Human Pyruvate Kinases. Role of the Divalent Cation in the Catalytic Mechanism of the Red Cell Enzyme†

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ABSTRACT: The kinetic properties of human erythrocyte pyruvate kinase vary depending on whether Mg^{2+} or Mn^{2+} is used as the essential divalent cation. In the Mg^{2+}-activated system catalytic activity is dependent on MgADP but not on the concentrations of uncomplexed Mg^{2+} or ADP. The Mn^{2+}-activated reaction is dependent on both free Mn^{2+} and MnADP, but not on uncomplexed ADP. Free P-enolpyruvate, rather than P-enolpyruvate complexed with metal, appears to be the preferred substrate. The velocity profile for P-enolpyruvate in the Mg^{2+}-activated system is sigmoid (Hill's n = 1.78) and the addition of low concentrations of fructose-1,6-P shifts the profile to a hyperbola (n = 0.92). In the Mn^{2+}-activated reaction a single hyperbolic curve is obtained with or without fructose-1,6-P (n = 0.95). Hill plots for free Mg^{2+} and free Mn^{2+} show slopes of 1.2 and 1.7, respectively. At limiting concentrations of metal, MgADP and MnADP have Hill's n values of 1.5 and 1.8, respectively. Dilution experiments indicate that the total order of reaction is the same (4.8) for both the Mg^{2+}- and Mn^{2+}-activated enzyme. Order with respect to P-enolpyruvate is 2.0 for the Mg^{2+}-activated enzyme and 1.1 for the Mn^{2+}-activated enzyme. It is proposed that Mn^{2+}, either as free metal or chelated to ADP, interacts with enzyme in such a way as to promote the binding of P-enolpyruvate thus accounting for the hyperbolic velocity profile. Phosphoryl transfer, however, is less efficient with Mn^{2+} resulting in a reduced maximum velocity compared to the Mg^{2+}-activated enzyme. The results suggest that Mn^{2+} and fructose-1,6-P effects are similar and may be mediated at the same or closely adjacent loci.

Pyruvate kinase (EC 2.7.1.40) catalyzes the conversion of P-enolpyruvate[1] and ADP to pyruvate and ATP; the reaction requires both monovalent and divalent cations (Boyer *et al.*, 1943). Cationic cofactors are required by many enzymes and apparently play a significant role in metabolic regulation (Wyatt, 1964; Bygrave, 1967). The observations that the cation specificity of glutamine synthetase changes from Mg^{2+} to Mn^{2+} on adenylylation (Kingdon *et al.*, 1967; Shapiro and Ginsburg, 1968), that *Escherichia coli* ADP: glucose synthetase has markedly different kinetics depending on whether Mg^{2+} or Mn^{2+} serves as cofactor (Gentner and Preiss, 1968) and that yeast phosphofructokinase shows a dependence on both uncomplexed and complexed Mg^{2+} (Mavis and Stellwagen, 1970) indicate the importance of specific cation effects.

A recent estimate of intracellular concentrations of free Mg^{2+} (Rose, 1968) in the human erythrocyte suggests that substantial amounts of ATP and ADP exist in the uncomplexed form, emphasizing the need to examine the effects of both free and bound ligands on red cell enzyme catalysis. The present communication describes the kinetic differences between Mg^{2+} and Mn^{2+} activation of a pyruvate kinase isolated from human erythrocytes.

Experimental Section

Methods

Materials. DPNH, Na_2ADP, trisodium P-enolpyruvate, and crystalline rabbit muscle pyruvate kinase, lactate dehydrogenase, aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase were purchased from Sigma; trisodium fructose-1,6-P from Calbiochem; triethaneolamine from Eastman. Reagent grade KCl, $MgSO_4$, or $MgCl_2$ and $MnCl_2$ were used.

Double glass-distilled water was used in preparing all glassware and solutions. Preparations of ADP were checked for contaminating ATP by the method of Beutler and Baluda (1964) and were found to contain as much as 7–10% ATP. No P-enolpyruvate was detected in solutions of ADP using the pyruvate kinase assay without added P-enolpyruvate. Up to 3% contamination of P-enolpyruvate with pyruvate was found using the lactate dehydrogenase assay of Kornberg (1955). No fructose 1,6-diphosphate contamination of P-enolpyruvate, ADP, or ATP was detectable using the coupled assay of Rutter *et al.* (1966). Concentrations were checked from the absorbance and the appropriate millimolar extinction coefficient: P-enolpyruvate, 2.93 at 230 nm; ADP; 15.4 at 259 nm. Since ADP was contaminated up to 10% by ATP, the pyruvate kinase assay using excess P-enolpyruvate was used to standardize ADP solutions. Identical kinetic results were obtainable with $MgCl_2$ or $MgSO_4$ in equimolar concentrations.

Purification of Erythrocyte Pyruvate Kinase. All steps were carried out at 4° unless otherwise specified. Outdated Red Cross blood collected in acid–citrate–dextrose anticoagulant 3–4 weeks earlier was used. Red cells separated from plasma and buffy coat were washed three times in three volumes of 0.15 M NaCl, buffered to pH 7.4 with 0.1 M K_2HPO_4, and hemolysed in 30 volumes of distilled water, with the stroma settling out overnight. A slurry of DEAE-cellulose (type 40; lot 1683), which had been equilibrated with 3 mM potassium phosphate buffer (pH 7.4) was added in the proportion of one

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[1] Abbreviations used are: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; FDP, fructose 1,6-diphosphate; TEA, tetraethylammonium; M^{2+}, divalent metal ion.

