LDH_5 molecule were prevented by the addition of an excess of NADH to the density gradient. This would explain why Bonavita and Guarneri (1963) found no effect of β-mercaptoethanol on the kinetic behavior of LDH since NADH was used in a substantial excess.

The role of an excess of NADH in preventing the β-mercaptoethanol-dependent changes in LDH_5 may be due to a tighter binding of the LDH_5 monomers. This would agree with the data of Fritz and Jacobson (1963), who observed fewer bands of LDH_5 activity when NADH was added in excess during electrophoresis performed in the presence of β-mercaptoethanol, and agrees with Zondag's (1963) finding that NADH protects against cold inactivation. It is interesting that the structure of LDH_1 is sufficiently different from the LDH_5 so that β-mercaptoethanol produced no detectable changes.

The physiological significance of these properties of LDH_5 cannot be assessed here. However, should similar reactions occur *in vivo*, they would have an important significance in the regulation of LDH activity.

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The Role of Metal Ions in the Pyruvic Kinase Reaction*

Jacklyn B. Melchior†

ABSTRACT: The velocity of the pyruvate kinase reaction has been measured over a wide range of concentrations of adenosine diphosphate (ADP), Mg^{2+}, and K^+. The data have been analyzed in terms of the equilibrium concentrations of the simple and the complex ions present in the solution. It is concluded that $MgADP^-$ is the specific substrate required, while K^+ functions to activate the enzyme. The data fit the mechanism proposed by Reynard and co-workers (A. M. Reynard, L. F. Haas, D. O. Jacobsen, and P. D. Boyer [1961], *J. Biol. Chem. 236*, 2277), except that the restriction of "equilibrium kinetics" does not appear to be justified.

The pyruvic kinase of muscle has been extensively studied (Boyer, 1962). In addition to requiring magnesium ion for activity (Lohman and Meyerhof, 1934), it represents one of the rare instances of a simple system which exhibits an absolute requirement for a monovalent cation (Boyer *et al.*, 1942; Boyer *et al.*, 1943; Lardy and Zeigler, 1945). Karchmar and Boyer (1953) have shown that rubidium or ammonium ions, but not sodium, can replace the activation by potassium. Reynard *et al.* (1961) have presented evidence that the enzyme possesses a binding site for each of its two substrates, and that the phosphoryl group is transferred directly from the donor to the acceptor. Evidence against an intermediate phosphoryl enzyme has been presented by Haas *et al.* (1961). From magnetic resonance studies of the Mn^{2+} activated system, Cohn (1963)

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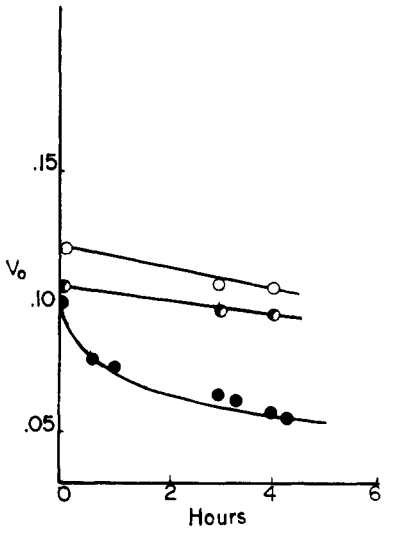

FIGURE 1: Stability of pyruvate kinase in dilute solutions. A suspension of the enzyme in ammonium sulfate was diluted at zero time as follows: ◐, in 0.2 M TMACl; ○, in 0.2 M KCl; ●, in water. These data were not compared to the standard assay solution. The units of V_0 are μM/min.

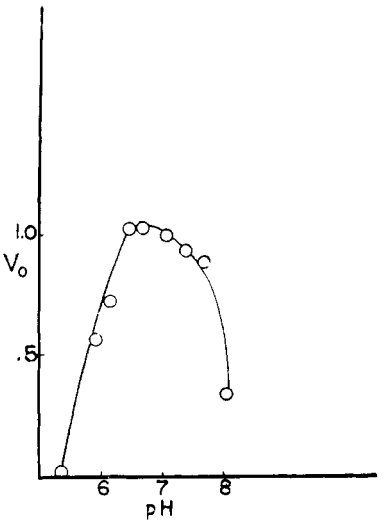

FIGURE 2: Effect of *p*H on the velocity of the pyruvate kinase reaction. The reaction mixture contained 1 mM PEP, 2 mM ADP_T, 5 mM Mg_T, and 0.2 M KCl.

has found evidence for a ternary complex of enzyme, PEP,[1] and ADP species.

