KINETIC, BINDING, AND CONFORMATIONAL STUDIES OF YEAST PYRUVATE KINASE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
JAMES RAY HUNSLEY

1970

This is to certify that the

thesis entitled

KINETIC, BINDING, AND CONFORMATIONAL STUDIES

OF YEAST PYRUVATE KINASE

presented by

James Ray Hunsley

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Clarence Schutt
Major professor

Date February 16, 1970

ABSTRACT

KINETIC, BINDING, AND CONFORMATIONAL STUDIES

OF YEAST PYRUVATE KINASE

By

James Ray Hunsley

A homogeneous preparation of bakers' yeast pyruvate kinase (EC 2.7.1.40) has been shown to exhibit cooperative steady-state kinetics for the stringently required monovalent cations K^+ and NH_4^+, Mg^{2+}, and phosphoenolpyruvate, but not for ADP, at saturating concentrations of all other substrates and metal ions, pH 6.2. Fructose-1,6-diphosphate was shown to heterotropically activate the enzyme, transforming the sigmoid saturation curves for monovalent cation, Mg^{2+}, and phosphoenolpyruvate to hyperbolic without affecting the V_m. In the presence of fructose diphosphate, Na^+ and Ca^{2+} were able to replace K^+ or NH_4^+ and Mg^{2+} or Mn^{2+} but at less efficiency. Inclusion of fructose diphosphate as well as increasing the phosphoenolpyruvate concentration increased activity of the enzyme in the basic pH range.

Strong kinetic interactions were noted for

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phosphoenolpyruvate or ADP and Mn^{2+} at pH 7.5. Fructose diphosphate abolished all ADP-Mn interactions, but high concentrations of phosphoenolpyruvate were additionally required to uncouple phosphoenolpyruvate-Mn interactions.

The Mn-enzyme and Mn-enzyme-substrate binary and ternary complexes were studied by the NMR proton relaxation rate and electron paramagnetic resonance techniques. An atypical binding curve for Mn^{2+} in the presence or absence of fructose diphosphate was suggestive of site-site interaction. Comparison of the effects of fructose diphosphate on the binding parameters of the phosphoenolpyruvate ternary complexes was consistent with the requirement of one filled Mn^{2+} binding site before the effector could lower the affinity for the substrate. As implied from comparison of binding and kinetic data, the enzyme-metal complex may bind phosphoenolpyruvate prior to the binding of ADP.

Preparative isoelectric focusing and microisoelectric focusing in polyacrylamide gel columns have resolved two conformers of the enzyme. Fructose diphosphate was shown to specifically convert the conformer of low isoelectric point to the high pH form which, upon removal of the effector, reverted to a mixture of both conformers. No kinetic differences were demonstrable for the two conformers and the

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tetrameric structure of the native enzyme was probably retained.

Metal analyses have revealed the presence of 0.14 moles of tightly bound Cu^{2+} of unknown function per 166,000 g of protein. Amino acid analysis has shown that the yeast enzyme has a relatively low aromatic amino acid content like the rabbit muscle enzyme but deviates significantly in amino acid content.

KINETIC, BINDING, AND CONFORMATIONAL STUDIES OF YEAST PYRUVATE KINASE

By

James Ray Hunsley

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1970

ACKNOWLEDGEMENT

I thank Dr. Clarence H. Suelter who provided the freedom and support required to make this thesis a reality.

Help from Miss Betty Baltzer, Miss Doris Bauer, Mrs. Kathy Ashworth, Mr. Bob Brown, Mr. Ron Kuczenski, Dr. John LaRue, Dr. John Wilson, Dr. Albert Mildvan, and Dr. Thomas Vogel at various stages of the work is gratefully appreciated.

TABLE OF CONTENTS

LIST OF FIGURES

Table

1. Proper

2. Multip
presum

3. Amino
kinase

4. Metal
pyruva

5. Requir
for al

6. Binary
kinase

LIST OF TABLES

ADP

AMP

ATP

CHA

ϵ

ϵ_b

EDTA

EPR

FDP

K_D

K_3

n_H

NADH

NMR

PEP

PMSF

Tris

LIST OF ABBREVIATIONS AND SYMBOLS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
CHA	cyclohexylammonium (cation)
ϵ	enhancement value
ϵ_b	enhancement value of binary Mn-enzyme complex
EDTA	ethylenediamine tetraacetic acid
EPR	electron paramagnetic resonance
FDP	fructose-1,6-diphosphate
K_D	dissociation constant of metal-enzyme complex
K_3	dissociation constant of ligand for metal-enzyme complex
n_H	Hill slope
NADH	nicotine adenine dinucleotide, reduced
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvic acid
PMSF	phenylmethanesulfonyl fluoride
Tris	tris(hydroxymethyl)amino methane

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INTRODUCTION

The glycolytic enzyme pyruvate kinase (ATP pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the following reaction: $H^+ + PEP + ADP \rightleftharpoons$ pyruvate + ATP. Preparations from rabbit muscle have, by far, been the most extensively studied though, in recent years, work has been widely extended to other organisms. This interest has been stimulated by reports that have established the enzyme as a glycolytic control point in several tissues and that have uncovered a spectrum of mechanisms by which pyruvate kinase activity is regulated.