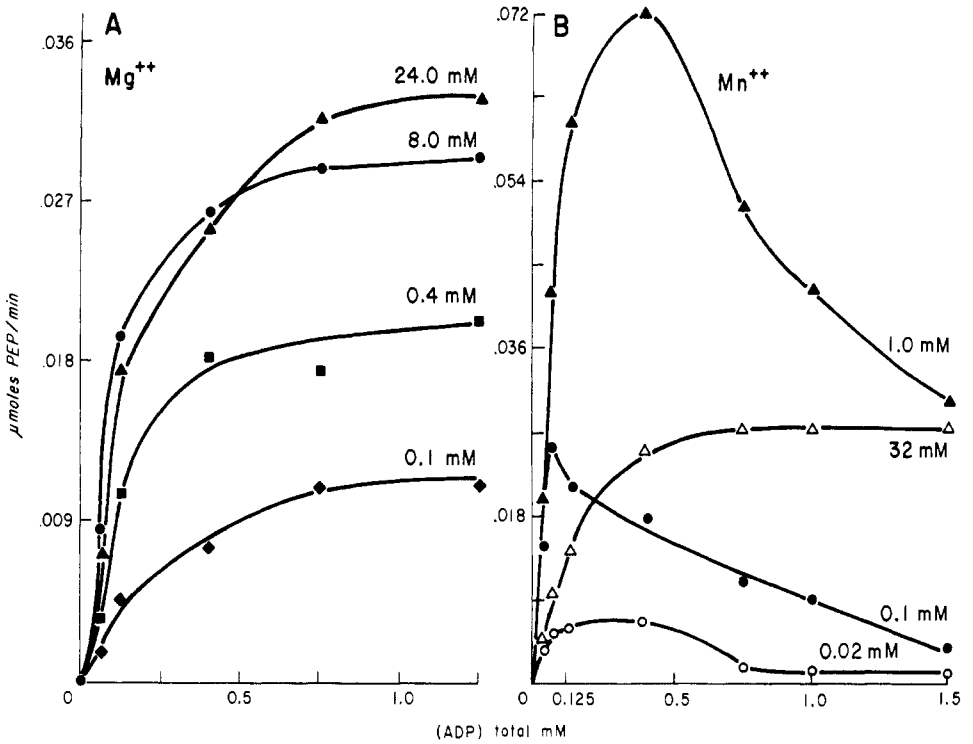


FIGURE 1: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on total ADP concentration at four concentrations of (A) Mg^{2+} and (B) Mn^{2+}. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, 0.5 mM P-enolpyruvate, enzyme, and Mg^{2+} or Mn^{2+} and ADP at the concentrations indicated.

volume to five volumes of the clear supernatant hemolysate and mixed for 1 hr. The DEAE-cellulose was filtered on a Büchner funnel, washed with six volumes of 3 mM phosphate buffer, and the enzyme eluted batchwise with three volumes of 10 mM KCl and then with three volumes of 0.1 M KCl. The 0.1 M KCl eluate was brought to 45% saturation by slow addition of crystals of solid enzyme grade $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 18,000*g*, and washed once with a small volume of a solution of 45% $(NH_4)_2SO_4$. The 45% $(NH_4)_2SO_4$ precipitate was dissolved in a small volume of distilled water, *e.g.*, 200 ml for precipitate from 10 l. of red cells, and brought to 20% saturation by slow addition of $(NH_4)_2SO_4$. The precipitate was removed by centrifugation, and the supernatant was brought to 38% $(NH_4)_2SO_4$, and the resulting precipitate was washed once with a small volume of 38% $(NH_4)_2SO_4$. The 20–38% $(NH_4)_2SO_4$ precipitate was dissolved in pH 5.5 buffer containing 12 mM citric acid, 28 mM dibasic potassium phosphate, 5 mM $MgSO_4$, 1 mM EDTA, and 1 mM mercaptoethanol, and dialyzed for 8–12 hr each against three changes of 100 volumes of the same buffer. The supernatant following centrifugation was heated to 40° for 1 hr. A 20–38% $(NH_4)_2SO_4$ cut from the supernatant following the heat step was collected as a slurry. When stored in this form at −15°, activity has remained stable for over 2 years. Samples for kinetic studies were prepared the same day by desalting on a G-25 Sephadex column in 0.15 M NaCl, or by dilution in 0.15 M NaCl. Since the final dilution of the latter preparations was 1500 ×, the maximum concentration of ammonium sulfate was less than 15 μM and of NaCl was less than 1.5 mM in the assay mixture. The specific activity of the enzyme varied from 20 to 50 units per mg.

Enzyme Assay. The direct spectrophotometric assay of Pon and Bondar (1967), which measures the disappearance of P-enolpyruvate at 230 nm, and the coupled assay of Bücher and Pfleiderer (1955) were used. The two methods gave identical results. Assays were done in triplicate in matched 5-mm light-path quartz cuvets using a Gilford Model 2400 multisample absorbance recorder equipped with single-channel offset controls. Temperature was maintained at 37° with a Haake circulating water bath. The reaction was initiated by adding 10 μl of diluted enzyme solution to a cuvet containing 1 ml of all other assay components which had been preequilibrated to 37°. These included 8 mM TEA buffer (pH 7.46), 75 mM KCl, and the desired concentrations of substrates as the sodium salt and divalent cations as the chloride salts. The initial velocity was obtained from the early slow phase of the progress curve for all assays. Recording was reproducible to within 0.002 optical density unit. Reaction velocities are expressed as micromoles of P-enolpyruvate consumed per minute. Where the coupled assay was used a 1:1 ratio between micromoles of DPNH and P-enolpyruvate was assumed. Doubling the concentration of enzyme did not alter any of the kinetic properties studied. All possible combinations of enzyme, P-enolpyruvate, ADP, and Mg^{2+}, with and without the effector fructose 1,6-diphosphate, were preincubated for 5 min at 37°. No changes occurred in the progress curve of the reaction on preincubating any combination of reactants and effector.

Dilution Experiments. In one set of experiments it was desired to study the order of reaction with regard to various ligands present as discussed by Atkinson *et al.* (1965). Starting with a 1.0-ml assay volume containing specified concentrations of all ligands, the volume of succeeding assays was increased by 0.05- or 0.1-ml increments containing identical concentrations of all ligands except those being tested.

