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Activation and Inhibition of Rabbit Muscle Pyruvate Kinase by Transition-Metal Ions

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The paper reports a comparative study of the effects of Mn^{2+}, Ni^{2+} and Co^{2+} on the reaction of ADP with phosphoenolpyruvate when catalysed by K^+-activated rabbit muscle pyruvate kinase. The activation and subsequent inhibition that occurs as the bivalent ion concentration is increased is taken as evidence that the substrates of the enzyme are phosphoenolpyruvate, uncomplexed ADP and free bivalent cation. Kinetic constants for the binding of the bivalent cation to the enzyme are reported.

Pyruvate kinase (EC 2.7.1.40) from rabbit muscle requires the presence of a bivalent cation, such as Mg^{2+}, and a univalent cation, preferably K^+, if catalysis of the reaction between phosphoenolpyruvate and ADP is to occur (Kachmar & Boyer, 1953). The enzyme is also activated by other bivalent transition-metal ions, such as Mn^{2+}, Ni^{2+} and Co^{2+} (Mildvan & Cohn, 1965, 1966; Bygrave, 1966). However, when the total concentrations of ADP and phosphoenolpyruvate are kept constant, the activation that is brought about by the bivalent cation is succeeded by inhibition as its concentration is increased (Ainsworth & Macfarlane, 1973; Bygrave, 1966). It has also been observed that the concentration of bivalent cation required for maximum activity depends both on the identity of the cation and on the concentrations of ADP and phosphoenolpyruvate used (Wimhurst & Manchester, 1972).

Maxima in the relationship of enzyme activity to bivalent cation concentration have been observed with other kinases and different interpretations of the effect have been suggested. Thus it has been postulated that creatine kinase is inhibited by high concentrations of bivalent cation because, at these concentrations, ancillary binding sites become occupied (Heyde & Morrison, 1969): these sites, however, have not been detected by electron-spin-resonance or proton-relaxation-rate measurements made by Cohn (1963). Again, Larsson-Raznikiewicz (1970) has suggested that the activation and inhibition of phosphoglycerate kinase by bivalent cations is a secondary phenomenon of unspecified character.

Similar maxima have been observed in the relationship of Mg^{2+} concentration to the activity of the pyruvate kinases from yeast and pig liver (Macfarlane & Ainsworth, 1972, 1974). In that connexion, as with the enzyme from rabbit muscle (Ainsworth & Macfarlane, 1973), it was suggested that the maxima arise because the true substrates of the enzyme are free phosphoenolpyruvate, ADP and Mg^{2+}. Thus the rate of catalysed reaction increases with Mg^{2+} concentration because Mg^{2+} acts as a substrate, but also diminishes with Mg^{2+} concentration because phosphoenolpyruvate and ADP are progressively removed as their metal-bound complexes: the two effects operating together provide the maximum observed. The purpose of this paper is to ascertain whether the role of Mn^{2+}, Ni^{2+} and Co^{2+} may be similarly described. It is convenient to use rabbit muscle pyruvate kinase for this purpose because evidence exists (Mildvan & Cohn, 1966; Mildvan *et al.*, 1971) that the mechanism of catalysis remains the same when Mg^{2+}, Mn^{2+} or Ni^{2+} is the added bivalent cation. To facilitate comparison with data for the Mg^{2+}-activated enzyme (Ainsworth & Macfarlane, 1973), reactions took place in solutions at 25°C and pH 6.2; K^+ was present throughout at a saturating concentration.

Experimental

Materials

ADP, NADH (as sodium salts), phosphoenolpyruvate (cyclohexylammonium salt), rabbit muscle lactate dehydrogenase (15371 ELAC) 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 from Pharmacia, Uppsala, Sweden. All other reagents were AnalaR grade as supplied by BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

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Methods

Preparation of substrates, products and enzymes for use. Na^+ was removed from ADP and NH_4^+ from pyruvate kinase and lactate dehydrogenase as described by Ainsworth & Macfarlane (1973). The purity and concentrations of the substrates were also determined as described by Ainsworth & Macfarlane (1973); in particular, the concentration of substrates as determined by direct absorption measurements and enzymic analysis were shown to be equal 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), lactate dehydrogenase (12i.u.), with substrates at the concentrations indicated in the Figures.

After addition of the necessary components, the reaction mixtures were incubated at 25°C for 5min before addition of NADH and the coupling enzyme. The reactions were initiated by addition of 10μl or less of pyruvate kinase solution (1mg/ml) and initial velocities were estimated from the linear slopes of progress curves recorded by an Optika spectrophotometer at 340nm. Control experiments showed that lactate dehydrogenase was not rate limiting under these conditions.

Determination of substrate concentrations. The substrates of pyruvate kinase, phosphoenolpyruvate, ADP and bivalent cation, interact in substrate-level equilibria. It is therefore necessary to determine the concentrations of different possible substrates by using the known equilibrium constants for their interconversions. This has been done at given free bivalent cation concentrations, by the method of Macfarlane & Ainsworth (1972), by assuming that the dissociation constants do not change with ionic strength over the range of our study (Macfarlane *et al.*, 1974). As a result we have evaluated A and B as the concentrations of phosphoenolpyruvate and ADP respectively that do not form a complex with bivalent cation (though K^+ may be bound), α and β as the concentrations of the corresponding complexed forms and A_t and B_t as their total concentration. C is also defined as the given free bivalent cation concentration and C_t as its total concentration.

