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THE EFFECT OF MONOVALENT AND DIVALENT CATIONS ON THE ACTIVITY OF *STREPTOCOCCUS LACTIS* C10 PYRUVATE KINASE

VAUGHAN L. CROW * and GRAHAM G. PRITCHARD

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North (New Zealand)

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Summary

The pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from *Streptococcus lactis* C10 had an obligatory requirement for both a monovalent cation and a divalent cation. NH_4^+ and K^+ activated the enzyme in a sigmoidal manner (n_H = 1.55) at similar concentrations, whereas Na^+ and Li^+ could only weakly activate the enzyme. Of eight divalent cations studied, only three (Co^{2+}, Mg^{2+} and Mn^{2+}) activated the enzyme. The remaining five divalent cations (Cu^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+} and Ba^{2+}) inhibited the Mg^{2+} activated enzyme to varying degrees. (Cu^{2+} completely inhibited activity at 0.1 mM while Ba^{2+}, the least potent inhibitor, caused 50% inhibition at 3.2 mM). In the presence of 1 mM fructose 1,6-diphosphate (Fru-1,6-P_2) the enzyme showed a different kinetic response to each of the three activating divalent cations. For Co^{2+}, Mn^{2+} and Mg^{2+} the Hill interaction coefficients (n_H) were 1.6, 1.7 and 2.3 respectively and the respective divalent cation concentrations required for 50% maximum activity were 0.9, 0.46 and 0.9 mM. Only with Mn^{2+} as the divalent cation was there significant activity in the absence of Fru-1,6-P_2. When Mn^{2+} replaced Mg^{2+}, the Fru-1,6-P_2 activation changed from sigmoidal (n_H = 2.0) to hyperbolic (n_H = 1.0) kinetics and the Fru-1,6-P_2 concentration required for 50% maximum activity decreased from 0.35 to 0.015 mM. The cooperativity of phospho*enol*pyruvate binding increased (n_H 1.2 to 1.8) and the value of the phospho*enol*pyruvate concentration giving half maximal velocity decreased (0.18 to 0.015 mM phospho*enol*pyruvate) when Mg^{2+} was replaced by Mn^{2+} in the presence of 1 mM Fru-1,6-P_2. The kinetic response to ADP was not altered significantly when Mn^{2+} was substituted for Mg^{2+}. The effects of pH on the

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* Present address: Laboratory of Microbiolgy and Immunology, National Institute of Dental Research, NIH, Bethesda, Md. 20014, U.S.A.

Abbreviations $FDP_{0.5V}$, fructose 1,6-diphosphate concentration giving half-maximal velocity; $PEP_{0.5V}$, phospho*enol*pyruvate concentration giving half-maximal velocity.

binding of phospho*enol*pyruvate and Fru-1,6-P_2 were different depending on whether Mg^{2+} or Mn^{2+} was the divalent cation.

Introduction

Factors affecting the activity of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from lactic streptococci have been reported for *Streptococcus lactis* ML3 by Collins and Thomas [1] and for *S. lactis* C10 by Crow and Pritchard [2]. The pyruvate kinases from these two *S. lactis* strains were activated by fructose-1,6-diphosphate (Fru-1,6-P_2) with the Fru-1,6-P_2 showing sigmoidal activation independent of the adenosine-5′-diphosphate (ADP) or phospho*enol*pyruvate concentration. Recently, Thomas [3] has shown that there is a broad activator specificity for pyruvate kinases from lactic streptococci, including *S. lactis* C10, and that several phosphorylated carbohydrates and glycolytic intermediates were better activators than Fru-1,6-P_2. As well as Fru-1,6-P_2, both potassium and magnesium were found to be required for pyruvate kinase activity [1,2]. Work on other microbial pyruvate kinases has shown that the type of cation, especially the divalent cation, can have a marked effect on kinetic and regulatory properties [4—6].

The purpose of this investigation was to determine the monovalent and divalent cation requirement of the purified pyruvate kinase from *S. lactis* C10 in some detail and to study the potential importance of cations in regulation of enzyme activity.

Materials and Methods

Chemicals and reagents. The monovalent and divalent metal chloride salts were obtained from British Drug Houses, Poole, U.K. as the AnalaR grade. All other chemicals and reagents used in this study have been described previously [2].