Equations for Estimation of Free and Bound Substrate and Divalent Cation. Both P-enolpyruvate and ADP have appre-

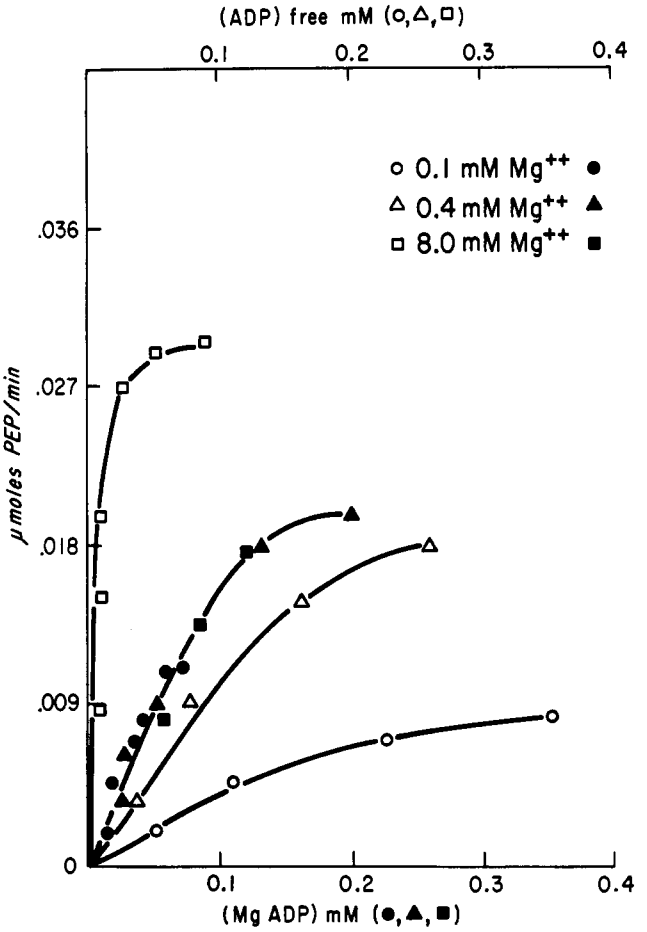


FIGURE 2: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the calculated concentrations of free ADP and MgADP. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl; 0.5 mM P-enolpyruvate, and enzyme. Concentrations of free ADP and MgADP were estimated by solution of the cubic equation described in the Experimental Section using a dissociation constant of 5×10^{-4} for MgADP (Melchior, 1965).

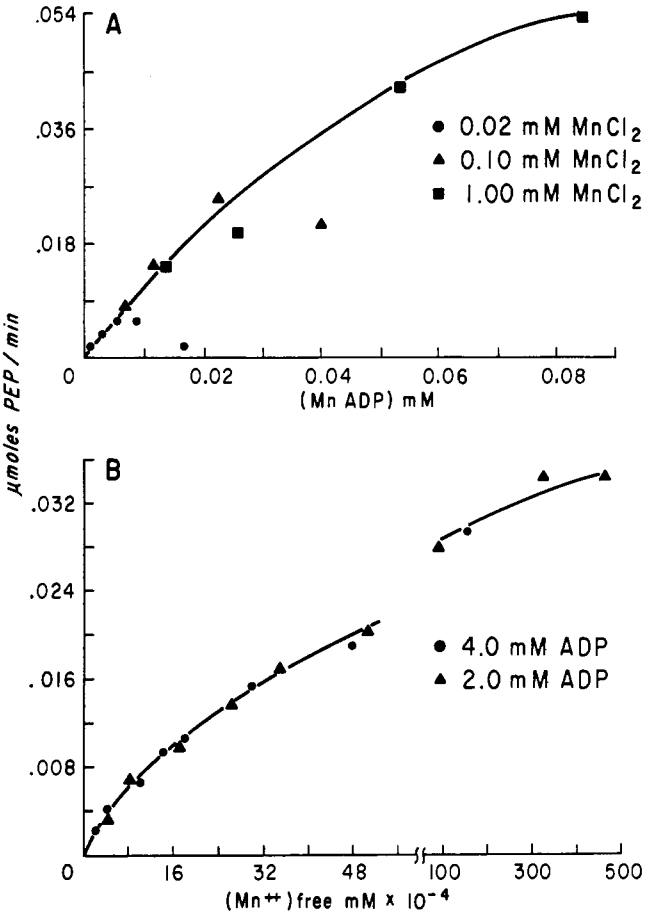


FIGURE 3: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the calculated concentrations of (A) MnADP and (B) free Mn^{2+}. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, and enzyme. P-enolpyruvate concentrations were 0.5 mM (A) and 4.0 mM (B). Concentrations of MnADP and free Mn^{2+} were estimated by solution of the cubic equation described in the Experimental Section using a dissociation constant of 1×10^{-4} for MnADP (Mildvan and Cohn, 1965). The coupled assay of Bücher and Pfleiderer (1955) was used for the data in part B.

ciable affinities for the divalent cations, Mg^{2+} and Mn^{2+}. The effect of chelation can be expressed by the following equilibria where M stands for metal, and S_1 and S_2 are the concentrations of substrates with their respective dissociation constants, K_1 and K_2. Simultaneous solution of eq 1 and 2

$$M + S_1 \xrightarrow{K_1} MS_1 \quad (1)$$

$$M + S_2 \xrightarrow{K_2} MS_2 \quad (2)$$

$$K_1 = \frac{(MS_1)}{((M) - (S_1) - (S_2))((S_1) - (MS_1))}$$

$$K_2 = \frac{(MS_2)}{((M) - (S_1) - (S_2))((S_2) - (MS_2))}$$

results in a cubic equation providing an estimate of the free and bound concentrations of metal and both substrates. The cubic equation was programmed in Fortran for use on a digital computer.

Results

As first reported in 1934 (Lohmann and Meyerhof), pyruvate kinase requires a divalent metal such as Mg^{2+} for activation. Double-reciprocal plots for free Mg^{2+} and free Mn^{2+} have demonstrated Michaelis constants of 1.0×10^{-4} M for Mn^{2+} and 5.5×10^{-4} M for Mg^{2+}.