Results

Macfarlane & Ainsworth (1972) proposed a test to identify the substrates of a kinase which requires the dependence of the reciprocal initial velocity on cation concentration (C) to be determined in solutions where the concentrations of different hypothetical substrates are kept constant.

Figs. 1–3 show the results of this test when applied to rabbit muscle pyruvate kinase activated by Mn^{2+}, Ni^{2+} and Co^{2+} respectively. It is evident that inhibition occurs, with increasing cation concentration (C), in every instance except where the free phosphoenolpyruvate (A) and free ADP (B) concentrations are kept constant. Further, Figs. 1(a), 2(a) and 3(a) show that the limiting slope at high C is proportional to C when either α and B or A and β

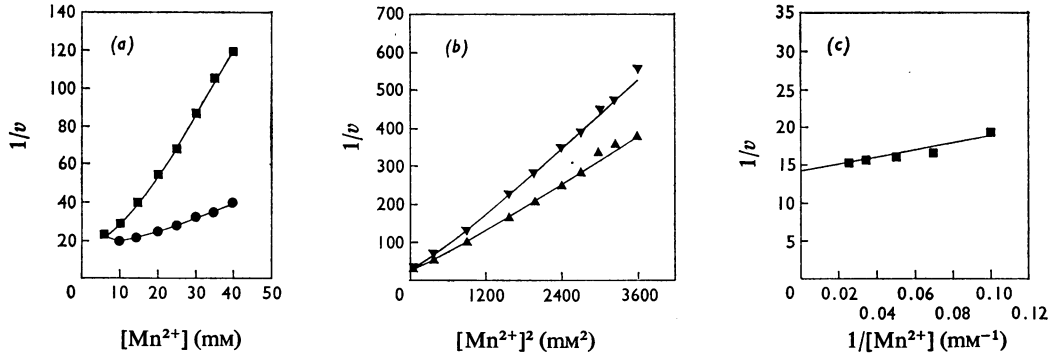

Fig. 1. *Dependence of the initial rate of the forward reaction catalysed by muscle pyruvate kinase on increasing Mn^{2+} concentrations*

Reaction mixtures contained tetrapropylammonium cacodylate buffer, pH6.2 (100μl), KCl (100μmol), NADH (0.15μmol) and 12i.u. of lactate dehydrogenase in a final volume of 1.0ml. In this and subsequent Figures (except for Fig. 4) v is expressed as μmol of NADH oxidized/min per μg of pyruvate kinase. Mn^{2+} was introduced as $MnCl_2$. The concentrations of phosphoenolpyruvate and ADP are defined by the relationships: total phosphoenolpyruvate $\equiv A_t = A + \alpha$, where $A \equiv [\Sigma\text{Pyr-}P] = [\text{Pyr-}P^{3-}] + [\text{HPyr-}P^{2-}] + [\text{KPyr-}P^{2-}]$, $\alpha \equiv [\Sigma\text{MnPyr-}P] = [\text{MnPyr-}P^{-}]$; total ADP $\equiv B_t = B + \beta$, where $B \equiv [\Sigma\text{ADP}] = [\text{ADP}^{3-}] + [\text{HADP}^{2-}] + [\text{KADP}^{2-}]$, $\beta \equiv [\Sigma\text{MnADP}] = [\text{MnADP}^{-}] + [\text{MnHADP}]$ and had the values: (a) ■, ΣPyr-P (26μM) and ΣMnADP (48μM), ●, ΣMnPyr-P (24μM) and ΣADP (1.65μM); (b) ▼, ΣMnPyr-P (24μM) and ΣMnADP (48μM); ▲, total Pyr-P (50μM) and total ADP (50μM); (c) ■, ΣPyr-P (26μM) and ΣADP (1.65μM).

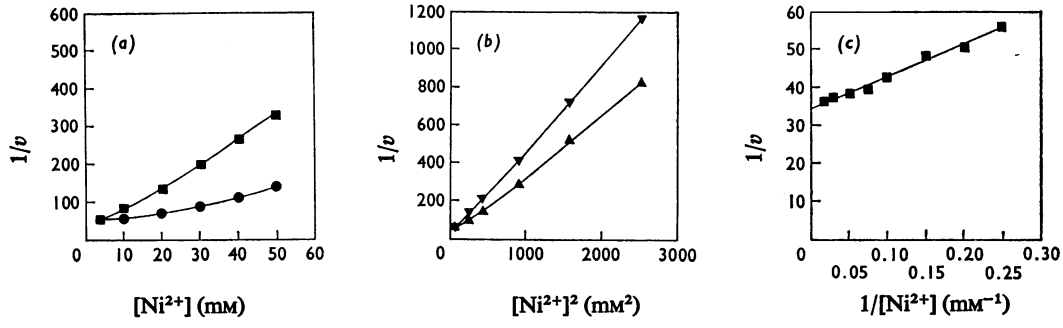

Fig. 2. *Dependence of the initial rate of the forward reaction catalysed by muscle pyruvate kinase on increasing Ni^{2+} concentrations*

Assay concentrations were as described in Fig. 1. Ni^{2+} was introduced as $NiSO_4$. The concentrations of phosphoenolpyruvate and ADP are defined as in Fig. 1 and were: (*a*) ■, ΣPyr-*P* (80.4 μM) and ΣNiADP (97.4 μM); ●, ΣNi Pyr-*P* (19.6 μM) and ΣADP (2.6 μM); (*b*) ▼, ΣNiPyr-*P* (19.6 μM) and ΣNiADP (97.4 μM), ▲, total Pyr-*P* (100 μM) and total ADP (100 μM); (*c*) ■, ΣPyr-*P* (80.4 μM) and ΣADP (2.6 μM).

