KINETIC STUDIES ON THE CATALYTIC AND REGULATORY MECHANISMS OF HUMAN ERYTHROCYTE PYRUVATE KINASE

by

Hubert A. Leonard, B. A.

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APPROVED:

. . [redacted] . . . .
(Professor in Charge of Thesis)

. . [redacted] . . .
(Chairman, Graduate Council)

AN ABSTRACT OF THE THESIS OF

Hubert A. Leonard (Name) for the Ph.D. (Degree) in Biochemistry (Major Department)

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Approved: Robert D Koler (signature)
(Professor in Charge of Thesis)

Pyruvate kinase is a glycolytic enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP. It is widely distributed in nature and has been detected in, and partially purified from, many different animal tissues. The enzyme has an absolute requirement for a monovalent and a divalent cation. Previous work has shown that the enzyme reaction involves a direct phosphoryl transfer rather than through a phospho-enzyme intermediate. Evidence suggests that there are at least two PEP molecules and two metal ions bound per molecule of enzyme. Kinetic data suggest that the binding site for PEP (or pyruvate) is distinct from the site for ATP (or ADP), with the transferable phosphoryl group overlapping both sites, and that the reaction is governed by equilibrium kinetics.

The present study is concerned with the kinetic and regulatory properties of human erythrocyte pyruvate kinase as they relate to:

(1) the mechanism of action of substrates, products and effectors,

(2) the mechanisms of action of the divalent cations, Mg^{++} and Mn^{++},

(3) the kind and number of interacting subunits,

(4) the role that this enzyme may play in control or regulation of erythrocyte metabolism.

The enzymatic activity was measured spectrophotometrically by a direct method which follows the disappearance of PEP at 230 mμ.

The following conclusions and interpretations have resulted from the data, as reviewed from the literature and presented in Results and Discussion:

Conclusions:

1. The divalent cations, Mg^{++} and Mn^{++}, and their ADP chelates interact with the enzyme in a positive cooperative manner ($n \rightarrow 2$) when metal is rate-limiting. When metal is saturating, no cooperative effect is observed.

2. PEP binds in a positive cooperative manner in the presence of Mg^{++} ($n \approx 2$), but non-cooperatively in the presence of Mn^{++} ($n \approx 1$).

3. FDP and ATP interact with the enzyme in a negative cooperative fashion ($n \rightarrow -2$), with FDP exerting its effect at or near the PEP subsite, and ATP exerting its effect on the nucleotide subsite.

4. The nucleotide subsite is specific for purine nucleoside mono-, di-, and triphosphates. Activation by mono- and triphosphates is evident in the magnesium-activated system but not in the manganese-activated system.

5. Activation by purine nucleoside mono- and triphosphates (nucleotide locus) predominantly affects Vm, whereas activation by FDP (PEP locus) predominantly affects the Km for PEP.

6. Two moles each (or some multiple) of PEP (or FDP), ADP (or ATP) and divalent metal can bind to the native enzyme.

Interpretations:

1. Human red cell pyruvate kinase is composed of two catalytic sites which can interact with each other.

2. Each catalytic site has four functionally important subsites, or loci:

   a. a locus for the enolpyruvate portion of PEP,

   b. a locus for the divalent metal,

   c. a locus for the nucleotide,

   d. a locus for the transferable phosphoryl group.

3. Activation by FDP involves the cation and PEP loci, whereas activation by purine nucleotides involves the cation and nucleotide loci.

4. The rate of catalysis can be regulated by both a precursor molecule (FDP), and the reaction product (ATP). Each regulator affects the enzyme in a unique manner depending on the concentrations of PEP, ADP and the concentration and type of divalent cation.

to Dr. Koler

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ABBREVIATIONS

AMP	adenosine 5' monophosphate
AMP (cyclic)	adenosine 3'5' monophosphate
ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
CTP	cytidine 5' triphosphate
DPNH (NAD)	nicotinamide adenine dinucleotide
ε	molar extinction coefficient
EDTA	ethylenediaminetetraacetic acid
FDP	fructose diphosphate
GDP	guanosine diphosphate
GTP	guanosine 5' triphosphate
Keq	equilibrium constant
Km	Michaelis constant
M^{++}	divalent metal
M.W.	molecular weight
P_i	inorganic phosphate
PEP	phosphoenolpyruvate
TEA	triethanolamine
TMA^+	tetramethylammonium ion
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine triphosphate
Vm	maximum velocity

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INTRODUCTION

I. Historical

Studies on pyruvate kinase encompass a vast and varied literature. Early work centered on the species of pyruvate kinase isolated from rabbit muscle. More recently, species of the enzyme have been isolated and studied from a variety of plant and animal sources.

A. *Studies on rabbit muscle enzyme.* Pyruvate kinase from rabbit muscle has been the prototype for all subsequent studies on this enzyme. The primary metabolic reaction catalyzed by pyruvate kinase is the transfer of a phosphoryl group from phosphoenolpyruvate to ADP as illustrated in equation (1)

$$\underset{\text{Phosphoenolpyruvate}}{CH_2=C(OPO_3^{2-})-C(=O)-O^-} + ADP \underset{}{\overset{(Mg^{++},K^+,H^+)}{\rightleftharpoons}} \underset{\text{Pyruvate}}{CH_3-C(=O)-C(=O)-O^-} + ATP \qquad (1)$$

The enzyme also has been referred to as pyruvate phosphoferase, pyruvate phosphokinase and phosphopyruvate transphosphorylase. The Commission on Enzymes has systematically named the enzyme ATP: pyruvate phosphotransferase (E.C. 2.7.1.40) and retained the trivial name, pyruvate kinase (1).

The first reports of activity attributable to pyruvate kinase were published by Lohmann and Meyerhof (2) and Parnas et al. (3) in 1934. Lohmann and Meyerhof also described the isolation and structural determination of phosphoenolpyruvate, but believed that it was involved in

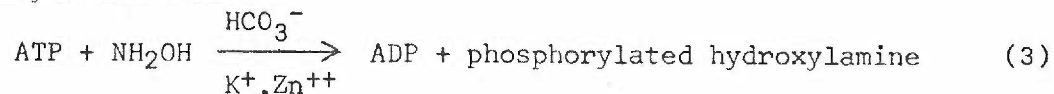
a dephosphorylation reaction yielding orthophosphate and pyruvate (2). However, Parnas et al. (4) regarded creatine as the acceptor of the phosphoryl group of phosphoenolpyruvate. In 1935 it was thought that phosphoenolpyruvate was involved in the phosphorylation of AMP producing ATP and pyruvate (5-7). Boyer et al. (8,9) subsequently showed that ADP was the true phosphate acceptor in the pyruvate kinase reaction. Phosphorylation of AMP was later recognized to result from the action of a second enzyme, adenylate kinase, operating in tandem with pyruvate kinase.

Pyruvate kinase can also catalyze "fluorokinase" (10) and "hydroxylamine kinase" (11,12) reactions. Both phosphorylation reactions require the presence of bicarbonate as illustrated in equations (2) and (3):

"Fluorokinase":

$$\text{ATP} + \text{fluoride} \xrightarrow{HCO_3^-} \text{ADP} + \text{fluorophosphate} \qquad (2)$$

"Hydroxylamine kinase":



$$\text{ATP} + NH_2OH \xrightarrow[K^+, Zn^{++}]{HCO_3^-} \text{ADP} + \text{phosphorylated hydroxylamine} \qquad (3)$$

Lohmann and Meyerhof (2) reported that pyruvate kinase requires a divalent metal such as Mg^{++} for activation. Later, Boyer et al. (8,9) showed that K^+ was also necessary for activity in rabbit muscle preparations. Kachmar and Boyer (13), using the sodium salts of ADP and PEP as substrates, and a Tris buffer at pH 7.4, found that activity was less than 1.5% of the maximal activity obtained with added K^+. Potassium also stimulates the "fluorokinase" and "hydroxylamine kinase"

reactions (10,12). Furthermore, K^+ (as well as Mg^{++}) is required for the exchange of the methyl hydrogens of pyruvate as reported by Rose (14). NH_4^+ and Rb^+ will activate pyruvate kinase in the absence of K^+, while Na^+ and Li^+ are generally inhibitory in the presence of K^+ (13,15). McQuate and Utter (16) and Reynard et al. (17) found that high concentrations of Mg^{++} did not inhibit the reaction in either direction.

The pyruvate kinase reaction was initially regarded as irreversible. The ability of the enzyme to catalyze the formation of PEP from ATP and pyruvate was first demonstrated by Lardy and Ziegler (18). Meyerhof and Oesper (19) determined the apparent equilibrium constant, defined by equation (4):

$$K_{eq} = \frac{(\text{ATP}) \cdot (\text{pyruvate})}{(\text{ADP}) \cdot (\text{phosphoenolpyruvate})} \qquad (4)$$

to be 2000. This was based on total concentrations of all forms of reactants present, at 30°C, in a bicarbonate buffer of unspecified pH. McQuate and Utter (16) later recorded a K_{eq} of about 6500 at pH 7.4 in a Tris buffer. Krimsky (20) reported a value of 2200 at pH 7.7 and 30°C under similar conditions but with a much lower concentration of Mg^{++}. As pointed out by Boyer (21), the higher K_{eq} values found by McQuate and Utter as compared to Krimsky at comparable pH values may reflect differences in Mg^{++} concentration. The affinity of Mg^{++} for ATP is approximately ten times greater than its affinity for ADP, displacing the equilibrium toward ATP and pyruvate formation.

Early kinetic work on rabbit muscle pyruvate kinase, has been summarized by Boyer (21). The data of Reynard et al. (17) showed that at 0°C and pH 8.5 the apparent K_m values for phosphoenolpyruvate and ADP were independent of the concentrations of ADP and phosphoenolpyruvate, respectively. McQuate and Utter (16), working at 30°C and pH 7.4, reported that the apparent K_m for phosphoenolpyruvate increased slightly with increasing ADP concentration, and vice versa. An important observation was that inhibition by ATP was competitive with both ADP and phosphoenolpyruvate (17). Various experimental approaches (22,23) have shown that phosphoryl enzyme formation does not occur in the pyruvate kinase reaction and it was concluded that a direct phosphoryl transfer must take place from donor to acceptor (23).

B. *Recognition of species differences and multiple forms in mammals.* Pyruvate kinase appears to exist as a constituent of all living cells. Boyer (24) first noted its wide distribution by demonstrating the presence of the enzyme in nineteen different tissues from eleven animal species, ranging from protozoa to mammals. As mentioned by Bücher and Pfleiderer (25), pyruvate kinase apparently was obtained first in crystalline form from rat muscle by Negelein whose data were lost during World War II. More recently, crystalline pyruvate kinase has been reported from rat muscle (25), human muscle (26), rabbit muscle (10,25), pig heart (12), and rat liver (27). Further studies have involved partially purified enzyme from erythrocytes (28-32), liver (33-36), skeletal muscle (33,36-38), kidney

(33,37), brain (11,33), heart (11,37), adipose tissue (36,39), tumor cells (40), and developing embryo (41) as well as from a protozoan (42), helminths (43), a locust (44), a crustacean (45), yeasts (46-50) and plant sources (51). The purification and physical properties of pyruvate kinases isolated prior to 1962 has been comprehensively reviewed by Boyer (21). Like the rabbit muscle enzyme, all pyruvate kinases studied to date have an absolute requirement for a monovalent and a divalent cation, and a specificity for PEP and nucleoside diphosphate (preferably ADP). Where studied, these enzymes show inhibition by high levels of both products.

Fellenberg et al. (33), in 1963, reported electrophoretic differences among pyruvate kinase preparations from kidney, liver, brain, cardiac muscle, and skeletal muscle of the rat, indicating that different species of pyruvate kinase are present in specific tissues. Two groups of investigators (52,53) reported that the pyruvate kinase level in liver was under hormonal and dietary regulation, like the levels of other key glycolytic enzymes. In contrast, the enzyme level in skeletal muscle was found to be independent of hormonal influences. Tanaka et al. (27,34) demonstrated the presence of two isozymes of pyruvate kinase in rat liver, named type M (similar to the single enzyme species found in muscle) and type L (a specific species of enzyme found in liver). The two types of pyruvate kinase were identified by electrophoretic and immunological procedures. The level of type L varied greatly in response to hormonal and dietary changes, whereas that of type M changed very little. Type M pyruvate kinase was found

by antibody neutralization to be present in skeletal muscle, brain, heart muscle, liver, kidney, fat pad and leucocytes, and type L was present only in liver and erythrocytes (27). Kinetically, type M enzyme resembled the rabbit muscle enzyme, whereas type L enzyme exhibited a sigmoid velocity profile for PEP and activation by low levels of FDP. More recently, Bigley et al. (37) demonstrated three electrophoretic species of human pyruvate kinase, designated PK I, PK II, and PK III based on decreasing anodal mobility. Brain, leucocytes and muscle (cardiac and skeletal) contain only PK III; erythrocytes appear to possess only PK I; liver - PK I and PK III; and kidney shows all three forms.

Koler et al. (30,54) studied the pyruvate kinases from leucocytes and erythrocytes extensively. The results indicated that the two enzymes differ in physiocochemical, antigenic, and kinetic characteristics. Furthermore, by examining the levels of enzyme in some patients with hereditary type II non-spherocytic hemolytic anemia, the erythrocyte enzyme was found to be deficient, whereas the level of leucocyte enzyme appeared normal. This result indicated that the two isozymic forms of the enzyme were under different genetic control in the two cell lines. Bigley and Koler (55) reported that a liver biopsy, performed on an individual with hereditary non-spherocytic hemolytic anemia due to erythrocyte pyruvate kinase deficiency, showed a deficiency in the "erythrocyte-like" species of pyruvate kinase (PK I), comparable to the red cell deficiency. The "leucocyte" or "muscle" type pyruvate kinase (PK III) from the individual's liver was

normal. This result supported the hypothesis that the erythrocyte enzyme and the PK I enzyme from liver were the same molecular species (37).

C. *Comparison of rabbit muscle enzyme with those from other sources.* Pyruvate kinase isozymes from all sources share certain common characteristics:

1. substrate specificity for PEP and nucleoside diphosphate (preferably ADP).
2. requirement for both a monovalent and a divalent cation activator.
3. product inhibition by high concentrations of pyruvate and ATP.

On the other hand, some species of pyruvate kinase (e.g. mammalian liver and erythrocytes, yeast and E. coli) differ from the rabbit muscle prototype in two important ways:

1. sigmoidal velocity profile for the substrate, PEP.
2. modulation of activity by the non-substrate, FDP.

II. Physicochemical Studies.

Physical and structural studies have been carried out predominantly on the rabbit muscle enzyme. In 1942 Bücher and Pfleiderer (25) estimated the molecular weight of rabbit muscle pyruvate kinase to be 166,000. More recently, however, the molecular weight has been redetermined and the reported value is about 237,000 (56-59). Morawiecki (60) first reported that 2-3 M urea dissociated pyruvate kinase into smaller fragments. This work was extended by Steinmetz and Deal (59)

who found that rabbit muscle pyruvate kinase (M.W. 237,000) was a tetramer composed of four polypeptide chains of 57,200 molecular weight. Furthermore, the data suggested that the tetramer consisted of two identical catalytic particles, or protomers (M.W. 115,000), with each protomer in turn consisting of two polypeptide chains. Such an interpretation is consistent with binding studies which show two moles of Mg^{++} or Mn^{++} bound to the enzyme in the K^+ activated state (61,62), and approximately two moles of PEP (17,63), and two moles of ADP (63). Peptide mapping of rabbit muscle pyruvate kinase indicated that the enzyme contains four highly similar, although not necessarily identical, subunits (64). It was also reconfirmed that the 7.35S (115,000 M.W.) species formed in 2M urea retains catalytic activity, whereas the 2.05S (57,000 M.W.) species formed in 4M urea is inactive (64). Johnson and Deal (65) have also shown that there is a specific binding of two to four moles of pyridoxal 5'-phosphate to rabbit muscle enzyme which produces total inactivation but no detectable conformational changes.

The molecular basis for cation activation is uncertain. Apparently both monovalent and divalent cations have direct effects on the protein. Suelter and Melander (66) first reported that both Mg^{++} and Mn^{++} gave ultraviolet difference spectra with rabbit muscle pyruvate kinase. This work was extended by Kayne and Suelter (67) and Suelter et al. (62) who showed that monovalent cations (K^+, NH_4^+, Rb^+), the substrate PEP and changes in temperature elicit similar protein difference spectra. Specific monovalent cations have been shown to

affect the immunoelectrophoretic patterns of rabbit muscle enzyme (68). Mildvan and Cohn (61,69) reported two moles of Mn^{++} bound per mole of pyruvate kinase when the reaction mixture contained 0.1 M K^{+}, an activating cation. However, when tetramethylammonium ion (TMA), a non-activating cation, was substituted for K^{+}, four Mn^{++} are bound per molecule of pyruvate kinase. Two moles of K^{+} were reported to bind per molecule of enzyme, and individual dissociation constants for Mn^{++} and K^{+} were determined. This led Mildvan and Cohn to postulate one divalent and one monovalent cation activator at each of two active sites on pyruvate kinase. Suelter et al. (62) calculated two moles of Mg^{++} bound per mole of rabbit muscle enzyme in the presence of TMA Cl. Recently, Cottam and Ward (70) reported four moles of Zn^{++} bound per mole of rabbit muscle pyruvate kinase in the presence of 0.5 M K^{+}; all four Zn^{++} binding sites appeared to have identical association constants and Zn^{++} and Mn^{++} were shown to compete for the same binding site on the enzyme.

Further work from Suelter's laboratory (71) has indicated that K^{+} and Mn^{++} alter the fluorescence emission and polarization of rabbit muscle pyruvate kinase. Temperature studies have revealed that the enzyme may exist in equilibrium between a high-temperature and a low-temperature form (72). Binding of cations favors the low-temperature form, whereas addition of protein structure disrupting agents favors the high-temperature form. Kuczenski and Suelter (73) have studied the "allosteric" pyruvate kinase from baker's yeast and have shown it to be inactivated by low temperatures. Addition of FDP, the allosteric

activator, markedly enhances the inactivation, both at room temperature and in the cold. Addition of Mg^{++} or Mn^{++} prevents inactivation. A mechanism was proposed which involved the binding of two moles of FDP, followed by dissociation into subunits during inactivation (decrease in the sedimentation coefficient from 8.6 to 3 to 1.75). Inactivation at 0°C appeared to follow the same mechanism. In view of the work on rabbit muscle and yeast enzymes, it is of interest whether this temperature dependent structural change is observed in other preparations of pyruvate kinase and whether or not such changes have physiological significance. Somero (45), working on pyruvate kinase isolated from Alaskan king crab, has presented evidence for a temperature-dependent interconversion between two forms (of a single protein species) having distinct and adaptive kinetic properties. Temperature changes mimicked the effects of FDP on "allosteric" pyruvate kinases.

It has generally been assumed that human erythrocytes possess one major molecular form of pyruvate kinase (30,37,55,74-76). Townes (77), and Townes and Harris (78), however, reported that human erythrocytes possessed two pyruvate kinase bands on starch gel electrophoresis at pH 8.6 in a triethanolamine buffer, the same conditions used by others who demonstrated one band (30,37). Gel filtration on Bio-Gel 200 and starch gel electrophoresis of the filtered fractions indicated that the two bands were of different molecular size (78), the slow being larger than the fast. An alternative explanation for two bands might be contamination of red cells by leucocytes (79), the leucocyte enzyme then accounting for the slow band. Careful attention to preparative

technique should eliminate the latter alternative.

III. Kinetic Studies.

A. *Rabbit muscle prototype.* Phosphoenolpyruvate is the only substrate that has been reported to phosphorylate ADP in the presence of pyruvate kinase, and among the α-keto acids only pyruvate appears to act as an acceptor of the γ-phosphoryl group of ATP. Rose (14) has shown that in the presence of Mg^{++}, K^{+}, and ATP or P_i, pyruvate was the only α-keto acid that exhibited an exchange of hydrogen ions with the β-carbon in the presence of pyruvate kinase. Davidson (80), using rabbit muscle enzyme, reported that guanosine diphosphate (GDP) and inosine diphosphate (IDP) showed about 60% and 75%, respectively, of the maximum activity obtained with ADP; pyrimidine diphosphates exhibited no activity. Plowman and Krall (81), using rabbit muscle enzyme prepared in a similar manner, reported broad specificity for nucleoside diphosphates with the purines being considerably better substrates than the pyrimidines. The importance of the sugar portion of purine nucleotides was noted by Klenow and Andersen (82), who showed that 2-deoxyadenosine diphosphate provided only 11% of the maximal activity obtained with ADP. Mildvan and Cohn (61) demonstrated that manganese could substitute for magnesium in the pyruvate kinase reaction, although the maximum rate was reduced. Ca^{++} was found to be a potent inhibitor of the reaction (13) and competitive with Mn^{++} (61). Furthermore, Kachmar and Boyer (13) and Solvonuk and Collier (28) noted that Ca^{++} inhibition could be overcome partially by increase in K^{+} concentration. It is interesting to note that the "hydroxylamine

kinase" activity requires divalent cations in the order of effectiveness: $Zn^{++} > Co^{++} > Mn^{++}$ and that activity is absent with Mg^{++} (11,83). "Fluorokinase" utilizes Mg^{++} and Mn^{++} but not Zn^{++} (11).

The role of Mg^{++} has been studied extensively but the results remain difficult to interpret. Magnesium complexes of the adenine nucleotides originally were implicated as the true substrates (16,17). This conclusion was supported by Melchior (84,85) following studies on rabbit muscle preparations. Free nucleotide did not appear to function as a substrate. Mildvan and Cohn (61,86), using purified rabbit muscle pyruvate kinase and nuclear magnetic resonance techniques, concluded that a metal nucleotide chelate bound more effectively to the enzyme than the free nucleotide to a metal-enzyme complex. Cleland (87), by replotting the kinetic data of Mildvan and Cohn (86), concluded that neither free manganese nor free ADP combines with the enzyme, and that there is no kinetic evidence that Mn^{++} (or Mg^{++}) plays any role in the mechanism other than to complex ADP and form the active substrate.

Mildvan et al. (88) recently compared the structure and role of Mn^{++} complexes of an allosteric pyruvate kinase from yeast with rabbit muscle enzyme using NMR techniques. They concluded that the active enzyme-Mn-ADP bridge can form by multiple pathways and that the yeast enzyme binds up to six moles of Mn^{++} cooperatively with apparent affinities varying by three orders of magnitude. The allosteric activator FDP appeared to raise the affinity of the yeast enzyme-Mn complex for ADP, and site-site interactions among the Mn^{++} binding sites altered their affinities for PEP. A comparison of the binding

and kinetic data for the yeast enzyme suggested a preferred order of binding: PEP prior to ADP. It was postulated that the affinity of the PEP binding sites might be controlled by the distance between two subsites: one for Mn^{++} and one for the carboxylate group of PEP.

The role of magnesium was examined by Kerson et al. (89) by computer simulation studies of data collected on rabbit muscle enzyme. Control by Mg^{++} appeared to these investigators as the most important among the activators of this enzyme.

Wood (90) challenged the competitive inhibition data of Reynard et al. (17) by showing that ATP inhibition depends on the level of Mg^{++} in the medium, and that high concentrations of ATP will chelate a large percentage of the free magnesium, thereby reducing the concentration of Mg ADP, and hence activity. Saturating Mg^{++} concentrations appeared to abolish the inhibition by ATP. Boyer (91), using a Mg^{++} buffer system to maintain free magnesium, reconfirmed the results of Reynard et al., and noted two possible explanations for the differences from Wood's data: 1) failure to correct for the presence of ADP in ATP which could account for the absence of inhibition in an assay using low ADP, and 2) a 30°C assay temperature, whereas Reynard et al. (17) worked at 0°C and Boyer at 25°C. The latter explanation could be important in view of the temperature effects noted by Kayne and Suelter (67,72) and Suelter (71) for a similar preparation of rabbit muscle enzyme. Recently, Holmsen and Storm (92) have shown that at very high Mg^{++} concentrations ATP inhibition is very weak. They suggested the ratio of ATP to magnesium as the critical factor governing ATP

inhibition.

B. *Other pyruvate kinases.* Hess et al. (48), Hunsley and Suelter (93), and Gancedo et al. (94) found that pyruvate kinase isolated from yeasts exhibited a sigmoid velocity profile for PEP and that FDP was a potent activator of the enzyme, similar to the results found for species of pyruvate kinase from rat liver (34, 95-98) and mouse liver (35). Comparable results have been obtained with pyruvate kinases from adipose tissue (39), E. coli (49), and human erythrocytes (76). Human liver also has been shown to possess a species of pyruvate kinase (55,99) that kinetically resembles the erythrocyte, rat liver (L-type) and yeast enzymes. The kinetic properties of yeast enzyme, erythrocyte enzyme, and the FDP-activatable enzymes from liver sources are similar and resemble those generally described as allosteric or regulatory enzymes (100-104). Llorente et al. (105) found that extracts of liver and kidney pyruvate kinase from the rat were desensitized to the homotropic cooperativity of PEP and FDP by low temperatures (0-2°C). Unfortunately, the samples used in these studies were crude extracts and both rat liver and kidney have been reported to contain more than one pyruvate kinase isozyme (27,33,95). Hence, low temperatures may selectively inactivate isozymes which exhibit "allosteric" properties while leaving other "non-allosteric" isozymes functional.

Campos et al. (74) studied species of pyruvate kinase isolated from human leucocytes and erythrocytes, respectively. The leucocyte enzyme resembled the rabbit muscle pyruvate kinase, and the Michaelis

constant observed for each substrate (PEP, ADP) was independent of the concentration of the second substrate. The erythrocyte enzyme, however, required at least ten-fold higher concentrations of PEP and the apparent Km value for each substrate varied with the concentration of the second substrate. Adenosine triphosphate was found to be a competitive inhibitor of PEP and ADP for both the leucocyte and erythrocyte isozymes (75).

Koler and Vanbellinghen (76) first reported the modulation of human erythrocyte pyruvate kinase by FDP, an effect similar to that observed with liver and yeast enzymes. The regulatory properties of this enzyme were further investigated by Bigley (106) who demonstrated an activating effect of ATP on the forward reaction rate at low concentrations of ADP and PEP. A similar result was noted by Haeckel et al. (107) with yeast pyruvate kinase. Rozengurt et al. (98), however, concluded that ATP has an inhibitory effect on rat "liver" type pyruvate kinase, considered to be qualitatively similar kinetically to the red cell enzyme used by Bigley (106). Ozaki and Shiio (108) demonstrated strong inhibition by ATP, but activation by AMP; however, they failed to examine the kinetic effects over a range of ATP concentrations.

Ibsen et al. (109) demonstrated kinetic properties of human erythrocyte pyruvate kinase different from those of Campos et al. (74) and more closely resembling muscle or leucocyte enzyme. Recently Ibsen and Schiller (110) reported isolation of multiple kinetic forms of the red cell enzyme which appear stable and interconvertible.

Whether these "multiple forms" represent unique molecular species remains doubtful. Variations in isolation and purification techniques could account for these differences.

C. *Models.* Various proposals have been made regarding the catalytic mechanism of pyruvate kinase. Initially, interest was focused on the rabbit muscle enzyme. Reynard et al. (17) and Boyer (21) postulated a rapid equilibrium random mechanism based on some or all of the following conditions:

1. competitive inhibition by ATP with both ADP and PEP (17).
2. direct transfer of the phosphoryl group from the donor to the acceptor (21).
3. a single locus for PEP and pyruvate (21).
4. a separate locus for ADP (ATP) which has specificity for the purine base and the ribose sugar moieties (80,82).
5. a common locus for the transferrable phosphate group.

The "common locus" postulate, however, may be an oversimplification since Kerson et al. (89) reported that AMP as well as ATP showed competitive inhibition with PEP and ADP for the rabbit muscle enzyme. Mildvan and Cohn (86) strengthened the rapid equilibrium random mechanism by demonstrating that the dissociation constants of the binary and ternary enzyme complexes (via NMR) were in good agreement with the kinetically calculated ones. The ternary complex represented an enzyme-metal-substrate bridge with PEP, ADP and enzyme, each donating a ligand to the metal. Enzyme and metal formed a functional binary complex.

Studies conducted on yeast (88), rat liver (27,98) and human erythrocyte (76) pyruvate kinases have indicated that these enzymes possess an added degree of complexity -- namely regulatory or allosteric properties (101,102). Each shows a sigmoid velocity profile with PEP and is strongly activated by FDP. Generally, it is assumed that allosteric phenomena are the result of conformational interactions between distinct and separate sites on a protein molecule (101,102, 104). Therefore, this does not necessarily restrict the application of the rapid equilibrium random mechanism to catalysis occurring at a given site. Campos et al. (74) presented evidence for the human red cell enzyme consistent with such a mechanism. The identical enzyme was found by Koler and Vanbellinghen (76) to possess allosteric properties as mentioned above.

Rozengurt et al. (98) fashioned their data collected from rat liver (type L) pyruvate kinase after the concerted transition model of Monod et al. (101). Koler and Vanbellinghen (76), working on the human red cell enzyme, proposed a model which would not require separate allosteric binding sites. In this model a change occurs in the enzyme following occupancy of the first catalytic site which increases the affinity for substrate at the remaining catalytic site(s). Activation by FDP can occur by binding to an empty catalytic site which then causes a change in affinity for substrate at the remaining (catalytic) site(s). Additional factors such as ATP activation (106), divalent cation effects and pH ultimately may require a more sophisticated model.

IV. Role in Metabolic Control.

Pyruvate kinase is one of three key glycolytic enzymes which control the rate and direction of glycolysis (111-116). Certain isozymes of pyruvate kinase in mammalian cells are under hormonal and dietary control as previously stated (52,53). Studies on the oscillations of glycolytic intermediates in yeast cells and beef heart have implicated phosphofructokinase as a primary oscillator due to its regulation by substances such as FDP, ADP, ATP and AMP (117,118). Computer studies, based on a simple feedback amplifier model, are consistent with these studies (119). The activities of allosteric pyruvate kinases are regulated by many of the same glycolytic intermediates. The data of Frenkel (118), which show that phosphofructokinase is at a cross-over point in the flux ratios of glycolytic intermediates, also demonstrates a similar cross-over point at the pyruvate kinase step.

It is the purpose of this thesis to examine in detail the kinetic mechanism of human erythrocyte pyruvate kinase, and where pertinent to study the human and rabbit muscle species of this enzyme. These studies were designed with the hope of integrating information from the vast literature on this enzyme regarding the catalytic and possible regulatory properties of mammalian pyruvate kinase isozymes.

EXPERIMENTAL

I. Materials

A. Reagents and substrates.

Source: DPNH, disodium ATP, disodium ADP, trisodium PEP, 3',5' AMP, GDP, GTP, UTP, CTP, crystalline lactic dehydrogenase, crystalline triose phosphate isomerase and crystalline α-glycerol-phosphate dehydrogenase were obtained from the Sigma Chemical Co. Trisodium FDP and crystalline rabbit muscle pyruvate kinase were purchased from either Sigma Chemical Co. or California Corporation for Biochemical Research. Triethanolamine was obtained from either Sigma Chemical Co. or Eastman Organic Chemicals. Tetramethyl-ammonium chloride was a product of Eastman Organic Chemicals. Tris and disodium dipotassium EDTA were purchased from Matheson Coleman and Bell. Sephadex G-25 was obtained from Pharmacia. $MgCl_2$, $MgSO_4$, $MnCl_2$, NaCl, and KCl were all reagent grade.

Determinations of Cantaminating Compounds in Various Substrate and Effector Solutions: Preparations of ATP were checked for the presence of ADP by adding ATP to the pyruvate kinase assay with excess PEP and allowing the reaction to reach equilibrium. The ATP preparations were contaminated with 1.5-3% of ADP. Preparations of ADP were checked for contaminating ATP by fluorimetry and were found to contain as much as 7-10% ATP. Contamination of ADP and ATP by PEP was checked, using the pyruvate kinase assay without adding PEP.

No detectable PEP was found in these nucleotide preparations. Pyruvate contamination by PEP was checked using the pyruvate kinase assay with excess ADP and no PEP was detected. Preparations of PEP, however, were contaminated with up to 3% pyruvate as measured in the lactic dehydrogenase assay with excess NADH. Solutions of PEP, ADP and ATP were assayed for the presence of FDP using the aldolase, triose phosphate isomerase, α-glycerolphosphate dehydrogenase coupled assay of Rutter et al. (120), and no FDP was detected.

Determination of Substrate Concentrations: All solutions were made up and diluted with distilled water. PEP, ADP, ATP and pyruvate concentrations were checked prior to use in kinetic assays by checking absorbancies and using the appropriate millimolar extinction coefficients: PEP, 2.93 at 230 mμ; pyruvate 0.65 at 230 mμ; ADP and ATP, 15.4 at 259 mμ. Since ADP was known to contain up to 10% contaminating ATP, a pyruvate kinase assay with excess PEP was used to standardize ADP solutions.

B. Enzymes.

Preparation of Human Erythrocyte Pyruvate Kinase: Red cell pyruvate kinase was prepared as previously described (76). Steps involved in the preparation include washing the leucocyte-free erythrocytes in 0.15 M NaCl, pH 7.4, buffered with sodium phosphate; hemolysis in 30 volumes of distilled water and allowing stroma to settle overnight; batch absorption and elution from DEAE cellulose; salting eluate to 45% with $(NH_4)_2SO_4$ followed by resuspension of the precipitate and reprecipitation at 25% $(NH_4)_2SO_4$; precipitation of the resulting

supernatant at 38% $(NH_4)_2SO_4$; heating the redissolved 38% fraction to 40°C for one hour in 0.0067 M EDTA, pH 5.0, and precipitating the enzyme from the resulting supernatant in 38% $(NH_4)_2SO_4$. The enzyme is stable in this form for many months at 4°C.

Aliquants for kinetic studies were passed through G-25 Sephadex columns to remove $(NH_4)_2SO_4$. The columns were pre-equilibrated with either 0.15 M NaCl or 0.104 M TMACl. The observed kinetic properties were identical for both the NaCl and TMACl exchanged preparations (subsequently referred to as Enzyme I). The NaCl stabilized samples lost very little activity over several weeks, but the TMACl stabilized samples lost 50% of initial activity within seven days. With very active 38% preparations, dilution with 0.15 M NaCl was considered sufficient to remove the effects of NH_4^+ ion (subsequently referred to as Enzyme II). Specific activities ranged from 20-50 units per mg protein. Protein (mg/ml) was determined by a modified Folin-Ciocalteu procedure described by Reiner and Cheung (121). Except where specifically stated, all steps were carried out at or near 4°C. All samples were stored at 4°C. Similar enzyme preparations exhibited single schlieren peaks in the analytic ultracentrifuge with approximate molecular weights of 230,000.[1] Disc gel and starch gel electrophoresis each demonstrated a single pyruvate kinase band, although other protein bands were present.[2]

[1]Ultracentrifuge runs were performed by Dr. D. A. Rigas.