Figure 1A represents a velocity profile with varying concentrations of ADP at several fixed concentrations of Mg^{2+}. No inhibition by excess ADP is observed, even when total ADP is increased to 12 times the total Mg^{2+} concentration, but at 24 mM Mg^{2+} slight inhibition is apparent at low concentrations of ADP. Figure 1B is a velocity profile with varying concentrations of ADP at several fixed concentrations of Mn^{2+}. At concentrations of total Mn^{2+} up to 1 mM, the velocity profile exhibits a peak with inhibition occurring as the ADP concentration is increased; 32 mM Mn^{2+} results in inhibition at low concentrations of ADP, with a plateau at the highest ADP concentrations studied.

Figures 2 and 3 illustrate velocity profiles plotted as MgADP, free ADP, MnADP, and free Mn^{2+}. The reaction velocity shows a common dependence on the concentrations of the MgADP and MnADP chelates, as well as on the free concentration of Mn^{2+}. Results not shown indicate that the reaction apparently does not have a dependence on free Mg^{2+}, or, in the Mn^{2+}-activated system, on free ADP.

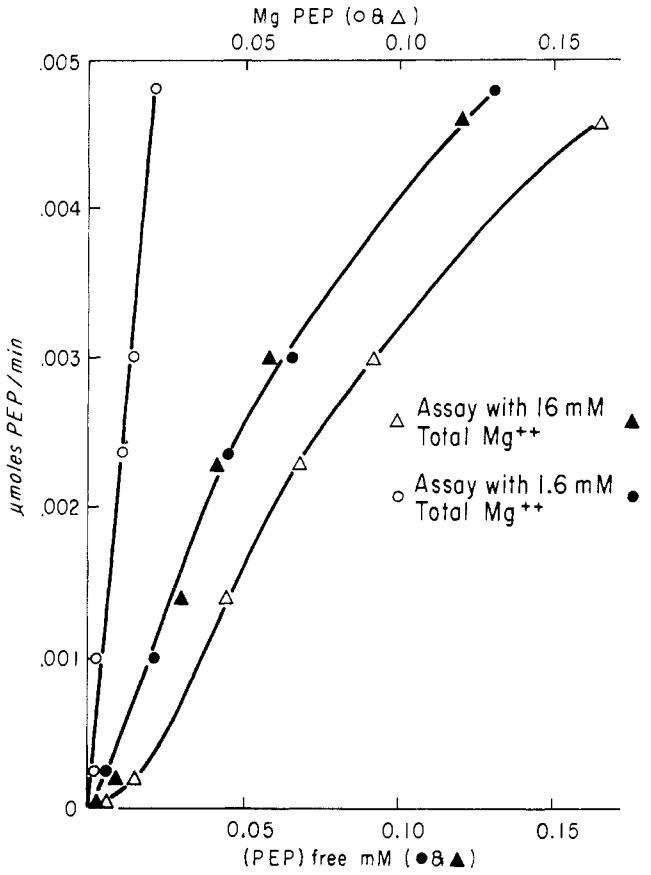


FIGURE 4: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the calculated concentration of free P-enolpyruvate and MgP-enolpyruvate. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, 0.125 mM ADP, and enzyme. Concentrations of free P-enolpyruvate and MgP-enolpyruvate were estimated by solution of the cubic equation described in the Experimental Section using a dissociation constant of 5.5×10^{-3} for MgP-enolpyruvate (Burton, 1961).

Since P-enolpyruvate also binds divalent metal to a significant degree, experiments were done to determine whether free or bound P-enolpyruvate is catalytically active. Figure 4 demonstrates a dependence of the rate on the calculated concentration of free P-enolpyruvate, but not on that of MgP-enolpyruvate. Data not shown indicate that the Mn^{2+}-activated system follows a similar pattern.

Figure 5 compares velocity profiles for P-enolpyruvate in the Mg^{2+}- and Mn^{2+}-activated pyruvate kinase reaction. Using Mg^{2+} the velocity profile is sigmoid and the addition of low concentrations of fructose-1,6-P results in a shift to a hyperbolic curve approaching the same maximum velocity. In the Mn^{2+}-activated system, a single hyperbolic curve is obtained in the presence and absence of fructose-1,6-P, but a lower maximum velocity is achieved.

Hill Plots; Order of Reaction. Table I summarizes Hill plot and order of reaction data gathered to examine the characteristics of cooperative interactions between the red blood cell enzyme and the ligands P-enolpyruvate, ADP, and divalent metal. Examples of Hill plot and order of reaction data are shown in Figures 6 and 7, respectively. Hill plots reveal that two kinds of n values[2] are found with human erythrocyte pyruvate kinase: $n = 1.0$, and $1 < n < 2$. These vary with the divalent cation and substrate being studied. The sums of Hill plot n values in the columns for the Mg^{2+}- and Mn^{2+}-activated systems are both 5.5, indicating that the total order of the reaction is probably the same in both. The dilution data support this conclusion demonstrating identical total orders of 4.8. Since the summations of Hill plot n values are redundant in terms of the metal and ADP contributions, the absolute values cannot be used as an estimate of the total order. However, assuming no ligand contributes more than 2.0 to the total order, there appear to be five potential contributors: (1) enzyme, (2) metal–ADP, (3) P-enolpyruvate, (4) free metal, and (5) an interaction term, which is dependent on the divalent cation.

TABLE I: Effects of Divalent Cation Activators on the Hill Coefficients and Orders of Reaction for the Substrates and Effectors in the Human Erythrocyte Pyruvate Kinase Reaction.