Pyruvate kinase assay. The pyruvate kinase from *S. lactis* C10 was purified and assayed as described by Crow and Pritchard [2]. One unit of enzyme activity is defined as that amount of enzyme which gives a rate of phospho*enol*-pyruvate utilization of 1 μmol per minute under the assay conditions described. The purified enzyme was stored in a stabilizing buffer (0.005 M phosphate buffer pH 7.0, 50% glycerol (w/v), 0.005 M $MgCl_2$ and 0.1% 2-mercaptoethanol) and prior to assays, was diluted at least 1 : 20 with 20% glycerol/deionised distilled water solution at 0° C and dialysed against the diluent for at least 2 h. This dialysed and diluted enzyme was stable for at least 10 h at 0° C, but was not used for periods longer than 10 h as activity started to decrease after 24 h. The dilution and dialysis ensured that the concentration of phosphate and magnesium ions carried over from the stabilising buffer was insufficient to affect activity. The essentially homogeneous enzyme [2] was used in this study. The specific activities of the preparations after dialysis against 20% glycerol in deionised distilled water were 75—85 units/mg protein.

Results

Effect of monovalent cations on activity

The monovalent cations (NH_4^+, Na^+, Li^+, and K^+), as their chloride salts, were tested for their effect on pyruvate kinase activity (Fig. 1). The maximum activity obtained at saturating concentrations of monovalent cations was similar for both K^+ and NH_4^+ (Fig. 1a). At unsaturating levels of NH_4^+, the activity was slightly higher than the activity obtained with the same K^+ concentrations. In contrast, Na^+ and Li^+ had only a weakly activating effect on the activity (Fig. 1b), as the maximum activity obtained with Na^+ or Li^+ was only 7—8% of that obtained at saturating concentrations of NH_4^+ or K^+. Both K^+ and NH_4^+ showed cooperative binding at concentrations greater than 3 mM; the n_H value of 1.55 was the same for both cations (Fig. 1c).

Effect of divalent cations on activity

Of the divalent cations tested (Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+} and Ba^{2+}) as their chloride salts, only Mg^{2+}, Mn^{2+} and Co^{2+} were able to activate the