[2]Electrophoresis was carried out by Dr. Robert Bigley and Mrs. Zelma Stocklen.

Preparation of Human Muscle Pyruvate Kinase: Muscle pyruvate kinase was prepared by Dr. Robert Bigley and Mrs. Irene Pierovich from human psoas muscle obtained at autopsy. The procedure will be described elsewhere (122). Specific activities ranged from 40-150 units/mg. Single enzyme bands were detected by starch gel and disc gel electrophoresis.

Preparation of Rabbit Muscle Pyruvate Kinase: Pyruvate kinase (type II; crystalline from rabbit skeletal muscle; Sigma Chemical Co.) a suspension (∿10 mg/ml) in $(NH_4)_2SO_4$ solution was used. Aliquots for use in kinetic assays were passed through Sephadex G-25 (pre-equilibrated with 0.15 M NaCl) to remove $(NH_4)_2SO_4$.

II. Methods

A. Assay at 230 mμ.

The enzymatic activity of pyruvate kinase is usually estimated by measurement of the pyruvate produced in the forward reaction by coupling to the DPNH dependent lactic dehydrogenase reaction and following the disappearance of DPNH at 340 mμ (10,25). A direct method follows the disappearance of phosphoenolpyruvate at 230 mμ (123), and has been used exclusively in the reported work, except where specifically stated. Table 1 summarizes the results of a pilot study comparing the two assay methods. The standard reaction mixture contained 8 mM TEA buffer, pH 7.46, 75 mM KCl, 16 mM $MgSO_4$,[3] 1.5 mM PEP, and 1.0

[3] $MgCl_2$ and $MgSO_4$ gave identical kinetic results over the concentration range of Mg^{++} used in these experiments.

TABLE 1

Substrate Concentrations: (ADP)	(PEP)	LDH coupled assay (340 mμ) ε = 6.22×10^3	Direct PEP assay (230 mμ) ε = 2.2×10^3
ADP (0.125 mM)	PEP (1.0 mM)	.032*	.029
ADP (0.125 mM)	PEP (0.25 mM)	.011	.011
ADP (1.0 mM)	PEP (1.0 mM)	.119	.121
ADP (1.0 mM)	PEP (0.25 mM)	.038	.034

* Activities are expressed as micromoles of DPNH (PEP) per min. Identical aliquots of diluted human RBC pyruvate kinase were added to each reaction mixture. Values are mean of triplicate determinations.

mM ADP in a total volume of 1.0 ml. The mixture was incubated for 4 to 5 minutes at 37°C and the enzyme (10 μl or 20 μl) was added last; the final volume being 1.01 or 1.02 ml, respectively.

Velocities of the pyruvate kinase catalyzed reaction were followed in matched 0.5 cm light path quartz cuvettes by measuring the change in optical density with time (ΔOD/min) at 230 mμ in either a model DU spectrophotometer equipped with a Gilford 2000 multichannel absorbance recorder, or a Gilford model 2400 recording spectrophotometer with multiple sample absorbance recorder. The temperature of the cuvette chamber was maintained with a Haake circulating water bath at 37°C except where stated otherwise. Assays were run in triplicate for all initial velocity determinations. Readings were accurate to within ± .002 optical density units. High precision was maintained by discarding any set of triplicates in which the deviation between the mean and any single determination exceeded twenty percent.

B. Estimation of rates.

The progress curves of the forward reaction have sigmoidal patterns, i.e., the rates are non-linear (in the absence of added effectors). In order to obtain accurate values of the rate of reaction at any time, t, the rate was determined by graphically drawing a tangent to the curve at 30 second intervals and computing the slope of the tangent from the graph. Tangents drawn to the curve at approximately one minute were operationally defined as "initial" velocities. The designation "maximum velocity" refers to the maximum change in

optical density with time for any given progress curve. The designation "maximum velocity, V_m," denotes the maximum rate achievable for a given enzyme preparation with saturating levels of the variable ligand, other ligand and cofactor concentrations remaining constant. "Standard assay velocity" is the maximum rate that can be achieved at optimal concentrations of all substrates and cations affecting the catalytic capacity of the enzyme (16 mM $MgSO_4$, 75 mM KCl, 8 mM TEA, pH 7.46, 1.0 mM ADP, 1.5 mM PEP). The change in optical density when the reaction reached equilibrium was determined by making a plot of reciprocal ΔOD against reciprocal time and extrapolating to infinite time.

Preincubation: In all kinetic studies performed the reaction was initiated by the addition of enzyme. Since the progress curve of the forward reaction was characterized by an initial slow phase resulting in sigmoidal curves, preliminary incubation studies were performed to ascertain whether the slow phase could be eliminated by previously incubating the reactants. All possible combinations of enzyme, PEP, ADP and Mg^{++}, with and without the effectors ATP and FDP, were preincubated for 5 minutes at 37°C. No changes occurred in the progress curve of the reaction on preincubating any combination of reactants and effectors.

Temperature Effects on Enzyme: Samples of enzyme used in kinetic assays were kept on ice following removal from storage (4°C). Preincubation of enzyme aliquots of different dilution for one, two, three or six hours at 25°C or 37°C showed no change in progress curve

profile when compared to enzyme kept near 4°C. Incubation of diluted enzyme samples at 37°C for longer than one hour resulted in considerable loss in activity. Incubation of diluted enzyme samples at 25°C caused no noticeable loss in activity up to six hours following dilution and incubation.

C. pH Optima.

All three species of pyruvate kinase were assayed at a pH of 7.4-7.5. This was found to be in the optimal range for each enzyme. Figure 1 illustrates the pH profile for human erythrocyte pyruvate kinase with various concentrations of PEP and magnesium using a TEA buffer. A profile of the enzyme in the presence of FDP is also presented.

D. Dilution experiments.

In one set of experiments it was desired to study the order of reaction with regard to the various ligands present (see discussion section). Starting with a 1.0 ml assay volume containing specified concentrations of all ligands, the volume of succeeding assays was increased by 0.05 ml or 0.1 ml increments until activity was no longer measurable.

III. Analysis of Data

A. Kinetic order of the reaction.

Order of Reaction; Hill Plots. A sigmoid curve of initial rates as a function of substrate concentration can indicate that a given enzyme interacts in some way with more than one molecule of ligand. A generalized equation for the overall order of such an enzyme catalyzed

Figure 1

Velocity of the pyruvate kinase reaction as a function of pH for the TEA buffered system. All assays contained 75 mM KCl and 0.125 mM ADP.

(ADP) = 0.125 mM
all circles = 1.6 mM Mg^{++}
all triangles = 16 mM Mg^{++}
0.15 mM PEP
1.0 mM FDP
0.75 mM PEP
0.15 mM PEP
μmoles PEP/min
0.8
0.6
0.4
0.2
5.5
6.5
7.5
8.5
pH

reaction can be written as

$$v = k(L)^n \tag{5}$$

where v = initial velocity observed, k is a rate constant and (L) is the concentration of ligand which is interacting with the enzyme. The value of n is equivalent to the number of ligands interacting with enzyme, but is typically neither integral nor constant except at low (non-saturating) concentrations of the ligand(s). In order to evaluate n graphically, eq (5) is transformed to eq (6):

$$\log(v) = \log k + n\log(L) \tag{6}$$

In the dilution experiments described in Section II D above, increasing volume is directly proportional to decreasing (L). Equation (6) has been used to evaluate the overall order of the pyruvate kinase reaction. From equation (5) and other assumptions discussed by Atkinson (124) and Bernhard (125), A. V. Hill arrived at an empirical equation which relates the fractional velocity of a reaction to the number of binding sites for a given ligand or substrate, S, on the protein molecule:

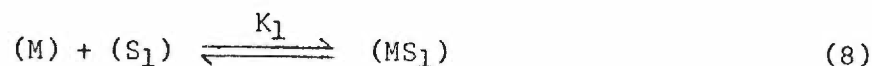
$$\log \frac{v}{V_m - v} = n\log(S) - \log K \tag{7}$$

where v = initial velocity, V_m = maximum velocity at optimal (S), and K is the product of the n-dissociation constants for the separate binding steps. A plot of log $\frac{v}{V_m - v}$ as a function of log(S) should give a

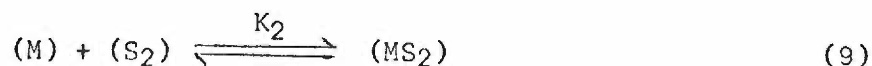
straight line of slope n. This slope is a function of the *number* of interacting substrate binding sites per enzyme molecule and the *strength of interaction* between these sites. When there is no interaction associated with the binding of a given ligand, n = 1 (in the non-saturating concentration range).

B. Equations for estimation of free and bound substrate and divalent cation.

Both PEP and ADP have appreciable affinities for the divalent cations, Mg^{++} and Mn^{++}. Dissociation constants for the various complexes have been determined by several investigators (61,126-127). Table 2 summarizes the constants used in conjunction with the present work. The effect of chelation can be expressed by the following equilibria:



$$(M) + (S_1) \underset{}{\overset{K_1}{\rightleftharpoons}} (MS_1) \quad (8)$$



$$(M) + (S_2) \underset{}{\overset{K_2}{\rightleftharpoons}} (MS_2) \quad (9)$$

therefore:

$$K_1 = \frac{(MS_1)}{(M-S_1-S_2)(S_1-MS_1)} \quad ; \quad K_2 = \frac{(MS_2)}{(M-S_1-S_2)(S_2-MS_2)}$$

Solving either equation in terms of M,S or MS results in a quadratic equation. Such an expression is good only for estimating the effects of one substrate. Simultaneous solution of equations (8) and (9) resulted in a cubic equation providing an estimate of the free and

TABLE 2

Metal	Substrate	$K_D \times 10^4$	Reference and Comments
Mg^{++}	PEP	55.0	(127) μ = 0.1; pH 7.0; 25°C
Mg^{++}	ADP	5.0	(85) pH = 7.0; 25°C; μ = 0.1
Mg^{++}	ATP	0.48	(85) pH 7.0; 25°C; μ = 0.1
Mg^{++}	ATP	0.55	(127) μ = 0.11; 25°C
Mn^{++}	PEP	19.1	(61) pH 7.5; 24°C; μ ≈ 0.1
Mn^{++}	PEP	17.8	(127) μ = 0.1; 25°C
Mn^{++}	ADP	1.0	(61) pH 7.5; 24°C; μ ≈ 0.1
Mn^{++}	ADP	1.0	(127) 23°C; μ = 0.1
Mn^{++}	ATP	0.14	(61) pH 7.5; 24°C; μ ≈ 0.1
Mn^{++}	ATP	0.13	(127) μ = 0.1; 23°C

bound concentrations of metal and both substrates. The cubic equation was programmed in Fortran for use on an 1130 digital computer.[4]

[4]The solution and programming was performed with the kind help of Dr. William Kimberling, Postdoctoral Fellow in Genetics.

RESULTS AND DISCUSSION

I. Initial Velocity Studies

A. Hill plot data.

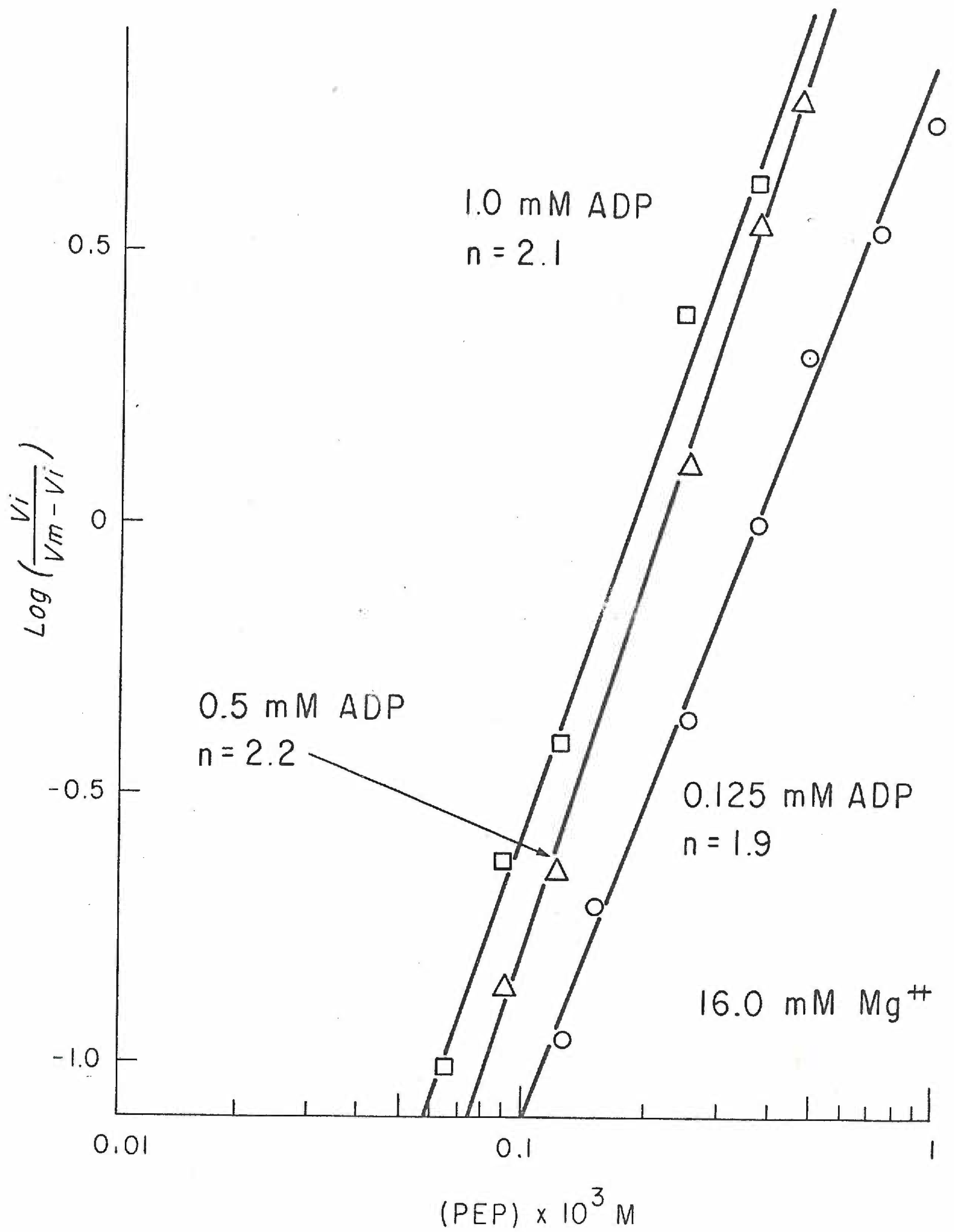
The kinetics of the reaction catalyzed by human erythrocyte pyruvate kinase are complex. The plot of initial rate against phosphoenolpyruvate concentration is sigmoid in the usual assay system using Mg^{++} (76). When plotted in the form of a Hill plot as described in Experimental, a straight line is obtained with a slope of approximately 2 (fig. 2). Varying the ADP concentration displaces the line, but does not alter the slope. Figure 2 illustrates this phenomenon at a Mg^{++} concentration of 16mM. Other experiments have shown an identical effect at 1.6mM magnesium. Figure 3 shows that FDP activates the magnesium containing system, as has been reported (76).

In contrast to the results using Mg^{++} as the divalent cation, figure 4 illustrates the results using Mn^{++}. A straight line with an approximate slope of 1 is obtained in the Hill plot, and the velocity profile with PEP is hyperbolic compared to the sigmoid profile in the presence of Mg^{++} (fig. 3). Figure 3 also demonstrates that FDP has no effect on the manganese-activated system.

It was suggested by Melchior (84,85), and Reynard et al. (17) that the true substrate of the pyruvate kinase reaction is the metal chelate of ADP. In figure 3 the concentrations of ADP and Mg^{++}, and of ADP and Mn^{++} were chosen so that the concentrations of MgADP and MnADP were

Figure 2

Order with respect to PEP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 16 mM $MgSO_4$; enzyme(I); and PEP and ADP at the concentrations indicated. The abscissa is plotted as total PEP.



1.0 mM ADP
n = 2.1
0.5 mM ADP
n = 2.2
0.125 mM ADP
n = 1.9
16.0 mM Mg⁺⁺
0.5
0
-0.5
-1.0
0.01
0.1
1
Log (Vi / (Vm - Vi))
(PEP) x 10³ M

Figure 3

Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the concentration of total PEP. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.3 mM ADP; enzyme(II); and PEP at the concentrations indicated. (□) 1.6 mM $MgSO_4$; (■) 1.6 mM $MgSO_4$ and 1.0 mM FDP; (Δ) 0.4 mM $MnCl_2$; (▲) 0.4 mM $MnCl_2$ and 1.0 mM FDP.

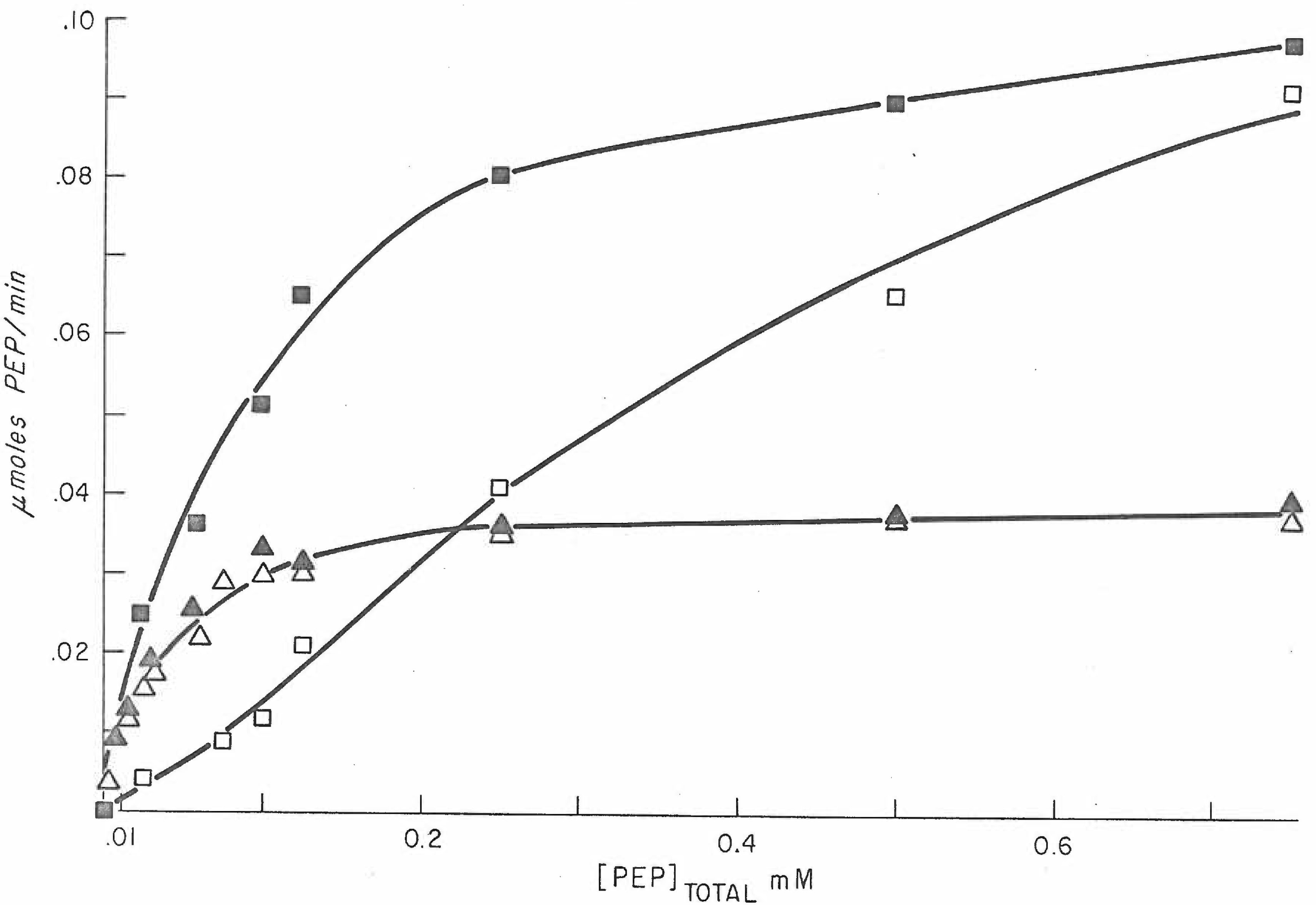
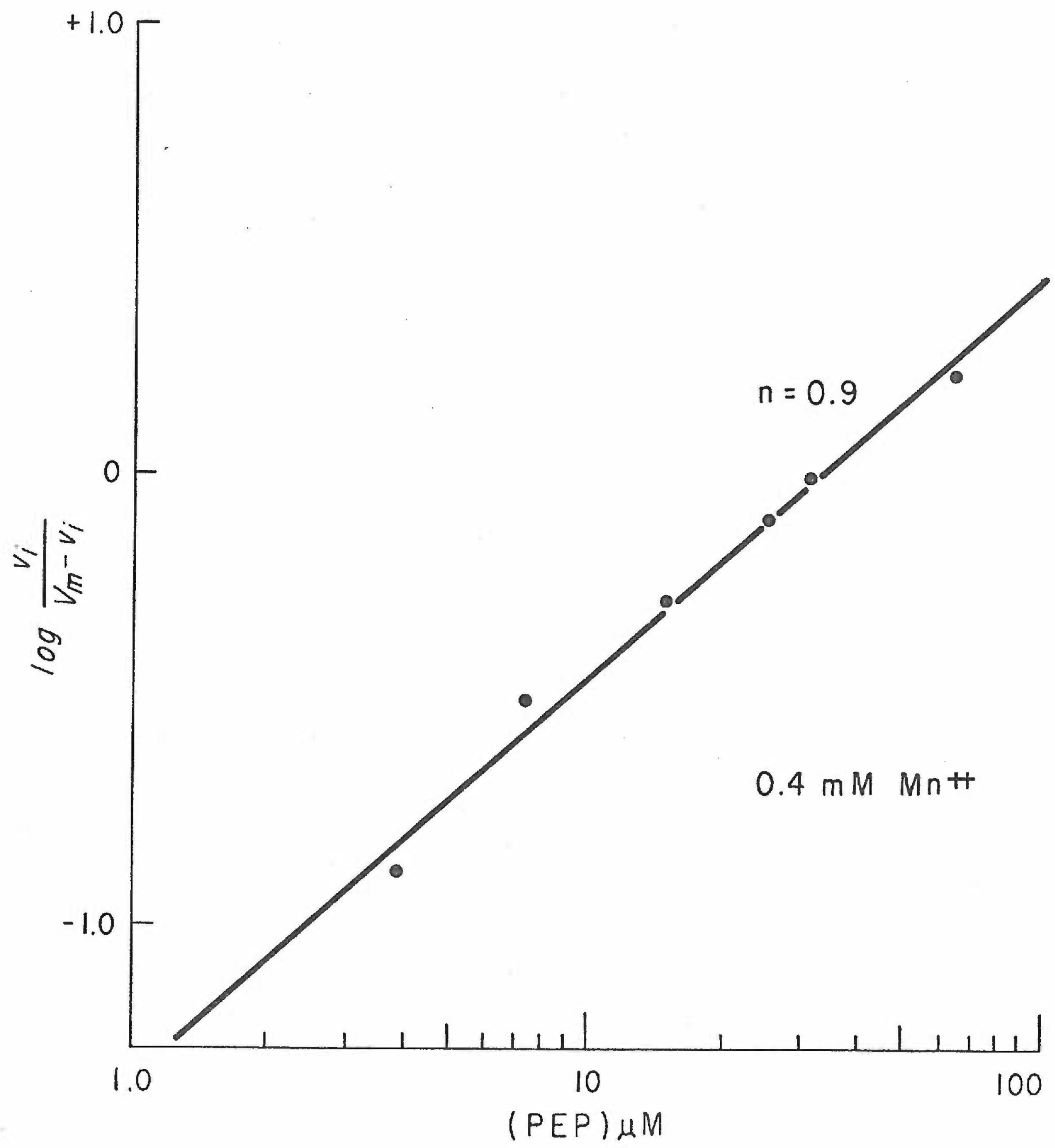

.10
.08
.06
.04
.02
μmoles PEP/min
.01
0.2
0.4
0.6
[PEP]TOTAL mM

Figure 4

Order with respect to PEP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.4 mM $MnCl_2$; 0.3 mM ADP; enzyme(II); and PEP at the concentrations indicated. The abscissa is plotted as total PEP.


+1.0
0
-1.0
$\log \frac{v_i}{V_m - v_i}$
n = 0.9
0.4 mM Mn++
1.0
10
100
(PEP) μM

approximately equal to each other at each value of PEP. The cubic equation described in Experimental and the formation constants listed in Table 2 were used to estimate the metal-ADP concentrations. The results show that the concentration of metal-ADP chelate is not the only factor which governs the rate of human erythrocyte pyruvate kinase, since the only difference in these studies is the type and amount of divalent cation present. Each divalent metal appears to have a qualitatively different effect on the catalytic mechanism. Subsequent results will help to define this difference.

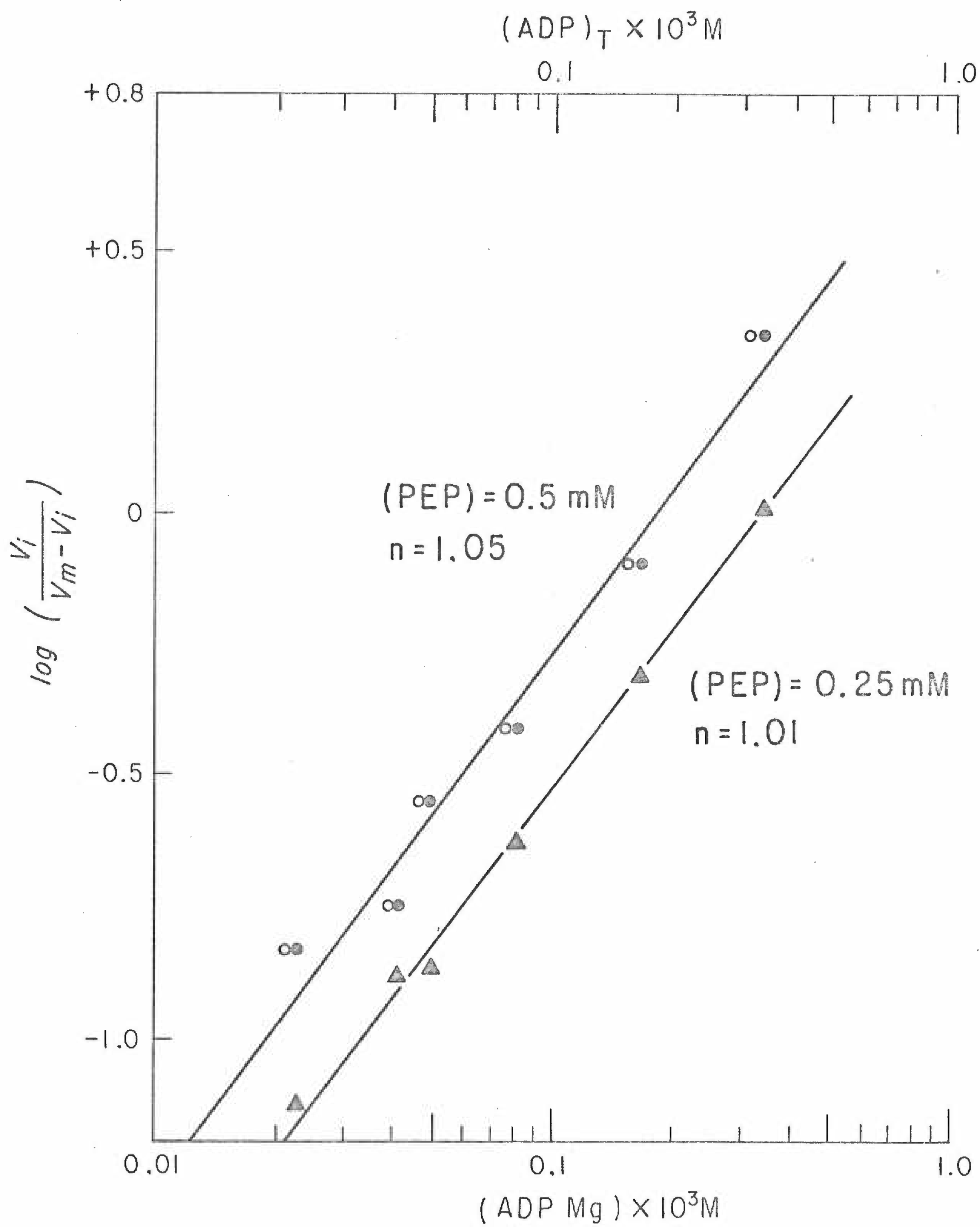
The results illustrated in figures 5 and 6 show the pyruvate kinase reaction to be effectively first order with respect to metal-ADP at fixed saturating levels of Mg^{++} or Mn^{++}. In figures 7 and 8 ADP is held constant and Mg^{++} and Mn^{++}, respectively, are rate-limiting. Plots of both free metal and metal-ADP chelate concentrations show Hill coefficients greater than 1 but less than 2. Cooperative effects, as measured by Hill plots, appear greater with free Mn^{++} and MnADP than with free Mg^{++} and MgADP. These data indicate that the divalent cation, either free or chelated to ADP, is essential to any cooperative interactions which occur during binding of the metal and nucleotide.

Figure 8 illustrates one anomalous finding: the Hill plot for MnADP shifts sharply from a slope of 1.8 to a slope of 5. Although no clear interpretation of this phenomenon is available, Ling (128) has suggested that such shifts in slope of log-log plots may be due to specific changes in the adsorption of small molecules to proteins.

Figure 9 illustrates the order with respect to free ATP, a known

Figure 5

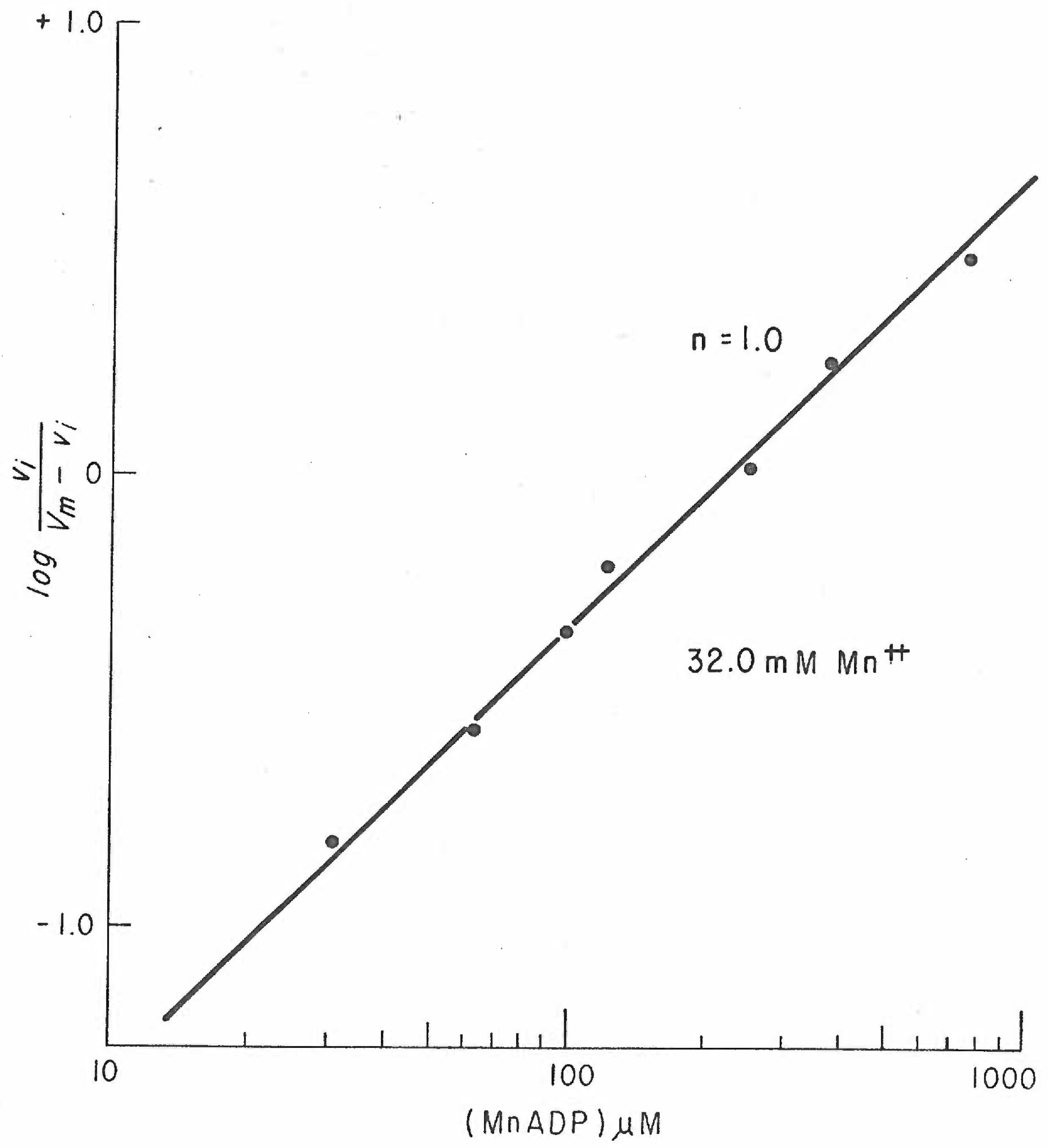
Order with respect to MgADP and total ADP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 16 mM $MgSO_4$; enzyme(I); and PEP, ADP (total) and MgADP at the concentrations indicated. The upper abscissa is expressed as total ADP and corresponds to the filled circles and triangles. The lower abscissa is expressed as MgADP and corresponds to the open circles. Concentrations of MgADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



(ADP)T × 10³M
0.1
1.0
+0.8
+0.5
0
-0.5
-1.0
log (Vi / (Vm - Vi))
(PEP) = 0.5 mM
n = 1.05
(PEP) = 0.25 mM
n = 1.01
0.01
0.1
1.0
(ADP Mg) × 10³M

Figure 6

Order with respect to MnADP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 32 mM $MnCl_2$; 0.125 mM PEP; enzyme(II); and ADP (as MnADP) at the concentrations indicated. Concentrations of MnADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



+ 1.0
0
- 1.0
$\log \frac{v_i}{V_m - v_i}$
n = 1.0
32.0 mM Mn^{++}
10
100
1000
(MnADP) μM

Figure 7

Order with respect to free Mg^{++} and MgADP 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 PEP and ADP; enzyme(I); and free Mg^{++} and MgADP at the concentrations indicated. Concentrations of free Mg^{++} and MgADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.