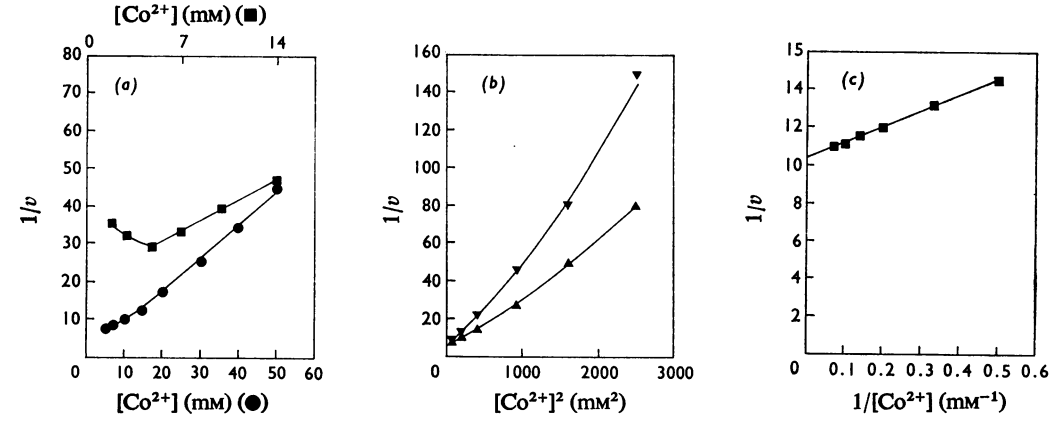

Fig. 3. *Dependence of the initial rate of the forward reaction catalysed by muscle pyruvate kinase on increasing Co^{2+} concentrations*

Assay conditions were as described in Fig. 1. Co^{2+} was introduced as $CoCl_2$. The concentrations of phosphoenolpyruvate and ADP are defined as in Fig. 1 and were: (*a*) ●, ΣPyr-*P* (66 μM) and ΣCoADP (96 μM); ■, ΣCoPyr-*P* (18 μM) and ΣADP (10 μM) (Co^{2+} concentration ranges: ●, 5–50 mM: ■, 2–14 mM; values on 1/*v* scale are ×5 for ■); (*b*) ▼, ΣCoPyr-*P* (33 μM) and ΣCoADP (96 μM); ▲, total Pyr-*P* (100 μM) and total ADP (100 μM); (*c*) ■, ΣPyr-*P* (82 μM) and ΣADP (10 μM).

are kept constant, whereas Figs. 1(*b*), 2(*b*) and 3(*b*) show that the limiting slope is proportional to C^2 when either α or β or A_t and B_t are kept constant.

It will be observed that the experiments where α and *B* or *A* and *B* were kept constant in the presence of Co^{2+} (Figs. 3*a* and 3*c* respectively) relate to a lower concentration range than that used in the remaining experiments with Co^{2+}. At the higher concentrations inhibition was observed: however, as *B* is less than 4% B_t under these conditions, it seems reasonable to assume that the linear relationship in Fig. 3(*c*) provides the correct qualitative information and that the inhibition at higher concentrations arose either from dead-end inhibition by CoADP complex or from error in its dissociation constant.

Fig. 4 shows that the initial rate of pyruvate formation passes through a maximum when α and *B* are kept constant and *C* is increased. The position of the maximum can be reasonably estimated when C is Co^{2+}, Ni^{2+} or Mn^{2+}, but is very ill-defined when C is Mg^{2+}.

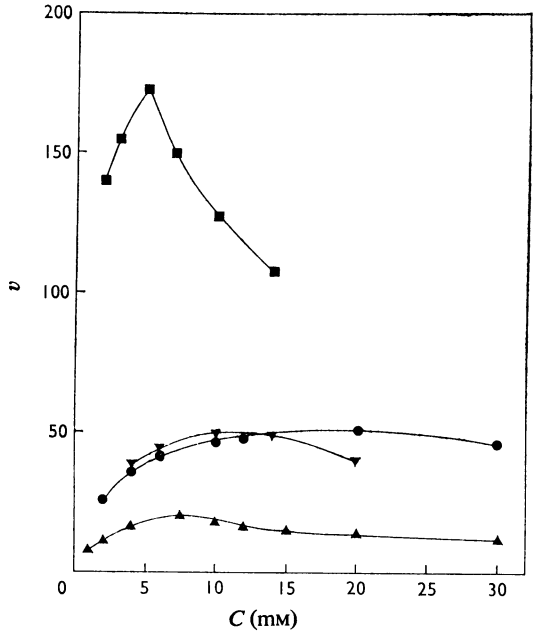

Fig. 4. *Dependence of the initial rate of the forward reaction catalysed by muscle pyruvate kinase at constant concentrations of complexed phosphoenolpyruvate (α) and free ADP (B) on increasing concentrations of bivalent cation*

Assay conditions were as described in Fig. 1. The salts of Mn^{2+}, Ni^{2+} and Co^{2+} used were as described earlier. Mg^{2+} was added as $MgCl_2$. v is expressed as μmol/min per mg of pyruvate kinase. ●, Mg^{2+} with ΣMgPyr-*P* (25.0 μM) and ΣADP (14.0 μM); ▼, Mn^{2+} with ΣMnPyr-*P* (26.0 μM) and ΣADP (1.65 μM); ▲, Ni^{2+} with ΣNiPyr-*P* (19.6 μM) and ΣADP (2.6 μM); ■, Co^{2+} with ΣCoPyr-*P* (18.0 μM) and ΣADP (10.0 μM).