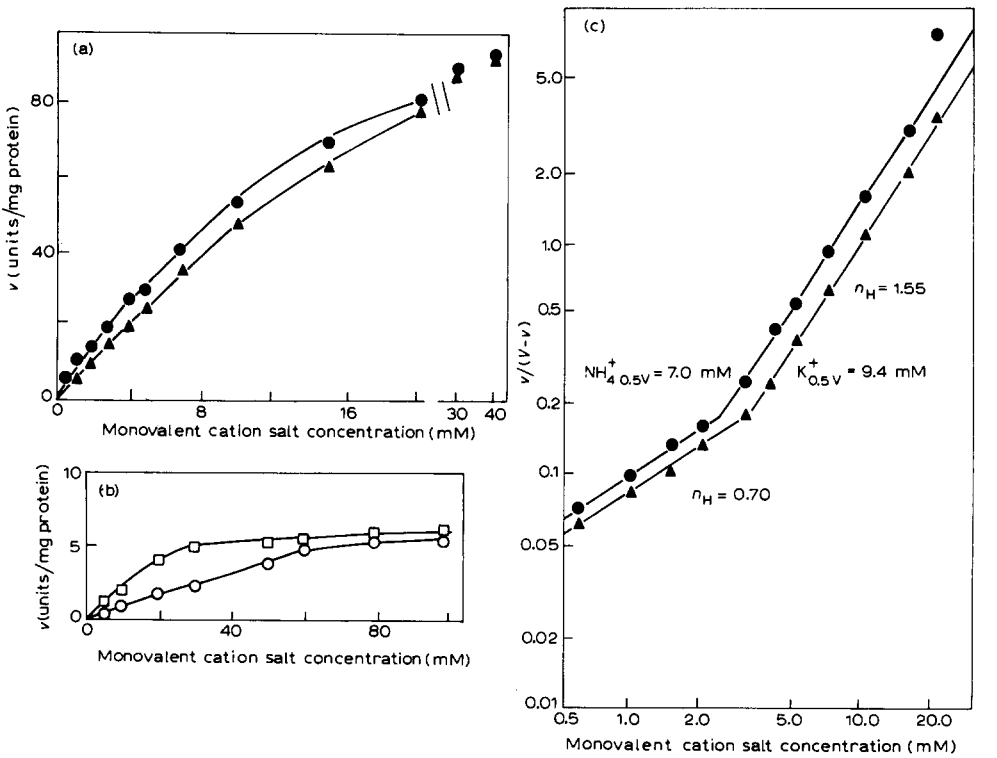

Fig. 1. The relationship between pyruvate kinase activity and different monovalent cations. (a) Reaction velocity as a function of NH_4Cl (●) and KCl (▲) concentrations. (b) Reaction velocity as a function of LiCl (□) and NaCl (○) concentrations. (c) The same data from (a) expressed as Hill plots of log $v/(V - v)$ versus log NH_4Cl (●) and log KCl (▲) concentrations. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 3.3 mM ADP, 1 mM phospho*enol*-pyruvate, 1 mM Fru-1,6-P_2, 3.3 mM $MgCl_2$, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 3 μg protein, monovalent cations at concentrations shown.

enzyme (using the standard assay system [2], minus $MgCl_2$). The relationship between pyruvate kinase activity and each of the three activating divalent cations is shown in Fig. 2a. At saturating concentrations of Mg^{2+} or Mn^{2+} the maximum activity was very similar, whereas the maximum activity obtained under the same conditions with Co^{2+} as the divalent cation was significantly less. With Mg^{2+} or Co^{2+} as the divalent cation, at concentrations ranging from 0.1 to 20 mM, no activity was found in the absence of Fru-1,6-P_2. However, with Mn^{2+}. considerable activity was found in the absence of Fru-1,6-P_2 (Fig. 2a). The maximum activity achieved with a saturating concentration of Mn^{2+} in the absence of Fru-1,6-P_2 was 55—60% of the maximum activity achieved if 1 mM Fru-1,6-P_2 was present.

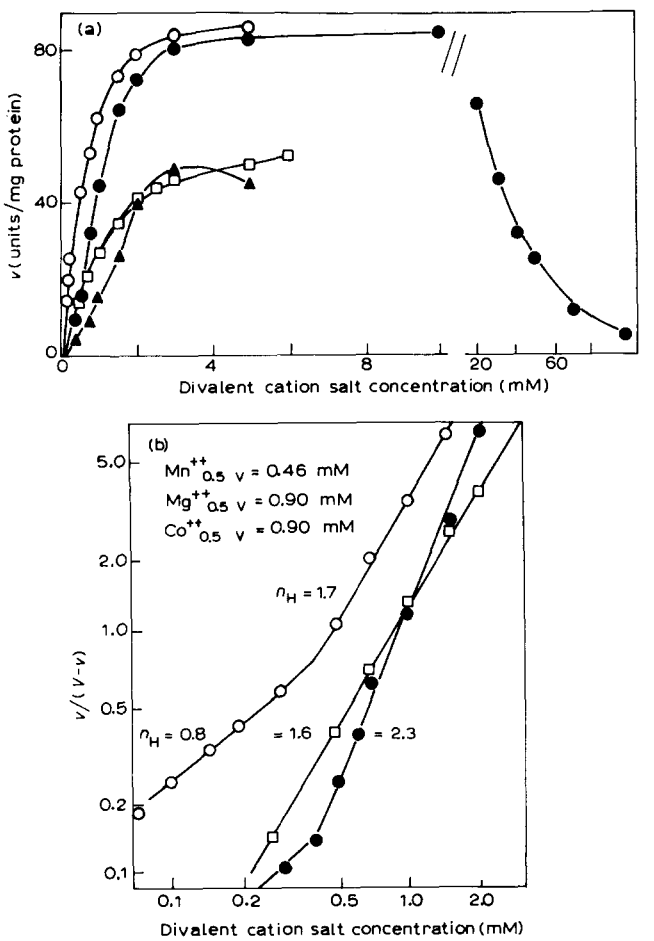

Fig. 2. The relationship between pyruvate kinase activity and different divalent cations. (a) Reaction velocity as a function of $MnCl_2$ (○), $MgCl_2$ (●), $CoCl_2$ (□), (all with Fru-1,6-P_2 present) and $MnCl_2$ (▲) (no Fru-1,6-P_2 present in reaction mixture). (b) The same data from (a) (except for $MnCl_2$ with no Fru-1,6-P_2 present) expressed as Hill plots of log $v/(V - v)$ versus log divalent cation salt concentration. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 3.3 mM ADP, 1 mM phospho*enol*pyruvate, 1 mM Fru-1,6-P_2 (except as noted). 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 3 μg protein, divalent cations at concentrations shown.