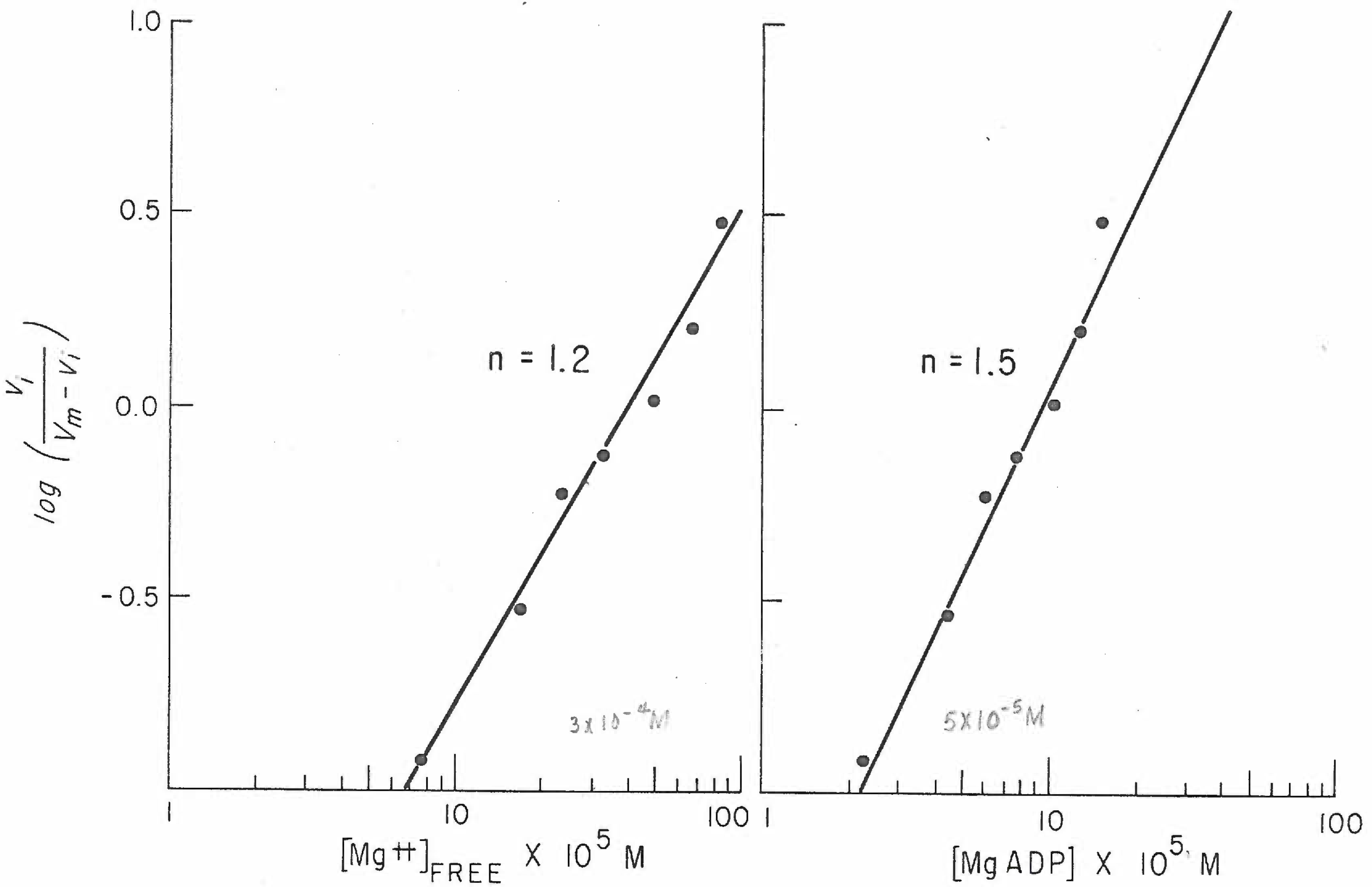
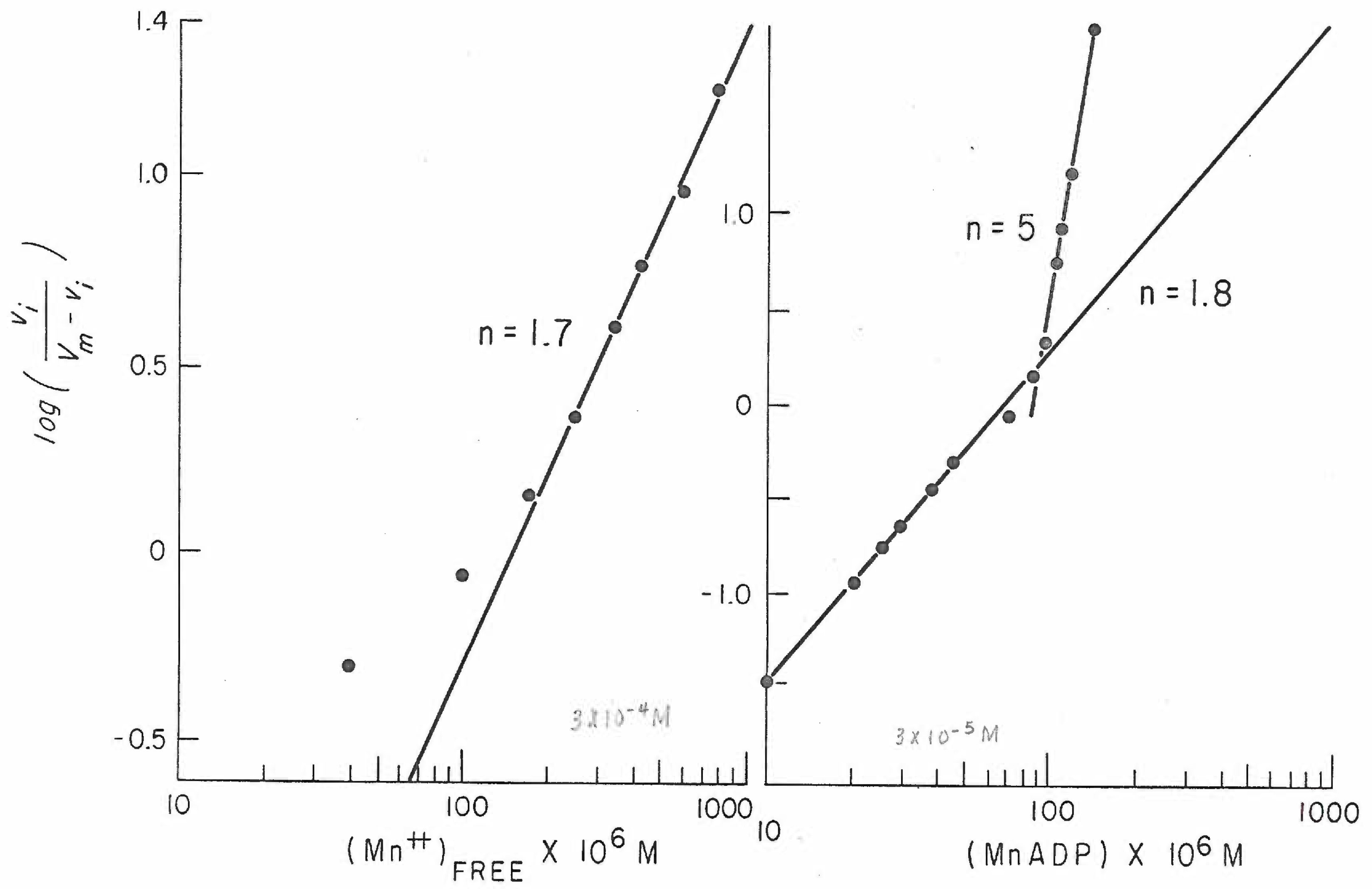

log (vi / (Vm - vi))
1.0
0.5
0.0
-0.5
n = 1.2
3x10⁻⁴M
1
10
100
[Mg++]FREE X 10⁵ M
n = 1.5
5x10⁻⁵M
1
10
100
[Mg ADP] X 10⁵ M

Figure 8

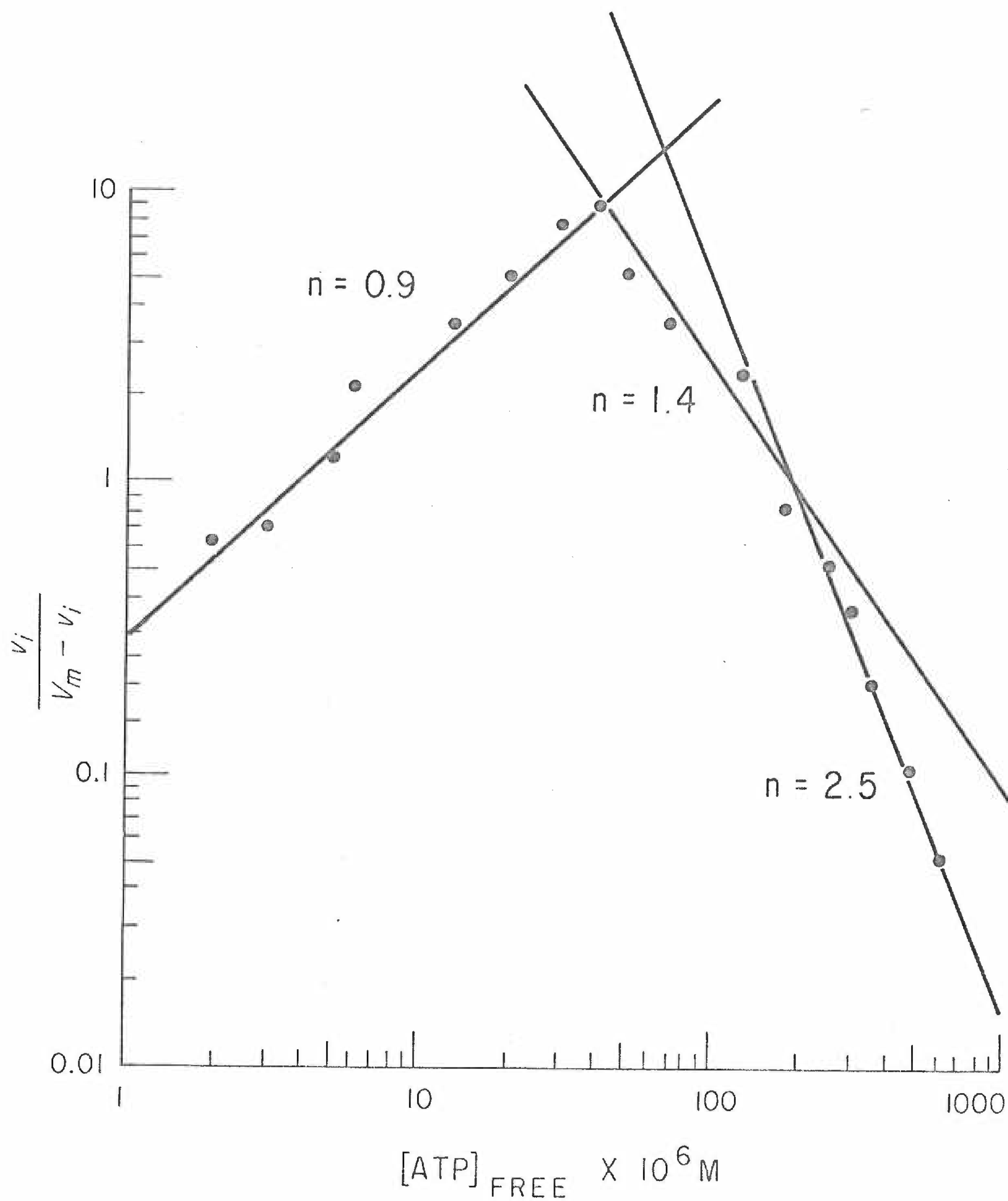
Order with respect to free Mn^{++} and MnADP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.125 mM PEP and ADP; enzyme(I); and free Mn^{++} and MnADP at the concentrations indicated. Concentrations of free Mn^{++} and MnADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



log (vi / (Vm - vi))
1.4
1.0
0.5
0
-0.5
n = 1.7
3x10-4M
10
100
1000
(Mn++)FREE X 10^6 M
1.0
0
-1.0
n = 5
n = 1.8
3x10-5M
10
100
1000
(MnADP) X 10^6 M

Figure 9

Order with respect to free ATP of the reaction catalyzed by human erythrocyte pyruvate kinase. Reaction mixtures contained 32 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.375 mM PEP; 0.125 mM ADP; enzyme(II); and free ATP at the concentrations indicated. Concentrations of free ATP were estimated by solution of the cubic equation described in Experimental. ATP and ADP were used as the two ligands in the equation using the stability constants reported in Table 2. PEP was assumed to have a negligible effect in complexing Mg^{++}.


10
1
0.1
0.01
n = 0.9
n = 1.4
n = 2.5
$\frac{v_i}{V_m - v_i}$
1
10
100
1000
[ATP]$_{FREE}$ X 10^6 M

positive effector of erythrocyte pyruvate kinase (106). An initial positive slope approaching 1 is followed by an inflection and a negative slope with a value of n approaching 2 as the concentration of ATP is increased. Similar results were noted with the effector, FDP, by Koler and Vanbellinghen (76). It should be pointed out that plotting ATP as total ATP gives a plot similar to figure 9, with only slight variations in the designated slopes. The observations that maximum values of n from Hill plots with PEP (fig. 2), and ATP (fig. 9) approach 2 supports the concept of two catalytic sites on the pyruvate kinase molecule (17,61,76). Furthermore, these are consistent with the interpretation of Hill plot data presented by Atkinson et al. (124), from studies on yeast isocitrate dehydrogenase, and by Taketa and Pogell (129), from studies on rat liver fructose diphosphatase.

In summary, Hill plots reveal that two kinds of n values are found with human erythrocyte pyruvate kinase: $n=1.0$, and $1 \leq n < 2$. These vary with the divalent cation, and the ligand being studied (substrate or effector). No n value exceeds 2, except with ATP, where cation becomes limiting at high concentrations of this effector. Table 3 summarizes the Hill plot data. The sum of all the substrate n values in each column are quite similar (5.3 and 5.7). This indicates that the total order of reaction should be about the same in both the magnesium- and manganese-activated systems, if the contribution of enzyme is the same in each. Since the summations are redundant in terms of the metal and ADP contributions, the absolute values cannot

Table 3

Effects of divalent cation activators on the Hill coefficients (n-values) for the substrates and effectors in the human erythrocyte pyruvate kinase reaction.

Substrate Contributing to Order	Divalent Cation (M^{++}) Mg	Mn
(PEP) free	≃ 2.0	≃ 1.0
($ADP-M^{++}$) (limiting ADP)	≃ 1.0	≃ 1.0
($ADP-M^{++}$) (limiting metal)	≃ 1.5	≃ 1.8
(M^{++}) free	≃ 1.2	≃ 1.5
sum	5.7	5.3

effector	Mg^{++}	Mn^{++}
ATP (activation)	≃ 1.0	none
(inhibition)	≃ 2.0	-
FDP (activation)	≃ 1.0 (ref. 76)	none
(inhibition)	≃ 2.0 (ref. 76)	-

be used as a prediction of the order. However, assuming no ligand contributes more than 2.0 to the total order, there appear to be five potential contributors to the total order: 1) enzyme; 2) metal-ADP; 3) PEP; 4) free metal; and 5) an interaction term, which is dependent on the specific divalent cation used.

B. Over-all kinetic order of reaction.

For most enzymic reactions it is impossible to observe an over-all kinetic order because various sequential steps (e.g. ordered or ping pong mechanisms) cannot all simultaneously be rate-limiting. As pointed out by Atkinson et al. (124), if the velocity of an enzymatic reaction is proportional to the concentration of one specific 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 rabbit muscle pyruvate kinase reaction generally has been assumed to be rapid equilibrium random (17). Previous data (74) on the human erythrocyte enzyme are consistent with this proposal although the authors interpreted the data as representing a strictly "ordered" mechanism, which has also been deduced for yeast enzyme by Mildvan et al. (88).

At sufficiently low concentrations of all components in the reaction, and assuming that free enzyme is virtually identical with the total enzyme concentration, simple dilution of the pyruvate kinase reaction mixture should produce a fifth order decrease in rate

with Mg^{++} as the divalent cation. Making only enzyme and PEP rate-limiting should produce a third order decrease in rate in the magnesium-activated system. These predictions are confirmed in figure 10. When concentrations are such as to give a reaction velocity about 1% of Vm, the over-all kinetic order for pyruvate kinase is approximately 5, and the order for PEP (and enzyme) is 3.

Figure 11 illustrates the kinetic order for PEP and enzyme at two different concentrations of Mg^{++}. Similar slopes were obtained with both 1.6mM and 16mM $MgSO_4$. Later experiments utilize these concentrations of Mg^{++} extensively, and the kinetic differences between systems containing either 1.6mM $MgSO_4$ or 16mM $MgSO_4$ can be assumed to be independent of changes in the kinetic order of the reaction.

Replacing Mg^{++} by Mn^{++} produces an identical total order ($n \approx 5$), but the order with respect to PEP and enzyme is reduced from three (with Mg^{++}) to two (fig. 12). The total order with respect to PEP and enzyme in the manganese-activated system is consistent with the Hill plot data of figure 3, which demonstrates a first order rate dependence on PEP in the manganese-activated system. Assuming enzyme is first order in both cation-activated systems and PEP contributes 1 to the total order in the Mn^{++} assay and 2 in the Mg^{++} assay, but the total orders are approximately the same for both cation-activated systems, the increment due to PEP in the Mg^{++} system must be compensated for in the Mn^{++} system by free metal, ADP or the metal-ADP chelate. A more thorough probe of divalent cation effects has helped to distinguish among these possibilities.

Figure 10

(●) Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the overall concentration of the reaction mixture using Mg^{++} as the divalent cation. Reaction mixtures contained volumes of 8 mM TEA buffer, pH 7.46 and 75 mM KCl as indicated on the abscissa plus 0.125 μmole PEP; 0.125 μmole ADP; 0.1 μmole $MgSO_4$; and 20 μliters enzyme(I). (○) Dependence of the rate on the concentration of PEP. Conditions were the same as above except that ADP and Mg^{++} were increased to saturating levels of 1.0 μmole and 1.6 μmoles, respectively. Lines were drawn according to the reasoning of Atkinson et al. (124).

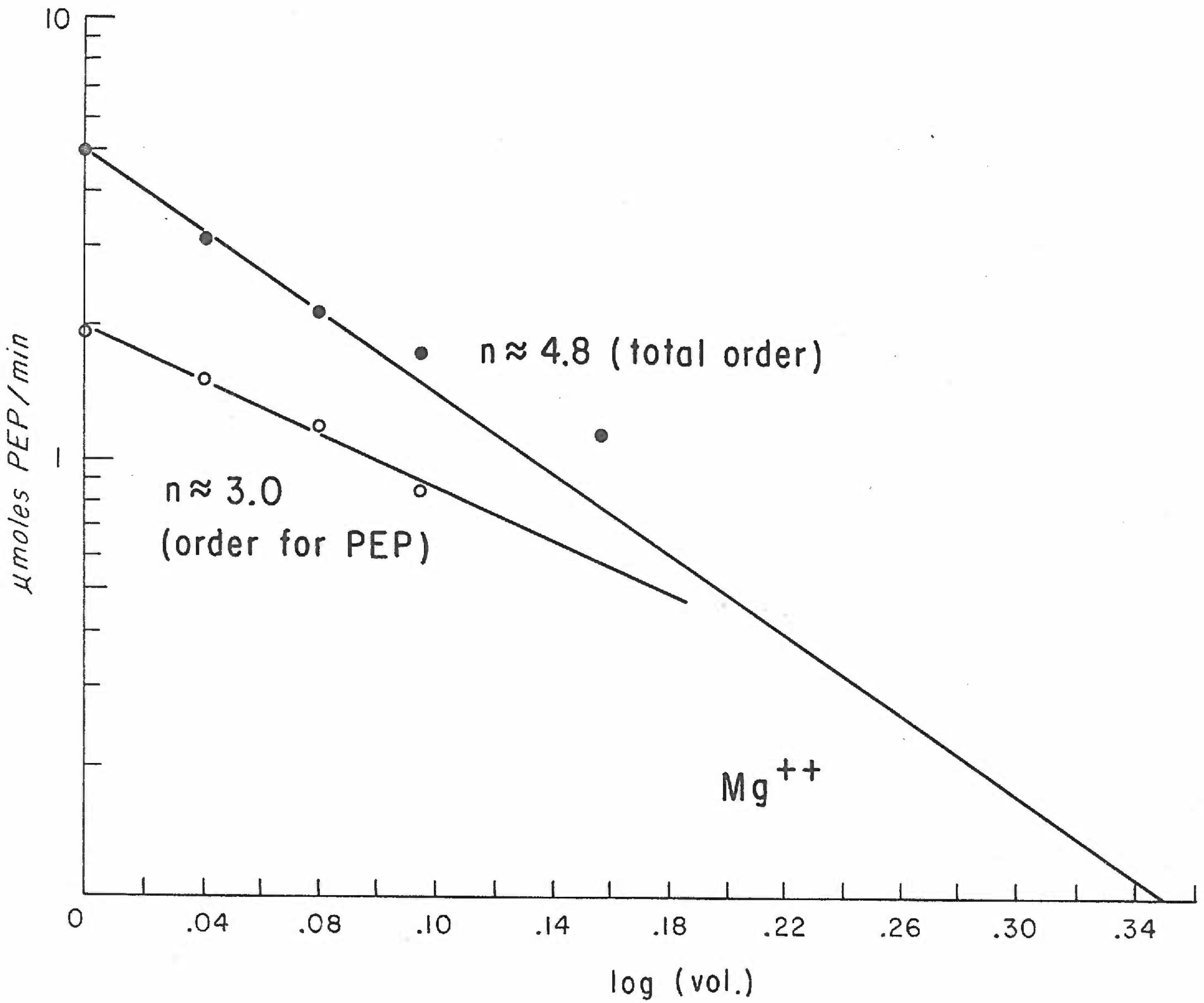

10
1
µmoles PEP/min
n ≈ 4.8 (total order)
n ≈ 3.0
(order for PEP)
Mg++
0
.04
.08
.10
.14
.18
.22
.26
.30
.34
log (vol.)

Figure 11

Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the concentrations of substrates ADP and PEP. Reaction mixtures contained volumes of 8 mM TEA buffer, pH 7.46 and 75 mM KCl as indicated on the abscissa plus 0.125 μmole PEP and ADP; 20 μliters enzyme(I); and Mg^{++} at the concentrations indicated.

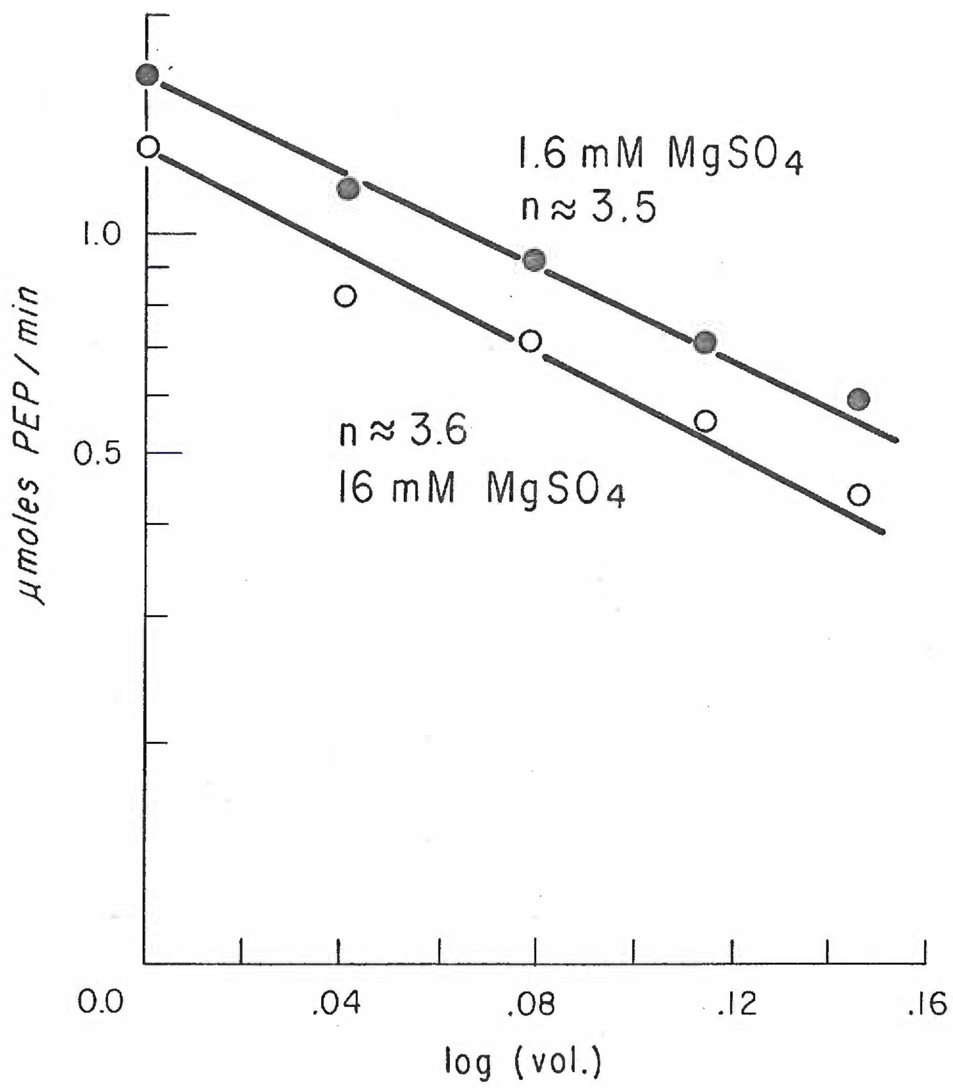
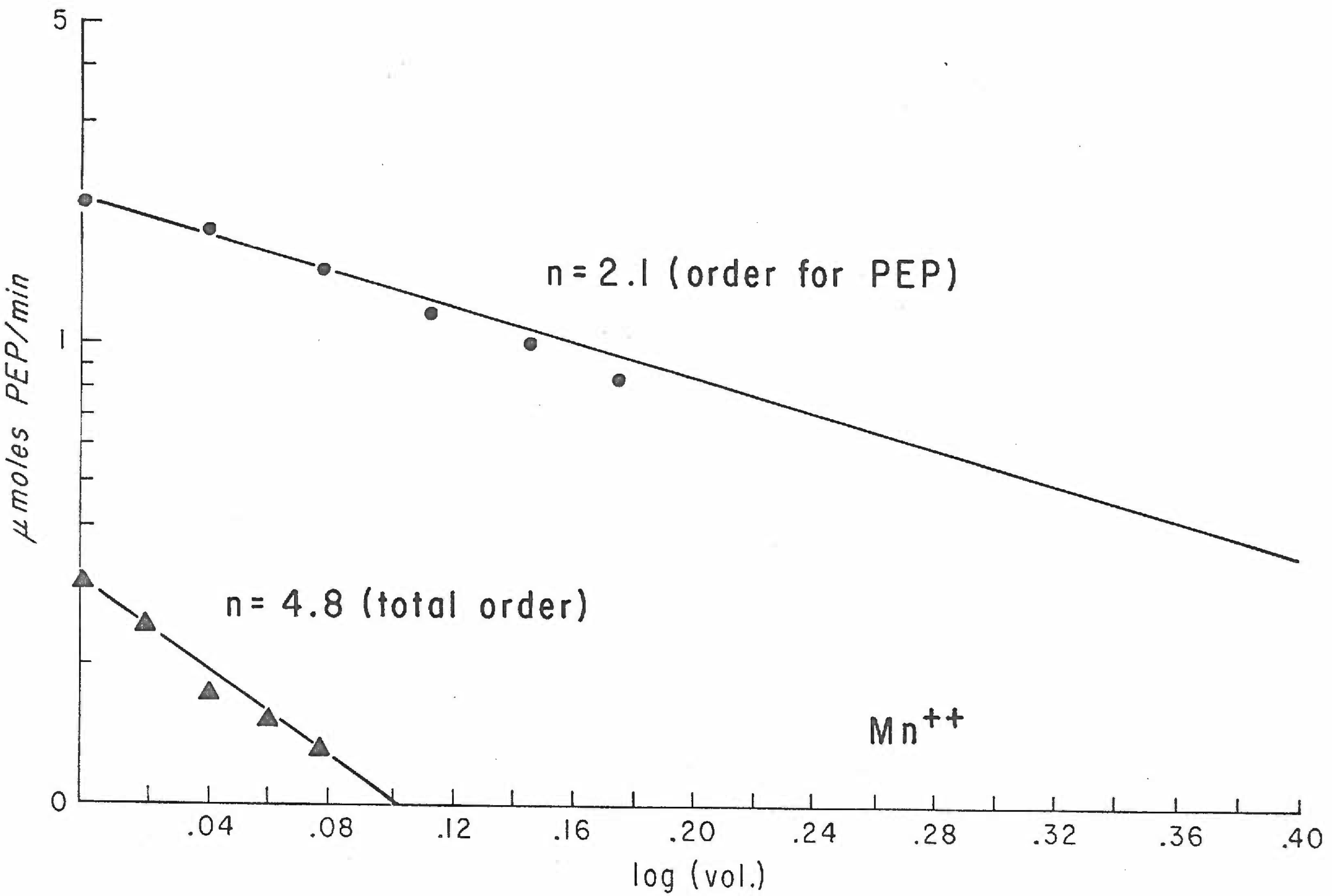

1.6 mM MgSO4
n ≈ 3.5
n ≈ 3.6
16 mM MgSO4
1.0
0.5
μmoles PEP / min
0.0
.04
.08
.12
.16
log (vol.)

Figure 12

(▲) Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the overall concentration of the reaction mixture using Mn^{++} as the divalent cation. Reaction mixtures contained volumes of 8 mM TEA buffer, pH 7.46 and 75 mM KCl as indicated on the abscissa plus 0.125 μmole PEP; 0.125 μmole ADP; 0.02 μmole $MnCl_2$; and 20 μliters enzyme(II). (●) Dependence of the rate on the concentration of PEP. Conditions were the same as above except that ADP and $MnCl_2$ were increased to saturating levels of 1.0 μmole and 4.0 μmoles, respectively.


5
1
0
μmoles PEP/min
n = 2.1 (order for PEP)
n = 4.8 (total order)
Mn++
.04
.08
.12
.16
.20
.24
.28
.32
.36
.40
log (vol.)

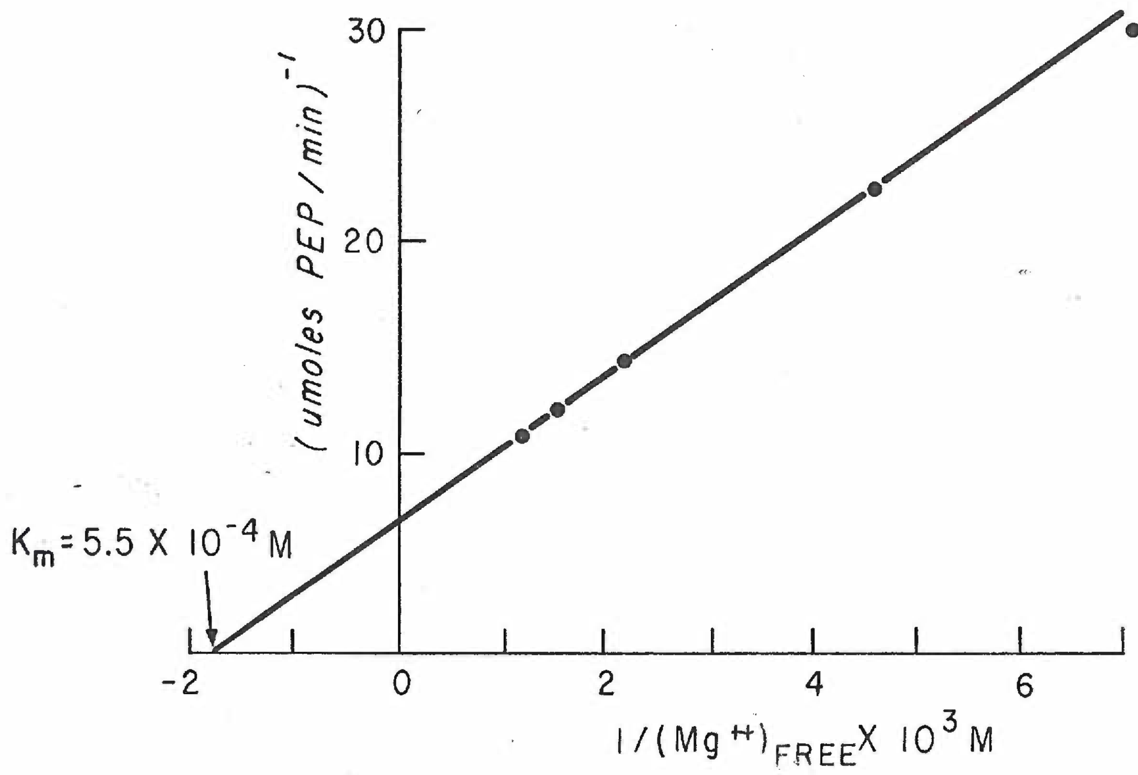
C. Divalent cation effects.

As was reported in 1934 (1), pyruvate kinase requires a divalent metal such as Mg^{++} for activation. Figure 13 shows double reciprocal plots for free Mg^{++} and free Mn^{++}, respectively. The extrapolated values for the Michaelis constants indicate that Mn^{++} has an affinity for the enzyme about five times that of Mg^{++}. The values are very similar to those reported by Mildvan and Cohn (61), who used spin resonance techniques to probe the direct interaction between free metal and rabbit muscle enzyme. They are also fair approximations to the chelation constants of Mg^{++} and Mn^{++} for ADP, and therefore it has been argued that binding is limited by the ability to form metal-nucleotide chelates, which then function as the true substrates (17,85,87).