Discussion

Mechanism of rabbit muscle pyruvate kinase

Previous studies (Mildvan & Cohn, 1966; Mildvan *et al.*, 1971; Ainsworth & Macfarlane, 1973) have established that the mechanism of rabbit muscle pyruvate kinase is equilibrium random order in type in the presence of Mg^{2+}, Mn^{2+} and Ni^{2+}. Ainsworth & Macfarlane (1973) also showed that it is both necessary and sufficient to assume that free phosphoenolpyruvate (A), free ADP (B) and Mg^{2+} (C) are the substrates of the enzyme and that other combinations of hypothetical substrates cannot, by themselves, explain the kinetic behaviour. The results, given above in Figs. 1–3, are identical in qualitative terms with those provided by the Mg^{2+}-activated enzyme and therefore indicate that the same mechanism operates when the enzyme is activated by the additional bivalent cations.

Table 1. *Apparent dissociation constants of complexed phosphoenolpyruvate (α) and complexed ADP (β)*

These apparent dissociation constants have been calculated for pH 6.2 in the presence of 100 mM-K^+ (Macfarlane *et al.*, 1974).

Metal ion ...	Mg^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}
K_1 (mM)	20	6.4	10.3	16.4
K_2 (mM)	1.5	0.23	0.22	0.1

Estimation of kinetic constants

On the basis established above, we shall assume that the enzyme forms all the possible binary, ternary and quaternary complexes with the substrates. The inverted rate equation for pyruvate formation in the absence of products is therefore:

$$\frac{E_0}{v} = \frac{K_A K_{AB} K_{ABC}}{V_f}\left(\frac{1}{ABC} + \frac{1}{K_C AB} + \frac{1}{K_B AC} + \frac{1}{K_A BC} + \frac{1}{K_B K_{BC} A} + \frac{1}{K_A K_{AC} B} + \frac{1}{K_A K_{AB} C} + \frac{1}{K_A K_{AB} K_{ABC}}\right) \quad (1)$$

which corresponds, term by term, with (Dalziel, 1969):

$$\frac{E_0}{v} = \frac{\phi_{ABC}}{ABC} + \frac{\phi_{AB}}{AB} + \frac{\phi_{AC}}{AC} + \frac{\phi_{BC}}{BC} + \frac{\phi_A}{A} + \frac{\phi_B}{B} + \frac{\phi_C}{C} + \phi_0 \quad (2)$$

where V_f is the turnover number and K_{XYZ} is the dissociation equilibrium constant for the binding of substrate Z to the enzyme complex containing substrates X and Y. E_0 is the concentration of enzyme. The ϕ terms are kinetic constants.

By using apparent dissociation constants:

$$K_1 = AC/\alpha \quad \text{and} \quad K_2 = BC/\beta \quad (3)$$

whose values are given in Table 1, eqn. (2) may be re-expressed as:

$$\frac{E_0}{v} = \phi_1 C + \frac{\phi_2}{C} + \phi_3 \ (\alpha \text{ and } B \text{ constant}) \quad (4)$$

so as to define the relationship $v^{-1} = f(C)$ in solutions where α and B are kept constant. An equation identical with eqn. 4, but with primed constants ($\phi'_1, \phi'_2, \phi'_3$) may be derived to represent the relationship $v^{-1} = f(C)$ in solutions where A and β are kept constant. The significance of the two sets of ϕ constants is given in Table 2.

Eqn. (4) shows that the limiting slope at high C is ϕ_1 or ϕ'_1. The two constants have been determined in this way and their values are given in Table 2.

Differentiation of eqn. (4) with respect to C shows the E_0/v has a minimum value (i.e. the initial velocity is maximum) when $C \equiv C_{m1}$ or C_{m2}, where:

$$C_{m1}^2 = \phi_2/\phi_1 \quad \text{and} \quad C_{m2}^2 = \phi'_2/\phi'_1 \quad (5)$$

Table 2. *Physical significance and evaluation of kinetic constant (ϕ) terms at constant concentrations of complexed phosphoenolpyruvate (α) and free ADP (B) and free phosphoenolpyruvate (A) and complexed ADP (β)*

ϕ values were calculated assuming that pyruvate kinase has a mol.wt. of 237000 (Warner, 1958) and that the enzyme has two active sites/molecule (Mildvan & Cohn, 1965).