On the other hand, the mechanism of the activation of this system by the metal ions has not been elucidated. In fact, the picture has been complicated by the discovery that adenylic acid polyphosphates form complex ions in the presence of both potassium (Melchior, 1954) and magnesium ions (Spicer, 1952; Hers, 1952; Martell and Schwartzenbach, 1956). It has been suggested (Melchior, 1954) that these complex ions differ in shape as well as charge, and thus it seemed probable that the enzyme would exhibit specificity toward one or another of the nucleotide ions which are present. This paper presents the results of an investigation of the specific role played by the metal ions in the pyruvic kinase reaction.

Methods

The PEP used in these experiments was procured as the tricyclohexylamine salt from Sigma Chemical Co. and was used directly. Barium ADP was obtained from Sigma, and was converted to the TMA^+ salt as described previously (Melchior and Melchior, 1958). TMACl was recrystallized twice from isopropyl alcohol and once from ethanol. All other reagents were Mallinckrodt AR grade. Potassium and magnesium were added to the various solutions as the chlorides.

All the experiments described herein were done using crystalline pyruvic kinase, prepared from rabbit skeletal muscle (California Biochemical Corp.). The enzyme was stored as a suspension in 2.1 M ammonium sulfate. It was diluted approximately 250-fold with 0.2 M TMACl on the day of an experiment, and stored on ice. No activity was apparent unless potassium ion was added to the system.

In the presence of excess Mg^{2+}, the reaction is:

$$PEP^{3-} + MgADP^- + H^+ \longrightarrow Pyruvate^- + MgATP^{2-}$$

and is conveniently followed by measurement of H^+ utilization. Because of the formation of complex ions, the utilization of H^+ is not generally equivalent to the disappearance of substrate.

In order to determine the exact stoichiometry in each case, the following constants were used: for K_F of $MgADP^-$, the value of 2.1×10^3 M^{-1}, and for K_F $MgATP^{2-}$, 2.2×10^4 M^{-1}, based on the measurements of Walaas (1958) but corrected for the KCl employed as a background electrolyte. For K_F of $KADP^{2-}$, 4.8 M^{-1} and for K_F of $KATP^{3-}$, 9.6 M^{-1} was used (Melchior, 1954), and K_3 for $HADP^{2-}$ was taken as 2.2×10^{-7} M and K_4 for $HATP^{3-}$, 1.26×10^{-2} M (Melchior, 1954). An IBM 7090 digital computer[2] was used to calculate the species present at various stages of the reaction for each set of conditions. The observed

[1] The following abbreviations are used in this paper: PEP, phosphoenolpyruvate; ADP, adenosine diphosphate; ADP^{3-}, $HADP^{2-}$, $KADP^{2-}$, $MgADP^-$ refer to specific ionic species which exist in certain aqueous solutions containing ADP; TMA^+, tetramethylammonium ion. Parentheses enclosing a molecular formula indicate the concentration of that species in moles/liter; the sum of the concentrations of all the molecular species containing a particular component is indicated by the subscript *T*, e.g., $(ADP)_T$.

[2] The equations used were analogous to those used in making similar calculations for the ATP-Na-Mg system (Melchior and Melchior, 1958). The Fortran program used to calculate these values, as well as the calculated results, will be sent upon request.

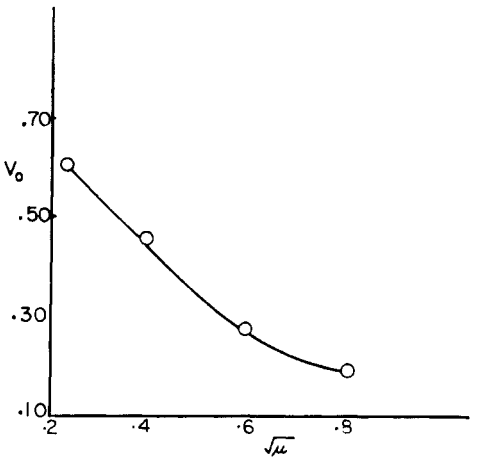

FIGURE 3: Effect of ionic strength on the activity of pyruvate kinase. The reaction mixture contained 1 mM PEP, 2 mM ADP_T, 0.2 M KCl, and 5 mM $(Mg)_T$.