<u>Results with magnesium.</u> Further kinetic studies were performed to discriminate between these two possibilities. Figure 14 illustrates a double reciprocal plot of free ADP and velocity at several fixed concentrations of Mg^{++}. The lines, fitted by a least squares estimation, demonstrate a "mixed" type of inhibition. The slopes and intercepts from figure 14 were replotted against the reciprocal of the free Mg^{++} concentration, as illustrated in figure 15. The intercept replot clearly intersects the ordinate above the origin. The slope replot is more complicated. A straight line drawn through the points can intersect at the origin, but the points appear to be fitted more precisely by a parabola which intersects the ordinate above the origin. According to Cleland (130), and Heyde and Morrison (131), this evidence

Figure 13

Lineweaver-Burk plot of the effects of free Mg^{++} and free Mn^{++} on the velocity of the reaction catalyzed by human erythrocyte pyruvate kinase. The reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.4 mM PEP; 0.25 mM ADP; enzyme(I); and free Mg^{++} (top) or free Mn^{++} (bottom) at the concentrations indicated. Concentrations of free Mg^{++} and free Mn^{++} were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.


30
20
10
(umoles PEP / min)$^{-1}$
$K_m = 5.5 \times 10^{-4}$ M
-2
0
2
4
6
$1/(Mg^{++})_{FREE} \times 10^3$ M

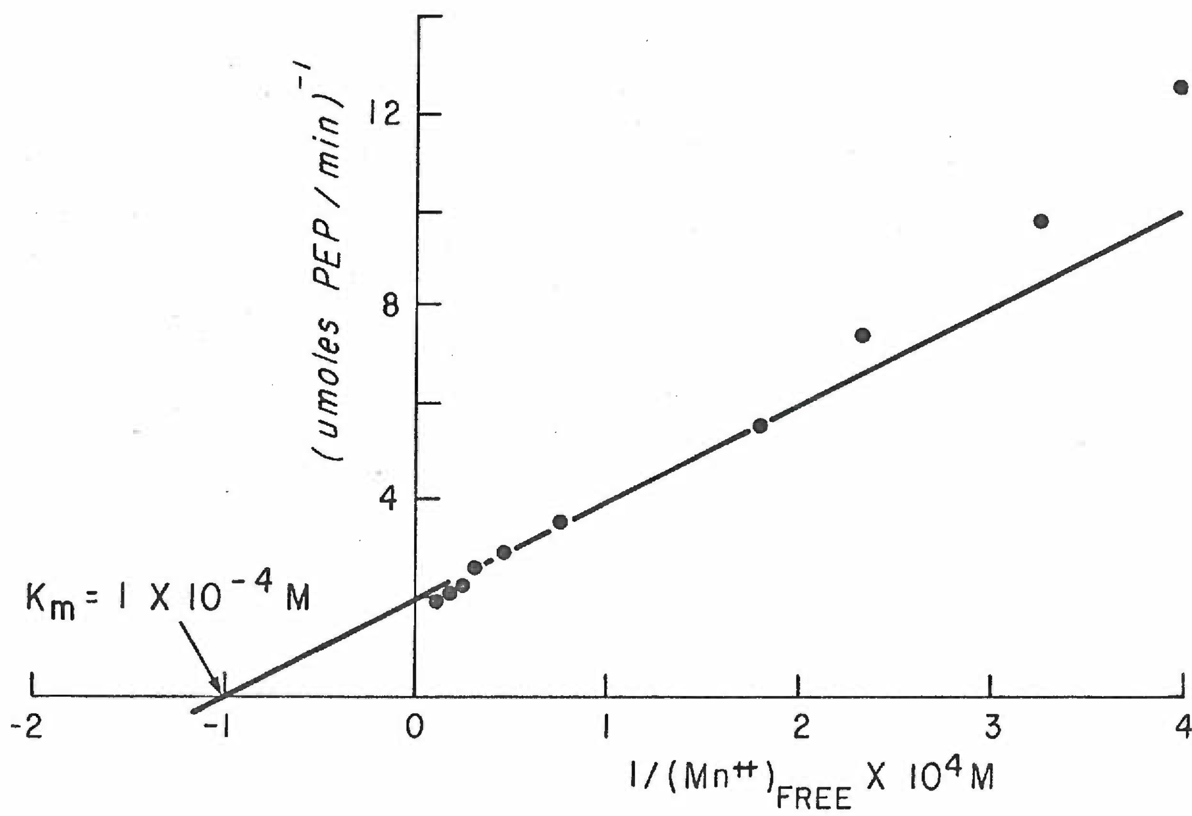
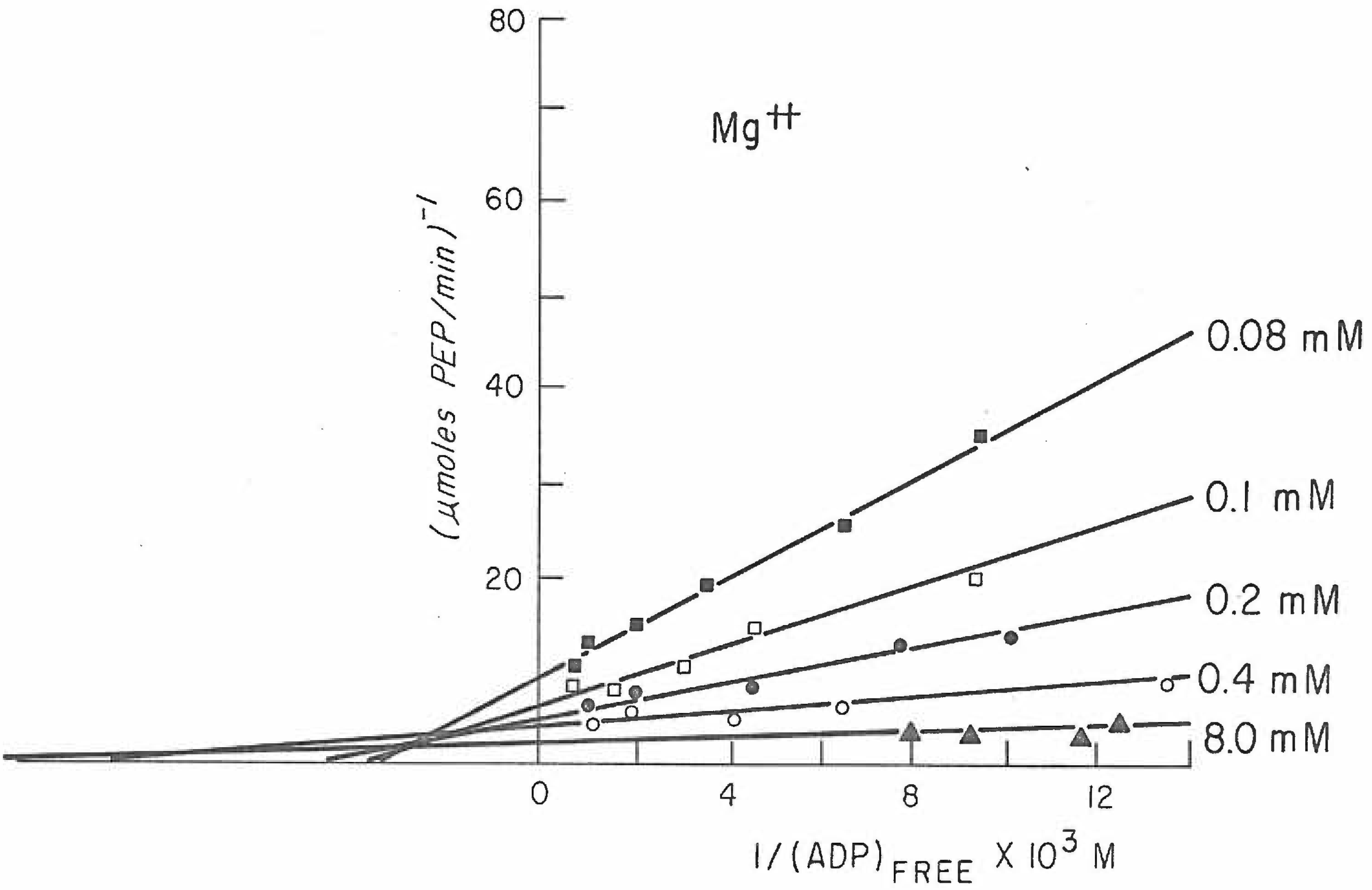

12
8
4
(umoles PEP / min)$^{-1}$
$K_m = 1 \times 10^{-4}$ M
-2
-1
0
1
2
3
4
$1/(Mn^{++})_{FREE} \times 10^4$ M

Figure 14

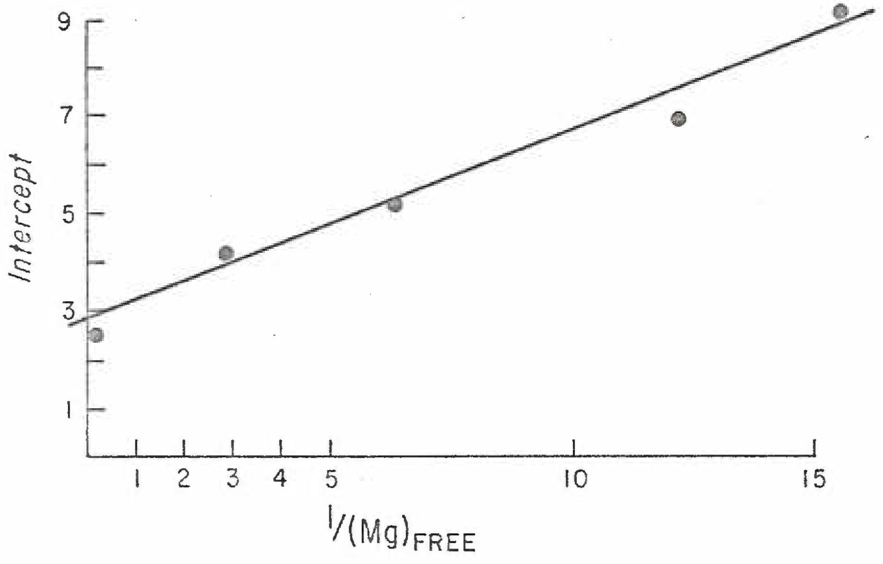
Lineweaver-Burk plot of free ADP concentration against initial velocity of human erythrocyte pyruvate kinase at varying levels of Mg^{++}. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.0 mM PEP (saturating); enzyme(I); and free ADP and Mg^{++} at the concentrations indicated. Concentrations of free ADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



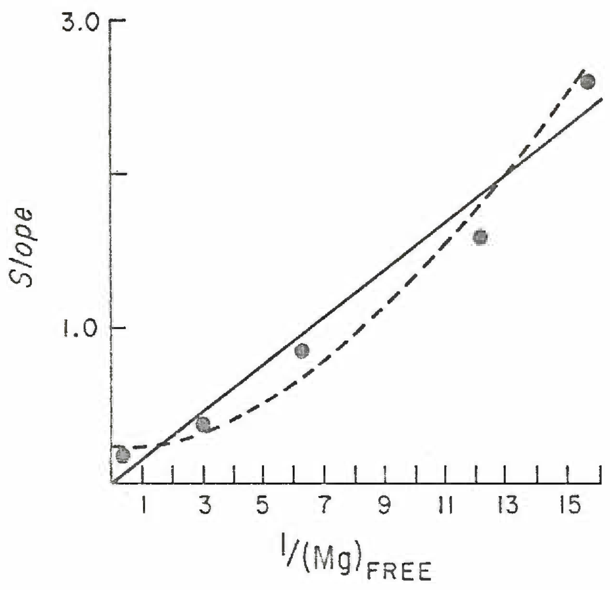
Mg⁺⁺
80
60
40
20
(µmoles PEP/min)⁻¹
0.08 mM
0.1 mM
0.2 mM
0.4 mM
8.0 mM
0
4
8
12
1/(ADP)FREE X 10³ M

Figure 15

Secondary plots of intercepts and slopes against the reciprocal of free Mg^{++} for the data presented in figure 14. Concentrations of free Mg^{++} were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



Intercept
9
7
5
3
1
1
2
3
4
5
10
15
1/(Mg)FREE



Slope
3.0
1.0
1
3
5
7
9
11
13
15
1/(Mg)FREE

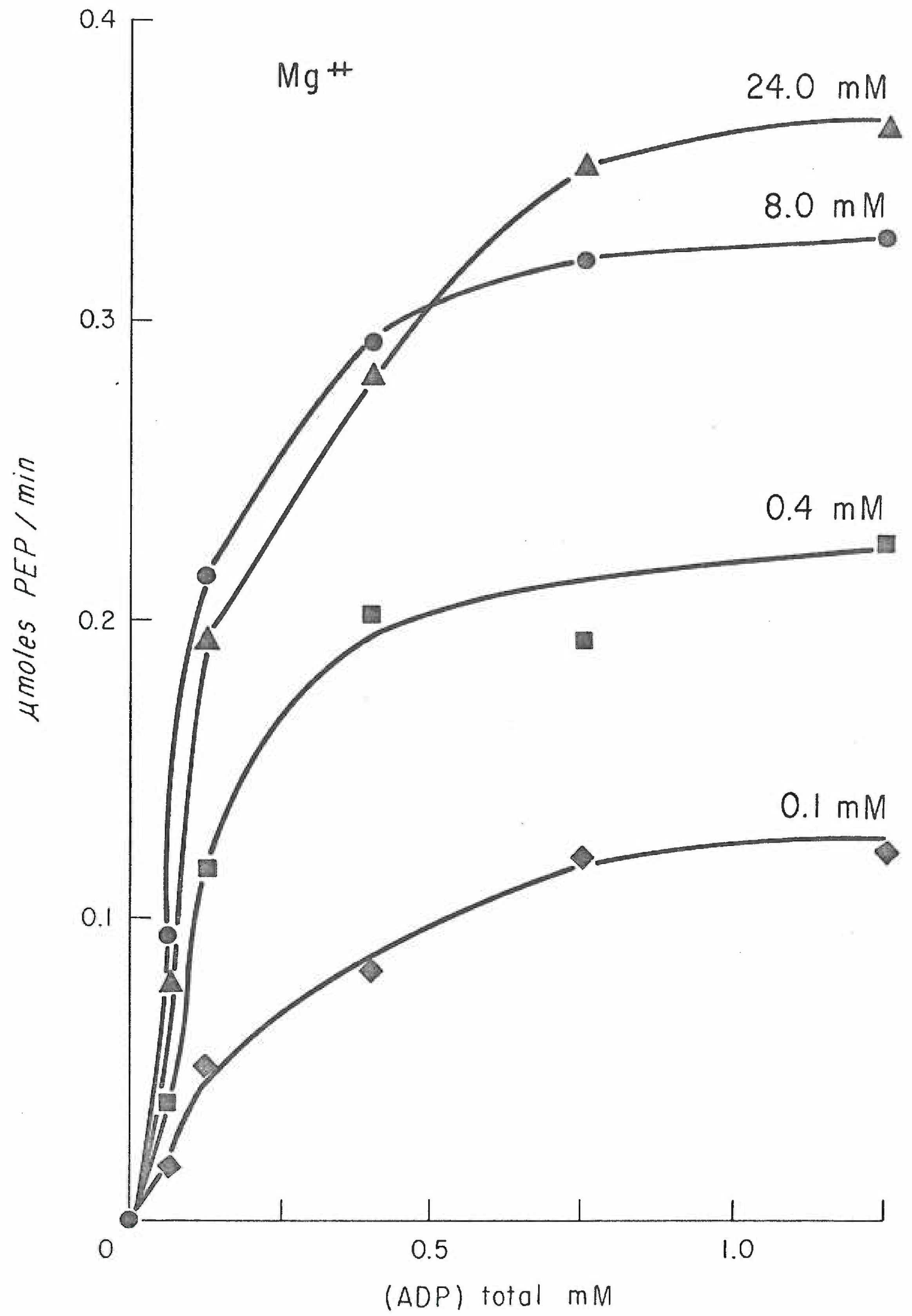
argues in favor of the presence of a significant concentration of enzyme-magnesium in the pyruvate kinase reaction. This interpretation supports the evidence of Mildvan and Cohn (61,86) and Mildvan et al. (88), that free divalent cation can interact with the enzyme independent of the nucleotide. It is possible, however, that E-magnesium could be derived from an E-Mg-ADP-PEP complex by dissociation of the substrates, and, therefore, be a side product of catalysis (131).

It has been postulated that Mg^{++} participates in the reaction as its nucleotide salt (17,85,87). Other investigators have shown that the divalent metals can interact directly with the enzyme protein without being chelated to nucleotide (61,86,88). Figure 16 represents a velocity profile with the substrate ADP at several fixed concentrations of Mg^{++}. No inhibition by excess ADP is observed, even when total ADP is increased to twelve times the total Mg^{++} concentration but, at very high levels of Mg^{++}, slight inhibition is apparent at low concentrations of ADP. A similar profile was found by Melchior (fig. 8, ref. 85) for the rabbit muscle enzyme.

Figure 17 illustrates velocity profiles for erythrocyte pyruvate kinase plotted as MgADP and free ADP. The reaction velocity shows a common dependence on the calculated concentration of MgADP, but not on that of uncomplexed ADP. Plotting the data with calculated free Mg^{++} as the variable substrate also shows no relationship to the observed reaction velocities.

Figure 16

Dependence of the rate of the human erythrocyte pyruvate kinase reaction on total ADP concentration at four concentrations of total Mg^{++}. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.5 mM PEP; enzyme(I); and Mg^{++} and ADP at the concentrations indicated.



0.4
0.3
0.2
0.1
0
0.5
1.0
Mg ++
24.0 mM
8.0 mM
0.4 mM
0.1 mM
μmoles PEP / min
(ADP) total mM

Figure 17

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 PEP; and enzyme(I). Increasing amounts of ADP were added to 0.1 mM, 0.4 mM and 8 mM $MgSO_4$. Concentrations of free Mg^{++} and MgADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.

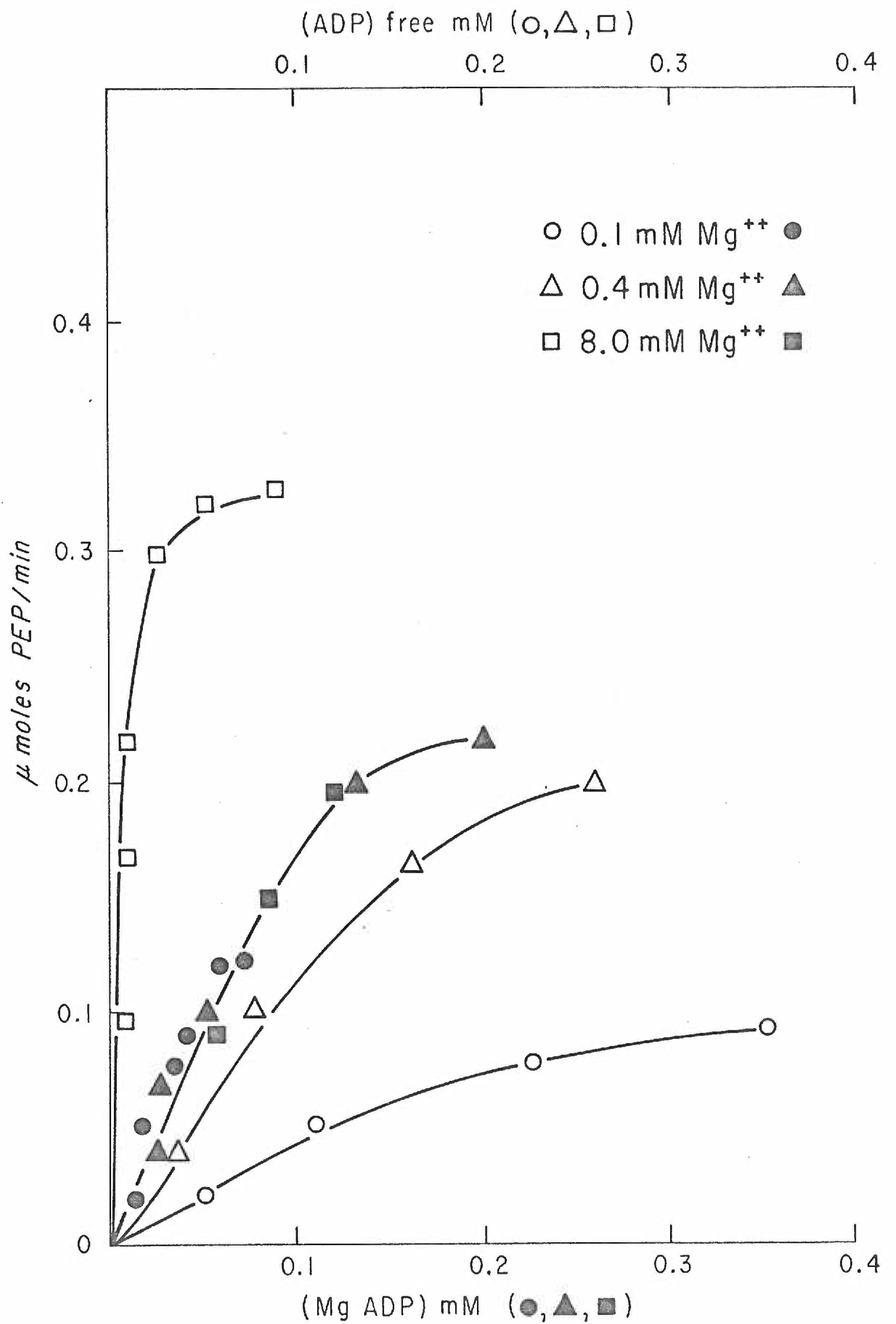

(ADP) free mM (○,△,□)
0.1
0.2
0.3
0.4
○ 0.1 mM Mg++ ●
△ 0.4 mM Mg++ ▲
□ 8.0 mM Mg++ ■
0.4
0.3
0.2
0.1
0
μ moles PEP/min
0.1
0.2
0.3
0.4
(Mg ADP) mM (●,▲,■)

Figure 18 demonstrates a common dependence of the rate on the calculated concentration of free PEP, but not on that of MgPEP. Furthermore, the rates of catalysis shown in figure 11 exhibit no common dependence on the concentrations of uncomplexed Mg^{++}. In the magnesium-activated system, therefore, it appears as though the preferred substrates are free PEP and MgADP. The Hill plots for these two ligands (figs. 2 and 7) indicate that both may interact with erythrocyte pyruvate kinase in a cooperative fashion, whereas free Mg^{++} has an extremely weak effect. Furthermore, over-all kinetic order is consistent with two molecules of PEP and two complexes of MgADP interacting with the enzyme.

Results with manganese. It was noted previously that Mn^{++} and Mg^{++} appear to have qualitatively different effects on human erythrocyte pyruvate kinase. Figure 19 is a velocity profile with ADP at several fixed concentrations of Mn^{++}. Comparison with the profile using Mg^{++} (fig. 16) points up two major differences. First, at low concentrations of total Mn^{++}, the velocity profile is biphasic, with inhibition occurring at high concentrations of ADP. Second, saturating levels of Mn^{++} cause a marked inhibition as compared to Mg^{++} at lower concentrations of ADP, but also prevent inhibition at higher ADP concentrations. Figure 20 demonstrates that the reaction velocity has an apparent common dependence on the calculated concentration of MnADP, similar to the dependence on MgADP. Figure 20 also indicates that the reaction velocity may be dependent on free Mn^{++} as well as on MnADP. Further experiments are required to confirm this

Figure 18

Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the calculated concentrations of free PEP and MgPEP. Reaction mixtures contained 8 mM TEA buffer pH 7.46; 75 mM KCl; 0.125 mM ADP; and enzyme(I). Increasing amounts of PEP were added to either 1.6 mM $MgSO_4$ or 16 mM $MgSO_4$. Concentrations of free PEP and MgPEP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.

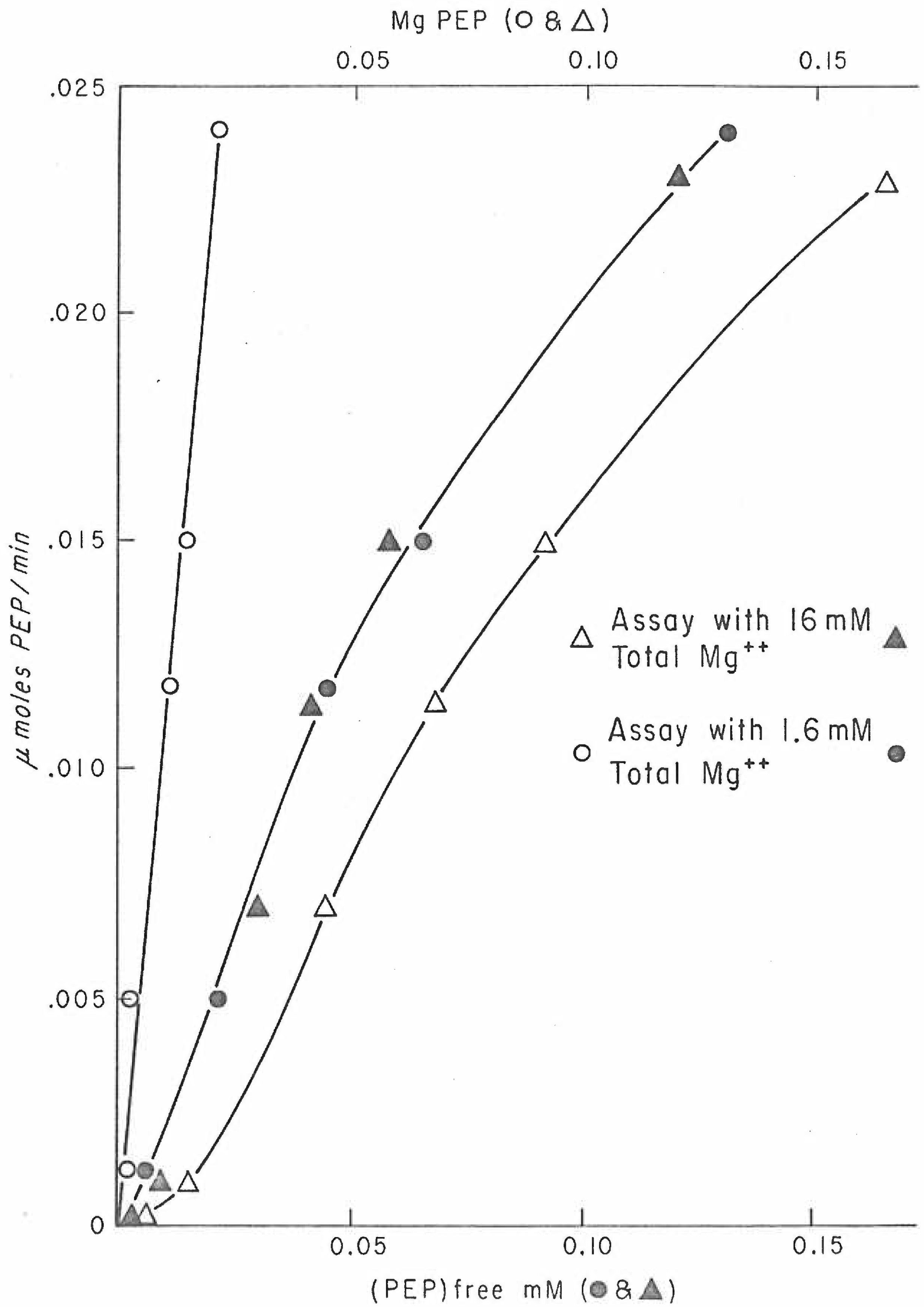
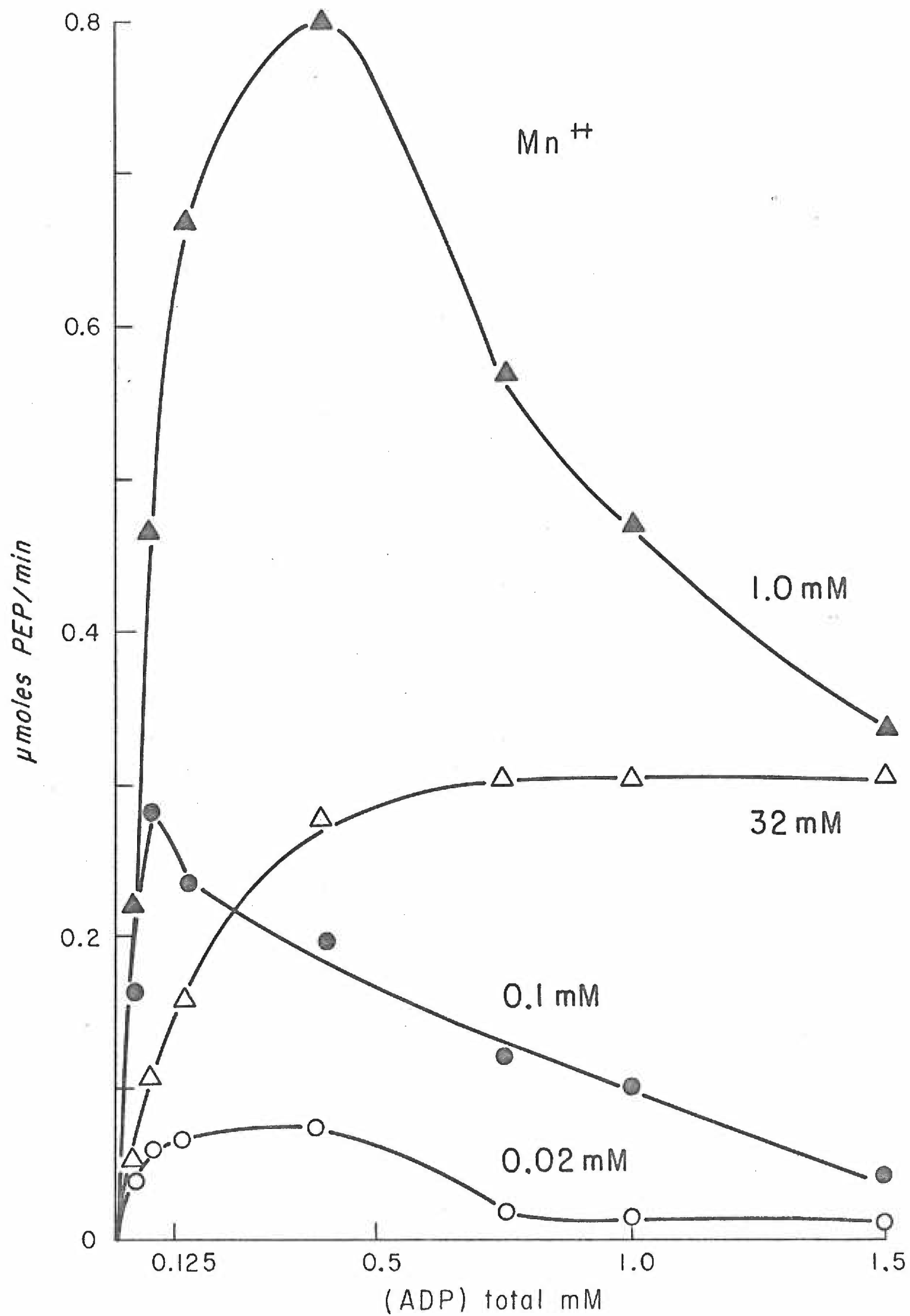

Mg PEP (O & △)
0.05
0.10
0.15
.025
.020
.015
.010
.005
0
μ moles PEP/min
Assay with 16 mM Total Mg⁺⁺
Assay with 1.6 mM Total Mg⁺⁺
0.05
0.10
0.15
(PEP) free mM (● & ▲)

Figure 19

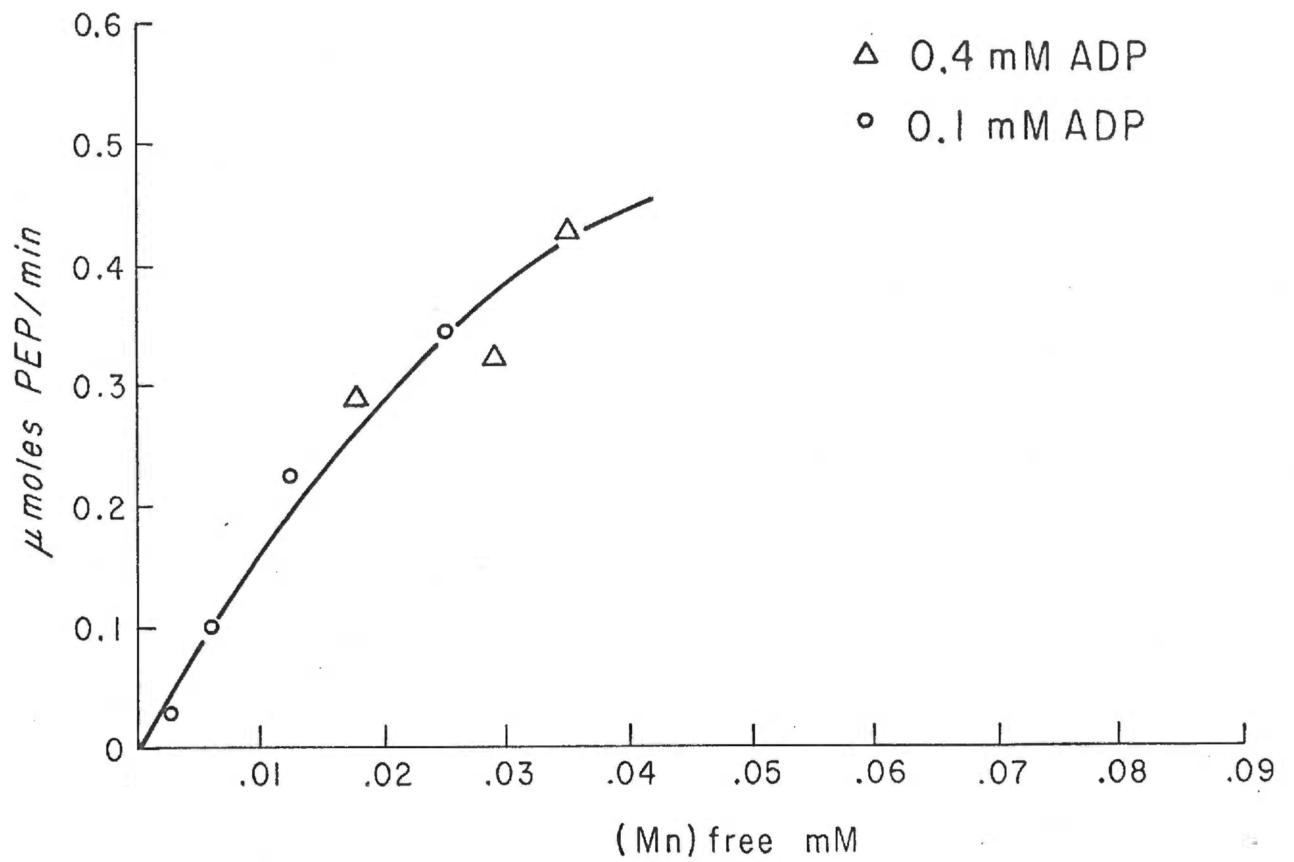
Dependence of the rate of the human erythrocyte pyruvate kinase reaction on total ADP concentration at four concentrations of total Mn^{++}. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.5 mM PEP; enzyme(I); and Mn^{++} and ADP at the concentrations indicated.