Hill plots of the data in Fig. 2a indicate cooperative binding of all three cations at concentrations above 0.5 mM (Fig. 2b). However, the strength of the cooperative binding as indicated by the n_H value was much higher for Mg^{2+} (n_H = 2.3) than for Mn^{2+} (n_H = 1.7) and Co^{2+} (n_H = 1.6). On the other hand the concentration of Mn^{2+} required to give half maximal activity (0.46 mM) was only half that for Co^{2+} and Mg^{2+} (0.9 mM).

$MgCl_2$ at concentrations greater than 10 mM inhibited enzyme activity (Fig. 2a). From a Hill plot (not shown) of the data the $Mg^{2+}_{0.5\,I}$ (Mg^{2+} concentration causing 50% inhibition of activity) was found to be 36 mM and the n_H value for Mg^{2+} binding over the inhibitor range was —2.5.

The chloride salts of Cu^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+} and Ba^{2+} were all unable to activate the enzyme using the standard assay system (minus $MgCl_2$) at concentrations ranging from 0.1 to 20 mM. However if the standard assay system 2 was used (i.e. 13.3 mM KCl/3.3 mM $MgCl_2$) and the concentrations of these non-activating salts were progressively increased from 0.1 mM, then inhibition occurred (Fig. 3a). Cu^{2+} completely inhibited activity at a concentration of 0.1 mM, Zn^{2+} was the next most potent inhibitor, followed by Ca^{2+}, then Ni^{2+} with Ba^{2+} inhibiting to the least extent. Hill plots of the data for Zn^{2+}, Ca^{2+}, Ni^{2+} and Ba^{2+} inhibition are shown in Fig. 3b. For all four divalent cations the Hill interaction coefficient value is —1.55 to —1.60. The concentration of the divalent cation required for 50% inhibition ranged from 0.07 mM Zn^{2+} to 3.2