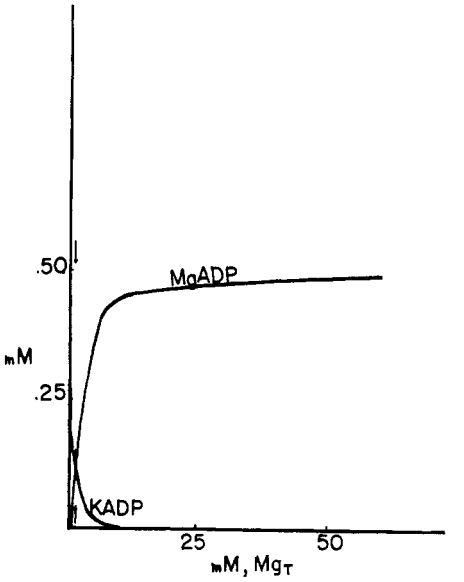

FIGURE 4: Effect of Mg_T on species present in a solution 0.2 M K_T, 0.5 mM ADP_T, *p*H 7.0. The line indicating free Mg^{2+} lies close to the ordinant axis. The arrow indicates where $(Mg)_T = (ADP)_T$.

velocities were then corrected by an appropriate factor. For example, the stoichiometry for conversion of 10% of the ADP present to product under the conditions of our standard assay is given by the equation:

$$\begin{aligned} 0.193\ H^+ + 0.2\ PEP^{3-} + 0.0366\ Mg^{2+} \\ + 0.154\ MgADP^- + 0.019\ ADP^{3-} \\ + 0.0087\ HADP^{2-} + 0.0181\ KADP^{2-} \\ \rightarrow 0.0025\ ATP^{4-} + 0.191\ MgATP^{2-} \\ + 0.0019\ HATP^{3-} + 0.0048\ KATP^{3-} \\ + 0.0133\ K^+ + 0.2\ Py^- \end{aligned}$$

and thus the observed acid disappearance is 4% lower than the true velocity of the reaction.

The reaction was followed using a Radiometer *p*H meter as a *p*H-stat. The reaction was carried out in a water-jacketed reaction vessel in which all the reactants were equilibrated prior to adding the enzyme. In the initial experiments the reaction was stirred by bubbling nitrogen through the solution; later a magnetic stirrer was found to produce smoother curves and greater precision. After temperature equilibration was obtained, the enzyme was added by micro pipet. The volume of the mixture was 6 ml, and aliquots of enzyme of 20–100 μl were used.

After the reaction had started, the automatic buret injected acid (0.003 M HCl) into the solution to maintain a pre-set *p*H, and the recorder registered the amount of acid added as a function of time. The total volume of acid added in a given run never exceeded 0.5 ml. The lines obtained were generally straight until a significant fraction of one of the substrates had been consumed. Thus, the slope of the line is directly proportional to the velocity of the reaction. In order to compare experiments from day to day, the enzyme solution was assayed under standardized conditions at the beginning, midway, and at the end of each day. These data showed that there was no appreciable loss of activity during the day by the diluted enzyme solution. With the exception of the data presented in Figure 1, the units of activity used were calculated by dividing the observed velocity by the velocity of the standard. Initial concentrations for the standardizing assays were: 0.2 M K_T, 5.0 mM Mg_T, 1.0 mM PEP, and 2.0 mM ADP_T.

Reynard *et al.* (1961) have mentioned that the enzyme loses activity in dilute solution unless KCl is added. The loss of activity upon dilution was confirmed, but, as shown in Figure 1, TMACl is as effective as KCl in protecting the enzyme from loss of activity. In all the experiments reported here, the enzyme was diluted with 0.2 M TMACl. Additional preliminary experiments included a study of the effect of *p*H and of ionic strength. Since the use of the *p*H-stat permits assays to be made without the necessity of adding a buffer, it becomes possible to study the system in the absence of extraneous specific ion effects. The effect of *p*H is illustrated in Figure 2. Because of this, we have made all measurements at a *p*H of 7.0. In Figure 3 the effect of ionic strength is demonstrated. Since it is obvious that ionic strength does affect this system, μ was adjusted to 0.5 by the addition of TMACl in all of the experiments reported herein. Other factors kept constant throughout this work include the concentration of PEP, which was 1 mM, and the temperature, which was 25°. Each point represents the average of from three to ten separate determinations.

Calculations. In order to express the composition of the solutions in terms of the actual species present, the digital computer[2] was used to calculate the concentrations over a large range of total concentrations of K, ADP, Mg, and *p*H. Typical curves showing the results of the calculations of the concentrations of the various species present resulting from an increasing $(Mg)_T$ and $(ADP)_T$ concentration, respectively, are shown in

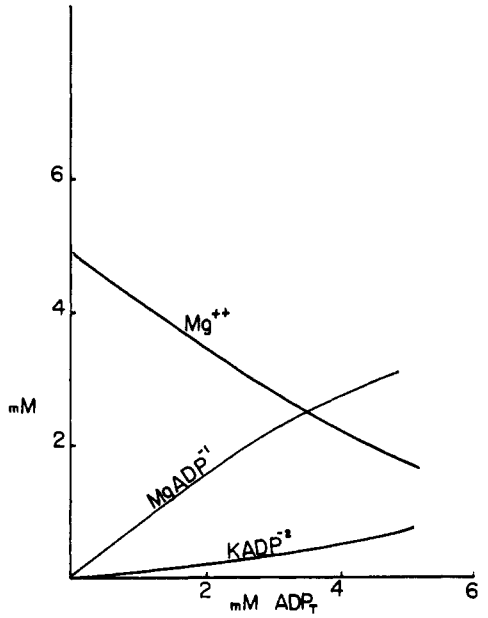

FIGURE 5: Effect of $(ADP)_T$ on the concentrations of species present in a solution containing 5 mM $(Mg)_T$, 200 mM $(K)_T$, *p*H 7.0.