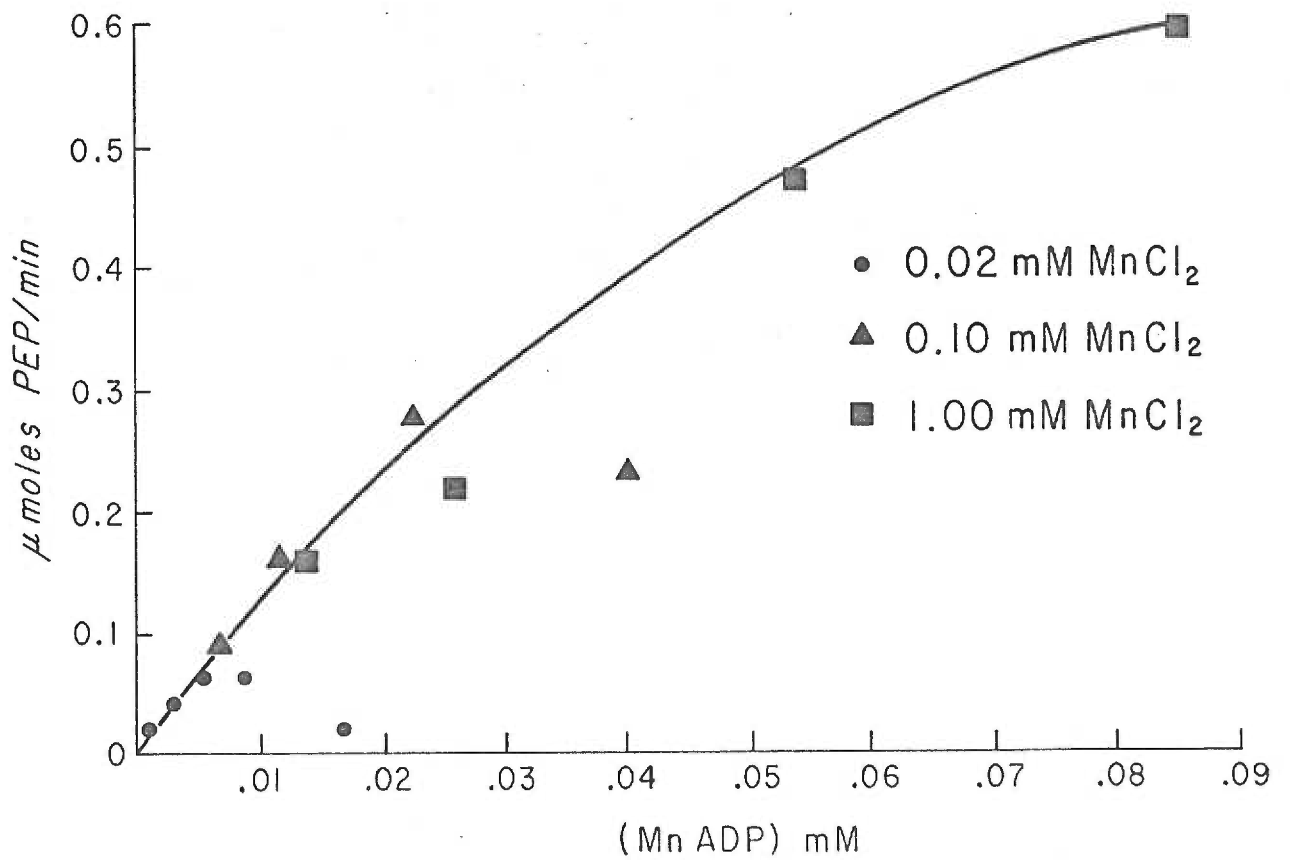
0.8
0.6
0.4
0.2
0
μmoles PEP/min
Mn ++
1.0 mM
32 mM
0.1 mM
0.02 mM
0.125
0.5
1.0
1.5
(ADP) total mM

Figure 20

Dependence of the rate of the human erythrocyte pyruvate kinase reaction on the calculated concentrations of free Mn^{++} and MnADP. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.5 mM PEP and enzyme(I). Increasing amounts of ADP were added to 0.02 mM, 0.1 mM and 1.0 mM $MnCl_2$. Concentrations of free Mn^{++} and MnADP were estimated by solution of the cubic equation described in Experimental using the stability constants reported in Table 2.



0.6
0.5
0.4
0.3
0.2
0.1
0
μ moles PEP/min
△ 0.4 mM ADP
○ 0.1 mM ADP
.01
.02
.03
.04
.05
.06
.07
.08
.09
(Mn) free mM



0.6
0.5
0.4
0.3
0.2
0.1
0
μ moles PEP/min
● 0.02 mM MnCl2
▲ 0.10 mM MnCl2
■ 1.00 mM MnCl2
.01
.02
.03
.04
.05
.06
.07
.08
.09
(Mn ADP) mM

preliminary data, and to substantiate the dependence on free Mn^{++}. The drop-off of points in the lower part of figure 20 is apparently due to limiting concentrations of the divalent cation, Mn^{++}. At low levels of total Mn^{++}, increasing the ADP concentration will tie up larger and larger amounts of manganese; free Mn^{++} concentration will decrease, and consequently the velocity will decrease. Saturating levels of Mn^{++} will prevent inhibition at high ADP concentration.

An alternative explanation for the ADP, Mn^{++} profile is that both free ADP and free Mn^{++} in excess of the catalytically preferred MnADP, inhibit the enzyme by forming E-ADP and E-manganese complexes. Why free ADP would inhibit the manganese-activated system but not the magnesium-activated system is the major difficulty with this alternative. However, if Mg^{++} and Mn^{++} each participated in the catalytic reaction in a unique way, as postulated to explain the Hill plot and reaction order data, many differences in catalysis between the two cation-activated systems can be envisaged.

The effect of replacing Mg^{++} by Mn^{++} has not been examined in sufficient detail to warrant a comparative analysis of the divalent cation-PEP relationship to catalysis.

Discussion of cation effects. Wold and Ballou (132) compared the activating effects of Mg^{++} and Mn^{++} on the enzyme enolase. They found significant differences between the metals in the maximum rate and minimum concentration necessary for catalysis, and noted that Mg^{++} and Mn^{++} differ in two important ways: ionic radius (Mg^{++}, 0.78 Å;

Mn^{++}, 0.62 Å) and electronegativity ($Mn^{++} > Mg^{++}$). Kayne and Reuben (133) recently demonstrated the importance of a third difference between Mg^{++} and Mn^{++}. Manganese is paramagnetic due to one unpaired electron, whereas Mg^{++} is diamagnetic. These authors presented evidence which strongly suggests that bound monovalent cation undergoes dipolar interactions with the unpaired electron spin of Mn^{++}. All three differences between divalent cations could affect the catalysis of the pyruvate kinase reaction. Studies on the "fluorokinase" (10,11) and "hydroxylamine kinase" (11,12,58,83) reactions indicate that slight differences in the properties of various divalent cations, e.g. electronegativity, have profound effects on the nature of the reaction catalyzed by rabbit muscle pyruvate kinase. "Hydroxylamine kinase" activity requires divalent metals in the decreasing order of effectiveness: $Zn^{++} > Co^{++} > Mn^{++}$, and no activity with Mg^{++}. "Fluorokinase" activity requires Mn^{++} or Mg^{++}, but is inactive with Zn^{++}. Pyruvate kinase activity is absent in the presence of Zn^{++} or Co^{++}. Cottam (83) has reported that rabbit muscle pyruvate kinase is capable of ATPase activity in the presence of Zn^{++} and Mn^{++}, but not Mg^{++}.

The available experimental evidence, therefore, does not permit the assignment of a purely chelating role to divalent metal ions in the pyruvate kinase reaction. The fact that Mg^{++} forms weaker nucleotide complexes than Mn^{++}, but is apparently more active catalytically (fig. 3) seems inconsistent with such a role. Dwyer (134) has postulated that a divalent cation exercises a supplementary polarizing influence

during the catalytic process. This effect can be mediated in a variety of ways. It may induce a favorable conformation of the enzyme, either by direct interaction, or as a chelate with the substrate. It could provide for electronic distortion of the substrate(s) by acting in conjunction with polar centers on the enzyme, or it may function solely to facilitate the bond making or bond breaking steps in the phosphoryl transfer reaction. Clearly, each metal ion can exert its own unique effect on catalysis, and this effect may be manifest in the presence or absence of chelated substrate.

Mildvan et al. (88) have proposed that the binding site for PEP on pyruvate kinase operationally may consist of two subsites. One subsite binds the carboxylate moiety and the other binds the phosphoryl group. The distance between these two sites is critical for the binding of PEP. Metal ion may have an important effect on adjusting this distance by its interaction with the phosphoryl group. Furthermore, the homotropic binding of the first mole of PEP to the erythrocyte enzyme may optimize the distance between subsites at the opposite catalytic center, and thereby facilitate the binding of the second mole of PEP. The difference between Hill plot slopes for PEP in the Mg^{++} ($n \sim 2$) and Mn^{++} ($n=1$) systems could be interpreted as evidence that Mn^{++} is capable of a favorable adjustment of the distance between subsites at any given catalytic center, whereas Mg^{++} is not.

As mentioned previously, PEP Hill plots in the presence of Mn^{++} give values of one, a result similar to FDP activated pyruvate kinase. These results may indicate that Mn^{++} mimics the FDP effect

(or vice versa) by inducing the same change in enzyme-substrate conformation. Such an effect could result from binding of Mn^{++} and FDP at or near the catalytic center. Indirect evidence supporting this postulate has been presented by Kuczenski and Suelter (73) who found that FDP activates yeast pyruvate kinase in the presence of divalent metal, but causes an inactivation when metal was absent. They found that two moles of FDP bind per mole of enzyme. It seems unlikely that two distinct types of sites exist on the enzyme for FDP, one promoting activation and one promoting inactivation. It is probable that FDP and divalent cation bind together to activate the enzyme, but, when metal is absent, the enzyme-FDP conformation is unstable. Since the critical site for divalent metal binding is the catalytic site, it is reasonable to postulate that FDP and divalent metal effects are mediated at this locus. Kinetic evidence to be presented in a later section indicates that FDP may be a competitive inhibitor with respect to PEP in the erythrocyte pyruvate kinase reaction. Although the kinetics may be considerably more complicated, the results are consistent with the hypothesis that Mn^{++} and FDP induce similar changes in the enzyme.

In summary, initial velocity studies on human erythrocyte pyruvate kinase have shown that the divalent metal activator is a critical factor in determining the catalytic and regulatory properties of this enzyme. The data indicate that the reaction shows a dependence on the concentration of metal-ADP chelate present in the system. The manganese-activated system may also be dependent on free Mn^{++}. The effects of

each metal evidently can be manifested when the metal is bound to nucleotide. The question then arises as to the role that nucleotides may play in the catalytic and regulatory mechanism.

D. Nucleotide interactions.

Reynard et al. (17) first reported that ATP was a competitive inhibitor of both PEP and ADP for the rabbit muscle pyruvate kinase reaction. Wood (90) suggested an alternative explanation by arguing that ATP inhibition depends on the level of free Mg^{++} in the medium, and that high concentrations of ATP, by chelating a large percentage of the free Mg^{++}, would reduce the concentration of MgADP and activity. He used saturating Mg^{++} which appeared to abolish ATP inhibition. Boyer (91), however, using a Mg^{++} buffer system to maintain free Mg^{++}, reconfirmed the original interpretation (17) that ATP inhibits the pyruvate kinase reaction by competing with both substrates. Recently, Holmsen and Storm (92) reexamined the ATP and Mg^{++} relationship to inhibition of the rabbit muscle enzyme. They found that ATP and EDTA had similar inhibitory effects when Mg^{++} was the variable, and interpreted this to mean that the ATP inhibition was due to a chelation phenomenon. However, these authors did not investigate the possibility that EDTA or ATP may have a direct effect on the enzyme.

Tietz and Ochoa (10) reported a broad specificity for the nucleoside diphosphate substrate in the pyruvate kinase catalyzed reaction. Davidson (80) questioned this by showing that rates obtained with pyrimidine nucleoside diphosphates resulted from contamination of rabbit muscle pyruvate kinase with nucleoside diphosphokinase and adenosine

phosphates, and that only purine nucleoside diphosphates functioned as substrates for the reaction.

Figure 21 illustrates the effects of various nucleoside triphosphates on the velocity of the human erythrocyte enzyme using a low (non-saturating) concentration of ADP and 1.6mM Mg^{++}. Both purine nucleotides, ATP and GTP activate the reaction in a non-competitive manner. EDTA also strongly activates the reaction. The pyrimidine triphosphate, CTP, appears to have no effect on the reaction velocity. Other experiments have shown that, like CTP, the pyrimidine triphosphate, UTP, has no effect on the reaction. A tenfold increase in the concentration of Mg^{++} (fig. 22) results in activation effects which are qualitatively the same, but the magnitude of the activation is substantially reduced.

There are three main possibilities which can account for the activation by ATP and GTP:

1) The nucleoside triphosphate solutions may be contaminated with ADP. Control studies (see Experimental) demonstrated only 1-3% ADP contamination of the triphosphate solutions. This would amount to less than ten micromoles of ADP, or an increment of less than one-tenth the concentration of ADP in the actual reaction.
2) As suggested by Wood, the triphosphate portion of the nucleotide may chelate significant amounts of divalent metal and this relieves a non-competitive inhibitory effect of free metal. This possibility seems unlikely since all of the

Figure 21

Velocity profiles with PEP for human erythrocyte pyruvate kinase in the presence and absence of several effectors. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP and effector at the concentrations indicated.

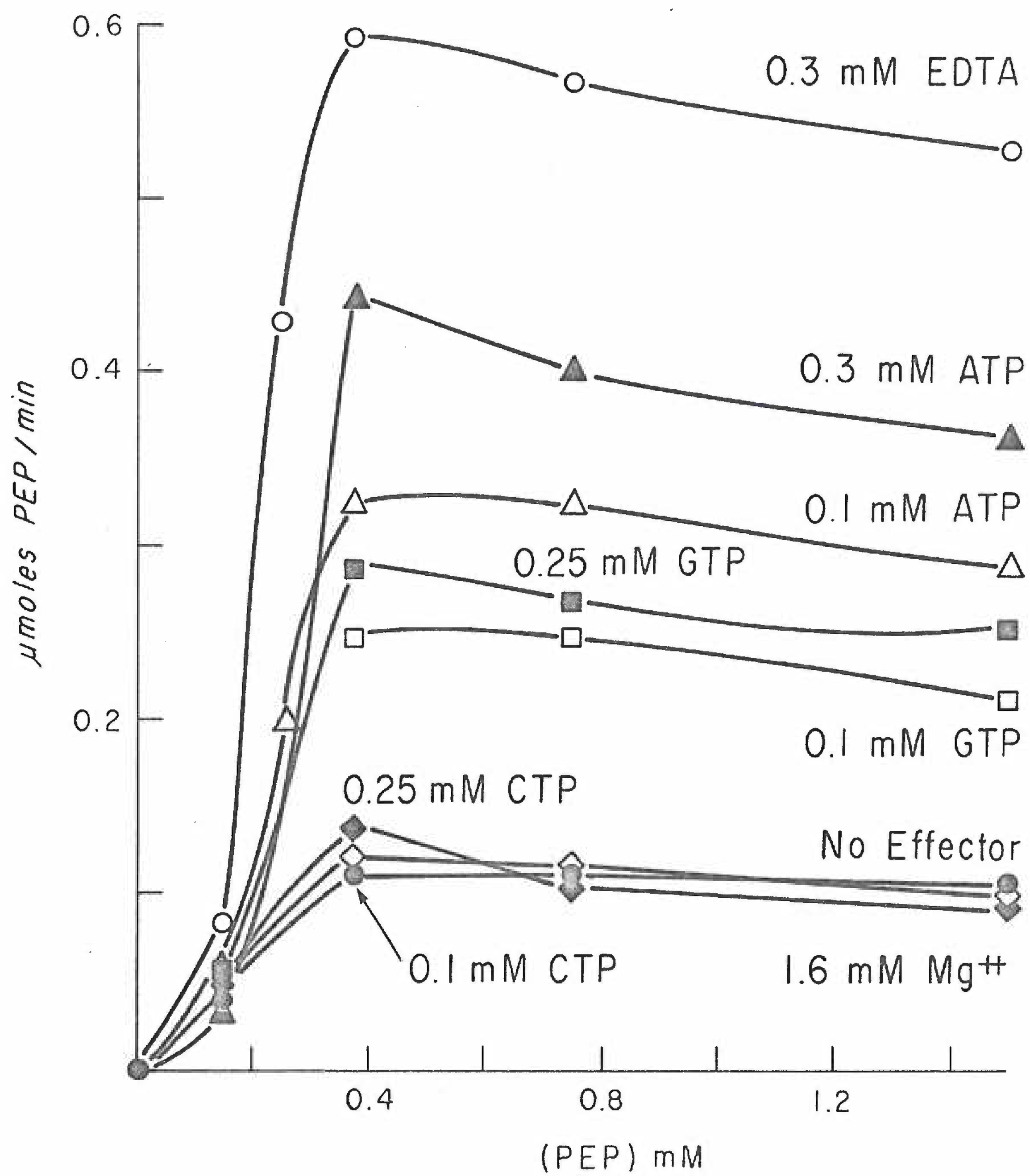
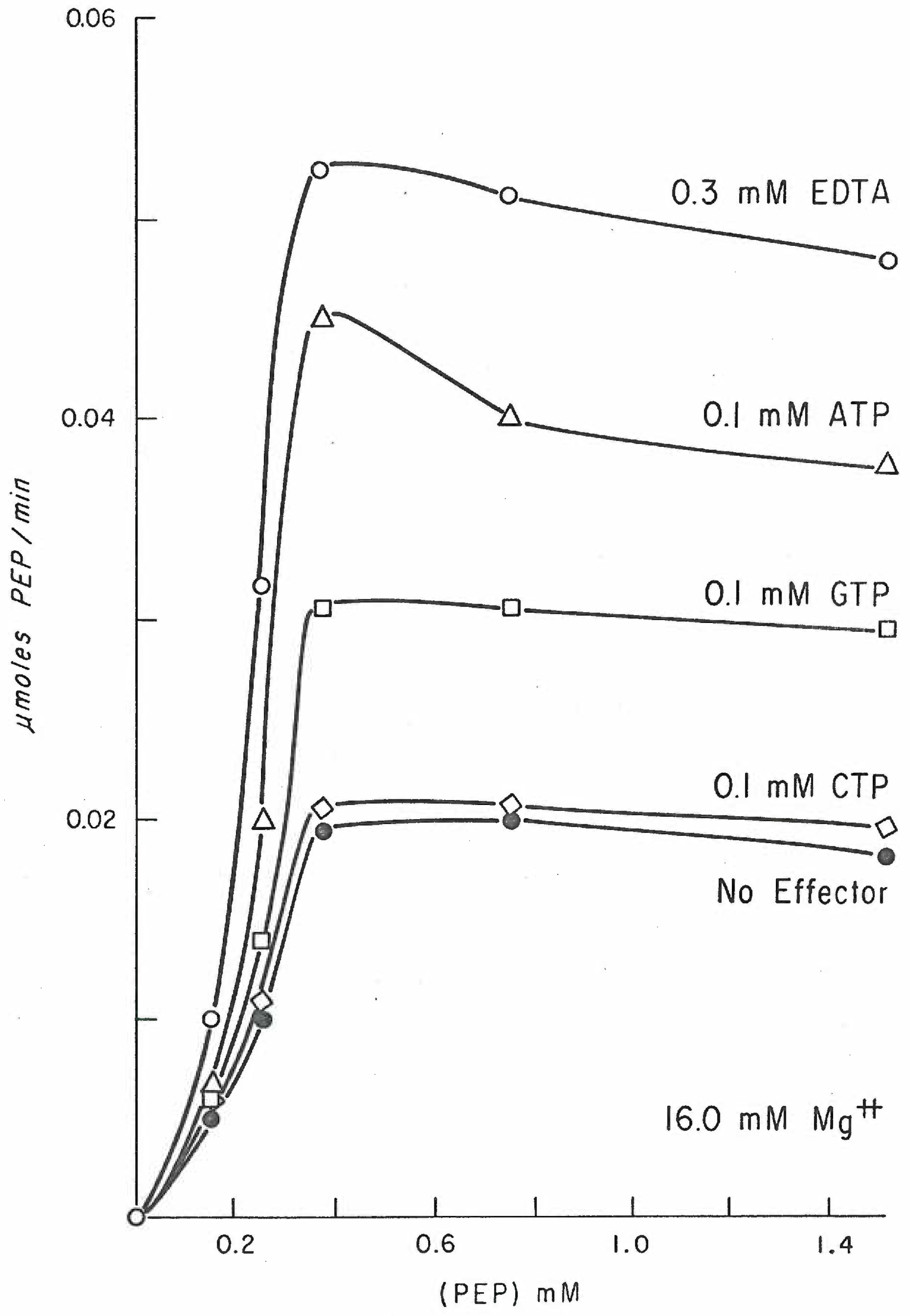

0.6
0.4
0.2
µmoles PEP / min
0.3 mM EDTA
0.3 mM ATP
0.1 mM ATP
0.25 mM GTP
0.1 mM GTP
0.25 mM CTP
No Effector
0.1 mM CTP
1.6 mM Mg++
0.4
0.8
1.2
(PEP) mM

Figure 22

Velocity profiles with PEP for human erythrocyte pyruvate kinase in the presence and absence of several effectors. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 16 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP and effector at the concentrations indicated.


0.06
0.04
0.02
μmoles PEP / min
0.3 mM EDTA
0.1 mM ATP
0.1 mM GTP
0.1 mM CTP
No Effector
16.0 mM Mg++
0.2
0.6
1.0
1.4
(PEP) mM

nucleoside triphosphates have approximately the same affinities for Mg^{++} (or Mn^{++}). If chelation were the major factor in the activation phenomenon it would be expected that all of the nucleoside triphosphates, pyrimidines as well as purines would affect the reaction in a similar manner. Furthermore, activation is observed even at 16mM $MgSO_4$. The presence of 0.1mM or 0.3mM nucleoside triphosphate would be able to chelate less than 2% of the cation present, which is a relatively insignificant amount.

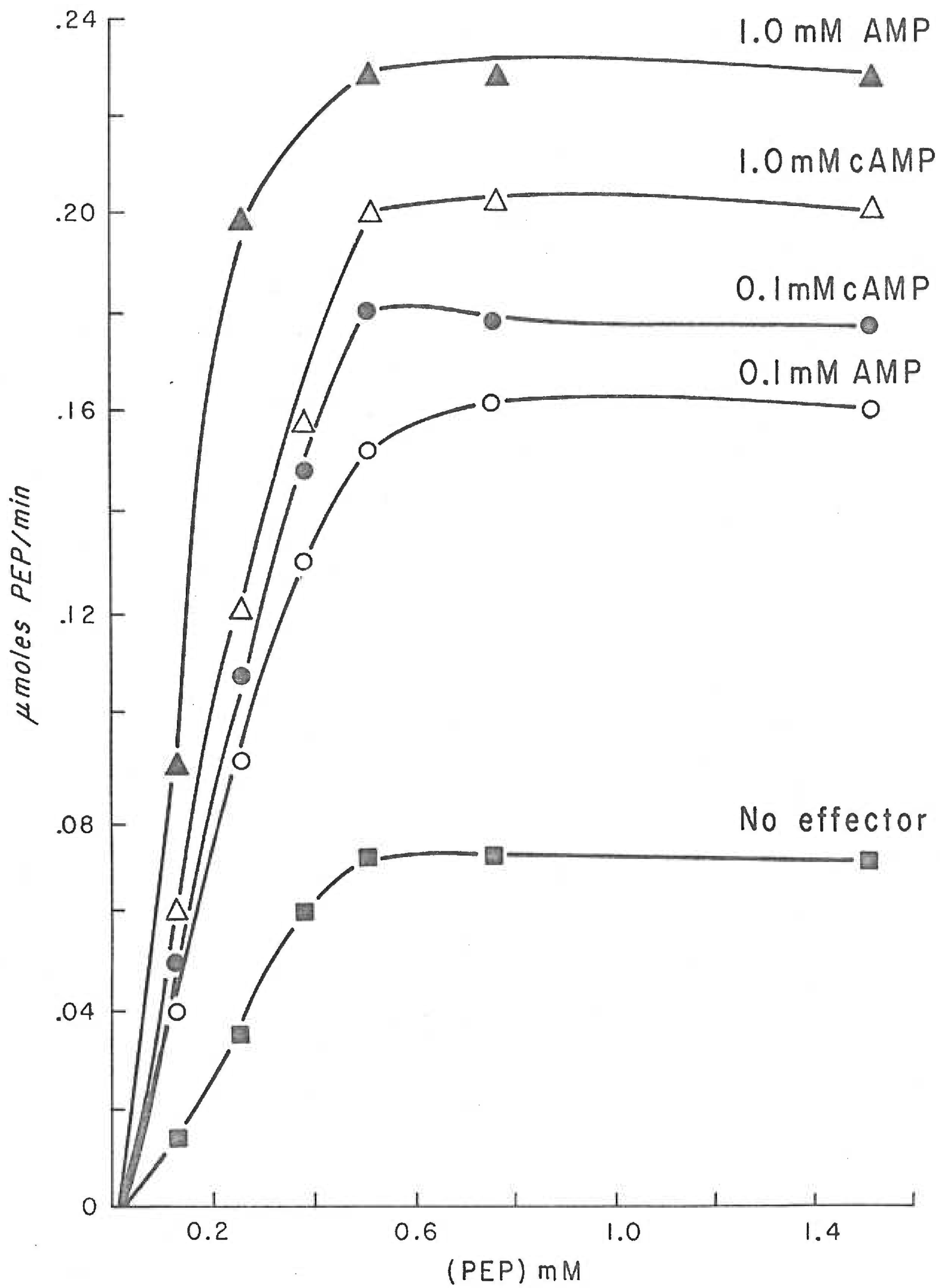
3) A specific binding locus may exist for the purine nucleoside moiety.

The evidence presented in figures 21 and 22 would, therefore, appear to indicate that the purine nucleoside triphosphates have a specific effect on the enzyme protein, and that the purine moiety is the critical factor in the activation phenomenon. Figure 23 shows that AMP and 3',5'-(cyclic) AMP have similar activating effects and that this activation is magnesium-dependent. Since neither AMP nor cAMP have appreciable affinity for Mg^{++}, it suggests that the nucleotide must interact directly with the enzyme. The reduced activation at high Mg^{++} may be due to the effects of Mg^{++} on the enzyme - nucleotide interaction. Neither AMP nor cAMP were assayed for contaminating ATP.

The activating effect of EDTA is similar to that observed with the purine nucleotides. Contamination and chelation of added divalent metal can both be ruled out by data and reasoning similar to that presented for purine nucleotides. One other possibility is that native pyruvate kinase has inhibitory trace metals which are removed by the

Figure 23

Velocity profiles with PEP for human erythrocyte pyruvate kinase in the presence and absence of either AMP or 3',5' (cyclic) AMP. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II) and PEP and effector at the concentrations indicated.



1.0 mM AMP
1.0 mM cAMP
0.1 mM cAMP
0.1 mM AMP
No effector
μmoles PEP/min
.24
.20
.16
.12
.08
.04
0
0.2
0.6
1.0
1.4
(PEP) mM

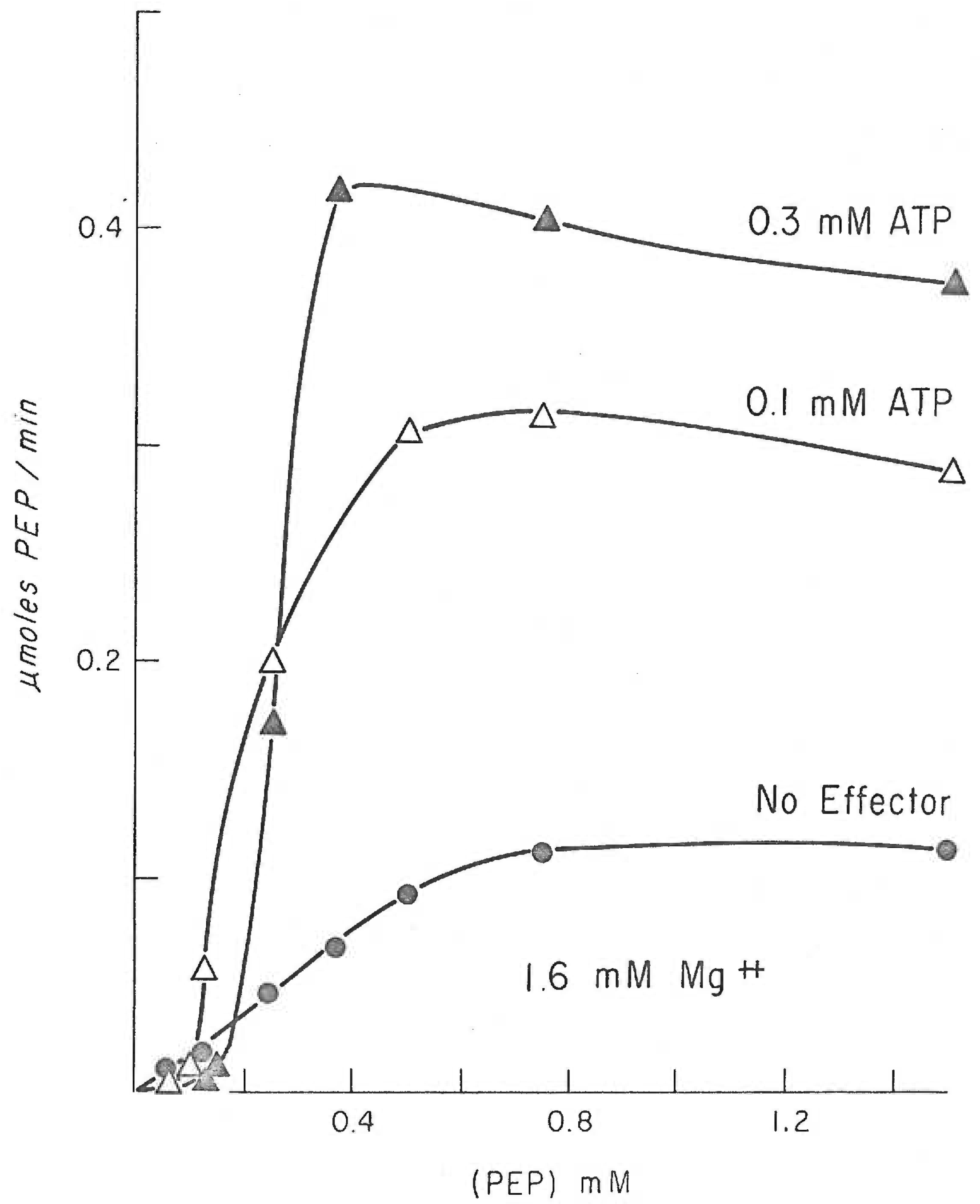
strong binding affinity of EDTA. This seems unlikely because the concentrations of EDTA necessary for activation were approximately two orders of magnitude greater than the estimated concentration of enzyme, which would mean that inhibitory metal must be bound very tightly. Should trace metals be bound tightly, it is reasonable to assume that they are present in the in vivo state. Furthermore, the removal of tightly bound metal could also be used to explain the ATP and GTP activations but the observed effects of AMP and cAMP would be inconsistent with this hypothesis.

Activation of pyruvate kinase by ATP was first observed by Haeckel et al. (107), using a yeast preparation. Figure 8 of that paper demonstrates that certain ratios of Mg^{++} to ATP concentrations result in activation of the enzyme. This data is similar in form to that collected by Holmsen and Storm (92) for their studies on ATP inhibition. Furthermore, it substantiates the data presented in figures 21 and 22 showing a dependence of the activation phenomenon on the Mg^{++} concentration. Figure 24 shows that ATP activation is also dependent on the PEP concentration. At low PEP concentrations, ATP inhibits the reaction but as the level of PEP is increased, ATP increases the maximum velocity, Vm, with apparently little effect on the Km for PEP. As seen in figure 9, ATP activation reaches a peak at a certain concentration (depending on the concentrations of PEP, ADP and Mg^{++}) and further increase in ATP leads to the inhibition previously discu[illegible] (17,74,98).

Haeckel et al. (107) interpreted the ATP activation phenomenon as a minimum ATP requirement for the maintenance of the active conformation

Figure 24

Velocity profiles with PEP for human erythrocyte pyruvate kinase in the presence and absence of the effector, ATP. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP and ATP at the concentrations indicated.