Substrate Contributing to Order	Hill Plot Data		Order of Reaction Data	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
(PEP) free	1.8	1.0	2.0[a]	1.1[a]
($ADP–M^{2+}$) (limiting ADP saturating metal)	1.0	1.0		
($ADP–M^{2+}$) (limiting metal saturating ADP)	1.5	1.8		
(M^{2+}) free	1.2	1.7		
Total	5.5[b]	5.5[b]	4.8	4.8

[a] Experimental values were 3.0 and 2.1 for Mg^{2+} and Mn^{2+} systems, respectively, and an order of 1.0 was subtracted for the contribution of enzyme. [b] Summated values of Hill plot data cannot be used as estimates of total order since the ADP and metal contributions are redundant.

Progress Curve Data. Figure 8 compares two progress curves with Mg^{2+} and Mn^{2+}, respectively, as the divalent cation at nearly equivalent calculation concentrations of metal–ADP chelate. The Mg^{2+}-activated reaction is sigmoid with a small initial slope followed by a steeper slope. Progress curves with Mn^{2+} are hyperbolic. Preincubation of the enzyme with all possible combinations of required ligands does not alter the shape of the progress curves. Fructose-1,6-P and ATP alter the sigmoid progress curves of the Mg^{2+}-activated reaction (Leonard, 1970). The difference between the Mg^{2+}- and Mn^{2+}-activated progress curves is consistent with the data in Figures 2, 3, and 5, and supports the idea that the two cations behave differently in activating the enzyme.

Discussion

Interpretations of enzyme kinetic data may involve cooperative effects between two or more sites as well as interactions occurring at a single catalytic site. Previous studies on

[2] The Hill plot for MnADP (Figure 7B) shows an inflection from a slope of 1.8–5 at higher concentrations of the chelate. This result is not readily explainable for the red cell enzyme, although yeast pyruvate kinase has been shown to bind up to 6 moles of divalent cation (Mildvan *et al.*, 1971; Hunsley and Suelter, 1969).

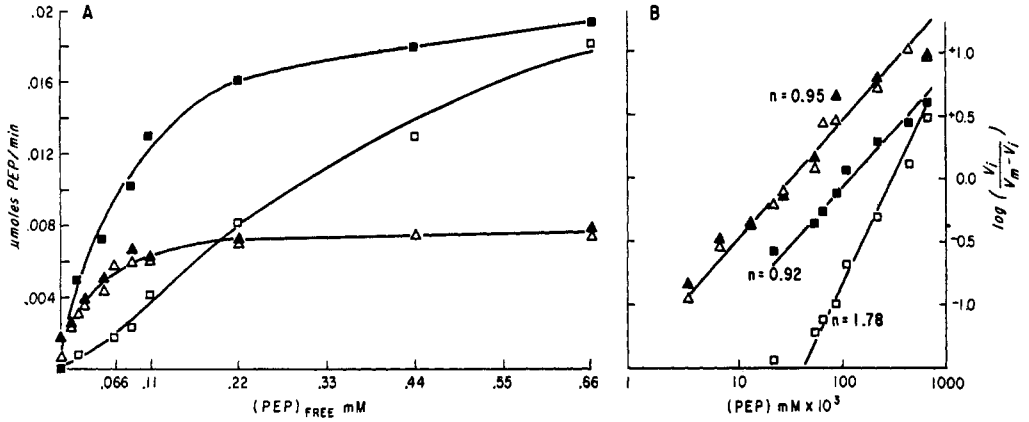


FIGURE 5: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the concentration of total P-enolpyruvate. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, 0.3 mM ADP, enzyme, and P-enolpyruvate at the concentrations indicated. (□) 1.6 mM $MgSO_4$; (■) 1.6 mM $MgSO_4$ and 1.0 mM fructose-1,6-P; (△) 0.4 mM $MnCl_2$; (▲) 0.4 mM $MnCl_2$ and 1.0 mM fructose-1,6-P. The data shown in part A were used in the Hill plot shown in part B.

human erythrocyte pyruvate kinase have indicated that both types of interactions must be considered (Campos *et al.*, 1965; Koler and Vanbellinghen, 1968; Black and Jacobsen, 1971). The differences in catalysis for Mn^{2+}- and Mg^{2+}-activated red cell enzyme probably reflect both single-site and cooperative effects.

Effects at a Single Catalytic Site. Wold and Ballou (1957) compared the activating effects of various divalent cations on the enzyme enolase. They found significant differences among the cations in maximum catalytic rate and in minimum concentration necessary for catalysis. Only those metals such as Mn^{2+} and Zn^{2+} which are more likely to complex with nitrogen are active in the hydroxylamine kinase reaction (Cottam, 1967; Solvonuk and Collier, 1955). "Fluorokinase" activity requires Mn^{2+} or Mg^{2+}, but is inactive with Zn^{2+} (Tietz and Ochoa, 1958) and the substrate, fluorophosphate, is directly coordinated to the enzyme-bound Mn of the rabbit muscle pyruvate kinase (Mildvan *et al.*, 1967). Human erythrocyte pyruvate kinase shows cation activation in the decreasing order $Mg^{2+} > Mn^{2+} > Co^{2+}$ and no activity with Zn^{2+} (Solvonuk and Collier, 1955). Activation could result from interaction with any of the obligatory ligands K^+, P-enolpyruvate, or ADP, as well as with the enzyme. A certain, percentage of P-enolpyruvate is bound to divalent cation, although results in Figure 4 suggests that free P-enolpyruvate is the active species in the pyruvate kinase reaction.

Another type of interaction might result from differences between the metal–nucleotide chelates. Values reported here for the Michaelis constants for Mg^{2+} and Mn^{2+} closely resemble those found for the rabbit muscle enzyme by Mildvan and Cohn (1965), and closely resemble the chelation constants of Mg^{2+} and Mn^{2+} for ADP. Mildvan and Cohn (1965, 1966), using purified rabbit muscle pyruvate kinase and nuclear magnetic resonance techniques, demonstrated a metal–enzyme complex, but concluded that a metal–nucleotide chelate bound more effectively to free enzyme than free nucleotide to a metal–enzyme complex. Cleland (1967), by replotting the kinetic data of Mildvan and Cohn (1966), concluded that neither free Mn^{2+} nor free ADP combines with the enzyme. However, enzyme–metal binding by rabbit muscle enzyme in the absence of nucleotide has been established by studies using proton relaxation rates (Mildvan and Cohn, 1966; Reuben and Cohn, 1970), protein difference spectroscopy (Kayne and Suelter, 1965; Suelter *et al.*, 1968), and enzyme–metal relaxation time (Hammes and Simplicio, 1970). The data of Figure 3B show a dependence of velocity on the level of free metal in the Mn^{2+}-activated erythrocyte pyruvate kinase reaction.