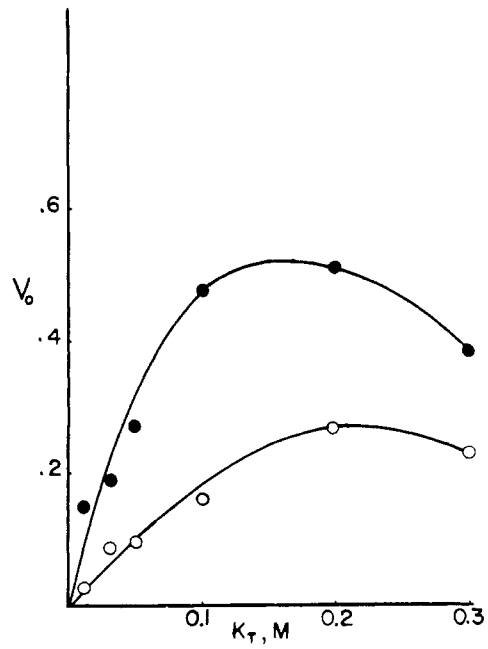

FIGURE 6: Effect of K_T on velocity of the pyruvate kinase reaction. Mg_T = 2 mM; ●, ADP = 2 mM; ○, ADP = 0.5 mM.

Figures 4 and 5. Although appearing qualitatively similar to those previously published for solutions containing ATP, Mg^{2+}, and Na^+ ions (Melchior and Melchior, 1958), they differ significantly because the formation constants for the complex ions are much lower for the ADP systems. For example, the maximal concentration of Mg complex is approached in most ATP solutions when the total Mg is equal to the total ATP, while in ADP solutions the concentration of complex increases significantly with Mg until the total Mg is ten to twenty times that of the ADP. This point is emphasized by the arrow in Figure 4.

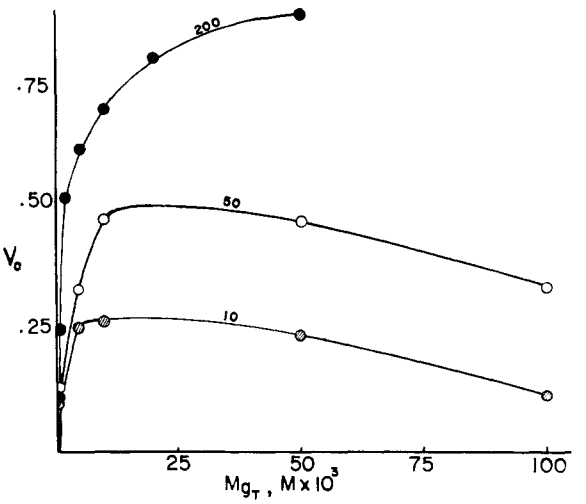

FIGURE 7: Effect of Mg_T on the velocity of the pyruvate kinase reaction. ADP_T = 2 mM; K_T in mM is indicated on each curve.

In much of the work done previously upon this system, ionic strength has not been controlled (Karchmar and Boyer, 1953), and ADP_T has been considered in the interpretation of the data (Reynard *et al.*, 1961). For these reasons it has been necessary to repeat experiments where these factors are of significance.

Results

The effect of (K^+) on the velocity of the reaction is shown in Figure 6. It is apparent that there is an inhibition at very high levels of potassium which cannot be attributed to the effect of the ionic strength.

Previous workers have not found an inhibition by excess Mg^{2+} in this system (Reynard *et al.*, 1961). As is shown in Figure 7, the effects of Mg^{2+} depend upon the level of K^+ present. A definite inhibition is obtained when $(K)_T$ is low. It should be mentioned that the velocity data shown in Figure 7, when plotted against *p*Mg instead of Mg_T, resemble the bell-shaped curves reported by Brintzinger and Fallab (1960) for yeast hexokinase.

As shown in Figure 8 no inhibition by ADP^{3-} was observed even when $(ADP)_T$ was increased to five times the $(Mg)_T$. The inhibition by excess (Mg^{2+}) is apparent in the low $(ADP)_T$ region of Figure 8.