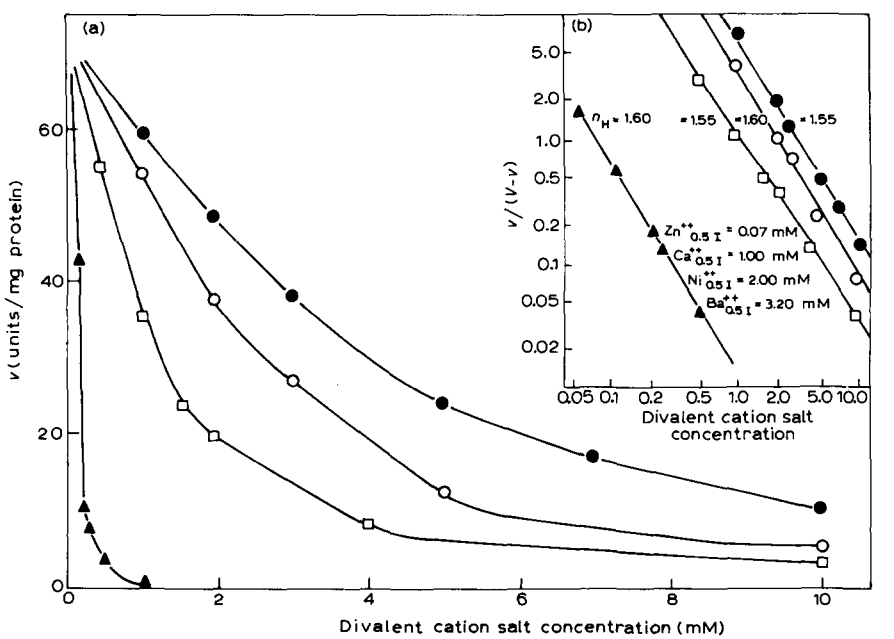

Fig. 3. The divalent cation inhibition of pyruvate kinase activity. (a) Reaction velocity as a function of $ZnCl_2$ (▲), $CaCl_2$ (□), $NiCl_2$ (○), and $BaCl_2$ (●), concentrations. (b) The same data from (a) expressed as Hill plots of log $v/(V - v)$ versus log divalent cation salt concentration. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 3.3 mM ADP, 1 mM phospho*enol*pyruvate, 1 mM Fru-1,6-P_2, 13.3 mM KCl, 3.3 mM $MgCl_2$, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 2.5 μg protein, divalent cations at concentrations shown.

mM Ba^{2+}. These values are low when compared with the corresponding value for inhibition by Mg^{2+} (36 mM).

Comparison of response to varying Fru-1,6-P_2 and substrate concentrations with either Mn^{2+} or Mg^{2+} as the divalent cation

Fig. 4 shows the results obtained when the Fru-1,6-P_2 concentration is varied in the presence of either $MgCl_2$ or $MnCl_2$. In the presence of $MnCl_2$, Fru-1,6-P_2 activates the enzyme in a hyperbolic manner (from Hill plot, not shown, n_H = 1.0) whereas with $MgCl_2$, Fru-1,6-P_2 activates in a sigmoidal manner (n_H = 2.0). The $FDP_{0.5V}$ value (0.015 mM) with Mn^{2+} as the activating divalent cation is low compared to the value (0.35 mM Fru-1,6-P_2) obtained with Mg^{2+} as the cation.

The Hill plots in Fig. 5 were obtained by varying the phospho*enol*pyruvate concentration in the presence of $MgCl_2$ or $MnCl_2$. With $MnCl_2$, the response to varying phospho*enol*pyruvate was examined in the presence and absence of Fru-1,6-P_2. The $PEP_{0.5V}$ and n_H values obtained from the Hill plots (Fig. 5) are summarised in Table I. With 1 mM Fru-1,6-P_2 present in the assay system, replacement of Mg^{2+} by Mn^{2+} increased the n_H value from 1.2 to 1.8 and decreased the $PEP_{0.05V}$ value from 0.18 to 0.015 mM. In the absence of Fru-1,6-P_2, with Mn^{2+} as the cation, the $PEP_{0.5V}$ value is 0.09 mM, which is half the value obtained with Mg^{2+} in the presence of Fru-1,6-P_2. With Fru-1,6-P_2 present, the V with either Mn^{2+} or Mg^{2+} is essentially the same, whereas the V obtained with Mn^{2+} in the absence of Fru-1,6-P_2 was significantly lower.

Fig. 6 shows the activity as a function of ADP concentration with different cation and Fru-1,6-P_2 combinations. For the three different combinations of cation/Fru-1,6-P_2, maximum activity was achieved between 2—4 mM ADP with activity dropping off at higher ADP concentrations. Inhibition by high concentrations of ADP was rather less with Mn^{2+} as the divalent cation than

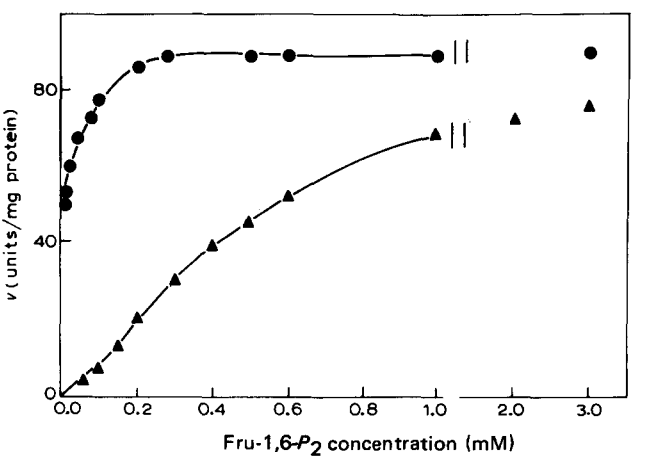

Fig. 4. The relationship between pyruvate kinase activity and Fru-1,6-P_2 concentration with 3.0 mM $MgCl_2$ (▲) and 3.0 mM $MnCl_2$ (●) as the essential divalent cations. For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 3.3 mM ADP, 1 mM phospho*enol*pyruvate, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 2.5 μg protein, Fru-1,6-P_2 at concentrations shown.