Terms	ϕ values with α and B kept constant
$\phi_1 = \frac{\phi_{AB}}{K_1 \alpha B} + \frac{\phi_A}{K_1 \alpha}$	8.27×10^{-3} M^{-1}·min (Mn^{2+}, Fig. 1*a*) 1.67×10^{-2} M^{-1}·min (Ni^{2+}, Fig. 2*a*) 3.08×10^{-3} M^{-1}·min (Co^{2+}, Fig. 3*a*)
$\phi_2 = \frac{\phi_{BC}}{B} + \phi_C$	6.8×10^{-7} M·min (Mn^{2+}, Fig. 5*a*) 1.15×10^{-6} M·min (Ni^{2+}, Fig. 5*b*) 7.59×10^{-8} M·min (Co^{3+}, Fig. 5*c*)
$\phi_3 = \frac{\phi_{ABC}}{K_1 \alpha B} + \frac{\phi_{AC}}{K_1 \alpha} + \frac{\phi_B}{B} + \phi_0$	2.91×10^{-5} min (Mn^{2+}, Fig. 5*a*) 1.24×10^{-4} min (Ni^{2+}, Fig. 5*b*) 1.54×10^{-5} min (Co^{2+}, Fig. 5*c*)
	ϕ values with A and β kept constant
$\phi_1' = \frac{\phi_{AB}}{K_2 A \beta} + \frac{\phi_B}{K_2 \beta}$	2.94×10^{-2} M^{-1}·min (Mn^{2+}, Fig. 1*a*) 5.65×10^{-2} M^{-1}·min (Ni^{2+}, Fig. 2*a*) 7.42×10^{-3} M^{-1}·min (Co^{2+}, Fig. 3*a*)
$\phi_2' = \frac{\phi_{AC}}{A} + \phi_C$	
$\phi_3' = \frac{\phi_{ABC}}{K_2 A \beta} + \frac{\phi_{BC}}{K_2 \beta} + \frac{\phi_A}{A} + \phi_0$	

Eqn. (5) may be used to eliminate either ϕ_2 or ϕ_1 from eqn. (4):

$$\frac{E_0}{v} = \phi_1\left(\frac{C^2+C_{m1}^2}{C}\right) + \phi_3 \qquad (6)$$

$$\frac{E_0}{v} = \phi_2\left(\frac{C^2+C_{m1}^2}{CC_{m1}^2}\right) + \phi_3 \qquad (7)$$

Visual estimates of C_{m1} were obtained from the plots of $v^{-1} = f(C)$, given in Fig. 4 for the Mn^{2+}-, Ni^{2+}- and Co^{2+}-activated reactions. These estimates were used to construct the plots, based on eqn. (7), which are displayed in Fig. 5. The values of ϕ_2 and ϕ_3, provided by Fig. 5, are given in Table 2.

Fig. 4 also shows that the maximum of $v^{-1} = f(C)$ for the Mg^{2+}-activated enzyme cannot be defined accurately by eye. However, by using kinetic constants that are already available for this reaction (Ainsworth & Macfarlane, 1973), ϕ_1 and ϕ_2 may be calculated by the definitions given in Table 2: eqn. (5) then provides C_{m1}. The value thus obtained, 17.8mM, is within the range expected from Fig. 4. Alternatively, C_{m1} may be determined by introducing putative values into eqn. (7) and testing the data for linearity. On this basis (Fig. 6) the best value for C_{m1} was found to be 15mM. The result obtained when C_{m1} was taken to be 20mM is shown for comparison.

The value of C_{m1} can also be determined if ϕ_1 is known by extrapolation by using paired observations of v and C. Thus eqn. (6) gives:

$$C_{m1}^2 = \left[E_0\left(\frac{1/v_1 - 1/v_2}{\phi_1}\right) - C_1 + C_2\right]\frac{C_1 C_2}{C_2 - C_1} \qquad (8)$$

On this basis C_{m1} for Mn^{2+}, Ni^{2+} and Co^{2+} is 9.9, 7.7 and 4.2mM respectively.

The results of Mildvan & Cohn (1966) and Mildvan *et al.* (1971) suggest that bivalent cations bind only at the active site of muscle pyruvate kinase and not elsewhere. If so, K_A and K_B, the equilibrium constants for the dissociation of phosphoenolpyruvate and ADP respectively from their enzyme complexes should be independent of the nature of the bivalent cation. The same must also be true of K_{AB} and K_{BA}, the dissociation constants for the equilibrium of ADP and phosphoenolpyruvate respectively with the ternary enzyme–ADP–phosphoenolpyruvate complex. Further constants can therefore be calculated by making use of values of K_B, K_{AB} and K_{BA} obtained in a study of the Mg^{2+}-activated enzyme under identical experimental conditions (Ainsworth & Macfarlane, 1973). Thus Table 2 gives:

$$\phi_2 = \phi_C\left(\frac{\phi_{BC}}{\phi_C B} + 1\right) \equiv \phi_C\left(\frac{K_{AB}}{B} + 1\right) \qquad (9)$$

$$\phi_2' = \phi_C\left(\frac{\phi_{AC}}{\phi_C A} + 1\right) \equiv \phi_C\left(\frac{K_{BA}}{A} + 1\right) \qquad (10)$$

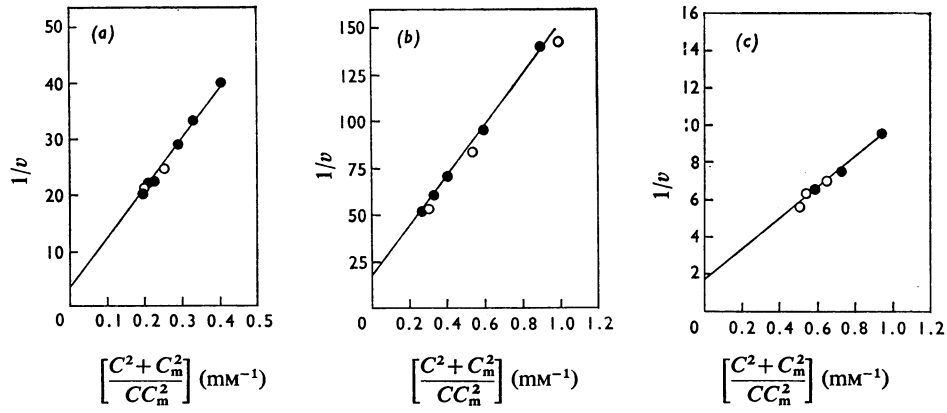

Fig. 5. *Replot of* v^{-1} *against* $(C^2+C_m^2)/CC_m^2$ *with increasing transition-metal ion concentrations when concentrations of complexed phosphoenolpyruvate* (α) *and free ADP* (*B*) *are kept constant*