Discussion

Reynard *et al.* (1961) have concluded that the mecha-

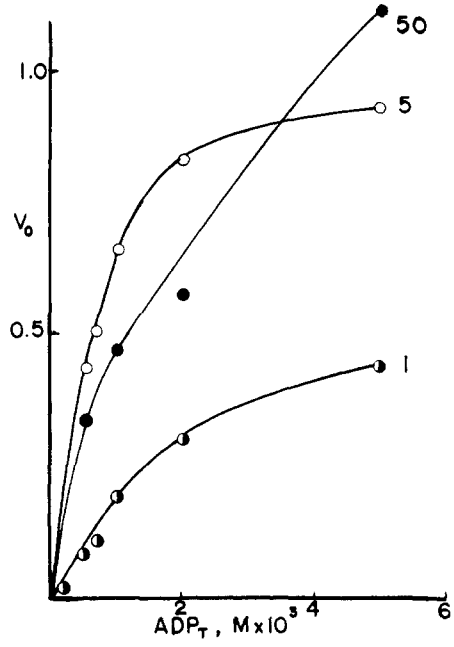

FIGURE 8: Effect of ADP on the velocity of the pyruvate kinase reaction. K_T = 200 mM; $(Mg)_T$ in mM is indicated on each curve.

nism of the pyruvate kinase reaction has the following characteristics: first, a direct transfer of the phosphoryl group from the donor to the acceptor occurs; second, there are two catalytic sites, one which can bind pyruvate and PEP and one which binds ATP or ADP; and third, there is a random combination of PEP and ADP with the enzyme, with "equilibrium kinetics" governing. We will use their proposed mechanism as the starting point in the analysis of our data. The mechanism is represented in Figure 9. It is assumed that M represents one of the metal activators present, i.e., K^+ or Mg^{2+}, and that A represents the active ADP species, i.e., ADP^{3-}, $KADP^{2-}$, or $MgADP^-$; B represents PEP. For reasons to be explained below we have found it necessary to consider the whole equation without the simplifying assumption that "equilibrium kinetics" governs the system.[3] The rate equation for the production of product or disappearance of substrate in Figure 9 is:

$$-dA/dt = -dB/dt = dC/dt = dD/dt = (k_{20} + k_{16})(\mathrm{M \cdot C \cdot D}) \quad (1)$$

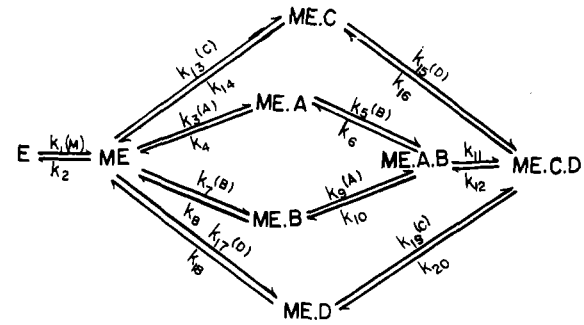

FIGURE 9: Proposed mechanism for the pyruvate kinase system. Symbols are explained in the text.

Using customary methods[4] (Alberty, 1953) for solving such equations, the rate equation relating the initial velocity to the constants and variables of the system was derived. This equation is shown in Figure 10. Although the complete equation appears formidable, for a given set of parameters it is much less complex. For example, since PEP is constant throughout our measurements, all terms involving B as the only variable can be collected with the constant terms. The equation of Figure 10 then becomes:[5]

$$\frac{V_0}{V_m} = \frac{(\mathrm{A})(\mathrm{M})(\alpha + \beta \mathrm{A})}{\gamma(\mathrm{A}) + \Delta(\mathrm{M}) + \epsilon(\mathrm{A})^2(\mathrm{M})} \quad (2)$$

In order to test the equation given in Figure 10 against the data, it is necessary to consider the variables singly. Since the amount of $KADP^{2-}$ present is always very small with respect to $(K)_T$, it is possible to assume that free (K^+) is equal to $(K)_T$. Perusal of the data quickly led to the exclusion of $(KADP^{2-})$ and (Mg^{2+}) as being related directly to the rate of the reaction. However, $(MgADP^-)$ was found to be closely related to the velocity when $(K)_T$ was constant. In order to test this, all measurements in which $(K)_T$ was 200 mM were collected; these experiments were done over a period of 1 year, and represent a wide range of variation of the parameters other than $(K)_T$. Figure 11 illustrates the close relationship of V_0 to $(MgADP^-)$ when $(K)_T$ is held constant.