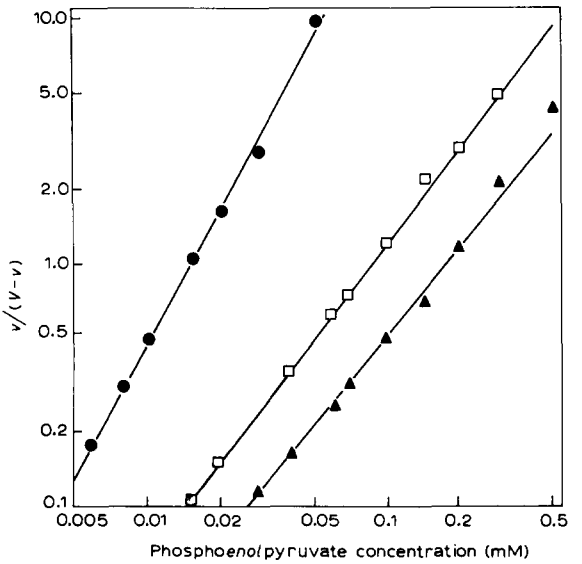

Fig. 5. Hill plots of log $v/(V - v)$ versus log phospho*enol*pyruvate concentration with 3.0 mM $MgCl_2$ + 1 mM Fru-1,6-P_2 (▲), 3.0 mM $MnCl_2$ + 1 mM Fru-1,6-P_2 (●) and 3.0 mM $MnCl_2$ with no Fru-1,6-P_2 present (□). For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 3.3 mM ADP, 13.3 mM KCl, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 2.5 μg protein, phospho*enol*pyruvate at concentrations shown.

with Mg^{2+}. For Mn^{2+} and Mg^{2+} in the presence of Fru-1,6-P_2, the Hill plots (not shown) were essentially identical. ADP showed no homotrophic interaction (n_H = 1.0) and the $ADP_{0.5V}$ value was 1.1 for both cations. However, when using Mn^{2+} in the absence of Fru-1,6-P_2 there was significant cooperative interaction of the ADP with the enzyme (n_H = 1.8). This is probably due to the absence of Fru-1,6-P_2 since it was previously shown [2] that decreasing the Fru-1,6-P_2 concentration increases the cooperative binding of ADP to the enzyme.

The effect of pH on the kinetic properties

The data so far described have all been determined at pH 7.5 (the pH optimum of the enzyme). The effect of the pH on phospho*enol*pyruvate and Fru-1,6-P_2 binding was also studied at two other pH values, 6.4 and 8.75 with Mn^{2+} and Mg^{2+} as the essential divalent cations. The results are shown in Tables II and III.

TABLE I

RESPONSE TO VARYING PHOSPHO*ENOL*PYRUVATE WITH Mg^{2+} OR Mn^{2+}

Values obtained from Hill plots shown in Fig. 5.

	n_H	$PEP_{0.5\,V}$ (mM)	V (units/mg protein)
3.0 mM $MgCl_2$ + 1.0 mM Fru-1,6-P_2	1.2	0.18	67.0
3.0 mM $MnCl_2$ + 1.0 mM Fru-1,6-P_2	1.8	0.015	66.0
3.0 mM $MnCl_2$; no Fru-1,6-P_2	1.3	0.09	48.0

