay conditions were as described in Fig. 8. The concentrations of pyruvate were: ○, 0.0 mM; □, 5.0 mM; ●, 10.0 mM; △, 20.0 mM. *v* is expressed as units (μmol of NADH oxidized/min)/μg of pyruvate kinase.

Discussion

The study described above suggests that the reaction mechanism of activated yeast pyruvate kinase is an ordered Tri Bi mechanism and that pyruvate inhibits, in part, through the formation of a dead-end complex. The relationship of this study to previous work is now briefly explored.

The conclusion that the enzyme-catalysed reaction proceeds through the formation of a quaternary complex is in agreement with the mechanism postulated for the muscle enzyme (Mildvan & Cohn, 1966). It has also been suggested by Mildvan *et al.* (1971) that phosphoenolpyruvate may bind to the yeast enzyme before ADP. The binding constant, $k_{-1}/k_{+1} = 0.087$ mM for phosphoenolpyruvate, given in Table 3, is in good agreement with the value of 0.069 mM found by Kuczenski & Suelter (1971) in direct binding studies with yeast pyruvate kinase. The inhibition constants for MgATP may be compared with an apparent constant of 0.6 mM determined, at an Mg^{2+} concentration of 2.5 mM, by Haekel *et al.* (1968) in a study of the enzyme from brewer's yeast, and with values of 5.6 mM and 4.5 mM obtained by Taylor *et al.* (1969) for liver and hepatoma pyruvate kinase respectively. The value of 21 mM for K_{DP} can also be compared with the dissociation constant of 4.5 mM for the release of pyruvate from an enzyme–Mn^{2+}

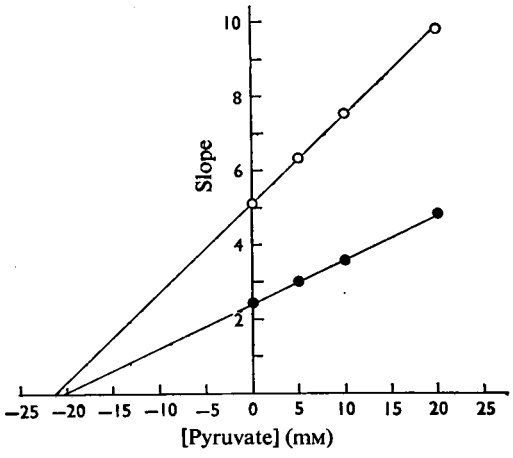


Fig. 10. *Effect of pyruvate on the slope of the relationship between v^{-1} and $[\Sigma Pyr\text{-}P]^{-1}$ for pyruvate kinase at two different ΣADP concentrations*

The concentrations of ΣADP were: o, 0.014mM; ●, 0.028mM.

complex determined by G. L. Cottam, A. S. Mildvan, J. R. Hunsley & C. H. Suelter (personal communication).

Several rate constants are available for comparison with the values given in Table 3. ADP binds to creatine kinase with a rate constant of $2.2\times10^{7}\,M^{-1}\cdot s^{-1}$ and dissociates with a rate constant of $1.8\times10^{4}\,s^{-1}$ (Hammes & Hurst, 1969). The comparable constants for the binding of NiADP to muscle pyruvate kinase are $4\times10^{6}\,M^{-1}\cdot s^{-1}$ and $1.26\times10^{2}\,s^{-1}$ respectively (Mildvan *et al.*, 1971).

Perhaps more interesting is the insight the postulated mechanism gives to the sequence of events at the active site. In solution Mg^{2+} is bound between the α- and β-phosphate groups of ADP and the β- and γ-groups of ATP (Cohn & Hughes, 1960, 1962; Hamms *et al.*, 1961). Thus if MgADP were to be a substrate there would be some difficulty in explaining the shift in position of Mg^{2+} during reaction. However, the separate binding of Mg^{2+} readily suggests that the cation bridges the phosphate group of phosphoenolpyruvate and the terminal phosphate group of ADP, assisting the phosphorylation of the latter, and ultimately being eliminated bound between the β- and γ-phosphate groups of ATP.

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APPENDIX

Substrate-Species Equilibria

The strategy adopted for the calculation of substrate concentrations may be exemplified by the simple equilibrium:

$$S + L \overset{K}{\rightleftharpoons} SL$$

where S and SL represent free and ligand-bound substrate respectively. Defining the concentrations L and S_t, the relationships:

$$S = \frac{S_t K}{K+L}$$

$$SL = \frac{S_t L}{K+L}$$

$$L_t = L + SL$$

enable S and SL to be calculated and also define the total amount of ligand that has to be added to the system.