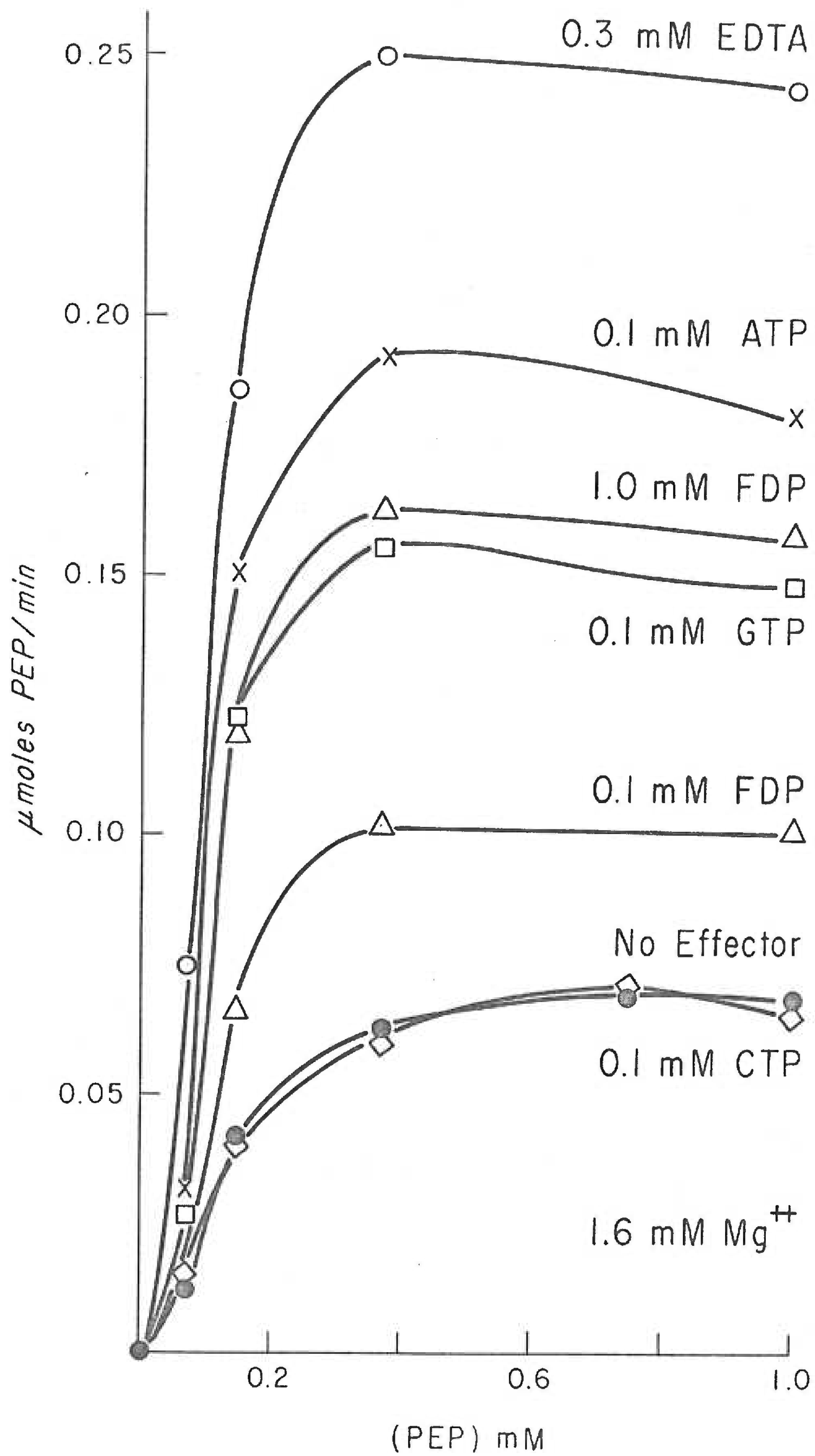

0.3 mM ATP
0.1 mM ATP
No Effector
1.6 mM Mg ++
µmoles PEP / min
0.4
0.2
0.4
0.8
1.2
(PEP) mM

state of the yeast enzyme. Since both the yeast and human erythrocyte enzymes are activated by FDP and are generally considered to be different from other mammalian isozymes, it was of interest to see if ATP activation was also a property of the human and rabbit skeletal muscle enzymes which previously have been thought to obey classical Michaelis-Menten kinetics (17,74). Preliminary evidence indicates that EDTA, ATP, GTP, CTP and UTP affect the rabbit muscle and human skeletal muscle species of pyruvate kinase in a qualitatively identical fashion to that observed for the human erythrocyte enzyme. Figure 25 illustrates this relationship for the human skeletal muscle pyruvate kinase. A Mg^{++} concentration of 1.6mM was generally optimal for observing these effects. The major difference between the muscle forms of pyruvate kinase and the erythrocyte form is the velocity response to FDP. A comparison of figures 3 and 25 demonstrates the difference. FDP affects the Km for the erythrocyte enzyme, whereas it appears to alter only the maximum velocity, Vm, of the muscle enzymes.

Campos et al. (74) studied the kinetics of PEP and ADP binding to the erythrocyte enzyme. The results showed that both the maximum velocity, Vm, and the Km changed when PEP was varied at several fixed concentrations of ADP. Generally, as the concentration of the constant substrate was increased, the Vm and Km both increased. The data are consistent with a rapid equilibrium random mechanism in which binding of one substrate hinders binding of the second. Analogous effects on the kinetic parameters are observed in figures 21 and 24 for activation by ATP and GTP when ADP and Mg^{++} are non-saturating, although changes

Figure 25

Velocity profiles with PEP for human skeletal muscle pyruvate kinase in the presence and absence of added effectors. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP and effectors at the concentrations indicated.

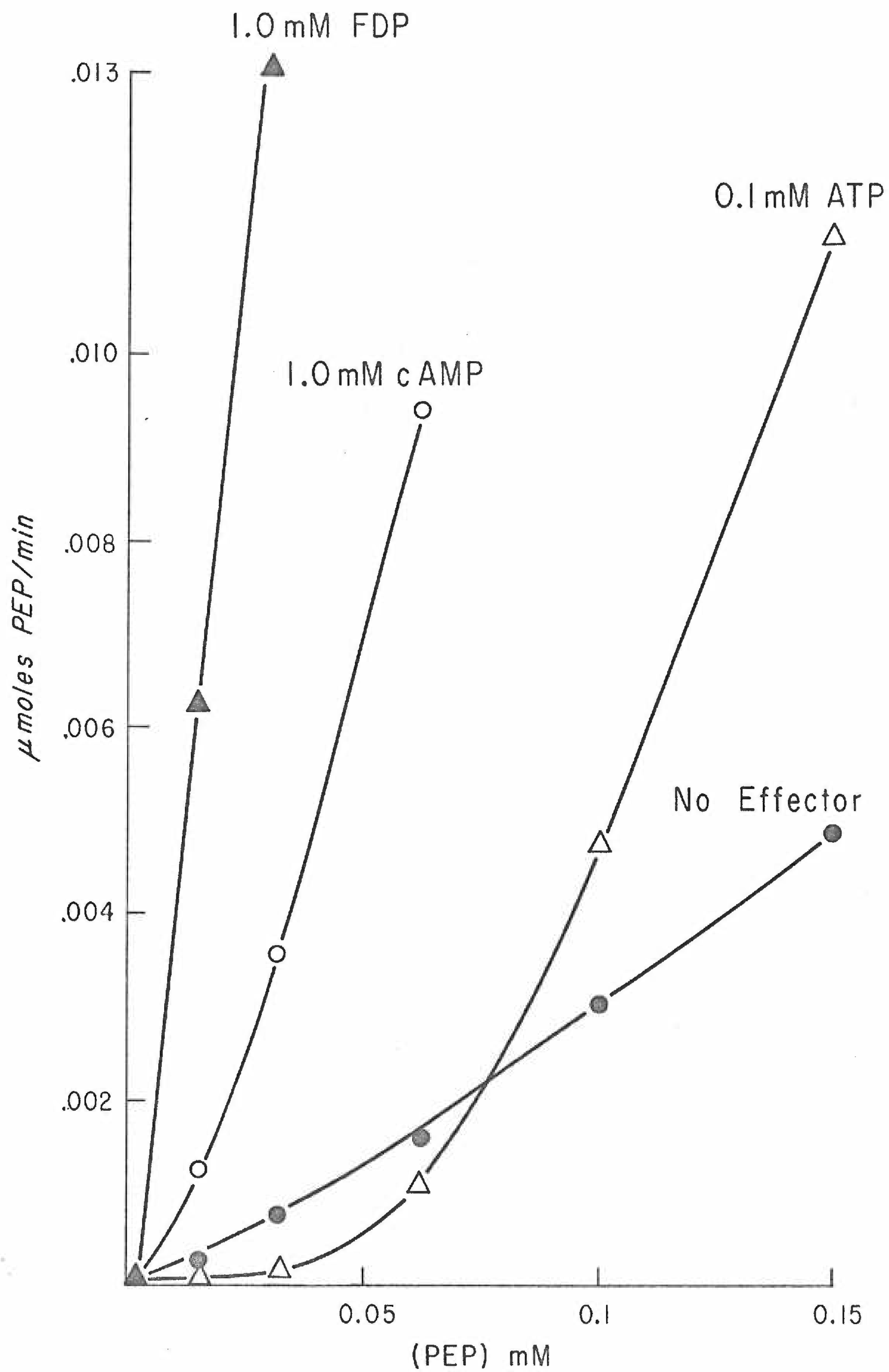

0.3 mM EDTA
0.1 mM ATP
1.0 mM FDP
0.1 mM GTP
0.1 mM FDP
No Effector
0.1 mM CTP
1.6 mM Mg++
0.25
0.20
0.15
0.10
0.05
µmoles PEP/min
0.2
0.6
1.0
(PEP) mM

in Km are very small. The only difference is that the triphosphates inhibit at very low PEP concentrations. Reexamination of the data from which figure 2 [as published by Campos et al. (74)] was drawn (135) reveals that the data could possibly be best fitted to a parabola rather than a straight line. This would allow for the sigmoid velocity profiles observed in figures 21 and 24. The similarity between these two sets of data allows one to postulate that ATP may bind to one ADP locus in place of MgADP and promote the binding of Mg^{++}, ADP or MgADP at a different catalytic site. The inhibition by ATP and low PEP (fig. 24) could simply be due to interference with PEP binding, and hence the ability of PEP to exert its homotropic effect. This argument seems plausible in view of the model proposed by Boyer (21) for the rabbit muscle pyruvate kinase in which there is a common locus for the γ-phosphoryl group of ATP and the phosphoryl group of PEP. Occupancy of this site by ATP may allow for activation, whereas occupancy by PEP may be responsible for its homotropic-cooperative effect.

Figure 26 is a representation of the activation by different positive effectors of human erythrocyte pyruvate kinase. The FDP effect has been discussed previously and elsewhere (76). The activation by 1.0mM cAMP is similar to that for AMP. No initial inhibitory phase is observed and a small decrease in Km is usually observed. The ATP effect shows the usual inhibition at low PEP with activation resulting from increase in the PEP concentration. Since the monophosphates do not show inhibition at low PEP, it is intriguing to speculate that the

Figure 26

Velocity profiles with PEP for human erythrocyte pyruvate kinase in the presence and absence of added effectors. All reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP and effectors at the concentrations indicated. Concentrations of the added effectors, FDP and cyclic AMP, were those found to give maximum activation.


1.0 mM FDP
0.1 mM ATP
1.0 mM cAMP
No Effector
.013
.010
.008
.006
.004
.002
μmoles PEP/min
0.05
0.1
0.15
(PEP) mM

transferable phosphate moiety of ATP is responsible for the inhibition observed at low PEP when ATP is the added effector. This hypothesis is consistent with the model proposed by Boyer (21). The important addition to the Boyer model would be the possibility for interactions between binding sites for the ligands PEP, MgADP and/or ATP.

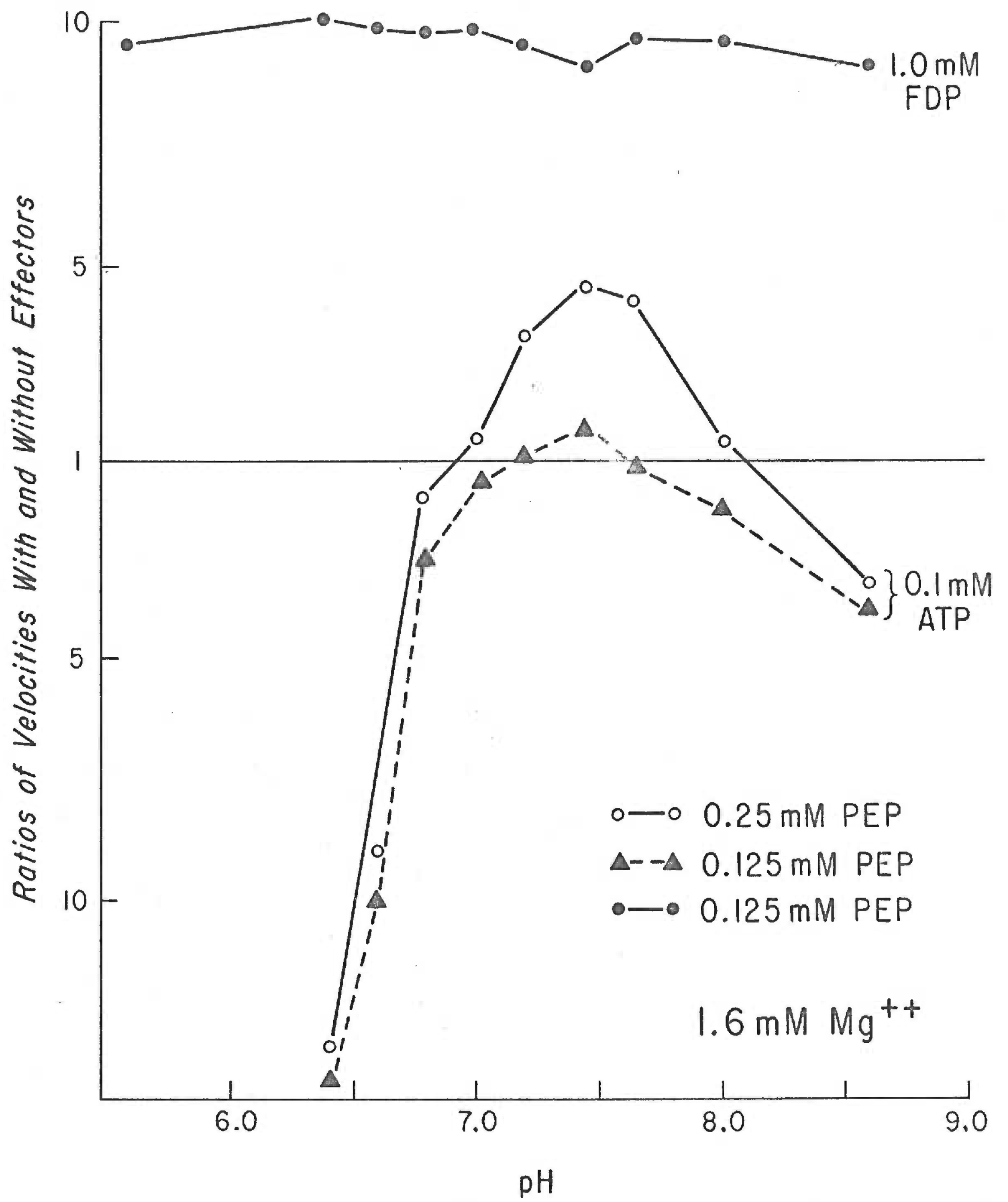
E. pH Effects.

As pointed out by Bygrave (40), pH may be an important factor influencing enzyme activity, because the values for the stability constants of metal ion chelates with adenine nucleotides, proteins and other substrates depend in large part on the pH of the medium. Figure 27 illustrates the effect of pH on the activation by ATP and FDP of human erythrocyte pyruvate kinase. Activation by FDP generally appears to be independent of pH over the entire range studied (5.6 - 8.5), whereas ATP shows a bell-shaped curve with activation occurring only in a narrow range between pH 6.8 and 8.0. Below pH 6.8 and above pH 8.0, the presence of ATP results in inhibition. The Mg^{++} concentration, 1.6mM, was that found to give optimal ATP activation at pH 7.46. The magnitude of the ATP activation is dependent on the level of PEP, as mentioned previously.

Examination of the activation portion of the pH-ATP profile indicates that the maximum activation is achieved between pH 7.4 and 7.6, which is noteworthy since human erythrocytes in a normal physiological setting are thought to function in this pH range (136). In view of the narrow pH range for ATP activation, the pH of each assay solution was checked following the reaction. At the ATP level used in this

Figure 27

Ratios of velocities in the presence and absence of added effectors and their relationship to pH, for human erythrocyte pyruvate kinase. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM TEA buffer; 1.6 mM $MgSO_4$; 0.125 mM ADP; enzyme(II); and PEP, ATP and FDP at the concentrations indicated.



Ratios of Velocities With and Without Effectors
10
5
1
5
10
1.0 mM
FDP
0.1 mM
ATP
0.25 mM PEP
0.125 mM PEP
0.125 mM PEP
1.6 mM Mg++
6.0
7.0
8.0
9.0
pH

study (0.1mM) virtually no change in pH was observed in either the control assays or the assays with added ATP.

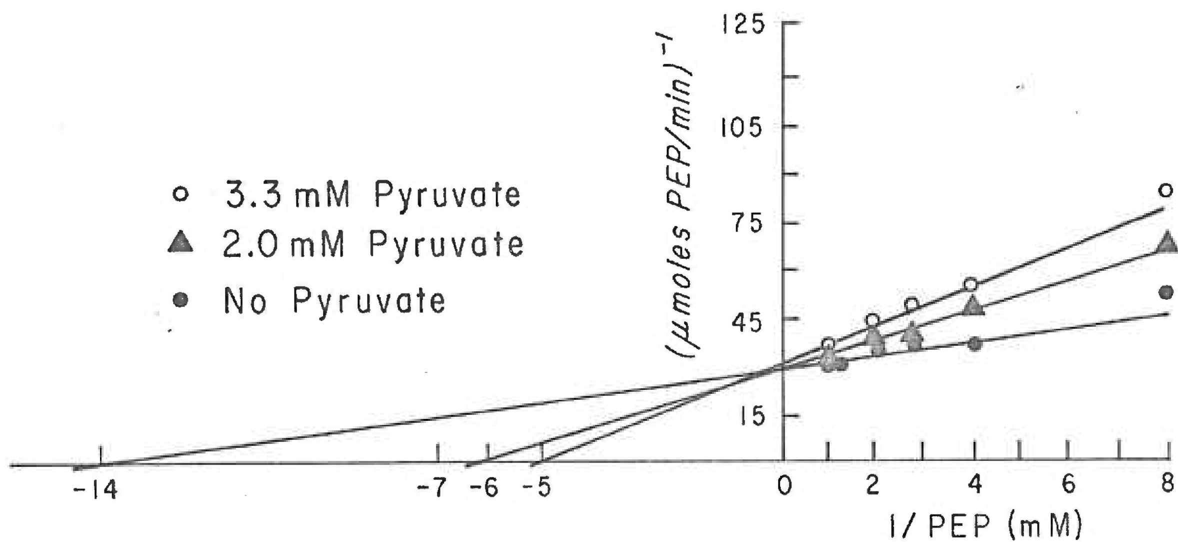
Several possibilities exist to explain the pH-ATP effects. First, the pH may affect a critical ligand on the enzyme necessary for ATP binding. Second, ATP itself may undergo ionization (or protonation) which alters its affinity for protein. Third, ATP ionization or protonation may alter its affinity for Mg^{++}, which in turn may affect its interaction with the enzyme. The latter seems a very likely possibility since Phillips (126) has indicated that ATP has a critical ionization near pH 7.5 which can affect its ability to chelate divalent cation.

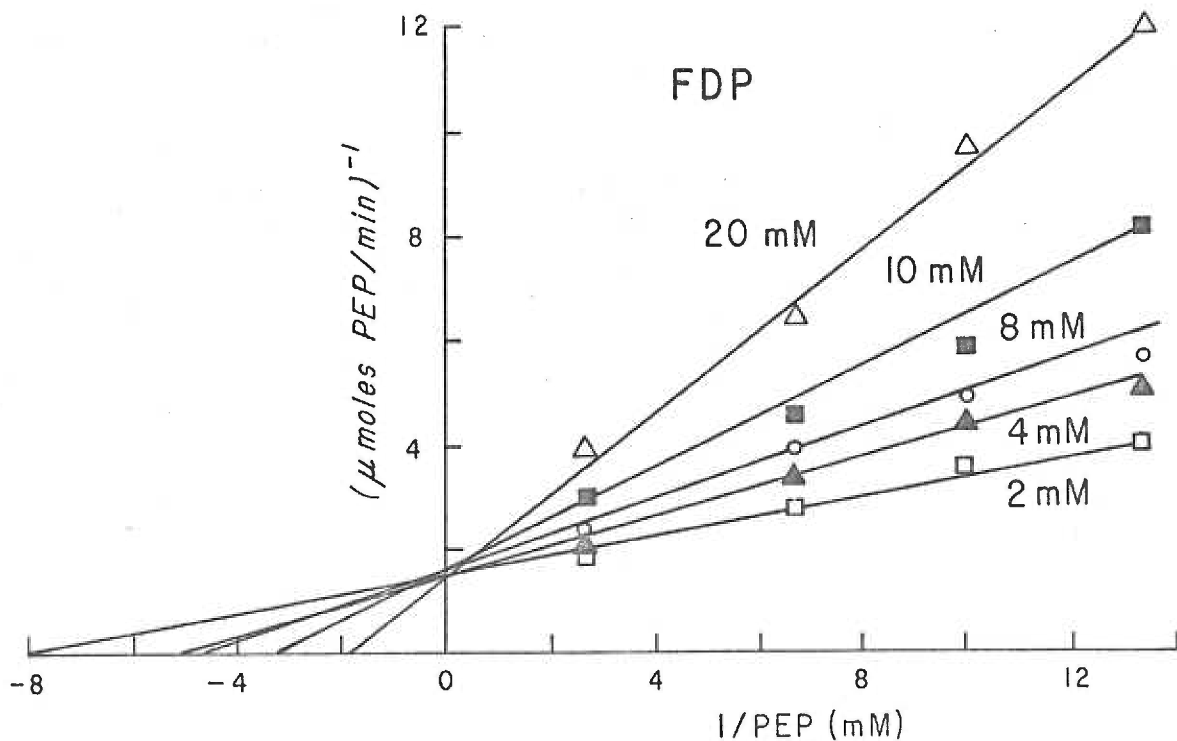
F. Pyruvate and FDP effects.

Mildvan and Cohn (86) reported that pyruvate was a competitive inhibitor with respect to PEP for the rabbit muscle pyruvate kinase. Figure 28 (top) shows that pyruvate is competitive with respect to PEP for the human erythrocyte pyruvate kinase as well. Earlier work on the effect of FDP on the binding of PEP gave no clear pattern of inhibition (76). The lower part of figure 28, however, indicates that FDP may also be a competitive inhibitor of PEP. If this proves to be the case, it would strongly support the model proposed by Koler and Vanbellinghen (76) in which FDP exerts its modulating effect by binding to the PEP locus on the enzyme. The idea that both PEP and FDP could bind to a common locus on erythrocyte pyruvate kinase is consistent by analogy with the work of Kirtley and Dix (137) on rabbit liver fructose 1,6-diphosphatase. They found that FDPase could

Figure 28

Lineweaver-Burk plot of total PEP concentration against initial velocity of human erythrocyte pyruvate kinase at varying levels of pyruvate (upper) and FDP (lower). Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 16 mM Mg^{++}; 0.125 mM ADP; enzyme(II); and PEP and ADP at the concentrations indicated.


3.3 mM Pyruvate
2.0 mM Pyruvate
No Pyruvate
(μmoles PEP/min)⁻¹
125
105
75
45
15
-14
-7
-6
-5
0
2
4
6
8
1/ PEP (mM)


FDP
(μmoles PEP/min)⁻¹
12
8
4
20 mM
10 mM
8 mM
4 mM
2 mM
-8
-4
0
4
8
12
1/PEP (mM)

catalyze the hydrolysis of PEP as well as FDP and that both hydrolyses were dependent on the presence of Mn^{++} or Mg^{++}. It was also shown that FDP inhibited PEP hydrolysis. Furthermore, PEP is a negative effector of phosphofructokinases from pea seed (138) and _E. coli_ (139), and the negative cooperative effects are dependent on the concentrations of Mg^{++} and fructose-6-phosphate.

II. Progress Curve Data

The use of complete reaction curves for the characterization of enzymatic reactions is particularly well suited to reactions which can be monitored continuously. Human erythrocyte pyruvate kinase in the absence of added effectors exhibits sigmoid progress curves with a small initial slope followed by a steeper slope. Figure 29 illustrates three progress curves at varying Mg^{++} and PEP concentrations. Divalent cation appears to be an important variable in determining the degree of sigmoidicity. Saturating levels of magnesium (16mM) reduce the sigmoidicity, making it difficult to observe if reactions are not monitored immediately after addition of enzyme. At lower levels of Mg^{++} (1.6mM) the degree of sigmoidicity is greatly enhanced. PEP generally does not alter the sigmoidicity, but at saturating Mg^{++} concentration and high concentrations of PEP, the slower initial phase of the reaction is almost totally obscured.

It should be pointed out that sigmoidicity is observed only when the concentration of ADP is non-saturating. This is true regardless of the Mg^{++} concentration.

Figure 30 shows that ATP has a distinct effect on the progress

Figure 29

Progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.125 mM ADP, and identical concentrations of enzyme(I). PEP and Mg^{++} concentrations were as indicated in the diagram.

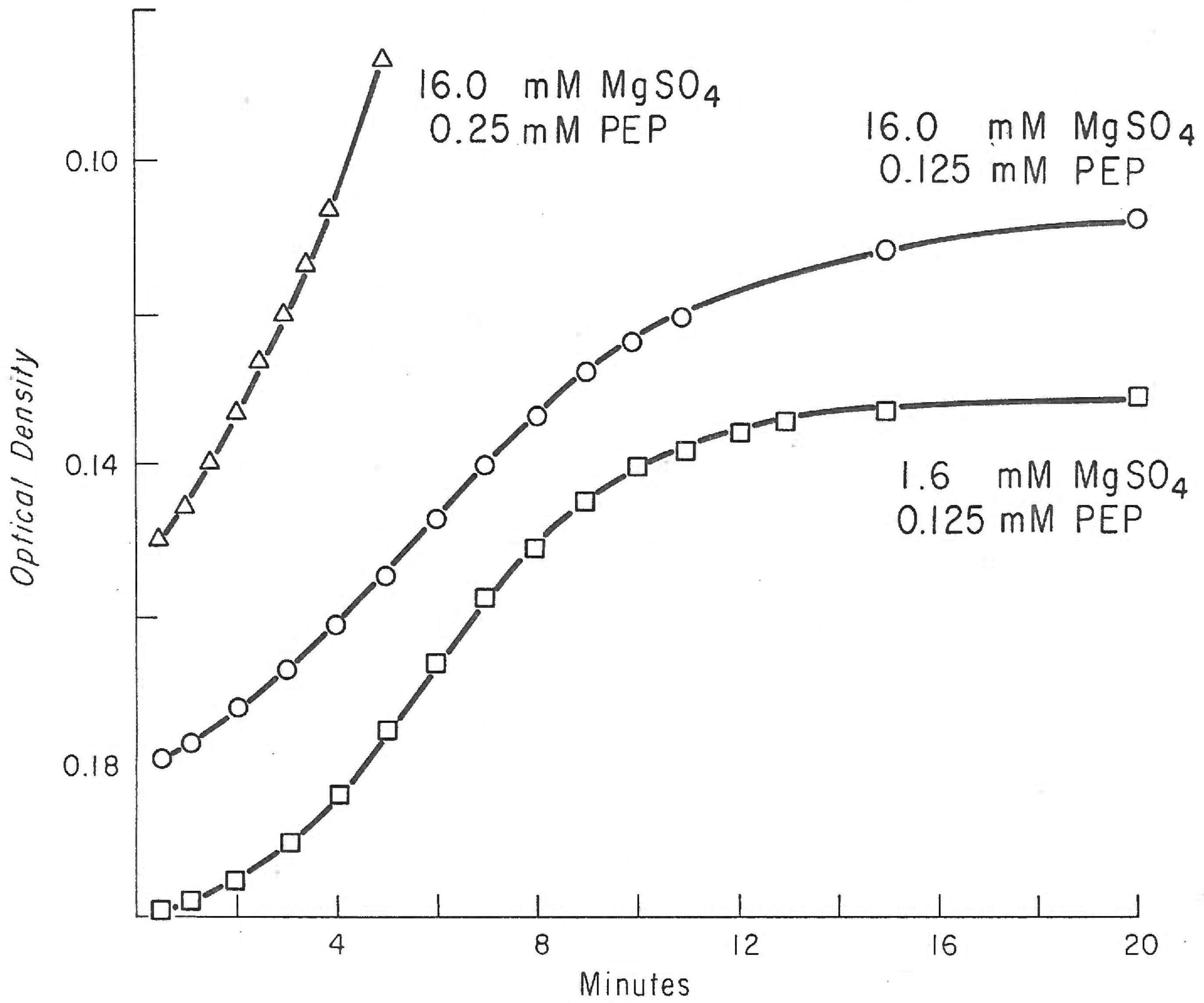
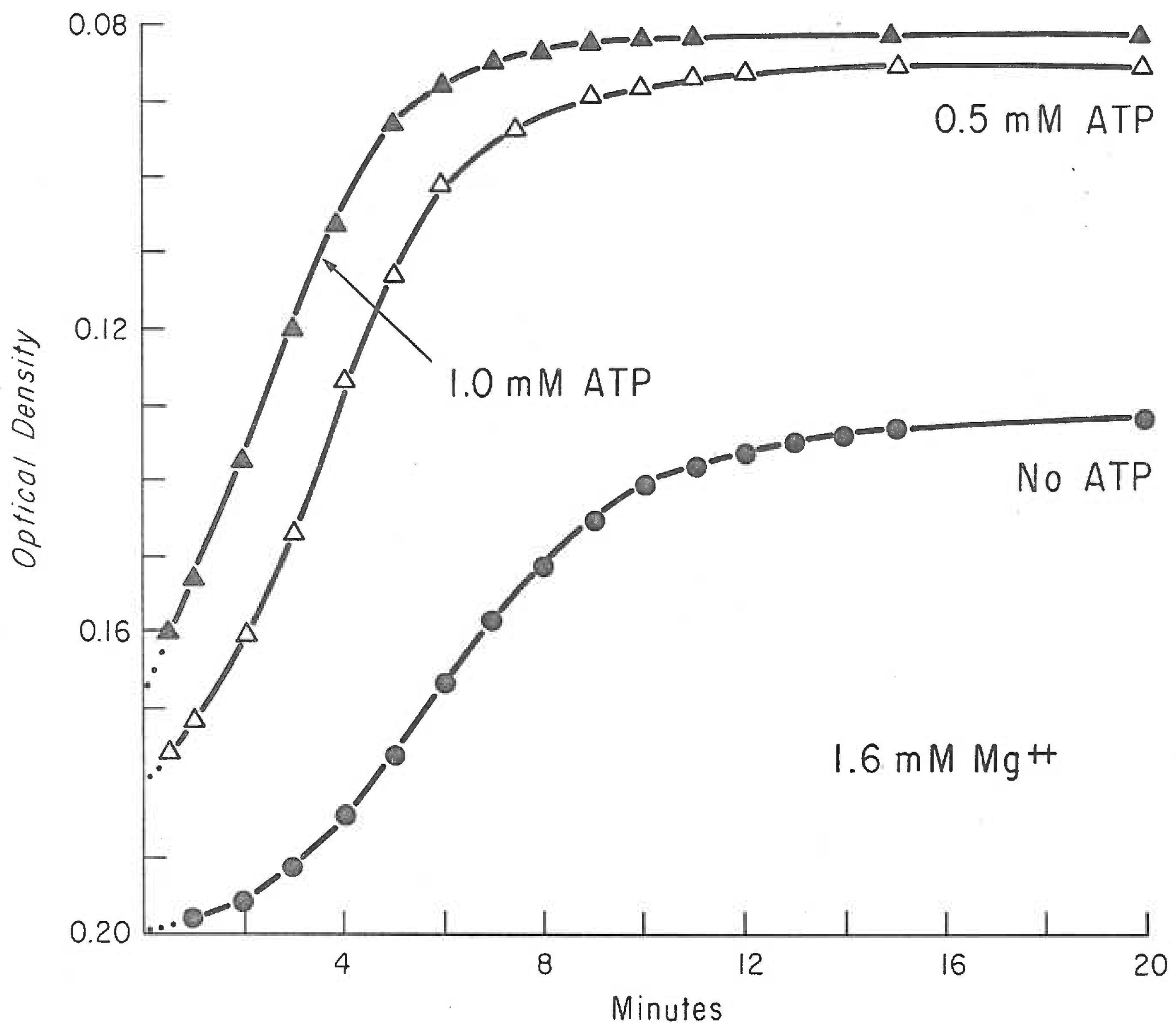

16.0 mM MgSO4
0.25 mM PEP
16.0 mM MgSO4
0.125 mM PEP
1.6 mM MgSO4
0.125 mM PEP
Optical Density
0.10
0.14
0.18
4
8
12
16
20
Minutes

Figure 30

Effects of ATP on the progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP and PEP, and identical concentrations of enzyme(I). ATP concentrations were as indicated in the diagram.


0.08
0.12
0.16
0.20
Optical Density
0.5 mM ATP
1.0 mM ATP
No ATP
1.6 mM Mg++
4
8
12
16
20
Minutes

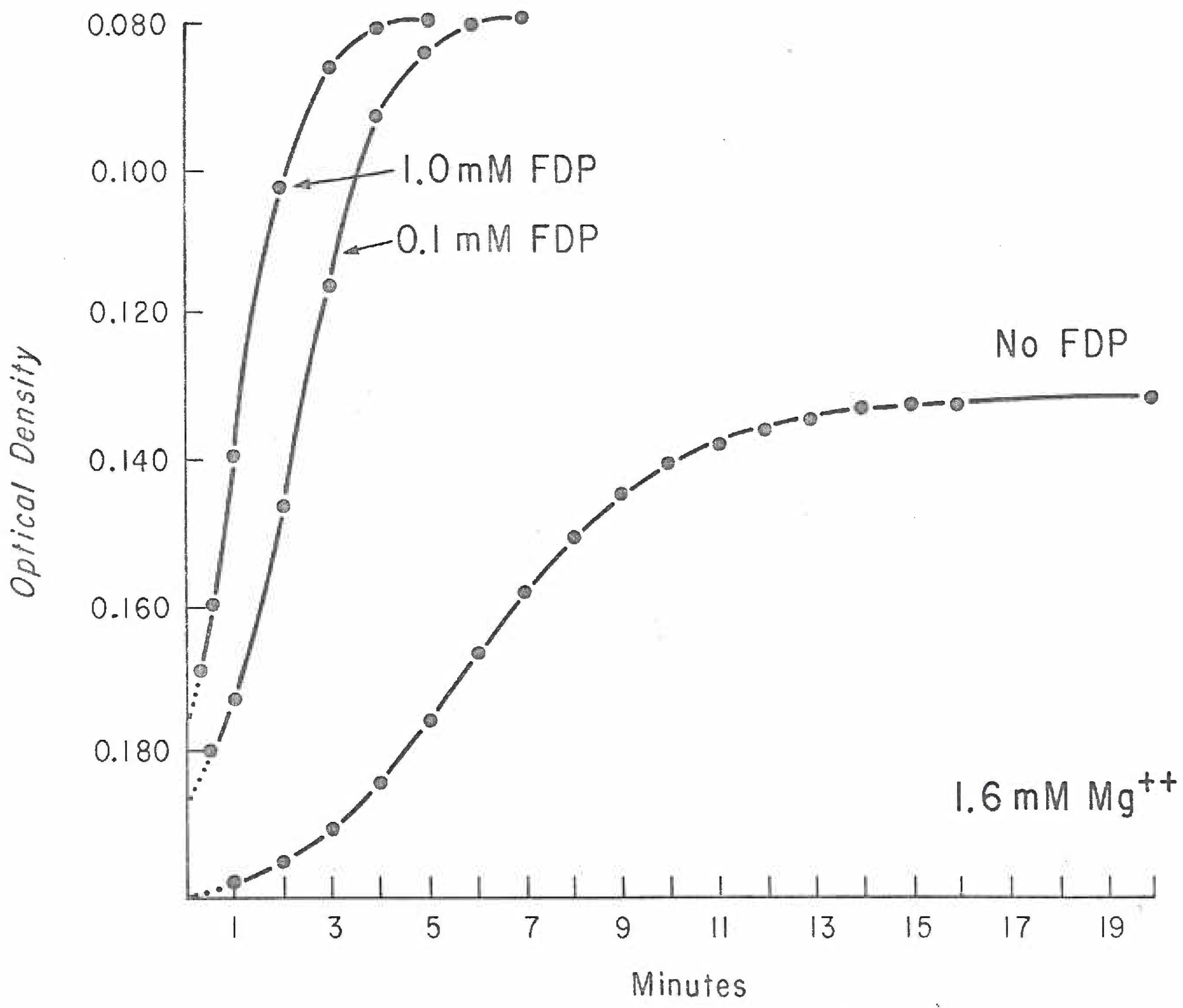
curves of erythrocyte pyruvate kinase. The sigmoidicity is markedly reduced when ATP is added to a system with low Mg^{++}. The initial rates are greater and the sigmoidicity is less in the presence of ATP at zero time, and this correlates with the ATP activation phenomenon observed in the initial velocity studies reported in section I of Results. Addition of FDP also dramatically reduces the sigmoidicity of the progress curves as seen in figure 31. The reduction in sigmoidicity is similar to the ATP effect, but the degree of activation is greater at optimal FDP concentrations than at optimal ATP concentrations. Other data indicate that FDP activation increases with increasing Mg^{++} concentration. These data would imply that FDP and ATP exert their effects at different loci on the enzyme. GTP mimics the ATP effect, but neither CTP nor UTP significantly alters the progress curve sigmoidicity.