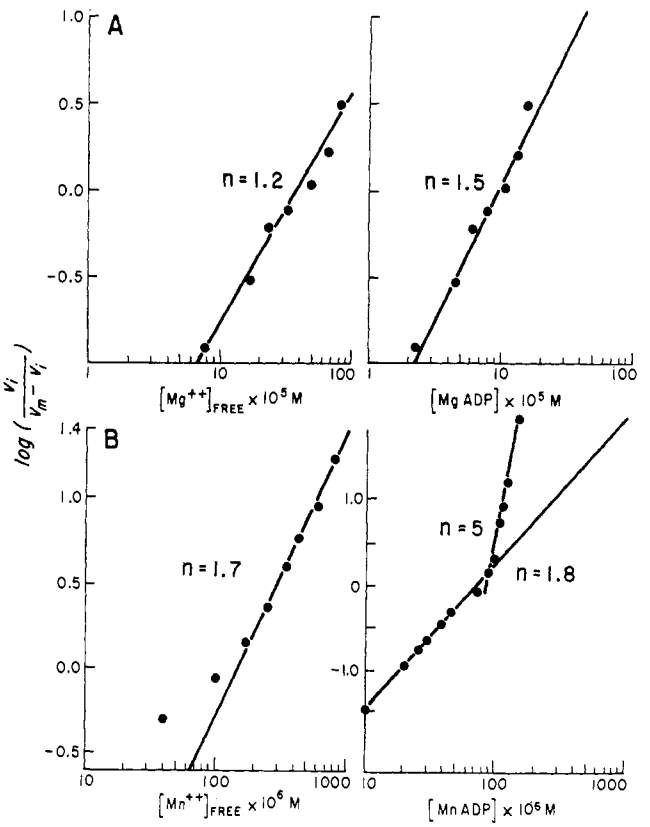


FIGURE 6: Hill plots with respect to (A) free Mg^{2+} and MgADP and (B) free Mn^{2+} and MnADP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, 0.125 mM P-enolpyruvate and ADP, and enzyme. Concentrations of free and bound divalent cations were estimated by solution of the cubic equation described in the Experimental Section using dissociation constants of 5×10^{-4} M for MgADP (Melchior, 1965) and 1×10^{-4} M for MnADP (Mildvan and Cohn, 1965); and 5.5×10^{-3} and 1.8×10^{-3} M for MgP-enolpyruvate and MnP-enolpyruvate, respectively (Burton, 1961).

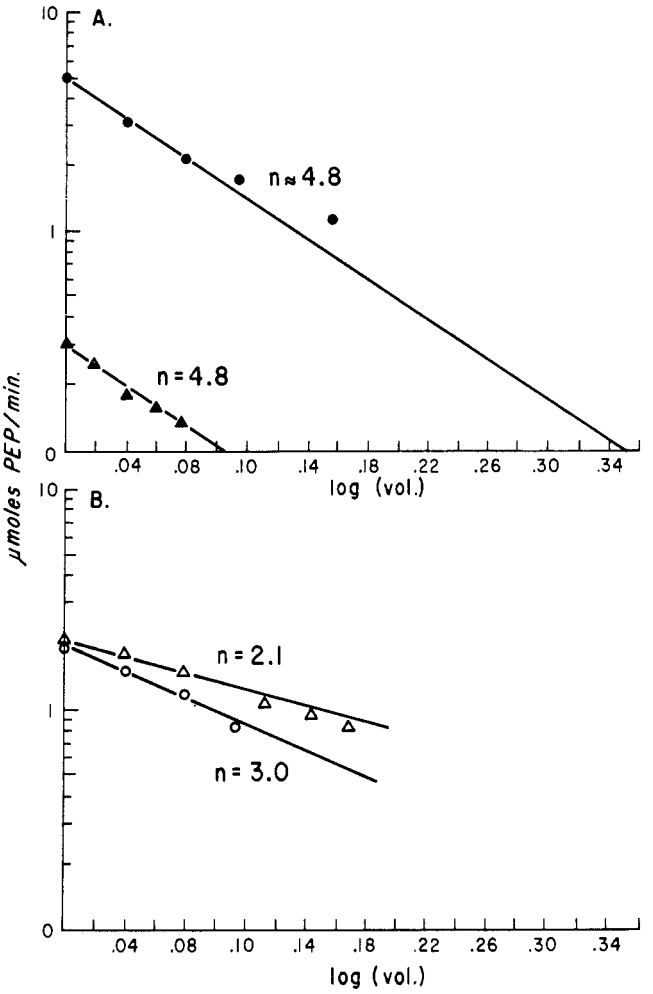


FIGURE 7: Dependence of the rate of the human erythrocyte pyruvate kinase reaction on (A) the overall concentrations of the reaction mixture: (●) Mg^{2+}-activated system; (▲) Mn^{2+}-activated system. Reaction mixtures contained 8 mM TEA buffer (pH 7.46) and 75 mM KCl, 0.125 μmole of P-enolpyruvate, 0.125 μmole of ADP, 0.1 μmole of $MgSO_4$ or 0.02 μmole of $MnCl_2$, and 20 μl of enzyme in a total volume as indicated on abscissa. (B) the concentration of P-enolpyruvate: (○) Mg^{2+}-activated system; (△) Mn^{2+}-activated system. Conditions were the same as above except that ADP concentration was held constant at 1.0 mM and Mg^{2+} and Mn^{2+} were held constant at 1.6 and 1.0 mM, respectively.