Schemes 1, 2 and 3 define the substrate and fructose diphosphate equilibria that are important in the pH range 5–9 and in the presence of Mg^{2+} and K^+. The ATP equilibria may also be represented by Scheme 1. The values of the dissociation constants that appear in the schemes are for an ionic strength of 0.2, except those for fructose diphosphate, which relate to an ionic strength of 0.1.

In direct analogy to the simple equilibrium, once pH and the concentrations of free K^+, free Mg^{2+} and total substrate have been defined, the concentrations of substrate species may be calculated. Taking Scheme 1 as an example, it is convenient to choose one substrate species (say $MgADP^-$) and express it in terms of the total substrate and ligand concentrations, thus:

$$[MgADP^-] = \frac{CB_t}{\frac{K_2 P}{K_1} + K_2 + \frac{K_2 H}{K_5} + \left(1 + \frac{H}{K_3}\right) C}$$

where P represents the concentration of free K^+ and H that of protons.

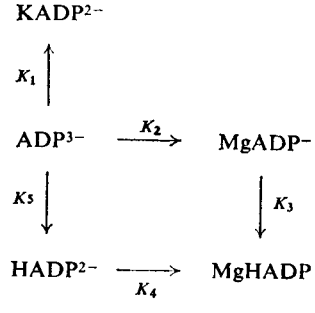
Therefore, with $[MgADP^-]$ known, the concentrations of all other nucleotide species may be calculated. For example:

$$[ADP^{3-}] = \frac{K_2[MgADP^-]}{C}$$

The conservation equation for Mg^{2+} in the presence of phosphoenolpyruvate, ADP and fructose diphosphate may therefore be written:

$$C_t = C + \frac{CA_t}{\frac{K_{10} P}{K_8} + K_{10} + \frac{K_{10} H}{K_9} + C} + \frac{CB_t(1 + H/K_3)}{\frac{K_2 P}{K_1} + K_2 + \frac{K_2 H}{K_5} + \left(1 + \frac{H}{K_3}\right) C} + \frac{C[\text{Fru-1,6-di-}P]_t(1 + HK_{13}/K_{11}K_{14})}{K_{13} + \frac{K_{13} H}{K_{11}} + \frac{K_{13} H^2}{K_{11} K_{12}} + \left(1 + \frac{K_{13} H}{K_{11} K_{14}}\right) C}$$

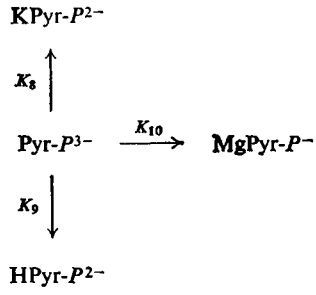
Computer programs based on these equations were employed to define the substrate concentrations employed in the study described in the main paper.



Scheme 1. *ADP species equilibria*

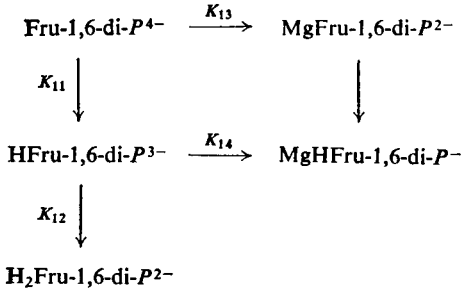
A directly analogous scheme may be set up for ATP species equilibria, each intermediate gaining an additional negative charge. The origin of the constants is as follows:

	For ADP (M)	For ATP (M)	Reference
K_1	1.81×10^{-1}	7.1×10^{-2}	Smith & Alberty (1956)
K_2	3.7×10^{-4}	3.47×10^{-5}	Phillips *et al.* (1966)
K_3	4.9×10^{-6}	6.1×10^{-6}	Phillips *et al.* (1966)
K_5	1.4×10^{-7}	9.1×10^{-8}	Phillips *et al.* (1966)



Scheme 2. *Phosphoenolpyruvate species equilibria* (*Wold & Ballou*, 1957)

$K_8(8.3\times10^{-2}\text{M})$; $K_9(4.4\times10^{-7}\text{M})$; $K_{10}(5.5\times10^{-3}\text{M})$.



Scheme 3. *Fructose diphosphate species equilibria* (*McGilvery*, 1965)

$K_{11}(1.7\times10^{-7}\text{M})$; $K_{12}(1.1\times10^{-6}\text{M})$; $K_{13}(1.8\times10^{-3}\text{M})$; $K_{14}(7.1\times10^{-3}\text{M})$.

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