An earlier investigation of the yeast enzyme (Washio and Mano, 1960) was hampered by instability. Later, Hommes (1964), Pye and Eddy (1965), and Hess and Brand (1965b) provided proof that the reaction was rate limiting in whole yeast or extracts. At the same time, the latter workers reported fructose-1,6-diphosphate activation of the enzyme. The function of this effector as a heterotropic activator toward the required monovalent and divalent cations and PEP was shown with purified enzyme by Hess et al. (1966), Hunsley and Suelter (1967), Hess and Haeckel (1967), Haeckel

et al. (1968), and Hunsley and Suelter (1969b).

Since convenient, stable preparations of high purity yeast pyruvate kinase in gram amounts are now available (Haeckel et al., 1968; Hunsley and Suelter, 1969a), it was chosen for study in an attempt to arrive at the origins of its cooperative effects and to compare the structural and functional relationships of it and the rabbit muscle enzyme which displays no allosteric behavior.

Preliminary reports of this work have been presented (Hunsley and Suelter, 1967, 1969a, 1969b; Mildvan et al., 1970).

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LITERATURE REVIEW

Aspects of control of pyruvate kinase

The enzyme pyruvate kinase lies at a critical point, aptly designated the "pyruvate crossroads" (Sols, 1968), in intermediary metabolism because of the role it plays in the conversion of phosphoenolpyruvate to pyruvate. In addition, since ATP, the chief primer of biosynthetic reactions, is formed in the reaction and the reaction is displaced far toward pyruvate (McQuate and Utter, 1959), the enzymatic activity might be expected to be carefully regulated. A perplexing number of possible controls have, in fact, now been documented for the enzyme from a variety of organisms and tissues. In both intact yeast cells and lysates (Hommes, 1964; Pye and Eddy, 1965; Hess and Brand, 1965b), in intact perfused rat heart under certain conditions (Williamson, 1965), and in guinea pig cerebral cortex slices (Takagaki, 1968), the pyruvate kinase reaction can be shown to be a glycolytic control point. Of equal importance are the reports of the probable modulation of the enzyme activity during gluconeogenesis in yeasts and liver (Fernández et al., 1967; Sillero et al., 1969; Weber et al., 1967a).

This review will attempt to classify some of the literature on this subject which has appeared since the last exhaustive review of pyruvate kinase (Boyer, 1962).

I. Reaction mechanism and kinetic control

1. Random versus ordered binding

Mutually independent Michaelis constants from steady-state kinetic studies of substrates for an enzyme may be taken as presumptive evidence that binding events are random. Kinetic data for rabbit muscle (McQuate and Utter, 1959; Reynard et al., 1961), types M and L rat liver (Tanaka et al., 1967), and both forms of the crude rat epididymal fat pad (Pogson, 1968) pyruvate kinases are consistent with a random binding mechanism for ADP and PEP. Results from the human erythrocyte enzyme are in conflict (Campos et al., 1965; Ibsen et al., 1968) perhaps due to isozymic differences resulting from purification. A slight dependence of Hill slope and apparent K_m of ADP on PEP concentration was found by Haeckel et al. (1968) with a preperation from brewers' yeast (Saccharomyces carlsbergensis). Mildvan and Cohn (1966) have shown excellent agreement of binding and kinetic constants of the substrates for the rabbit muscle enzyme substantiating a preferred pathway for ADP and PEP binding. Inconsistencies in kinetic and binding data for bakers' yeast pyruvate kinase (Mildvan et al.,

1970) may mean that this enzyme has a preferred order of binding PEP.