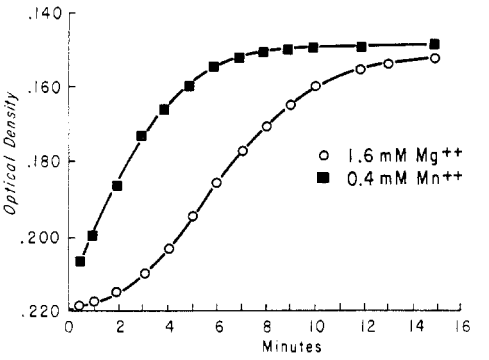
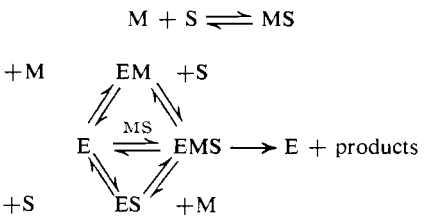


FIGURE 8: Progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase. Optical density changes were recorded at 230 nm in matched 0.5-cm quartz cuvets. Starting points were arbitrary and were positioned by linear offset control within 30 sec after addition of enzyme to start the reaction. Reaction mixtures contained 8 mM TEA buffer (pH 7.46), 75 mM KCl, 0.125 mM P-enolpyruvate and ADP, and identical concentrations of enzyme. (■) 0.5 mM Mn^{2+}; (○) 1.6 mM Mg^{2+}.

Studies on the interaction of divalent cations and substrates with rabbit muscle pyruvate kinase have suggested a quaternary bridge structure of enzyme, metal, P-enolpyruvate, and ADP (Mildvan and Cohn, 1966). The data of Figures 1, 2, and 3 on the red cell enzyme, however, allow no clear distinction between the relative importance of free metal or metal–nucleotide chelate in the formation of an active complex. Figure 1A demonstrates that the magnesium activation is similar to that found for the rabbit muscle enzyme (Melchior, 1965), and the data in Figure 2 support the conclusion from rabbit muscle enzyme studies that the magnesium chelate of ADP is the active substrate. The drop-off of experimental points for 0.02 and 0.1 as compared to 1.0 mM Mn^{2+} in Figure 3A suggests that Mn^{2+} is limiting. Alternatively, free ADP in excess of the catalytically preferred MnADP, may inhibit the enzyme by forming an E–ADP complex. Why free ADP would inhibit only the Mn^{2+}-activated system is the major difficulty with this alternative. Figure 1 is consistent with a generalized kinetic model (Mildvan and Cohn, 1966; London and Steck, 1969) in which modifier (M), substrate (S), and modifier–substrate complex (MS) may combine with the enzyme (E) to form the final complex.



Individually, the reactions are

$$M + S \overset{K_c}{\rightleftharpoons} MS$$

$$E + MS \overset{K_{ms}}{\rightleftharpoons} EMS$$

$$E + S \overset{K_s}{\rightleftharpoons} ES$$

$$ES + M \overset{K'_m}{\rightleftharpoons} EMS$$

$$E + M \overset{K_m}{\rightleftharpoons} EM$$

$$EM + S \overset{K'_s}{\rightleftharpoons} EMS$$

$$EMS \overset{k}{\longrightarrow} E + \text{products}$$

The K's represent association constants, and k is a rate constant for an assumed steady-state system. Letting M represent divalent cation and S the substrate, ADP, at constant P-enolpyruvate concentrations, both the Mg^{2+} and Mn^{2+} data of Figures 1, 2, and 3 are qualitatively consistent with the model. The observed differences between Mg^{2+}- and Mn^{2+}-activated pyruvate kinase could be explained by variations in the association constants K_c, K_s, K_m, K_{ms}, K'_m, and K'_s. A rate equation for the generalized kinetic model has been derived (London and Steck, 1969). If the ascending limb of the velocity profile with fixed total metal (M_t) and increasing total substrate (ADP_t) is sigmoid (Figure 1A) it predicts

that $K_m > K_{ms}$, implying that free metal has a significant affinity for enzyme and is inhibitory when $(M_t) > (ADP_t)$. If the ascending limb is hyperbolic then $K_{ms} > K_m$. Figure 1B shows apparent hyperbolic initial segments and peak velocities at nonsaturating metal concentration, implying that $K_{ms} > K_m$ and $K_s = 0$. As ADP_t is increased, the inhibition would be from E + S to ES. Sigmoidicity at low concentrations of ADP, though not apparent in Figure 1B, may exist; if it does the interpretation from the generalized rate equation is that free Mn^{2+} has a significant affinity for enzyme and at $(M_t) > (ADP_t)$ results in inhibition. Inhibition is observed at high total Mn^{2+} (Figure 1B).

In order to eliminate one variable from the complex interactions of enzyme, metal, ADP, and metal–ADP, the experiments in Figure 5 used concentrations of MgADP and MnADP that were calculated to be equivalent. The results can be interpreted in two ways. First, free Mn^{2+} may activate the enzyme and result in the hyperbolic velocity profile with varying P-enolpyruvate. It could also be postulated that the lower level of free Mn^{2+} limits the maximum velocity. Mildvan and Cohn (1965) reported that the turnover number of Mn^{2+}-activated rabbit muscle pyruvate kinase was about one-half that of the Mg^{2+}-activated enzyme. A second interpretation based on events at a single catalytic site is that the intrinsic properties of the MnADP chelate, compared to MgADP, may facilitate P-enolpyruvate binding, accounting for the apparent activation at low concentrations of P-enolpyruvate, but may limit the maximum rate of phosphoryl transfer to account for the lower V_m. In the Mg^{2+}-activated system, MgADP may not facilitate P-enolpyruvate binding resulting in lower velocities at low P-enolpyruvate concentrations, but the MgADP chelate may allow a greater rate of phosphoryl transfer, and a higher V_m.