In order to emphasize the lack of relationship to free Mg^{2+}, the value for (Mg^{2+}) in the particular experiment is placed in the circle representing the experiment. It seems clear that, when (K^+) and PEP are

[3] The term "equilibrium kinetics" or "quasiequilibrium" (see Hearon *et al.*, 1959) refers, in the system shown in Figure 9, to the specific case in which k_{11} and k_{12} are very low with respect to the other rate constants. In this case, the species ME·C·D, ME·D, and ME·C can be considered negligible and set equal to zero. When this assumption is not made these species enter into the final equation; however, terms containing concentration of product (i.e., (C) and (D)) can be equated to zero when V_0 is being considered (see King and Altman, 1956).

[4] The schematic method of King and Altman (1956) becomes increasingly cumbersome as the number of enzyme species involved in the proposed mechanism increases. We have found it more practical to solve the simultaneous linear equations by the method of Gauss (Fox, 1963), using the procedure of King and Altman to check the results for algebraic errors.

[5] In this and succeeding equations the Greek letters are used to represent combinations of constants and any variables which have been assigned specific values. They thus represent constants for the particular situation under consideration.

$$V_0/V_{MAX} = \{[M][A][B][k_{16}+k_{20}][k_1k_{11}k_{14}k_{18}][k_4k_7k_9+k_5k_7k_9(B)+k_3k_5k_8+k_3k_5k_9(A)]\} \div$$
$$[k_2k_8k_{14}k_{18}][k_4(k_6k_{20}+k_{10}k_{20}+k_{11}k_{20}+k_6k_{16}+k_{10}k_{16}+k_{11}k_{16}+k_6k_{12}+k_{10}k_{12})+k_5(B)(k_{10}k_{20}$$
$$+k_{11}k_{20}+k_{10}k_{16}+k_{11}k_{16}+k_{10}k_{12})]+[A][k_2k_9k_{14}k_{18}][k_4(k_{11}k_{20}+k_6k_{16}+k_{11}k_{16}+k_6k_{12}+k_6k_{20})$$
$$+k_5(B)k_{11}(k_{16}+k_{20})]+[M][k_1k_{14}k_{18}][k_4k_8(k_6k_{20}+k_{10}k_{20}+k_{11}k_{20}+k_6k_{16}+k_{10}k_{16}+k_{11}k_{16}+k_6k_{12}$$
$$+k_{10}k_{12})+k_5k_8(B)(k_{11}k_{20}+k_{10}k_{20}+k_{11}k_{16}+k_{10}k_{16}+k_{10}k_{12})+k_4k_7(B)(k_{11}k_{18}+k_6k_{20}+k_{10}k_{20}+k_6k_{16}$$
$$+k_{10}k_{16}+k_{11}k_{16}+k_8k_{12}+k_{10}k_{12})+k_5k_7(B^2)(k_{10}k_{20}+k_{11}k_{20}+k_{10}k_{16}+k_{11}k_{16}+k_{10}k_{12})]$$
$$+[A][M][k_1][(k_4k_9k_{14}k_{18})(k_6k_{20}+k_{11}k_{20}+k_6k_{16}+k_6k_{12}+k_{11}k_{16})+(k_3k_8k_{14}k_{18})(k_6k_{20}$$
$$+k_{10}k_{20}+k_{11}k_{20}+k_6k_{16}+k_{10}k_{16}+k_{11}k_{16}+k_6k_{12}+k_{10}k_{12})+(B)\{(k_5k_9k_{11}k_{14}k_{18}(k_{16}+k_{20})$$
$$+(k_5k_7k_9k_{11}(B)+k_3k_5k_8k_{11}+k_4k_7k_9k_{11})(k_{16}k_{18}+k_{14}k_{18}+k_{14}k_{20})+(k_4k_7k_9+k_6k_7k_9$$
$$+k_3k_5k_8+k_5k_7k_9(B)+k_3k_5k_{10})(k_{20}+k_{16}+k_{12})(k_{14}k_{18})\}]+[A^2][M](k_1k_3k_9)[k_6k_{14}k_{18}(k_{12}$$
$$+k_{18}+k_{20})+(k_{14}k_{18})(k_5(B)+k_{11})(k_{16}+k_{20})+(k_5k_{11}k_{18}(B)(k_{14}+k_{16})+k_5k_{14}(B)(k_{12}k_{18}+k_{11}k_{20})]$$

FIGURE 10: Rate equation corresponding to the mechanism shown in Figure 9.

constant, the velocity is related to ($MgADP^-$) and not to free (Mg^{2+}).

The wide range of other variables should be emphasized. For example, at about 0.5 mM MgADP there are two points with a thousandfold variation in free (Mg^{2+}). In these solutions, (ADP^{3-}) was 0.00237 and 1.90 mM, while ($HADP^-$) was 0.00108 and 0.864 mM, respectively. It is obvious that other possible factors, such as $MgPEP^-$, are also varying by about a thousandfold in these two solutions. It is thus apparent that (K^+) and ($MgADP^-$) are the critical factors in determining the velocity.