Furthermore, FDP does not appear to affect the equilibrium point of the reaction as measured by total change in optical density. High concentrations of ATP, a product of the reaction, reduce the total change in optical density. Low concentrations of ATP, however, do not noticeably reduce the total change in optical density which is reasonable in view of the apparently large equilibrium constant for the forward reaction.

The sigmoid progress curves in the absence of added effector and the change in sigmoidicity upon addition of ATP support the conclusion that ATP activates erythrocyte pyruvate kinase, and that the acceleration of rate observed when ATP is not added initially is due to the

Figure 31

Effects of FDP on the progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 1.6 mM $MgSO_4$; 0.125 mM ADP and PEP, and identical concentrations of enzyme(I). FDP concentrations were as indicated in the diagram.


0.080
0.100
0.120
0.140
0.160
0.180
Optical Density
1.0 mM FDP
0.1 mM FDP
No FDP
1.6 mM Mg++
1
3
5
7
9
11
13
15
17
19
Minutes

build-up of ATP as the reaction progresses. Further studies at carefully selected concentrations of PEP and ATP would enable a more quantitative evaluation of the ATP effect on the reaction rate.

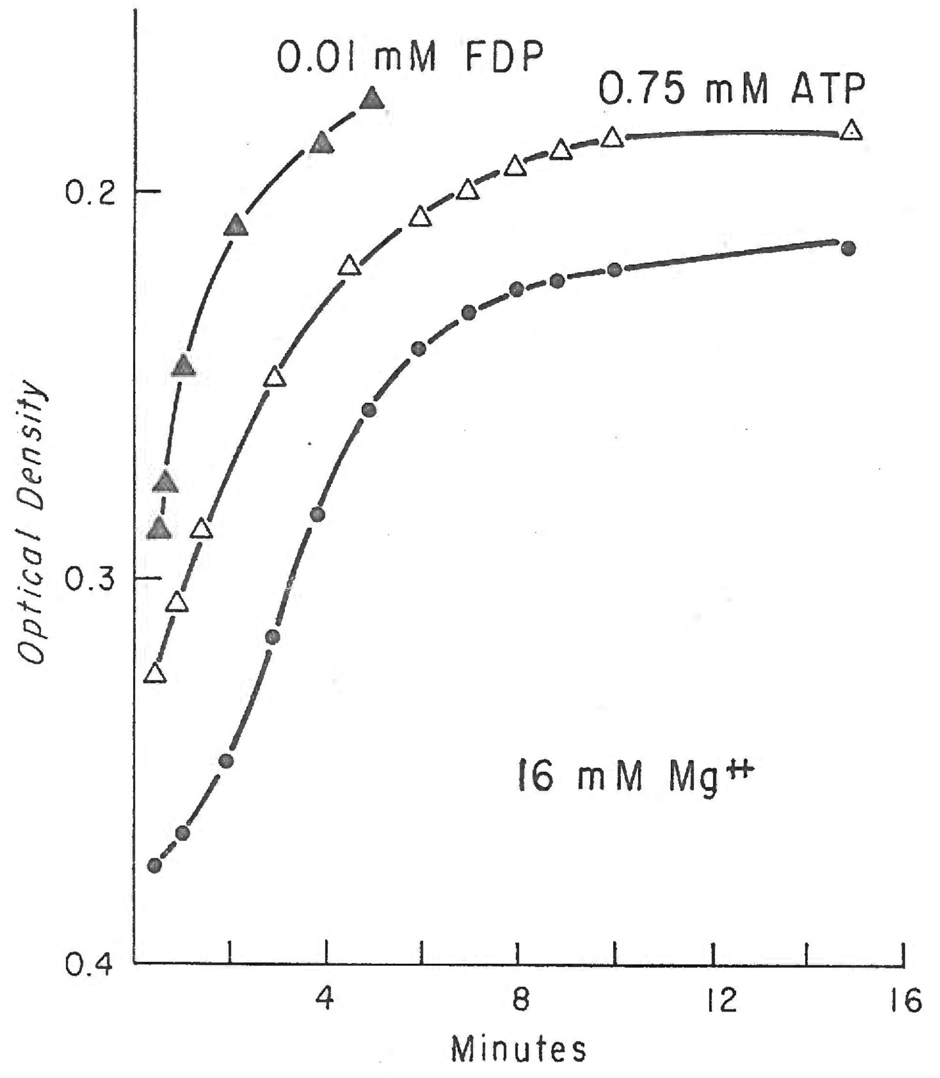
An alternative explanation has been considered. One of the substrates or cations in the reaction mixture may have a time-dependent interaction with the enzyme which results in conversion of the enzyme into a more active state. Rabin (140) has presented a simple kinetic model based on substrate-induced conformation isomerization to account for apparent cooperative effects. Furthermore, the extensive work conducted on glutamine synthetase from E. coli (141,142) has shown that catalytically inactive enzyme regains activity in a time-dependent process following addition of Mn^{++} or Mg^{++}. Preliminary incubation of glutamine synthetase with Mg^{++} or Mn^{++} eliminates the lag phase associated with activation. Control studies were conducted with erythrocyte pyruvate kinase to determine if Mg^{++}, PEP, ADP or some combination of these ligands may be involved in such a time-dependent reaction. Preincubation studies with these ligands have revealed that sigmoidicity is not changed. Only the presence of non-substrate effectors (ATP, GTP, AMP, FDP) or Mn^{++} (q.v. infra) alters the sigmoidicity.

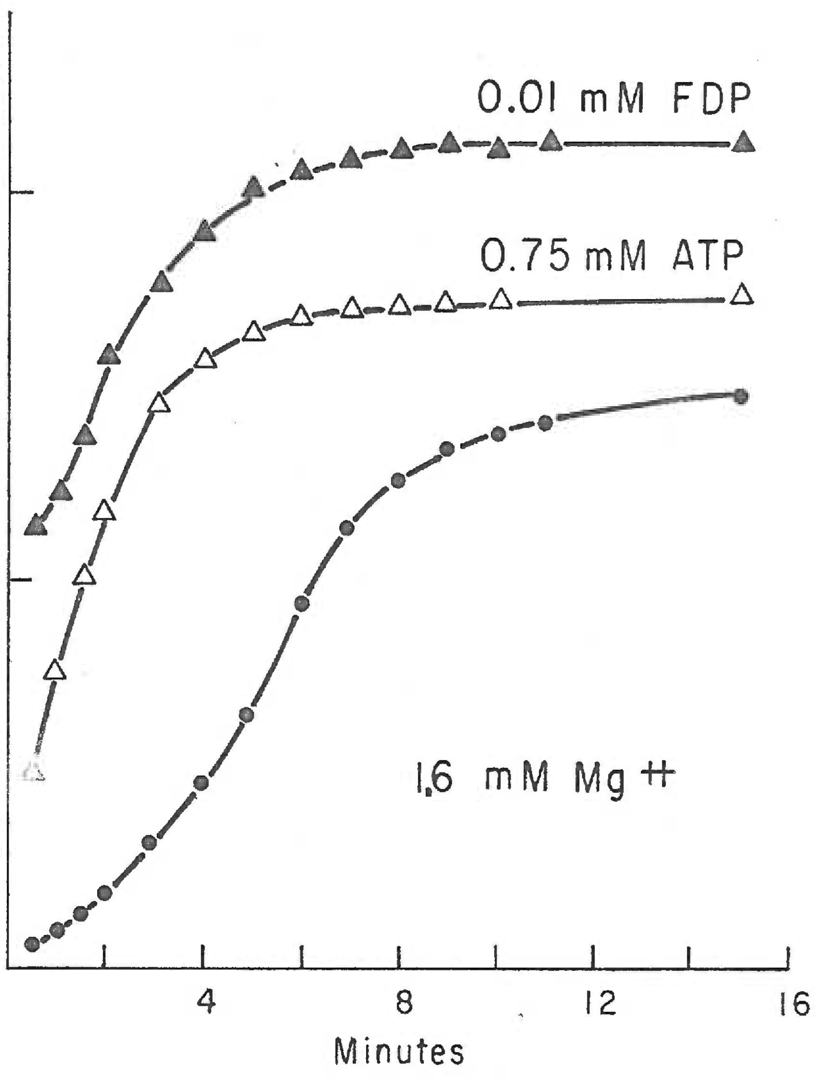
Figure 32 demonstrates the effect of Mg^{++} on the progress curves of erythrocyte pyruvate kinase. At high Mg^{++} (16mM) ATP has only a small effect on the initial rate, whereas at low concentration FDP strongly activates the reaction.

At low Mg^{++} (1.6mM), on the other hand, all other conditions

Figure 32

Effects of Mg^{++} on the progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase in the presence and absence of FDP or ATP. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.125 mM ACP and PEP, and identical concentrations of enzyme(I). FDP, ATP and Mg^{++} concentrations were as indicated in the diagram.


0.01 mM FDP
0.75 mM ATP
16 mM Mg++
Optical Density
0.2
0.3
0.4
4
8
12
16
Minutes


0.01 mM FDP
0.75 mM ATP
1.6 mM Mg++
4
8
12
16
Minutes

remaining the same, the effects of ATP and FDP are reversed. The nucleotide has a large effect on the initial portion of the progress curve, whereas FDP alters it only slightly. These data indicate that the presence of Mg^{++} may stabilize the binding of FDP or increase its affinity for the enzyme as discussed on page 83.

Another effect on the progress curve is that mediated by pyruvate. Figure 33 shows that pyruvate increases progress curve sigmoidicity, with little or no effect on the maximum velocity. These effects were most noticeable at high concentrations of Mg^{++}. Pyruvate, therefore, appears to act in a manner directly opposite to that of ATP, the other product of the pyruvate kinase reaction.

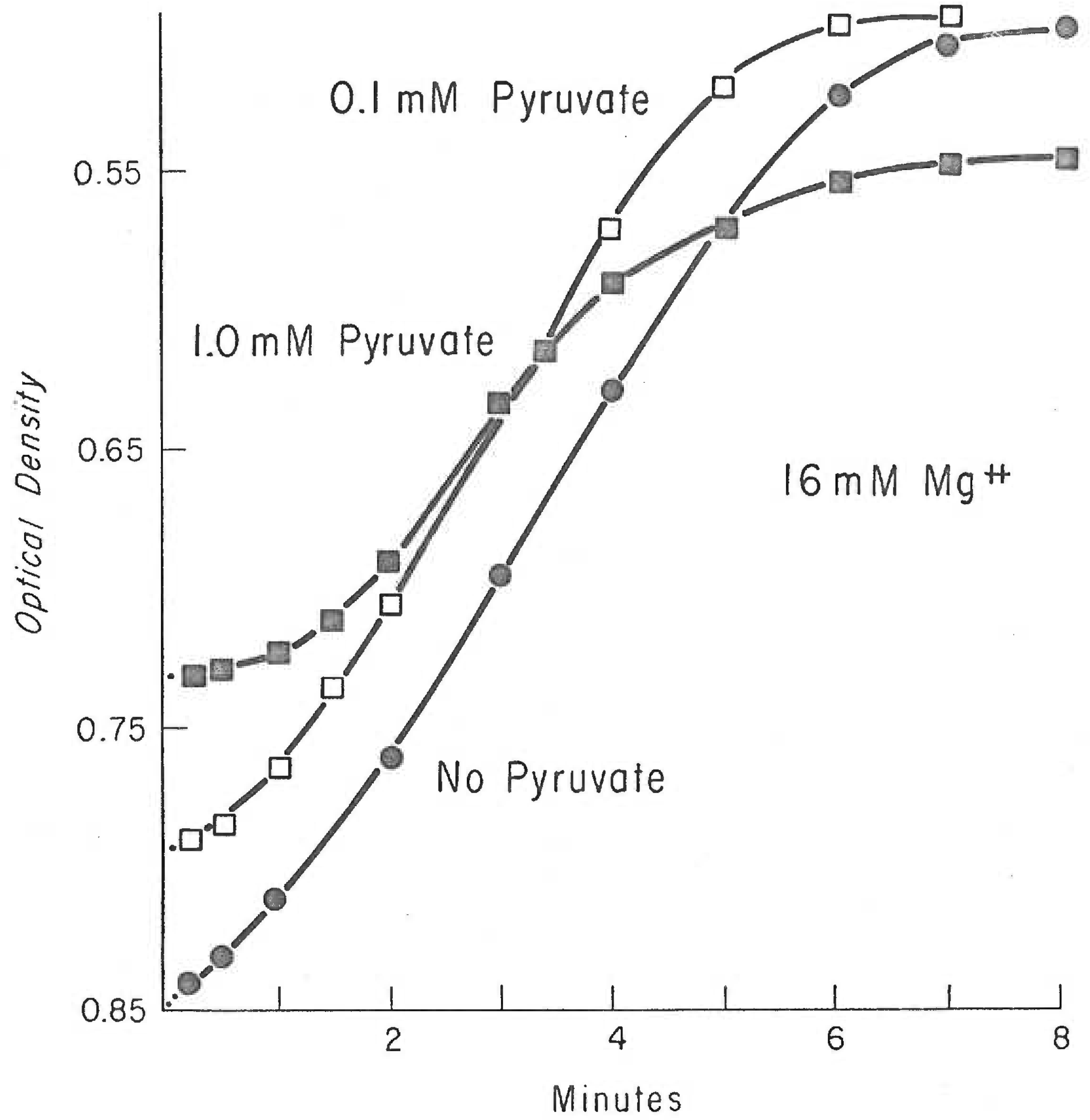
1. Pyruvate increases sigmoidicity, whereas ATP decreases sigmoidicity.
2. Pyruvate shows its major effect of increasing sigmoidicity at high Mg^{++}, whereas ATP shows its effect of decreasing sigmoidicity at low Mg^{++}.

The second point offers another clue to the mechanism, because the positive effect of FDP also shows a Mg^{++} dependence. Initial velocity studies have indicated that both pyruvate and FDP may be competitive inhibitors with respect to PEP, and therefore may bind to a common locus on the enzyme.

It was observed in the initial velocity studies that when Mn^{++} is substituted for Mg^{++} in the erythrocyte pyruvate kinase reaction the kinetic properties of the enzyme are dramatically changed (cf fig. 3). It would be predicted from these observations and the progress

Figure 33

Effects of pyruvate on the progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 16 mM $MgSO_4$; 0.125 mM ADP and PEP, and identical concentrations of enzyme(I). Pyruvate concentrations were as indicated in the diagram.



0.1 mM Pyruvate
1.0 mM Pyruvate
16 mM Mg++
No Pyruvate
Optical Density
0.55
0.65
0.75
0.85
2
4
6
8
Minutes

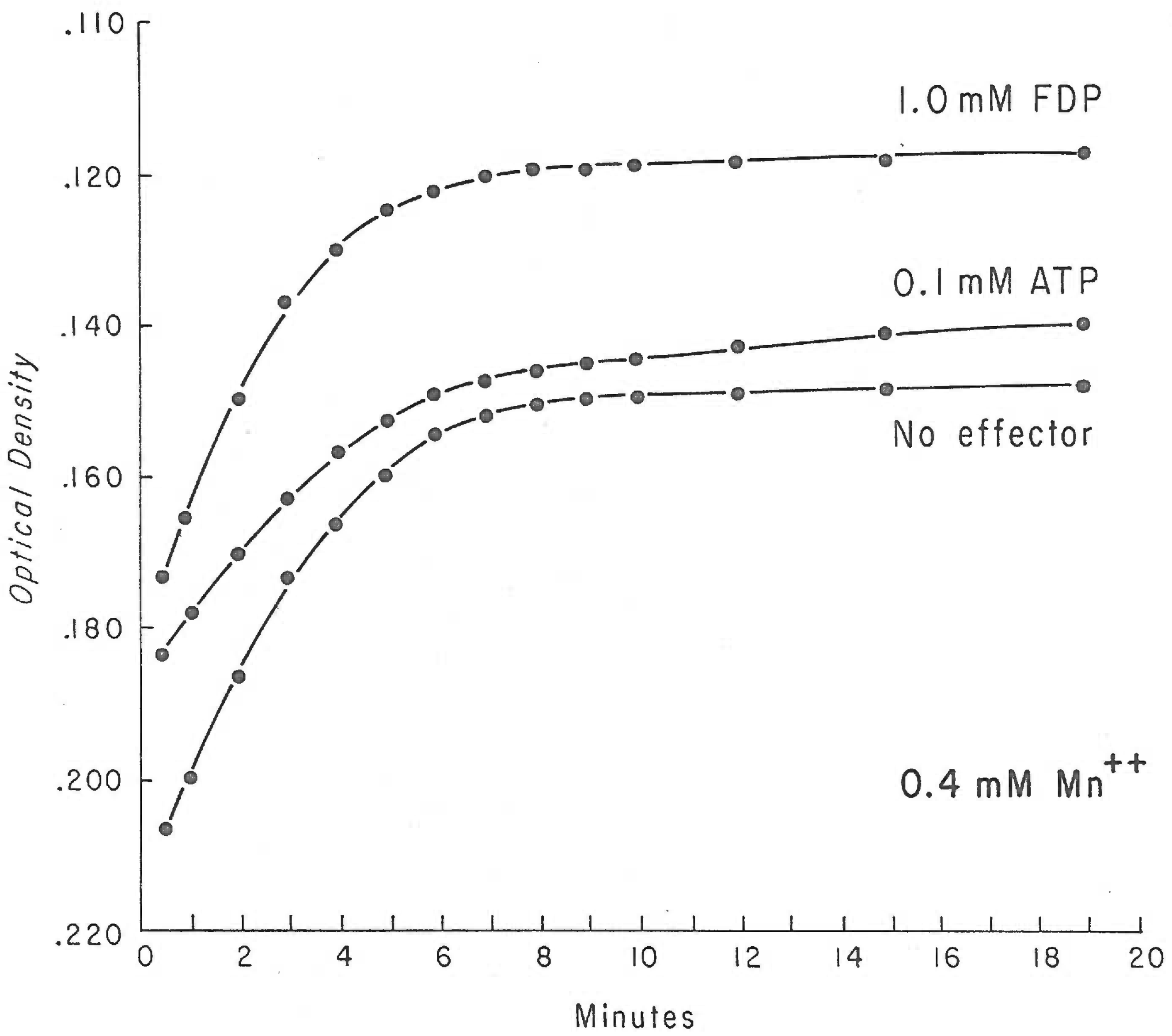
curve data with Mg^{++} just presented that the manganese-activated pyruvate kinase reaction should show no progress curve sigmoidicity in the presence or absence of added effectors. The progress curves in figure 34 are rectangular hyperbolas. The only effect exerted by ATP and GTP is inhibition, and FDP shows no effect over the concentration range which strongly activates the enzyme in the presence of Mg^{++}.

Assuming that the sigmoid progress curve is the result of ATP generated during the reaction, the progress curve data is generally consistent with the initial velocity studies. Four different comparisons support this idea:

1. Hill plots for MgADP have a slope of 1.6 at rate-limiting Mg^{++}, and 1.0 at saturating Mg^{++}. This implies that cooperative interactions can take place involving the Mg-ADP locus at low, but not high Mg^{++} concentrations. The proposed homotropic effects of ATP at the ADP locus would then be expected to be stronger at low Mg^{++}. Progress curve data are consistent with this interpretation.
2. Initial velocity studies show that ATP and GTP activate the enzyme, whereas CTP and UTP do not. Progress curve sigmoidicity is reduced by ATP and GTP, but is unaffected by CTP and UTP, supporting the hypothesis that purine nucleotides have a specific locus for binding.
3. The FDP activation in initial rate studies is dependent on Mg^{++}. The ability of FDP to reduce progress curve sigmoidicity

Figure 34

Effects of Mn^{++} on the progress curves of the reaction catalyzed by human erythrocyte pyruvate kinase in the presence and absence of FDP or ATP. Optical density changes were recorded at 230 mμ in matched 0.5 cm quartz cuvettes. Starting points were arbitrary and were positioned by linear offset control. Reaction mixtures contained 8 mM TEA buffer, pH 7.46; 75 mM KCl; 0.125 mM ADP and PEP, and identical concentrations of enzyme(II). FDP, ATP and Mn^{++} concentrations were as indicated in the diagram.



1.0 mM FDP
0.1 mM ATP
No effector
0.4 mM Mn++
Optical Density
.110
.120
.140
.160
.180
.200
.220
0
2
4
6
8
10
12
14
16
18
20
Minutes

is similarly dependent on Mg^{++}.

4. Initial velocity studies demonstrate that the manganese-activated reaction is first order with respect to PEP, unaffected by FDP, and inhibited by ATP. These data are consistent with the non-sigmoid progress curve observed in the manganese-activated system. Since ATP does not activate in this system, no acceleration of rate would be expected to occur during the course of the reaction, and none is observed experimentally. Similarly, a decrease in rate (i.e., inhibition by ATP) would be expected, and is observed experimentally.

<u>Summary</u>. For the magnesium-activated system it appears that the nucleotide product of the pyruvate kinase reaction, ATP, acts as an effector of the enzyme. When Mg^{++} and ADP concentrations are initially low, the formation of ATP during the reaction causes an increase in the rate of catalysis. Adenosine-triphosphate evidently exerts its effect through binding to a locus which is shared with ADP since high ADP abolishes the ATP effect. Fructose-diphosphate alters the shape of the progress curve, as does ATP, but apparently exerts its positive homotropic effect either directly or indirectly on the PEP and Mg^{++} loci. Pyruvate increases sigmoidicity under conditions of high Mg^{++}, and apparently acts as a negative effector of the enzyme. When Mn^{++} is substituted for Mg^{++}, no sigmoidicity is observed and ATP and FDP do not activate the reaction. Concentrations of ATP equivalent to the 7-10% contamination of ADP by ATP noted in Experimental had no effect on progress curves.

GENERAL DISCUSSION

In order for a small molecule to modulate enzymatic activity, it must alter some property of the protein which is directly related to catalysis. Assuming that human erythrocyte pyruvate kinase (M.W., 230,000, ref 76) is a multisubunit protein like the rabbit muscle enzyme (57,59,64,143), several possibilities exist for substrate or effector mediated changes in catalytic activity: (a) dissociation, association or aggregation phenomena; (b) multiple reaction pathways at a single active site; and (c) subunit interactions.

The ultracentrifuge studies and average molecular weight determinations described previously (76) for erythrocyte pyruvate kinase in the presence and absence of FDP argue against the first possibility. Sucrose density gradient experiments utilizing either FDP or ATP likewise showed no gross alteration in molecular weight following addition of these effectors.[5] On the basis of these data association-dissociation phenomena have been ruled out as factors in modulation of activity.

Some random substrate-enzyme interactions produce non-linear double reciprocal plots of velocity against substrate concentration (144-146) and can be misinterpreted as evidence of allosteric or regulatory phenomena. Sweeney and Fisher (147) have shown that a single independent active site, with more than one reaction pathway

[5]Sucrose gradients were run by Dr. Robert Koler and Dr. Robert Bigley.

leading to the binding of substrate, can also produce sigmoid kinetics. The work of Reynard et al. (17) and Koler and Vanbellinghen (76) argue against a simple random mechanism. The Hill plot and progress curve data presented in Results and Discussion in conjunction with binding studies on rabbit muscle (17,61,62,86,88) and yeast (88) enzymes indicate that pyruvate kinases possess more than a single active site.

Subunit interactions, which control enzymatic activity, and are modulated by substrates and effectors, remain as the most plausible mechanism to explain the kinetic results with human erythrocyte pyruvate kinase. Kinetic evidence from Hill plot studies indicates that this enzyme binds a maximum of two molecules of PEP, ATP or FDP. Since ATP is a competitive inhibitor with respect to PEP (75), binding of PEP and ATP evidently occurs at catalytic sites on the enzyme. Postulating separate allosteric or regulatory sites to account for ATP activation is unnecessary and inconsistent with the data. Consequently, the complex kinetics of human erythrocyte pyruvate kinase can be interpreted assuming only two sites on the enzyme, both of which are catalytic sites.[6]

Two important properties of human erythrocyte pyruvate kinase, unique among the pyruvate kinases so far studied, are 1) its

[6]Since the stoichiometry for the binding of these ligands to the enzyme has not been determined, it is still a possibility that four or more moles of substrate or effector could bind per mole of enzyme. This would not necessarily alter the kinetic interpretation. Similar conclusions were drawn by Kuczenski and Suelter (73) from studies on the yeast enzyme.

susceptibility to product-induced modulation of activity, and 2) its different kinetic properties depending on whether magnesium or manganese is used as the required divalent cation. Each of these properties will be analyzed in the following two sections.

I. Modulation of Activity by ATP and Purine Homologs

The over-all modulating effect of ATP, a product of the forward reaction catalyzed by pyruvate kinase, is dependent on four variables: 1) the type of divalent cation; 2) the concentration of divalent cation; 3) the concentration of ADP; and 4) the concentration of ATP. The results from initial velocity and progress curve studies are consistent with regard to all four. Other purine nucleotides (GTP, AMP, cAMP) function in the same manner as ATP, except that the triphosphates inhibit the reaction at low levels of PEP, whereas the monophosphates do not. Furthermore, three species of pyruvate kinase (from human red cells, human skeletal muscle and rabbit skeletal muscle) were examined and all three showed qualitatively identical results:

1) Low concentrations of purine nucleotide mono- and triphosphates activate in the magnesium-containing system at low concentrations of ADP. Pyrimidine nucleotides showed no effect.
2) Purine nucleotide activation was greater at lower concentrations of Mg^{++} than at saturating concentrations. This was true for monophosphates as well as triphosphates, ruling out chelation as the primary event.
3) High concentrations of purine nucleotide mono- and triphosphates inhibited the reaction at all concentrations of Mg^{++}

and ADP used.

4) In the manganese-containing system the activation phenomenon was not observed. Inhibition was observed with all purine nucleotide mono- and triphosphates tested.

5) When activation was observed (Mg^{++}; low ADP) it was generally manifested as an increase in Vm with only small changes (if any) in Km.

These findings provide suggestive evidence for a specific binding locus for purine nucleotides. Furthermore, the occupancy of this locus apparently causes a non-competitive type of activation. Generally, pure non-competitive behavior would be expected to result only if equilibrium conditions prevail in the formation of the quaternary complex.

Mildvan and Cohn (61) demonstrated agreement between dissociation constants derived from kinetic studies and from binding studies of enzyme or enzyme-metal with PEP and ADP, which supported the proposed scheme of equilibrium kinetics, and the random binding of metal and substrate to the rabbit muscle pyruvate kinase. More recently, however, Mildvan et al. (88) suggested that the yeast pyruvate kinase-metal complex binds PEP prior to ADP in a preferred order. They found agreement of the dissociation constants obtained from binding studies with K-values for PEP, but not for ADP. Since several distinctly different effectors produce a non-competitive activation of the red cell pyruvate kinase, equilibrium between enzymic conformations is consistent with all of these results.

In view of the evidence that mammalian pyruvate kinases consist of only two catalytic sites, it is important to discriminate between events occurring at a single catalytic site (intrasite relationships) and those resulting from interaction between the two catalytic sites (intersite relationships). The latter are best documented for the red cell enzyme, but may also be true of the muscle enzyme. Each individual site can be divided operationally into four subsites (or loci):

1. a locus for the enolpyruvate portion of PEP.
2. a locus for the divalent metal.
3. a locus for ADP.
4. a locus for the transferrable phosphoryl group (γ-phosphoryl of ATP, or phosphoryl group of PEP).

Figure 35 illustrates in greater detail the various loci composing a hypothetical catalytic site of red cell pyruvate kinase. Occupancy of the enolpyruvate[7] and the phosphoryl subsites by PEP, and of the metal subsite by magnesium, at one catalytic site, from the evidence presented, brings about a structural change in the protein such that the affinity at the opposite catalytic site for PEP is increased (positive homotropic-effect). This would explain the positive Hill slope of

[7]The enolpyruvate and phosphoryl subsites may be occupied by phosphoenolpyruvate, phosphoenol-α-ketobutyrate, and phosphoenol-α-ketovalerate but not by homologs with six or more carbons or the homolog with two methyl groups on the β-carbon (148).

Figure 35

Proposed model for the catalytic site of pyruvate kinase based on data reviewed from the literature and presented in Results and Discussion. Estimated bond distances were those of Mildvan et al. (88,154). Kayne and Reuben have postulated a K^+-M^{++} bond distance of 5-7 Å (133). The assignment of oxygen ligands to the metal follows from the reasoning of Mildvan et al. (154).

Enolpyruvate Locus

Metal Locus

Nucleotide Locus

Phosphoryl Locus

M^{++}

K^{+}

H_2O

~6Å

~3.5Å

purine

ribose

2.0 for PEP in the magnesium-activated assay.

Occupancy of the nucleotide locus at one catalytic site apparently has one of two effects on the opposite catalytic site:

1) the rate constant governing the magnesium-mediated phosphoryl transfer at the opposite site must be altered in such a way that catalysis is enhanced.
2) the affinity for ADP (or MgADP) may be increased. This possibility is consistent with the Hill plots for MgADP at limiting Mg^{++}. However, it is inconsistent with the negative Hill slope approaching 2.0 for free ATP (indicating a negative cooperative effect) because AMP, ADP and ATP are assumed to occupy the same nucleotide locus and it appears improbable that AMP and ATP affinity for the opposite site would be decreased at the same time that ADP affinity would be increased.

The second possibility, however, overlooks an effect of occupancy of the nucleotide subsite on two important variables at the second site: the divalent cation and PEP.

It has been shown that saturating levels of Mg^{++} substantially reduce the non-competitive type of activation by all nucleotide effectors. This result may be due to chelation of free ATP, the true effector, forming MgATP which has a low affinity for the enzyme. Furthermore, high concentrations of Mg^{++} may saturate the divalent metal locus and interfere with the cooperative binding of PEP or MgADP. This would be important in explaining the similar results with AMP

and cAMP as compared to ATP and GTP at high and low Mg^{++}. At low Mg^{++} concentrations a substantial amount of free ATP will be present. Free ATP (GTP or AMP) may bind in a negative cooperative fashion, but the binding of MgADP may be enhanced, since Hill plot data demonstrate a slope of 1.5 for MgADP at non-saturating Mg^{++}. The progress curve data is consistent with either of the two possible effects ascribed to ATP.

The proposed scheme of equilibrium kinetics predicts that PEP must be present simultaneously with metal and nucleotide in order for phosphoryl transfer to occur. The presence of PEP at one catalytic center, therefore, may stabilize the binding of metal, ADP or metal-ADP complex at the same site, the opposite site or both. Mildvan and Cohn (86) showed that ADP is required to obtain competition between pyruvate and PEP suggesting that the nucleotide must be present for proper binding of pyruvate. Evidence that PEP and ADP binding is influenced by the type of metal at the divalent cation subsite has already been cited. Consequently, occupancy of the metal and nucleotide or metal and PEP subsites may be influenced by PEP and nucleotide, respectively.

Assuming a rapid equilibrium mechanism, inhibition by purine nucleotide triphosphates, but lack of inhibition by monophosphates at very low concentrations of PEP is a plausible result since nucleotide triphosphates have an overlapping locus with the phosphoryl group of PEP. Competition for this common locus could displace the cooperative binding of PEP to higher concentrations. It is of interest to

note that Rozengurt et al. (98) demonstrated negative cooperative effects at low pH and high PEP concentrations for ATP using Type L (red cell type) pyruvate kinase from rat liver. They postulated that ATP behaves kinetically as an allosteric inhibitor. Activation by ATP was not observed in these studies but the concentrations of ATP and Mg^{++} were high and could have masked the activating effect.

Three different pyruvate kinases were examined with regard to product activation. The human muscle and rabbit muscle enzymes showed results with purine nucleotides nearly identical to the red cell enzymes at low concentrations of magnesium. This result is of considerable importance because it suggests a homology with regard to catalytic mechanism. The more complete physicochemical and kinetic information described in the literature on the rabbit muscle enzyme, therefore, may be more readily applicable to other mammalian pyruvate kinases. The homology may extend further if it is recalled that Haeckel et al. (107) observed similar kinetic results with yeast pyruvate kinase, i.e. a magnesium-dependent ATP activation.

The modulation of reaction rate by a product of the reaction provides a built-in regulatory mechanism. At low concentrations ATP stimulates its own production, and at high concentrations it slows down ATP production. Such a mechanism involving ATP as a substrate has been proposed previously for phosphofructokinase (149) and led to the designation of this enzyme as the control point in glycolysis. Kaloustian and Kaplan (150) found that lobster muscle lactic dehydrogenase was modulated by DPNH, when DPNH was the product formed in the

forward reaction (lactate to pyruvate). Their results, using entire progress curves, were similar to those presented for human erythrocyte pyruvate kinase, and they proposed a model for this enzyme consisting of four interacting catalytic sites.

II. Kinetic Properties with Magnesium or Manganese

Divalent cations are required by many enzymes. For some regulatory enzymes the response of reaction velocity to cation concentration is sigmoidal (124), and frequently the concentration of cation affects the affinity of the enzyme for one or more of its substrates. Although no consistent pattern is evident, metal cations appear to play a significant role in metabolic regulation as pointed out by Bygrave (40). Studies by Kingdon et al. (151) on glutamine synthetase have demonstrated a change in cation specificity from Mg^{++} to Mn^{++} following adenylylation of the enzyme, and Gentner and Preiss (152) have reported differences in kinetic behavior of _E. coli_ ADP-glucose synthetase depending on whether Mg^{++} or Mn^{++} is used as divalent cation activator.