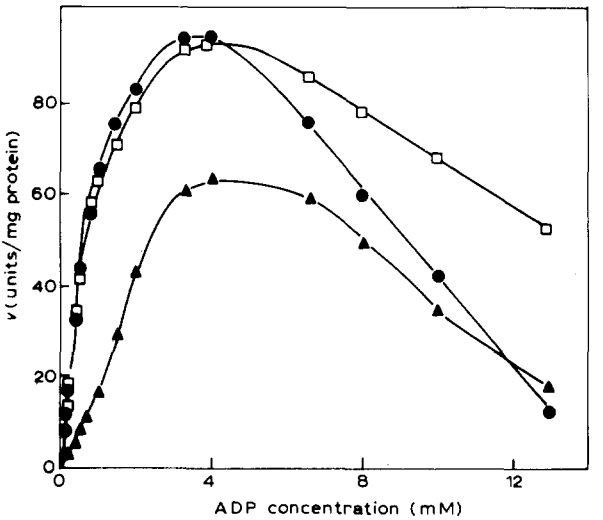

Fig. 6. The relationship between pyruvate kinase activity and ADP concentration with 3.0 mM $MnCl_2$ + 1 mM Fru-1,6-P_2 (□), 3.0 mM $MnCl_2$ with no Fru-1,6-P_2 present (▲) and 3.0 mM $MgCl_2$ + 1 mM Fru-1,6-P_2 (●). For each assay the reaction mixture contained (in a total volume of 3 ml): 80 mM triethanolamine/HCl buffer (pH 7.5), 13.3 mM KCl, 1.0 mM phospho*enol*pyruvate, 0.167 mM NADH, 20 units lactate dehydrogenase, 0.1 ml diluted pyruvate kinase containing 3 μg protein, ADP at concentrations shown.

The affinity of pyruvate kinase for phospho*enol*pyruvate (Table II) with Mg^{2+} as the divalent cation was highest at pH 8.75. However, with Mn^{2+} as the divalent cation, the affinity of the enzyme for phospho*enol*pyruvate was lowest at pH 8.75. The cooperativity of phospho*enol*pyruvate binding seen at pH 6.4 and 7.5 in the presence of Mn^{2+} was no longer evident at pH 8.75.

With Mg^{2+} as the divalent cation (Table III) the $FDP_{0.5V}$ value was nearly the same at pH 6.4 and 7.5 and showed a two-fold increase at pH 8.75. There is a

TABLE II

EFFECT OF pH ON PHOSPHO*ENOL*PYRUVATE ACTIVATION

For each pH and in the presence of either 3.3 mM $MnCl_2$ or 3.3 mM $MgCl_2$, with 1 mM Fru-1,6-P_2 present, the phospho*enol*pyruvate concentration was varied to give at least eight usable points for the respective Hill and Lineweaver-Burk plots. Standard assay conditions were 80 mM triethanolamine/HCl buffer; 3.3 mM ADP; 13.3 mM KCl; 2.5 μg purified pyruvate kinase.

Conditions		Lineweaver-Burk plot		Hill plot
pH	Divalent cation (3.3 mM)	K_m (mM phospho*enol*pyruvate)	V (units/mg protein)	n_H
6.4	$MgCl_2$	0.40	45.0	1.0
6.4	$MnCl_2$	0.02 *	42.0	1.7
7.5	$MgCl_2$	0.18	73.0	1.0
7.5	$MnCl_2$	0.02 *	77.0	1.8
8.75	$MgCl_2$	0.13	36.0	1.0
8.75	$MnCl_2$	0.13	36.0	1.1

* Values determined as $PEP_{0.5V}$ values from Hill plots.

TABLE III

EFFECT OF pH ON FRUCTOSE 1,6-DIPHOSPHATE ACTIVATION

For each pH and in the presence of either 3.3 mM $MgCl_2$ or 3.3 mM $MnCl_2$ the Fru-1,6-P_2 concentration was varied to give at least eight points for the respective Hill and Lineweaver-Burk plots. Standard assay conditions were 80 mM triethanolamine/HCl buffer; 3.3 mM ADP; 1 mM phospho*enol*pyruvate; 13.3 mM KCl; 2.5 μg purified pyruvate kinase.

Conditions		Hill plot		Lineweaver-Burk plot	Activity
pH	Divalent cation (3.3 mM)	n_H	$FDP_{0.5\,V}$ (mM Fru-1,6-P_2)	V (units/mg protein)	(in absence of Fru-1,6-P_2) (units/mg protein)
6.4	$MgCl_2$	1.9	0.18	42.0	0.0
6.4	$MnCl_2$	1.0	0.0065	41.0	24.0
7.5	$MgCl_2$	1.9	0.20	74.0	0.0
7.5	$MnCl_2$	1.0	0.02	74.0	30.0
8.75	$MgCl_2$	1.0	0.45	30.0	0.0
8.75	$MnCl_2$	1.0	0.23	24.0	8.4

relatively much greater increase in the $FDP_{0.5V}$ values as the pH was increased from 7.5 to 8.75 when Mn^{2+} was the divalent cation. The cooperative interaction of Fru-1,6-P_2 binding, evident only with Mg^{2+}, was affected by pH, since at pH 6.4 and 7.5 the n_H value was 1.9, while at pH 8.75 the n_H value was 1.0. At the three pH values the only activity found in the absence of Fru-1,6-P_2 was with Mn^{2+} as the divalent cation.