When M is held constant, the equation of Figure 10 becomes:

$$\frac{V_0}{V_m} = \frac{(\alpha'(A) + \beta'(A)^2)}{\gamma' + \Delta'(A) + \epsilon'(A)^2} \qquad (3)$$

Thus a linear reciprocal plot would not be expected when $1/(A)$ is plotted against $1/V_0$. In Figure 12 such a plot is shown, and it has the shape to be expected from the presence of the squared terms. In this figure the ($KADP^{2-}$) present in the solution is indicated in each circle. It is apparent that no relationship to ($KADP^{2-}$) exists, but that it is the concentration of ($MgADP^-$) that governs the rate of the reaction when $(K)_T$ is constant. In fact, the wide range of ($KADP^{2-}$) possible without affecting the rate would appear to justify excluding it as a possible competitive inhibitor of the reaction.

When (A) is held constant, the equation becomes:

$$V_0 = \frac{V_m(M)}{\alpha'' + \beta''(M)} \qquad (4)$$

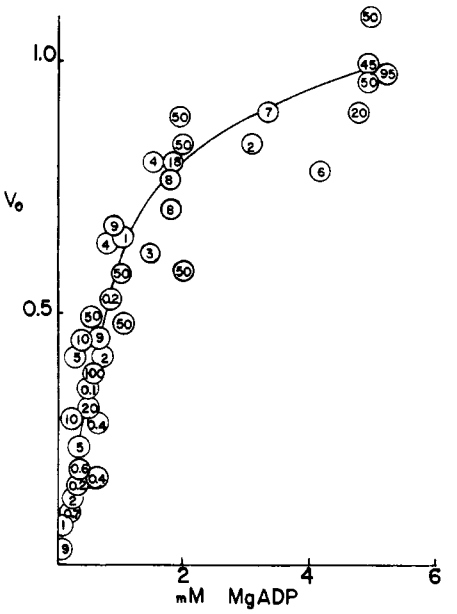

FIGURE 11: Initial velocity of the pyruvate kinase reaction as a function of (MgADP). K_T = 200; the number in each circle represents (Mg^{2+}) in mM.

Equation 4 is of the form of the classic equation of enzyme kinetics and a Lineweaver-Burke (1934) double-reciprocal plot should produce straight lines. It is difficult to arrange for constancy of ($MgADP^-$) while other factors are varied. However, a number of experiments were done in which (K^+) varied while

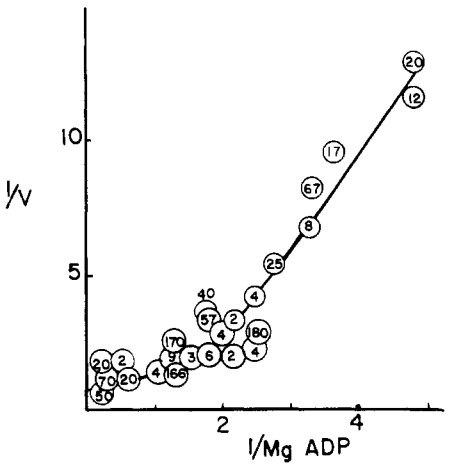

FIGURE 12: Reciprocal plot of the data in Figure 11. The number in each circle represents (KADP) in M × 10^5.

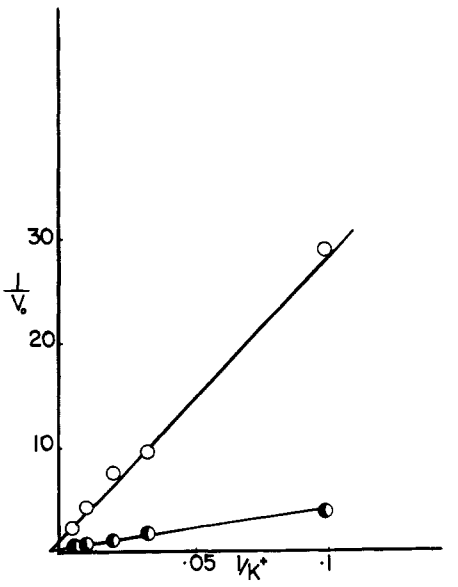

FIGURE 13: Reciprocal plot of the effect of K^+ on the velocity of the pyruvate kinase reaction. Abscissa is in M^{-1}; ○, (MgADP) = 0.47–0.48 mM; ◐, (MgADP) = 1.75–1.84 mM.