The role of the essential divalent metal in the reaction catalyzed by pyruvate kinase has not been completely documented. The emphasis has been on kinetic studies with magnesium as the activating ion. These led to the conclusion that the probable primary function of the metal ion was to form an active metal-ADP substrate and that direct interaction of the metal ion with the active site of the enzyme was not likely to contribute greatly to the reaction (17,85). Studies of the interaction of divalent cations and substrates with rabbit muscle pyruvate kinase (61,62,88) and yeast pyruvate kinase (88) using kinetic

and magnetic resonance methods have provided evidence suggesting a quaternary bridge structure of enzyme, metal, and substrates (PEP and ADP). Enzyme-metal binding has been demonstrated directly (without nucleotide present) using electron spin resonance (61), proton relaxation rate (86,88) and protein difference spectroscopy (62,67).

It should be pointed out that the monovalent cation, K^+, is also required for activity by all but two (49,108) pyruvate kinases studied to date. Its possible catalytic role, and relationship to the divalent cation have recently been reviewed (153). Potassium concentration has been held constant at a saturating level in all of the present investigations and it is assumed that the results are independent of a K^+ effect.

In the magnesium-activated system, PEP exhibits a positive cooperative effect ($n \approx 2$), purine nucleotide mono- and triphosphates produce a non-competitive type of activation with respect to PEP, and FDP shifts the PEP velocity profile from a sigmoidal to a hyperbolic curve. The FDP effect is manifested chiefly as a change in Km for PEP compared to the change in Vm noted for nucleotide activation. Activation by low concentrations of FDP increases when Mg^{++} concentration is increased. It was pointed out in Results and Discussion that FDP may bind to the PEP locus.

Binding of FDP at the enolpyruvate locus of one catalytic site may result in a structural change in the protein such that the affinity at the opposite center for PEP is increased (Km reduced), similar to the effect of PEP itself. Both PEP and FDP effects are stabilized by

high concentrations of Mg^{++}. Hence, both the enolpyruvate and metal loci appear to be involved in the mediation of cooperative phenomena by PEP or FDP.

The concentration of divalent metal, therefore, is apparently a critical factor involved in all potential cooperative interactions between the catalytic sites of red cell pyruvate kinase. At high concentrations of Mg^{++}, cooperative effects are mediated predominantly through events at the enolpyruvate locus, whereas at low concentrations of Mg^{++}, cooperative effects involving the nucleotide locus predominate. Cooperative events at the PEP locus mainly affect the Km for PEP and similar events at the nucleotide locus mainly affect the maximum velocity, Vm. This apparent selectivity for altering either Km or Vm may be significant with respect to metabolic control in vivo.

When Mn^{++} occupies the divalent cation subsite the effects on catalysis are different. First, the Hill plot for free Mn^{++} is 1.5 and for Mn^{++} bound to ADP is 1.8, indicating a considerable homotropic effect. Second, PEP does not exhibit a positive homotropic effect as observed in the magnesium-activated system. The Hill coefficient of ≈ 1.0 indicates that binding of PEP at one catalytic center does not affect PEP binding at the opposite active site. Mn^{++} could function in three ways to account for this phenomenon:

1) it may act independently at the divalent cation locus of each active site and alter the over-all topography such that PEP affinity is increased. Then the rate of catalysis will depend on the rate constant for the manganese-mediated

phosphoryl transfer.

2) it may bind to the enzyme in a positive cooperative manner, as mentioned above, and in so doing it may alter or mask the potential ability of PEP to add cooperatively to the enzyme.

3) a combination of (1) and (2) above.

The alteration or masking effect mentioned in (2) above could result from a different three-dimensional array of ligands induced by Mn^{++} as compared to Mg^{++} at the catalytic site. As was seen in figure 3, FDP does not activate the manganese-containing system. Assuming that FDP induces a structural change upon binding to one active site on the magnesium-activated enzyme, and reduces the PEP Hill coefficient to ≈1.0, it is possible that Mn^{++} may produce a similar structural alteration by either an intrasite or intersite mechanism.

Table 4 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 whether Mn^{++} or Mg^{++} serves as divalent cation, the molecular basis for this difference cannot be predicted from the present data. Further work is necessary in order to elucidate the exact catalytic role of both divalent cations.

Table 4

Summary of differences between magnesium-activated and manganese-activated systems.

	Divalent Cation	
	Mg^{++}	Mn^{++}
1. PEP-velocity profile	sigmoidal; n≃2	hyperbolic; n≃1
2. FDP (low concentration)	activation; shifts PEP profile from sigmoidal (n≃2) to hyperbolic (n≃1)	insensitive
(high concentration)	inhibition (n≃2)	inhibition
3. purine nucleotides (low concentration)	activation: non-competitive	inhibition
(high concentration)	inhibition	inhibition
4. divalent cation (low concentration)	nucleotide activation enhanced	-
	FDP activation reduced	-
(high concentration)	nucleotide activation reduced	-
	FDP activation enhanced	

CONCLUSION AND SUMMARY

This thesis has examined the kinetics of human erythrocyte pyruvate kinase. The following conclusions and interpretations have resulted from the data, as reviewed from the literature and presented in Results and Discussion:

Conclusions:

1. The divalent cations, Mg^{++} and Mn^{++}, and their ADP chelates interact with the enzyme in a positive cooperative manner ($n \rightarrow 2$) when metal is rate-limiting. When metal is saturating, no cooperative effect is observed.

2. PEP binds in a positive cooperative manner in the presence of Mg^{++} ($n \approx 2$), but non-cooperatively in the presence of Mn^{++} ($n \approx 1$).

3. FDP and ATP interact with the enzyme in a negative cooperative fashion ($n \rightarrow -2$), with FDP exerting its effect at or near the PEP subsite, and ATP exerting its effect on the nucleotide subsite.

4. The nucleotide subsite is specific for purine nucleoside mono-, di-, and triphosphates. Activation by mono- and triphosphates is evident in the magnesium-activated system but not in the manganese-activated system.

5. Activation by purine nucleoside mono- and triphosphates (nucleotide locus) predominantly affects the Km for PEP.

6. Two moles each (or some multiple) of PEP (or FDP), ADP (or ATP)

and divalent metal can bind to the native enzyme.

Interpretations:

1. Human red cell pyruvate kinase is composed of two catalytic sites which can interact with each other.

2. Each catalytic site has four functionally important subsites, or loci:

   a. a locus for the enolpyruvate portion of PEP.

   b. a locus for the divalent metal.

   c. a locus for the nucleotide.

   d. a locus for the transferrable phosphoryl group.

3. Activation by FDP involves the cation and PEP loci, whereas activation by purine nucleotides involves the cation and nucleotide loci.

4. The rate of catalysis can be regulated by both a precursor molecule (FDP), and the reaction product (ATP). Each regulator affects the enzyme in a unique manner depending on the concentrations of PEP, ADP and the concentration and type of divalent cation.

Bibliography

1. Report of the Commission on Enzymes of the International Union of Biochemistry, 1961. Macmillan Co., New York, 1961. (page 98)

2. Lohmann, K., and Meyerhof, O. Über die enzymatische umwandlung von phosphoglycerinsäure in brenztraubensäure und phosphorsäure. Biochem. Z. (1934) 273, 60-72.

3. Parnas, J. K., Ostern, P., and Mann, T. Über die verkettung der chemischen vorgange in muskel. Biochem. Z. (1934) 272, 64-70.

4. Parnas, J. K., Ostern, P., and Mann, T. Über die verkettung der chemische vorgänge in muskel, II. Biochem. Z. (1935) 275, 74-86.

5. Lahmann, H. Über die enzymatische synthese der kreatinphosphorsäure durch umesterung der phosphobrenztraubensäure. Biochem. Z. (1935) 281, 271-291.

6. Lutwak-Mann, C., and Mann, T. Über die verkettung der chemischen umsetzungen in der alkoholischen gärung. Biochem. Z. (1935) 281, 140-156.

7. Needham, D. M., and Heyningen, W. E. The linkage of chemical changes in muscle extract. Biochem. J. (1935) 29, 2040-2050.

8. Boyer, P. D., Lardy, H. A., and Phillips, P. H. The role of potassium in muscle phosphorylations. J. Biol. Chem. (1942) 146, 673-682.

9. Boyer, P. D., Lardy, H. A., and Phillips, P. H. Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. J. Biol. Chem. (1943) 149, 529-541.

10. Tietz, A., and Ochoa, S. "Fluorokinase" and pyruvate kinase. Arch. Biochim. Biophys. (1958) 78, 477-493.

11. Kupiecki, F. P., and Coon, M. J. Bicarbonate- and hydroxylamine-dependent degradation of adenosine triphosphate. J. Biol. Chem. (1959) 234, 2428-2432.

12. Kupiecki, F. P., and Coon, M. J. "Hydroxylamine kinase" and pyruvate kinase. J. Biol. Chem. (1960) 235, 1944-1947.

13. Kachmar, J. F., and Boyer, P. D. Kinetic analysis of enzyme reactions. J. Biol. Chem. (1953) 200, 669-682.

14. Rose, I. A. Studies on the enolization of pyruvate by pyruvate kinase. J. Biol. Chem. (1960) 235, 1170-1177.

15. Hunsley, J. R., and Suelter, C. H. Yeast pyruvate kinase. II. Kinetic studies. J. Biol. Chem. (1969) 244, 4819-4822.

16. McQuate, J. T., and Utter, M. F. Equilibrium and kinetic studies of the pyruvic kinase reaction. J. Biol. Chem. (1959) 234, 2151-2157.

17. Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. The correlation of reaction kinetics and substrate binding with the mechanism of pyruvate kinase. J. Biol. Chem. (1961) 236, 2277-2283.

18. Lardy, H. A., and Ziegler, J. A. The enzymatic synthesis of phosphopyruvate from pyruvate. J. Biol. Chem. (1945) 159, 343-351.

19. Meyerhof, O., and Oesper, P. The enzymatic equilibria of phospho(enol)pyruvate. J. Biol. Chem. (1949) 179, 1371-1385.

20. Krimsky, I. Phosphorylation of pyruvate by the pyruvate kinase reaction and reversal of glycolysis in a reconstructed system. J. Biol. Chem. (1959) 234, 232-236.

21. Boyer, P. D. Pyruvate kinase, in "The Enzymes" (Boyer, P. D., Lardy, H. A., and Myrbäck, K., Ed's) New York: Academic Press, 1962. 6, 95-113.

22. Harrison, W. H., Boyer, P. D., and Falcone, A. B. The mechanism of enzyme phosphate transfer reactions. J. Biol. Chem. (1955) 215, 303-317.

23. Hass, L. F., Boyer, P. D., and Reynard, A. M. Studies on possible phosphoryl enzyme formation in catalysis by hexokinase, pyruvate kinase, and glucose-6-phosphatase. J. Biol. Chem. (1961) 236, 2284-2291.

24. Boyer, P. D. The activation by K^+ and occurrence of pyruvic phosphoferase in different species. J. Cellular Comp. Physiol. (1953) 42, 71-77.

25. Bücher, T., and Pfleiderer, G. Pyruvate kinase from muscle, in Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., Ed's) New York: Academic Press, 1955. 1, 435-440.

26. Kubowitz, F., and Ott, P. Isolierung von gärungsfermenten aus menschlichen muskeln. Biochem. Z. (1944) 317, 193-203.

27. Tanaka, T., Harano, Y., Sue, F., and Morimura, H. Crystallization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. (Japan) (1967) 62, 71-91.

28. Solvonuk, P. F., and Collier, H. B. The pyruvic phosphoferase of erythrocytes. I. Properties of the enzyme and its activity in erythrocytes of various species. Can. J. Biochem. Physiol. (1955) 33, 38-45.

29. Aebi, H., Schneider, C. H., Gang, H., and Weismann, U. Separation of catalase and other red cell enzymes from hemoglobin by gel filtration. Experimentia (1964) 20, 103-104.

30. Koler, R. D., Bigley, R. H., Jones, R. T., Rigas, D. A., Vanbellinghen, P., and Thompson, P. Pyruvate kinase: molecular differences between human red cell and leucocyte enzyme. Cold Spring Harbor Symp. Quant. Biol. (1964) 29, 213-221.

31. Powell, R. D., and DeGowin, R. L. Relationship between activity of pyruvate kinase and age of the normal human erythrocyte. Nature (1965) 205, 507.

32. Whigham, W. R. Purification of pyruvate kinase from human red cells. Unpublished doctor's dissertation, Baylor University, 1965.

33. Fellenberg, R. von, Richterich, R., and Aebi, H. Electrophoetische verschieden wandernde pyruvat-kinasen aus einigen organen der ratte. Enzymol. Biol. Clin. (1963) 3, 240-250.

34. Tanaka, T., Harano, Y., Morimura, H., and Mori, H. Evidence for the presence of two types of pyruvate kinase in rat liver. Biochem. Biophys. Res. Commun. (1965) 21, 55-60.

35. Passeron, S., Asua, L. J., de, and Carminatti, H. Fructose 1,6-diphosphate, a reactivator of Cu^{++}- inhibited pyruvate kinase from liver. Biochem. Biophys. Res. Commun. (1967) 27, 33-38.

36. Leveille, G. A. The influence of fructose diphosphate on pyruvate kinase activity in liver, muscle, and adipose tissue of the rat, mouse, pig, and chicken. Comp. Biochem. Physiol. (1969) 28, 733-740.

37. Bigley, R. H., Stenzel, P., Jones, R. T., Campos, J. O., and Koler, R. D. Tissue distribution of human pyruvate kinase isozymes. Enzymol. Biol. Clin. (1968) 9, 10-20.

38. Somero, G. N., and Hochachka, P. W. The effect of temperature on catalytic and regulatory functions of pyruvate kinases of the rainbow trout and the antarctic fish Trematomus bernacchii. Biochem. J. (1968) 110, 395-400.

39. Pogson, C. I. Two interconvertible forms of pyruvate kinase in adipose tissue. Biochem. Biophys. Res. Commun. (1969) 30, 297-302.

40. Bygrave, F. L. The ionic environment and metabolic control. Nature (1967) 214, 667-671.

41. Yurovitskii, Y. G., and Mil'man, L. S. Regulation of pyruvate oxidase activity by fructose 1,6-diphosphate in developing embryos. Doklady Akademii Nauk SSSR (1967) 175, 1404-1406.

42. Reeves, R. E. A new enzyme with the glycolytic function of pyruvate kinase. J. Biol. Chem. (1968) 243, 3202-3204.

43. Bueding, E., and Saz, H. J. Pyruvate kinase and phosphoenol-pyruvate carboxykinase activities of ascaris muscle, Hymenolepsis diminuta and Schisotosoma mansoni. Comp. Biochem. Physiol. (1968) 24, 511-518.

44. Bailey, E., and Walker, P. R. A comparison of the properties of the pyruvate kinases of the fat body and flight muscle of the adult male desert locust. Biochem. J. (1969) 111, 359-364.

45. Somero, G. N. Pyruvate kinase variants of the Alaskan king-crab. Biochem. J. (1969) 114, 237-241.

46. Damodaran, M., Jagannathan, V., and Singh, K. Carbohydrate metabolism in citric acid fermentation. Enzymologia (1955) 17, 199-206.

47. Washio, S., Mano, Y., and Shimazono, N. Purification and assay method of pyruvate kinase from baker's yeast. J. Biochem. (Japan) (1959) 46, 1661-1663.

48. Hess, B., Haeckel, R., and Brand, K. FDP-activation of yeast pyruvate kinase. Biochem. Biophys. Res. Commun. (1966) 24, 824-831.

49. Maeba, P., and Sanwal, B. D. The regulation of pyruvate kinase of Escherichia coli by fructose diphosphate and adenylic acid. J. Biol. Chem. (1968) 243, 448-450.

50. Hunsley, J. R., and Suelter, C. H. Yeast pyruvate kinase. I. Purification and some chemical properties. J. Biol. Chem. (1969) 244, 4815-4818.

51. Miller, G., and Evans, H. J. The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiol. (1957) 32, 346-354.

52. Krebs, H. A., and Eggleston, L. V. The role of pyruvate kinase in the regulation of gluconeogenesis. Biochem. J. (1965) 94, 3c-4c.

53. Weber, G., Singhal, R. L., Stamm, N. B., and Srivastava, S. K. Hormonal induction and suppression of liver enzyme biosynthesis. Fed. Proc. (1965) 24, 745-754.

54. Koler, R. D., Bigley, R. H., and Stenzel, P. Biochemical properties of human erythrocyte and leucocyte pyruvate kinase, in Hereditary Disorders of Erythrocyte Metabolism (Beutler, E., Ed.) New York: Grune and Stratton, Inc., 1968. (pages 249-264)

55. Bigley, R. H., and Koler, R. D. Liver pyruvate kinase (PK) isozymes in a PK-deficient patient. Ann. Hum. Genet., Lond. (1968) 31, 383-388.

56. Warner, R. C. Physical properties of crystalline "fluorokinase". Arch. Biochem. Biophys. (1958) 78, 494-496.

57. Steinmetz, M. A. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Fed. Proc. (1966) 25, 223. (abstract)

58. Cottam, G. L., Kupiecki, F. P., and Coon, M. J. A study of the mechanism of O-phosphorylhydroxylamine synthesis catalyzed by pyruvate kinase. J. Biol. Chem. (1968) 243, 1630-1637.

59. Steinmetz, M. A., and Deal, W. C., Jr. Metabolic control and structure of glycolytic enzymes. III. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Biochemistry (1966) 5, 1399-1405.

60. Morawiecki, A. Dissociation of pyruvate kinase in urea solution. Biochim. Biophys. Acta (1960) 44, 604-605.

61. Mildvan, A. S., and Cohn, M. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. I. Divalent metal complexes of pyruvate kinase. J. Biol. Chem. (1965) 240, 238-246.

62. Suelter, C. H., Singleton, R., Jr., Kayne, F. J., Arrington, S., Glass, J., and Mildvan, A. S. Studies on the interaction of substrate and monovalent and divalent cations with pyruvate kinase. Biochemistry (1966) 5, 131-138.

63. Betts, J. R., and Evans, H. J. The effect of cations on the electrophoretic mobility and substrate binding properties of pyruvate kinase. Biochim. Biophys. Acta (1968) 167, 190-193.

64. Cottam, G. L., Hollenberg, P. F., and Coon, M. J. Subunit structure of rabbit muscle pyruvate kinase. J. Biol. Chem. (1969) 244, 1481-1486.

65. Johnson, G. S., and Deal, W. C., Jr. Inactivation of tetrameric rabbit muscle pyruvate kinase by specific binding of 2 to 4 moles of pyridoxal phosphate. J. Biol. Chem. (1970) 245, 238-245.

66. Suelter, C. H., and Melander, W. Use of protein difference spectrophotometry to determine enzyme-cofactor dissociation constants. J. Biol. Chem. (1963) 238, PC4108-4109.

67. Kayne, F. J., and Suelter, C. H. Effects of temperature, substrate, and activating cations on the conformations of pyruvate kinase in aqueous solutions. J. Amer. Chem. Soc. (1965) 87, 897-900.

68. Sorger, G. J., Ford, R. E., and Evans, J. H. Effects of univalent cations on the immunoelectrophoretic behavior of pyruvate kinase. Proc. Natl. Acad. Sci. (1965) 54, 1614-1621.

69. Mildvan, A. S., and Cohn, M. Divalent metal complexes of pyruvate kinase. Sixth International Congress of Biochemistry (1964) IV-111. (abstract)

70. Cottam, G. L., and Ward, R. L. ^{35}Cl-NMR Studies on zinc-pyruvate kinase complexes. Arch. Biochem. Biophys. (1969) 132, 308-314.

71. Suelter, C. H. Effects of temperature and activating cations on the fluorescence of pyruvate kinase. Biochemistry (1967) 6, 418-423.

72. Kayne, F. J., and Suelter, C. H. The temperature-dependent conformational transitions of pyruvate kinase. Biochemistry (1968) 7, 1678-1684.

73. Kuczenski, R. T., and Suelter, C. H. Effect of temperature and effectors on the conformations of yeast pyruvate kinase. Biochemistry (1970) 9, 939-945.

74. Campos, J. O., Koler, R. D., and Bigley, R. H. Kinetic differences between human red cell and leucocyte pyruvate kinase. Nature (1965) 208, 194-195.

75. Campos, J. O. Anemia hemolítica não-esferocítica por deficiência de quinase pirúvica. Unpublished doctor's dissertation, Belo Horizonte, Brazil, 1966.

76. Koler, R. D., and Vanbellinghen, P. J. The mechanism of precursor modulation of human pyruvate kinase I by fructose diphosphate. Advances in Enzyme Regulation (1968) 6, 127-142.

77. Townes, P. L. Electrophoresis of human pyruvate kinase. Lancet #7548, April, 1968 (page 529).

78. Townes, P. L., and Harris, H. personal communication to Dr. Bigley. 1968.

79. Hanel, H. K. Pyruvate kinase in human erythrocytes. Scand. J. Haemat. (1969) 6, 173-174.

80. Davidson, E. A. Specificity of pyruvate kinase. Biochim. Biophys. Acta (1959) 33, 238-240.

81. Plowman, K. M., and Krall, A. R. A kinetic study of nucleotide interactions with pyruvate kinase. Biochemistry (1965) 4, 2809-2814.

82. Klenow, H., and Andersen, B. Some enzyme reactions with adenine deoxyribose polyphosphates. Biochim. Biophys. Acta (1957) 23, 92-97.

83. Cottam, G. L. Catalytic mechanism and subunit structure of pyruvate kinase. Unpublished doctor's dissertation, Univ. of Michigan, 1967.

84. Melchior, J. B. Role of the metal ions in the pyruvate kinase system. Fed. Proc. (1965) 24, 285. (abstract)

85. Melchior, J. B. The role of metal ions in the pyruvate kinase reaction. Biochemistry (1965) 4, 1518-1525.

86. Mildvan, A. S., and Cohn, M. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. II. Complexes of enzyme, metal, and substrates. J. Biol. Chem. (1966) 241, 1178-1193.

87. Cleland, W. W. Enzyme kinetics. Ann. Rev. Biochem. (1967) 77-112.

88. Mildvan, A. S., Hunsley, J. R., and Suelter, C. H. Structure and the role of the metal complexes of an allosteric pyruvate kinase from yeast and a non-allosteric pyruvate kinase from muscle, in the Johnson Research Symposium of Probes for Macromolecular Structure and Function (Chance, B., Yonetani, T., Cohn, M., and Lee, C. P., Ed's.) Academic Press, 1969, In press.

89. Kerson, L. A., Garfinkel, D., and Mildvan, A. S. Computer simulation studies of mammalian pyruvate kinase. J. Biol. Chem. (1967) 242, 2124-2133.

90. Wood, T. The inhibition of pyruvate kinase by ATP. Biochem. Biophys. Res. Commun. (1968) 31, 779-785.

91. Boyer, P. D. The inhibition of pyruvate kinase by ATP: a Mg^{++} buffer system for use in enzyme studies. Biochem. Biophys. Res. Commun. (1969) 34, 702-706.

92. Holmsen, H., and Storm, E. The adenosine triphosphate inhibition of the pyruvate kinase reaction and its dependence on the total magnesium ion concentration. Biochem. J. (1969) 112, 303-316.

93. Hunsley, J. R., and Suelter, C. H. Allosteric properties and preparation of homogeneous yeast pyruvate kinase. Fed. Proc. (1967) 26, 559. (abstract)

94. Gancedo, J. M., Gancedo, G. C., and Sols, A. Regulation of the concentration or activity of pyruvate kinase in yeasts and its relationship to gluconeogenesis. Biochem. J. (1967) 102, 23c-24c.

95. Susor, W. A., and Rutter, W. J. Some distinctive properties of pyruvate kinase purified from rat liver. Biochem. Biophys. Res. Commun. (1968) 30, 14-20.

96. Bailey, E., Stirpe, F., and Taylor, C. B. Regulation of rat liver pyruvate kinase. The effect of preincubation, pH, copper ions, fructose 1,6-diphosphate and dietary changes on enzyme activity. Biochem. J. (1968) 108, 427-436.

97. Susor, W. A., and Rutter, W. J. Multiple forms of mammalian pyruvate kinase. Pacific Slope Biochemical Conference. Seattle, 1969. (abstract)

98. Rozengurt, E., Asua, L. J., de, and Carminatti, H. Some kinetic properties of liver pyruvate kinase. J. Biol. Chem. (1969) 244, 3142-3147.

99. Bigley, R. H., Koler, R. D., and Leonard, H. A. unpublished observations.

100. Monod, J., Changeux, J. P., and Jacob, F. Allosteric proteins and cellular control systems. J. Mol. Biol. (1963) 6, 306-329.

101. Monod, J., Wyman, J., and Changeux, J. P. On the nature of allosteric transitions: a plausible model. J. Mol. Biol. (1965) 12, 88-118.

102. Atkinson, D. E. Regulation of enzyme activity. Ann. Rev. Biochem. (1966) 35, 85-124.

103. Stadtman, E. R. Allosteric regulation of enzyme activity. Advan. Enzymol. (1966) 28, 41-148.

104. Koshland, D. E., and Neet, K. E. The catalytic and regulatory properties of enzymes. Ann. Rev. Biochem. (1968) 37, 359-410.

105. Llorente, P., Marco, R., and Sols, A. Regulation of liver pyruvate kinase and the phosphoenolpyruvate crossroads. Eur. J. Biochem. (1970) 13, 45-54.

106. Bigley, R. H. Manuscript in preparation.

107. Haeckel, R., Hess, B., Lauterborn, W., and Wuster, K. Purification and allosteric properties of yeast pyruvate kinase. Hoppe-Seyler's Z. Physiol. Chem. (1968) 349, 699-714.

108. Ozaki, H., and Shiio, I. Regulation of the TCA cycle and glyoxylate cycle in Brevibacterium flavum. J. Biochem. (Japan) (1969) 66, 297-311.

109. Ibsen, K. H., Schiller, K. W., and Venn-Watson, E. A. Stabilization, partial purification, and effects of activating cations, ADP, and phosphoenolpyruvate on the reaction rates of an erythrocyte pyruvate kinase. Arch. Biochem. Biophys. (1968) 128, 583-590.

110. Ibsen, K. H., and Schiller, K. Multiple kinetic forms of human erythrocyte pyruvate kinase. Amer. Chem. Soc. Meetings (Biol. Chem.) New York, 1969. (abstract)

111. Weber, G., Stamm, N. B., and Fisher, E. A. Insulin: inducer of pyruvate kinase. Science (1965) 149, 65-67.

112. Weber, G., Singhal, R. L., Stamm, N. B., Lea, M. A., and Fisher, E. A. Synchronous behavior pattern of key glycolytic enzymes: glucokinase, phosphofructokinase, and pyruvate kinase. Advances in Enzyme Regulation (1966) 4, 59-81.

113. Weber, G., Lea, M. A., Fisher, E. A., and Stamm, N. B. Regulatory carbohydrate metabolizing enzymes: insulin as inducer of key glycolytic enzymes. Enzymol. Biol. Clin. (1966) 7, 11-24.

114. Weber, G., Convery, J. H., and Stamm, N. B. Regulation of gluconeogenesis and glycolysis: studies of mechanisms controlling enzyme activity. Advances in Enzyme Regulation (1967) 5, 257-299.

115. Minikami, S., Suzuki, C., Saito, T., and Yoshikawa, H. Studies on erythrocyte glycolysis. J. Biochem. (Japan) (1965) 58, 543-550.

116. Minikami, S., and Yoshikawa, H. Studies on Erythrocyte glycolysis. J. Biochem. (Japan) (1966) 59, 139-144.

117. Chance, B., Hess, B., and Betz, A. DPNH oscillations in a cell-free extract of S. carlsbergensis. Biochem. Biophys. Res. Commun. (1964) 16, 182-187.

118. Frenkel, R. DPNH oscillations in glycolyzing cell-free extracts from beef heart. Biochem. Biophys. Res. Commun. (1965) 21, 497-502.

119. Higgins, J. A chemical mechanism for oscillations of glycolytic intermediates in yeast cells. Proc. Natl. Acad. Sci. (1964) 51, 989-994.

120. Rutter, W. J., Hunsley, J. R., Groves, W. E., Calder, J., Rajkumar, T. V., and Woodfin, B. M. Fructose diphosphate aldolase. Methods in Enzymology (1966) IX, 479-493.

121. Reiner, M., and Cheung, H. L. Fibrinogen. Standard Methods of Clin. Chem. (1961) 3, 114-123.

122. Bigley, R. H., Koler, R. D., and Pierovich, I. Manuscript in preparation.

123. Pon, N. G., and Bondar, R. J. L. A direct spectrophotometric assay for pyruvate kinase. Anal. Biochem. (1967) 19, 272-279.

124. Atkinson, D. E., Hathaway, J. A., and Smith, E. C. Kinetics of regulatory enzymes; kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. J. Biol. Chem. (1965) 240, 2682-2690.

125. Bernhard, S. The Structure and Function of Enzymes. New York: W. A. Benjamin, 1968. (pages 284-287)

126. Phillips, R. Adenosine and the adenine nucleotides. Ionization, metal complex formation, and conformation in solution. Chem. Rev. (1966) 66, 501-527.

127. Burton, K. Formation of complexes between metabolites and certain bivalent metals, in Biochemist's Handbook (Long, C., Ed.). London: E. and F. N. Spon, Ltd., 1961. (page 97)

128. Ling, G. N. A new model for the living cell: a summary of the theory and recent experimental evidence in its support. International Review of Cytology (1969) 26, 1-61.

129. Taketa, K., and Pogell, B. M. Allosteric inhibition of rat liver fructose 1,6-diphosphatase by adenosine 5'-monophosphate. J. Biol. Chem. (1965) 240, 651-662.

130. Cleland, W. W. The kinetics of enzyme catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim. Biophys. Acta (1963) 67, 188-196.

131. Heyde, E., and Morrison, J. F. The interaction of Mg^{++} and ATP^{4-} with ATP: creatine phosphotransferase. Biochim. Biophys. Acta (1969) 178, 47-60.

132. Wold, F., and Ballou, C. E. Studies on the enzyme enolase. J. Biol. Chem. (1957) 227, 313-328.

133. Kayne, F. J., and Reuben, J. Thallium-205 nuclear magnetic resonance as a probe for studying metal ion binding to biological macromolecules. Estimate of the distance between the monovalent and divalent activators of pyruvate kinase. J. Amer. Chem. Soc. (1970) 92, 220-222.

134. Dwyer, F. P., and Mellor, D. P., Chelating Agents and Metal Chelates. New York: Academic Press, 1964. (pages 335-382)

135. Koler, R. D. Personal communication, 1969.

136. West, E. S., Todd, W. R., Mason, H. S., and Van Bruggen, J. T. Textbook of Biochemistry (4th Ed.). New York: Macmillan Co., 1966. (page 710)

137. Kirtley, M. E., and Dix, J. C. The hydrolysis of phosphoenolpyruvate catalyzed by rabbit liver fructose 1,6-diphosphatase. Biochem. Biophys. Res. Commun. (1969) 37, 634-639.

138. Kelly, G. J., and Turner, J. F. The regulation of pea-seed phosphofructokinase by phosphoenolpyruvate. Biochem. J. (1969) 115, 481-487.

139. Blangy, D., Buc, H., and Monod, J. Kinetics of the allosteric interactions of phosphofructokinase from Escherichia coli. J. Mol. Biol. (1968) 31, 13-35.

140. Rabin, B. R. Cooperative effects in enzyme catalysis: a possible kinetic model based on substrate-induced conformation isomerization. Biochem. J. (1967) 102, 22c-23c.

141. Kingdon, H. S., Hubbard, J. S., and Stadtman, E. R. Regulation of glutamine synthetase. XI. The nature and implication of a lag phase in Escherichia coli glutamine synthetase reaction. Biochemistry (1968) 7, 2136-2142.

142. Shapiro, B. M., and Ginsburg, A. Effects of specific divalent cations on some physical and chemical properties of glutamine synthetase from Escherichia coli. Taut and relaxed enzyme forms. Biochemistry (1968) 7, 2153-2167.

143. Johnson, G. S., Kayne, M. S., and Deal, W. C., Jr. Metabolic control and structure of glycolytic enzymes. VIII. Reversal of the dissociation of rabbit muscle pyruvate kinase into unfolded subunits. Biochemistry (1969) 8, 2455-2462.

144. Westley, J. Enzymic Catalysis. New York: Harper and Row, 1969. (page 99)

145. Cleland, W. W. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochem. Biophys. Acta (1963) 67, 104-137.

146. Bridger, W. A., Millen, W. A., and Boyer, P. D. Substrate synergism and phosphoenzyme formation in catalysis by succinyl coenzyme A synthetase. Biochemistry (1968) 7, 3608-3616.

147. Sweeney, J. R., and Fisher, J. R. An alternative to allosterism and cooperativity in the interpretation of enzyme kinetic data. Biochemistry (1968) 7, 561-565.

148. Woods, A. E., O'Bryan, J. M., Mui, P. T. K., Crowder, R. D. Synthetic homologs of phosphoenolpyruvate and specificity of pyruvate kinase. Biochemistry (1970) 9, 2334-2338.

149. Lardy, H. A., and Parks, R. E., Jr. Phosphofructokinase, in Enzymes: Biological Structure and Function (Gaebler, O. H., Ed.) New York: Academic Press, 1956. (page 584)

150. Kaloustian, H. D., and Kaplan, N. O. Lactic dehydrogenase of lobster tail muscle. II. Kinetic and regulatory properties. J. Biol. Chem. (1969) 244, 2902-2910.

151. Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R. Regulation of glutamine synthetase. VIII. ATP: glutamine synthetase adenylyltransferase, an enzyme that catalyzes alterations in the regulatory properties of glutamine synthetase. Proc. Natl. Acad. Sci. (1967) 58, 1703-1710.

152. Gentner, N., and Preiss, J. Biosynthesis of bacterial glycogen. VI. Differences in the kinetic properties of the Escherichia coli B adenosine diphosphate glucose pyrophosphorylase depending on whether Mg^{++} or Mn^{++} serves as divalent cation. J. Biol. Chem. (1968) 243, 5882-5891.

153. Suelter, C. H. Enzymes activated by monovalent cations. Science (1970) 168, 789-795.

154. Mildvan, A. S., Leigh, J. S., and Cohn, M. Kinetic and magnetic resonance studies of pyruvate kinase. III. The enzyme-metal-phosphoryl bridge complex in the fluorokinase reaction. Biochemistry (1967) 6, 1805-1818.