v is expressed as μmol/min per mg of pyruvate kinase. In each Figure ● and ○ refer to initial rates obtained at bivalent cation concentrations above and below C_m, the bivalent cation concentration at which the initial velocity is maximum respectively. Initial rates are taken from Figs. 1, 2, 3 and 4. The estimates of C_m were obtained visually from Fig. 4. The substrate concentrations are defined as in Fig. 1. (*a*) Mn^{2+} ($C_m = 10$mM), ΣMnPyr-*P* (26μM) and ΣADP (1.65μM); (*b*) Ni^{2+} ($C_m = 7.5$mM), ΣNiPyr-*P* (19.6μM) and ΣADP (2.6μM); (*c*) Co^{2+} ($C_m = 5$mM), ΣCoPyr-*P* (18μM) and ΣADP (10μM).

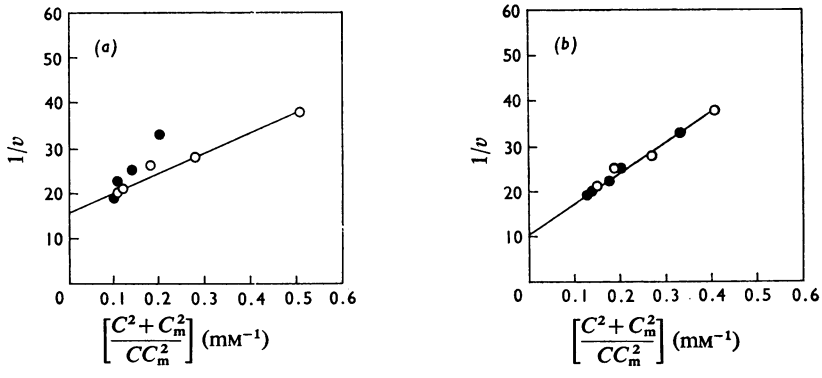

Fig. 6. *Replot of* v^{-1} *against* $(C^2+C_m^2)/CC_m^2$ *with* Mg^{2+} *as bivalent cation when* α *and B are kept constant*

(*a*) $C_m = 20$mM; (*b*) $C_m = 15$mM. ● and ○ represent initial rates obtained at Mg^{2+} concentrations above and below C_m respectively. The data are taken from Fig. 4. Also included are initial rates, taken from Ainsworth & Macfarlane (1973), obtained at higher Mg^{2+} concentrations than those shown in Fig. 4. Fig. 6*a* shows that eq. 7 is not obeyed when C_m is incorrectly chosen.

and therefore provides two methods for the evaluation of ϕ_C. With ϕ_C known, ϕ_{BC} may be obtained from the relationship:

$$\phi_{BC} = K_{AB}\phi_C \qquad (11)$$

and ϕ_{AC} and ϕ_{ABC} can be evaluated by the successive application of

$$\phi_{AC} = K_{BA}\phi_C \qquad (12)$$

$$\phi_{ABC} = K_B\phi_{AC} \qquad (13)$$

Values of ϕ_C, ϕ_{BC}, ϕ_{AC} and ϕ_{ABC} obtained by using eqns. (9)–(13) are given in Table 4.

Eqn. (2) can also be expressed either in terms of α and β or in terms of A_t and B_t (primed constants) by equations with the form:

$$\frac{E_0}{v} = \phi_4 C^2 + \phi_5 C + \frac{\phi_6}{C} + \phi_7 \qquad (14)$$

The detailed composition of the ϕ constants is given in Table 3. Eqn. (14) shows that ϕ_4 can be obtained as the limiting slope at high C from the relations $v^{-1} = f(C^2)$ (Figs. 1*b*, 2*b* and 3*b*). As Table 3 shows, ϕ_{AB} may be evaluated directly from ϕ_4: the values so

Table 3. *Physical significance and evaluation of ϕ terms at constant concentrations of complexed phosphoenolpyruvate and ADP (α and β) and total phosphoenolpyruvate and ADP (A_t and B_t)*

ϕ values were calculated by assuming that pyruvate kinase has a mol.wt. of 237000 (Warner, 1958) and that the enzyme has two active sites/molecule (Mildvan & Cohn, 1965).