(MgADP$^-$) was relatively constant. In contrast to the scatter pattern obtained when (Mg^{2+}) was used for M, plots of 1/K versus $1/V_2$ (see Figure 13 for typical examples) showed the linear relationship predicted by the equation, supporting the idea that the free potassium concentration is an important factor in the activity of the enzyme. The complexity of the constants of equation (4) do not permit prediction as to where the curves will intersect, and it was found that there is no constant point of intersection.

Thus the data supports the scheme shown in Figure 9 with M representing (K^+) and A representing (MgADP$^-$). However, as shown in Figure 7, free Mg^{2+} becomes inhibitory when it is increased to high levels with respect to $(K)_T$. This indicates that there is a relatively weak inhibition exerted by Mg^{2+} and that it is competitive with K^+. It is also apparent (Figure 6) that a large excess of K^+ inhibits the enzyme. These relationships are indicated as follows:

$$\underset{\text{(inactive)}}{MgE} \overset{Mg^{+2}}{\rightleftharpoons} \underset{\text{(inactive)}}{E} \overset{K^+}{\rightleftharpoons} \underset{\text{(active)}}{KE} \overset{K^+}{\rightleftharpoons} \underset{\text{(inactive)}}{K_2E}$$

It is possible that there are two binding sites for positive ions which can affect the activity of the enzyme. When one is occupied by K^+, the enzyme is active. When both are occupied, either by Mg^{2+} or by two K^+'s, the enzyme becomes less active or inactive.

Our measurements differ from those of Reynard *et al.* (1961) in that ionic strength was rigorously controlled, and our interpretation is in terms of the individual ionic species present rather than the total concentration of a nucleotide species. However, neither of these considerations affect the conclusions drawn by these workers concerning the effects of PEP on the rate of the reaction. The linear double reciprocal plots obtained by Reynard *et al.* when PEP was varied are explainable by the equation (Figure 10) on the basis of several assumptions concerning the rate constants; for example, the case in which K_7 is very small. In this case, the squared terms in B (representing PEP) are small with respect to the other terms and contribute little to the equation. With this restriction (when all factors are constant except PEP) the equation of Figure 10 becomes:

$$\frac{V_0}{V_m} = \frac{(B)}{\alpha''' + \beta'''(B)} \tag{5}$$

Thus the equation predicts linear reciprocal plots when PEP is varied, as reported by Reynard *et al.*, but nonlinear plots when (MgADP) is varied, as shown in this paper. The proposed mechanism appears to be consistent with all the data available for this system. The lack of linearity of the reciprocal plot for (MgADP) data seems to preclude the conclusion that the phosphorylation step is the only rate-limiting step, i.e., that k_{11} and k_{12} are small with respect to all other constants and that "equilibrium kinetics" or quasiequilibrium can be assumed to govern.

To our knowledge, the equation shown in Figure 10 has not been presented before. However, except for the activation of the enzyme by the metal ion, it closely resembles the two-substrate mechanism which has been widely discussed (Segal *et al.*, 1952; Alberty, 1953; Danziel, 1957; King and Altman, 1956). Most of the discussions have concerned only the case where quasiequilibrium is assumed to exist. In fact, deviations from linearity of reciprocal plots have been attributed to inhibition by excess substrate (Danziel, 1957) rather than deviations from quasiequilibrium. In the present case,

the equation appears to fit the data without the assumption of quasiequilibrium.

The data indicate further that $MgADP^-$ is the only ADP species which interacts appreciably with the enzyme. The wide range of $KADP^{2-}$ possible without affecting the rate is evident in Figure 12. The lack of any inhibition by excess ADP^{3-} similarly indicates little or no interaction by this species.

Although much attention has been paid to the binding of anions (Klotz, 1946a; Scatchard *et al.*, 1950) and of divalent cations (Klotz and Curme, 1948; Klotz, 1946b) by proteins, little attention has been paid to the binding of monovalent cations. Velick (1949) found no binding of K^+ by aldolase. An instance of an interaction of an enzyme protein with an alkali metal cation has been reported by Melchior and Melchior (1958), who found the inhibition of yeast hexokinase by Na^+ to be related only to the concentration of free sodium.

Based on magnetic resonance studies of the Mn-activated enzyme, Cohn (1963) has suggested that the conformation of the active site of pyruvate kinase is changed by K^+ in such a way as to influence the binding of the other substrate, an observation which is in agreement with the conclusion presented here on the basis of kinetic evidence. It is perhaps significant that Klotz and Ming (1954) have demonstrated that metal ions can mediate the binding of small molecules by proteins. A molecular explanation of the great specificity of K^+ as an activator of this enzyme while Na^+ (Karchmar and Boyer, 1953), excess Mg^{2+}, and excess K^+ are inhibitory would appear to require further investigation.

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