Discussion

It has been shown that a number of monovalent and divalent cations will activate or inhibit the activity of the pyruvate kinase from *S. lactis* C10, usually in a sigmoidal manner, when saturating Fru-1,6-P_2, ADP and phospho*enol*pyruvate concentrations are present.

Of the four monovalent cations studied, NH_4^+ and K^+ activate the enzyme in a sigmoidal manner (n_H = 1.55) to a similar extent. Na^+ and Li^+ can only weakly activate the enzyme. A requirement for K^+ is common to most pyruvate kinases although some bacterial kinases do not have an obligatory requirement for a monovalent cation, for example the Fru-1,6-P_2-activated pyruvate kinase from *E. coli* [6] and the pyruvate kinases from *Acetobacter xylinum* [7] and *Brevibacterium flavum* [8].

Only three (Mg^{2+}, Mn^{2+} and Co^{2+}) of the eight divalent cations studied could activate the enzyme. Of these three, only with Mn^{2+} as the divalent cation was activity detectable in the absence of Fru-1,6-P_2. The mechanism of this activation by Mn^{2+} alone is not clear. While Mn^{2+} enhances the binding of the activator Fru-1,6-P_2 to a greater extent than does Mg^{2+}, it apparently also acts by a mechanism which is independent of Fru-1,6-P_2, possibly by enhancing the binding of phospho*enol*pyruvate. Both the affinity for phospho*enol*pyruvate and the cooperativity of phospho*enol*pyruvate binding in the presence of a saturating concentration of Fru-1,6-P_2 are much higher if Mn^{2+} is the divalent

cation rather than Mg^{2+}. The pyruvate kinases from *Bacillus licheniformis* [4] and from rabbit muscle [9] which are not activated by Fru-1,6-P_2 were shown to have higher affinities for phospho*enol*pyruvate, but not ADP, when Mn^{2+} replaced Mg^{2+} as the divalent cation. This is also true for the Fru-1,6-P_2-activated pyruvate kinases from *Mucor rouxii* [5] and *E. coli* [8]. However, with the *M. rouxii* enzyme, Fru-1,6-P_2 did not activate the enzyme in the presence of Mn^{2+}. By contrast, the activity of the *S. lactis* pyruvate kinase in the presence of a saturating Mn^{2+} concentration is further increased by addition of Fru-1,6-P_2. The *S. lactis* pyruvate kinase is also different from the Fru-1,6-P_2-activated *E. coli* enzyme in that, for the latter enzyme, V in the presence of Fru-1,6-P_2 and Mn^{2+} is only half of the V when Mg^{2+} is the divalent cation. Also cooperative binding of the two cations by the *E. coli* pyruvate kinase is only evident in the absence of Fru-1,6-P_2.

Yamada and Carlsson [10] have recently described the properties of pyruvate kinase from *Streptococcus mutans* JC-2. This enzyme has an absolute and specific requirement for glucose 6-phosphate which cannot be replaced by 0.4 mM Fru-1,6-P_2. The activity in the presence of 10 mM Mn^{2+} is only 60% of that obtained when 10 mM Mg^{2+} is used as the divalent cation.

Thus the interaction of divalent cations and activator with the enzyme shows a distinctive pattern for each of the different pyruvate kinases which have been investigated, and there is clearly a need for further study to establish the underlying reasons for these differences. The activation of rabbit muscle pyruvate kinase [11] by thallium was dependent to a small degree on the concentration of the divalent cation. The interrelationship between monovalent and divalent cations and the possible effects of activator and substrate concentrations on cation activation needs further investigation with the *S. lactis* enzyme.

The availability of Mn^{2+} in vivo could clearly have important regulatory consequences particularly at low Fru-1,6-P_2 concentrations which may prevail when carbohydrate is the limiting nutrient [12].

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