Terms	ϕ values with α and β kept constant
$\phi_4 = \frac{\phi_{AB}}{K_1 K_2 \alpha\beta}$	$1.24\,M^{-2}\cdot min$ (Mn^{2+}, Fig. 1*b*) $3.94\,M^{-2}\cdot min$ (Ni^{2+}, Fig. 2*b*) $0.60\,M^{-2}\cdot min$ (Co^{2+}, Fig. 3*b*)
$\phi_5 = \frac{\phi_{ABC}}{K_1 K_2 \alpha\beta} + \frac{\phi_A}{K_1 \alpha} + \frac{\phi_B}{K_2 \beta}$	
$\phi_6 = \phi_C$	
$\phi_7 = \frac{\phi_{AC}}{K_1 \alpha} + \frac{\phi_{BC}}{K_2 \beta} + \phi_0$	
	ϕ values with A_t and B_t kept constant
$\phi'_4 = \frac{\phi_{AB}}{K_1 K_2 A_t B_t}$	$0.84\,M^{-2}\cdot min$ (Mn^{2+}, Fig. 1*b*) $2.83\,M^{-2}\cdot min$ (Ni^{2+}, Fig. 2*b*) $0.25\,M^{-2}\cdot min$ (Co^{2+}, Fig. 3*b*)
$\phi'_5 = \frac{\phi_{ABC}}{K_1 K_2 A_t B_t} + \frac{\phi_{AB}(K_1+K_2)}{K_1 K_2 A_t B_t} + \frac{\phi_A}{K_1 A_t} + \frac{\phi_B}{K_2 B_t}$	
$\phi'_6 = \frac{\phi_{ABC}}{A_t B_t} + \frac{\phi_{AC}}{A_t} + \frac{\phi_{BC}}{B_t} + \phi_C$	
$\phi'_7 = \frac{\phi_{ABC}(K_1+K_2)}{K_1 K_2 A_t B_t} + \frac{\phi_{AB}}{A_t B_t} + \frac{\phi_{AC}}{K_1 A_t} + \frac{\phi_{BC}}{K_2 B_t} + \frac{\phi_A}{A_t} + \frac{\phi_B}{B_t} + \phi_0$	

obtained for the several ions are recorded in Table 4. Finally, by using the definitions of ϕ_1 and ϕ'_1, given in Table 2, and the known values of ϕ_{AB}, values of ϕ_A and ϕ_B respectively may be calculated. These values are also given in Table 4.

Significance of kinetic constants

In the preceding section values have been ascribed to all the ϕ constants of eqn. (1) except for ϕ_0. The error of the estimates has not been calculated but obviously must be greater than that ascribed to similar constants by Ainsworth & Macfarlane (1973), not only because of the limited data used but also because the calculations depend in part on assumed values of dissociation constants (eqns. 9–13). Nonetheless, the real values obtained support the validity of the postulated mechanism: for example an equilibrium random-order mechanism in which the bivalent cation does not bind at the active site except in complex with A or B does not predict the constants ϕ_{AB} and ϕ_C which we have shown to be present.

Several constants are available for comparison with the cation dissociation constants given in Table 4. Thus the value of K_C given for Mn^{2+} is several orders of magnitude greater than that provided by measurements of the proton relaxation rate and electron spin resonance made by Mildvan & Cohn (1965). A similar discrepancy is observed between values of K_C for Mg^{2+} provided by kinetic studies (Ainsworth & Macfarlane, 1973) and difference spectroscopy (Kayne & Suelter, 1965). A conclusion that the lower-affinity site observed in kinetic studies is the one essential to catalysis is supported by the observation of Reed & Cohn (1973) that the tight binding of Mn^{2+} by muscle pyruvate kinase is unaffected by nucleotides. Such a conclusion does, however, weaken the validity of our assumption that K_A, K_B, K_{AB} and K_{BA} are independent of the nature of the bivalent metal ion C, for if the bivalent cation is able to bind tightly at ancillary sites of the enzyme, it is at least arguable that the binding constants of the active site will be affected thereby.

With this reservation in mind, note that there is an approximate correlation between K_1 and K_{AC} and between K_2 and K_{BC}, as the identity of C is altered, in terms of both the relative and absolute magnitude of the constants. In contrast, K_{CA} and K_{CB} are generally much smaller than K_1 and K_2 respectively, and there is no longer any correlation in their relative values as C alters. It appears from this comparison that the binding of C in triple complexes of the enzyme depends far more on

Table 4. *Constants for the uninhibited reaction catalysed by muscle pyruvate kinase activated by Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}*

Kinetic constants were calculated by assuming that pyruvate kinase has a mol.wt. of 237000 (Warner, 1958) and that the enzyme has two active sites/molecule (Mildvan & Cohn, 1965). Kinetic constants for the Mg^{2+}-activated enzyme have been taken from Ainsworth & Macfarlane (1973) and the above values for K_B, K_{AB} and K_{BA} were used in calculating several of the kinetic constants for each transition-metal-activated enzyme reaction. Of the two values for ϕ_{AB}, the upper one was obtained at constant A_t and B_t and the lower one was obtained at constant α and β.