Interactions between Two or More Sites. Rabbit muscle pyruvate kinase has been shown to be composed of four apparently identical subunits (Cottam *et al.*, 1969), and to have four binding sites for Mn^{2+} (Reuben and Cohn, 1970; Cottam and Mildvan, 1971), Zn^{2+} (Cottam and Ward, 1969), P-enolpyruvate (Kayne, 1971), and ADP (Hollenberg *et al.*, 1971). A minimum or two catalytic sites per molecule has been estimated from dissociation (Steinmetz and Deal, 1966), protein difference spectroscopy (Suelter *et al.*, 1968) and kinetic studies (Mildvan *et al.*, 1971; Hunsley and Suelter, 1969; Rozengurt *et al.*, 1969) for pyruvate kinases from yeast and from mammalian sources. Human erythrocyte enzyme, if activated by Mg^{2+} (Figure 5) resembles yeast and mammalian liver preparations in having a sigmoid velocity profile at varying concentrations of P-enolpyruvate; this is converted to a hyperbolic velocity profile by fructose-1,6-P. Saturating levels of Mg^{2+} have been used for all but two of these studies. In one, a magnesium Hill plot for the yeast enzyme showed the binding of 5–6 moles of metal. In the other, Mildvan *et al.* (1971) using nuclear magnetic resonance techniques demonstrated that yeast pyruvate kinase binds up to 6 moles of Mn^{2+} cooperatively with apparent affinities varying by three orders of magnitude. Fructose-1,6-P appears to raise the affinity of the yeast enzyme–Mn^{2+} complex for ADP and it was postulated that the site–site interactions between Mn^{2+} binding sites alters the affinity for P-enolpyruvate. Figure 5 supports previous work (Campos *et al.*, 1965; Koler and Vanbellinghen, 1968) showing that, in the Mg^{2+}-activated system, P-enolpyruvate binding to human red cell pyruvate kinase is cooperative ($n = 1.78$) whereas in the presence of fructose-1,6-P cooperativity is abolished ($n = 0.92$). In the Mn^{2+}-activated system no sigmoidicity is observed and the Hill plots suggest no cooperativity due to P-enolpyruvate ($n = 0.95$). The analysis of possible differences in the role of Mn^{2+} and Mg^{2+} at a single catalytic site cannot explain observations, such as those presented in Figures 5 and 6. Extending the analysis to cooperative effects between two or more catalytic sites may account both for the reported differences in divalent cation activation, and for similarities in the Mn^{2+}-activated enzyme and the fructose-1,6-P-modified Mg^{2+}-activated enzyme (Figure 5). The Mn^{2+} effect, whether mediated by an E–M complex or by an E–M–ADP complex may facilitate P-enolpyruvate binding both at a single site and cooperatively at a second or larger number of catalytic sites. Although catalysis at low levels of P-enolpyruvate is accelerated, Figure 5 shows that Mn^{2+} is less efficient than Mg^{2+} at saturating concentrations of P-enolpyruvate. However, the latter may be due to limiting concentrations of free Mn^{2+}.

TABLE II: Summary of Differences between Magnesium-Activated and Manganese-Activated Human Erythrocyte Pyruvate Kinase.

	Divalent Cation	
	Mg^{2+}	Mn^{2+}
1. PEP–velocity profile	Sigmoidal; $n \simeq 2$	Hyperbolic; $n \simeq 1$
2. FDP (1 mM or less)	Activation; shifts PEP profile from sigmoidal ($n \simeq 2$) to hyperbolic ($n \simeq 1$)	Insensitive
3. Progress curves (nonsaturating PEP, ADP)		
High (M)	Hyperbolic	Hyperbolic
Low (M)	Sigmoid	Hyperbolic
4. Dependence of rate on		
Free metal	No	Yes
Metal–ADP chelate	Yes	Yes

The similarities between Mn^{2+}-activated pyruvate kinase and the fructose-1,6-P-modified Mg^{2+}-activated enzyme indicates that Mn^{2+} and fructose-1,6-P may induce similar changes in the enzyme. Since one obligatory site for divalent metal binding is the catalytic site, the fructose-1,6-P and divalent metal effects could be mediated at the same or closely adjacent loci. Indirect evidence supporting this postulate has been presented by Kuczenski and Suelter (1970) from studies on a pyruvate kinase from yeast. Kinetic evidence demonstrates both that fructose-1,6-P may be a competitive inhibitor of P-enolpyruvate and that fructose-1,6-P abolishes progress curve sigmoidicity, similar to the effects of Mn^{2+} (Leonard, 1970).

As pointed out by Atkinson *et al.* (1965), if the velocity of an enzymatic reaction is proportional to the concentration of a particular enzyme–substrate complex, and if this complex is virtually at equilibrium with the free enzyme and free substrates, it follows that at sufficiently low concentrations of all substrates the reaction order should reflect the total number (n) of components in the rate-determining complex. The mechanism of the human erythrocyte pyruvate kinase reaction generally has been assumed to be ordered binding with rapid equilibrium (Campos *et al.*, 1965).

Table I summarizes the results bearing on the contribution of individual ligands to the total order of the erythrocyte pyruvate kinase reaction, and the differences attributable to Mg^{2+} *vs.* Mn^{2+} activation. The concentration of monovalent cation (K^+) has been kept constant; ligands which were varied are divalent cations and the two substrates of the forward reaction, ADP and P-enolpyruvate. Absence of cooperativity would predict a fourth order reaction dependent on contributions of enzyme, two substrates and the divalent cation. Interaction between two catalytic sites would add one to the total order resulting in a fifth-order reaction. The data presented are consistent with a fifth-order which is due to divalent cation–enzyme interaction in the Mn^{2+}-activated system, and to a P-enolpyruvate–enzyme interaction in the Mg^{2+}-activated system.

Table II summarizes the differences between the magnesium-activated and the manganese-activated systems. Although the kinetic properties of human erythrocyte pyruvate kinase differ in several important aspects depending on which divalent cation is used, a clear understanding of the molecular basis for this difference must await further study.

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