Kinetic constants	Metal ion ... Mg^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}
ϕ_A (M·min)	4.64×10^{-10}	2.19×10^{-10}	1.41×10^{-10}	7.71×10^{-10}
ϕ_B (M·min)	6.54×10^{-10}	2.25×10^{-10}	7.95×10^{-11}	4.01×10^{-10}
ϕ_C (M·min)	1.85×10^{-8}	7.97×10^{-10}	5.24×10^{-10}	2.09×10^{-9}
ϕ_{AB} (M^2·min)	3.58×10^{-14}	3.1×10^{-15}	5.7×10^{-15}	4.6×10^{-14}
		2.1×10^{-15}	4.3×10^{-15}	1.2×10^{-14}
ϕ_{AC} (M^2·min)	2.62×10^{-12}	1.11×10^{-13}	7.44×10^{-14}	2.97×10^{-13}
ϕ_{BC} (M^2·min)	2.66×10^{-11}	1.12×10^{-12}	7.55×10^{-13}	3.01×10^{-12}
ϕ_{ABC} (M^3·min)	2.12×10^{-15}	8.98×10^{-17}	6.02×10^{-17}	2.40×10^{-16}
Dissociation and equilibrium constants (M)				
$\phi_{ABC}/\phi_{BC}=K_A$	7.97×10^{-5}			
$\phi_{ABC}/\phi_{AC}=K_B$	8.09×10^{-4}			
$\phi_{ABC}/\phi_{AB}=K_C$	5.92×10^{-2}	3.45×10^{-2}	1.20×10^{-2}	8.28×10^{-3}
$\phi_{BC}/\phi_C=K_{AB}$	1.44×10^{-3}			
$\phi_{AC}/\phi_C=K_{BA}$	1.42×10^{-4}			
$\phi_{BC}/\phi_B=K_{AC}$	4.07×10^{-2}	4.98×10^{-3}	9.50×10^{-3}	7.51×10^{-3}
$\phi_{AB}/\phi_B=K_{CA}$	5.47×10^{-5}	1.16×10^{-5}	6.29×10^{-5}	7.23×10^{-5}
$\phi_{AC}/\phi_A=K_{BC}$	5.64×10^{-3}	5.07×10^{-4}	5.28×10^{-4}	3.85×10^{-4}
$\phi_{AB}/\phi_A=K_{CB}$	7.71×10^{-5}	1.19×10^{-5}	3.55×10^{-5}	3.76×10^{-5}

Table 5. *Interactions between substrates at the active site of muscle pyruvate kinase*

For details see the text.

Metal ion	A–C interaction $\frac{K_A}{K_{CA}}=\frac{K_C}{K_{AC}}$		B–C interaction $\frac{K_B}{K_{CB}}=\frac{K_C}{K_{BC}}$	
Mg^{2+}	1.46	1.45	10.49	10.50
Mn^{2+}	6.87	6.93	67.98	68.05
Co^{2+}	1.27	1.26	22.79	22.73
Ni^{2+}	1.10	1.10	21.52	21.51

charge interactions with the second substrate than does the binding of the second substrate itself.

A similar conclusion may be drawn from the ratios $K_C/K_{AC}\equiv K_A/K_{CA}$ and $K_C/K_{BC}\equiv K_B/K_{CB}$ whose values are given in Table 5. A value of 10, say, for the first ratio indicates that the prior binding of A or C by the enzyme decreases the constant for the dissociation of C or A from the enzyme complex by a factor of 10, that is, the binding of the second substrate is enhanced. Examination of Table 5 shows that all the bivalent cations enhance the binding of the second substrate but to different extents, which depend on the identities of both substrates. It is evident from this that enhancement does not depend solely on charge attraction, as was suggested by Ainsworth & Macfarlane (1973). In this connexion, it is noteworthy that the marked difference in the enhancing influence of Mg^{2+} and Mn^{2+} (Table 5) is reflected by equally marked differences in the fluorescence properties of the Mg^{2+} and Mn^{2+} ternary complexes with phosphoenolpyruvate and rabbit muscle pyruvate kinase (Kayne & Price, 1972).

Turning now to more qualitative aspects of our results, note that the differences in the influence of the bivalent cations, referred to above, have been observed in solutions with comparable ionic strengths. We can therefore, in agreement with Wimhurst & Manchester (1972), set aside ionic-strength effects as the possible cause of the inhibitions observed at high concentrations of bivalent cations.

A previous study by Bygrave (1966) has established that the concentration of bivalent cation at the maximum of the relationship $v=f(C)$ decreases in the order $[Mg^{2+}]$, $[Mn^{2+}]$, $[Co^{2+}]$ when A_t and B_t are kept constant. Bygrave (1966) also noted that both the velocity at the maximum and the inhibitory effect of the cation decreased in the reverse order. Fig. 4 shows the same orders for the effects in solutions where α and B were kept constant. In addition, by using the constants given in Table 4, we have calculated the values of C_m that arise when $A_t=B_t=$ 0.1mM; these are: Mg^{2+} 6.3mM, Mn^{2+} 1.2mM, Co^{2+} 1.3mM and Ni^{2+} 0.8mM. This result is consistent with Bygrave's (1966) finding. We have

also shown, by calculation, that C_m decreases as A_t or B_t decreases, in agreement with findings of Wimhurst & Manchester (1972).

It has already been noted that the maxima in the plots $v = f(C)$ are particularly broad when C is Mg^{2+} and when either α and B or A and β are kept constant (Fig. 4 and Ainsworth & Macfarlane, 1973). This is a potentially misleading situation, for the apparent independence of the velocity on C over a substantial range could lead to the conclusion that the non-varied substrates are the true substrates of the enzyme. The conclusion has been avoided by extending the range of C and the combinations of hypothetical substrates kept constant, but it is especially valuable that the other activating cations point the error by displaying much sharper maxima.

Conclusion

The results above described show that the kinetics of rabbit muscle pyruvate kinase can be satisfactorily explained by assuming that its substrates are free phosphoenolpyruvate, free ADP and the activating bivalent cation: in addition, it is clear that a mechanism which prevents the binding of the essential bivalent cation at the active site except in complex with one of the other substrates cannot apply to this enzyme. In terms of this picture, the inhibitions that occur with increasing cation concentration are accounted for by substrate-level equilibria.

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