2. Metal complexes and the nature of the true substrates

ADP and PEP readily form divalent and monovalent cation complexes (Melchior, 1954; Smith and Alberty, 1956; Wold and Ballou, 1957) within the physiological range of concentration of the more important cations, Mg^{2+}, K^{+}, and Na^{+}. Furthermore, the secondary acid dissociation constants of the phosphate moities of ADP and PEP have pK_a values of 6.7 and 6.4, respectively, and, at least for ADP, these values are dependent on the nature of the metal complex (Wold and Ballou, 1957; Smith and Alberty, 1956). Because of the limitations of equilibrium kinetics and the complexity of determining the various equilibria involving the substrates, studies of the possible catalytically active species of substrates have been limited. For the rabbit muscle preparation, Melchior (1965) concluded that $MgADP^{-}$ was the principal interacting ADP species with the enzyme and that $KADP^{2-}$ could be varied over a wide range with no effect on rate. In binding and kinetic studies with the same enzyme, Mildvan and Cohn (1966) saw no conflict with the hypothesis of random binding of Mn^{2+}, ADP, and $MnADP^{-}$. A computer simulation of the enzyme (Kerson et al., 1967) was consistent with the

Mildvan and Cohn model, and indicated, because of the limitations of free Mg^{2+} in cells, that activity was controlled by Mg^{2+} availability. In a reply to a criticism (Cleland, 1967) of this model, Mildvan et al. (1970) in a rapid quenching kinetic approach to the problem, showed conclusively that the reaction, NiADP + enzyme, was not the only productive pathway to a quaternary complex.

3. Activators

Monovalent cation-A pyruvate kinase from Acetobacter xylinum (Benziman, 1969) has now been described which shows no requirement for the monovalent cations K^+ or NH_4^+. Data for two other bacterial sources are suggestive (Maeba and Sanwal, 1968; Ozaki and Shiio, 1969) but inconclusive because of NH_4^+ and Na^+ contamination. It would be of interest to know if both the constituitive and inducible pyruvate kinases of Escherichia coli require K^+ for activity (Malcovati and Kornberg, 1969).

The activation mechanism of monovalent cations is under controversy. The predominant thinking is that monovalent cations are necessary to maintain an enzyme conformation necessary for catalysis (Evans and Sorger, 1966). More recently, Suelter (1970) has theorized a specific mechanistic role for these cations: interaction with the enol-keto tautomers of pyruvate at the active site. Results exist which

support both hypotheses; a variety of phenomena such as enzyme stability, sedimentation, electrophoretic behavior, fluorescence, and binding studies (Wilson et al., 1967; Sorger et al., 1965; Mildvan and Cohn, 1964; Kayne and Suelter, 1965; Suelter et al., 1966; Suelter, 1967), have been interpreted via the conformational model. Recently, however, Kayne and Reuben (1970) have shown by direct nuclear magnetic resonance that the activating univalent cation $^{205}Th^{+1}$ binds rabbit muscle pyruvate kinase in very close proximity to the diamagnetic Mn^{2+} site, and therefore, the active site itself. Kinetically, strong interaction occurs between the allosteric activator FDP and K^+ for yeast pyruvate kinase (Hess and Haeckel, 1967; Hunsley and Suelter, 1969b). NMR proton relaxation rate binding studies have shown that for the bakers' yeast enzyme, K^+ raises the affinity of the enzyme-Mn complex for ADP (Mildvan et al., 1970).

pH-Mechanistically, little can be said about the effects of pH on pyruvate kinase activity. It should be noted, however, that a proton is taken up in the reaction in the direction of pyruvate formation. Rose (1960) concluded that the ATP-activated enolization of pyruvate catalyzed by the rabbit muscle enzyme, which rose steeply above pH 8, was dependent on the ionization state of the protein since no

new ionizing groups were available in the nucleotide above pH 7. With two cooperative enzymes from yeast and rat liver (Haeckel et al., 1968; Rozengurt et al., 1969), variations in the K_m for PEP were pH dependent (although in an opposite manner) and pK_a values of 7.0 and 6.9, respectively, could be extrapolated from the data for the ionizable groups responsible. These observations fail to explain, however, the double peaked pH activity profiles seen for several cooperative pyruvate kinases from brewers' and bakers' yeast, desert locust fat body, and rat liver (Haeckel et al., 1968; Hunsley and Suelter, 1969b; Bailey and Walker, 1969; Bailey et al., 1968).

Metabolically derived activators-Fructose-1,6-diphosphate was first identified by Hess and Brand (1965a) as an allosteric activator of yeast pyruvate kinase. The enzyme from many sources, including rat liver, loach embryos, E. coli, fish muscle, human erythrocytes, mouse liver, fish embryos, Euglena gracilis, pig and chicken liver, and desert locust fat body and flight muscle (Tanaka et al., 1967; Milman and Yurowitzki, 1967; Maeba and Sanwal, 1968; Somero and Hochachka, 1968; Koler and Vanbellinghen, 1968; Carminatti et al., 1968; Mil'man and Yurovitskii, 1969; Ohmann, 1969; Leveille, 1969; Bailey and Walker, 1969) has similarly been shown to be FDP activated.

For most cases reported, FDP acts as a heterotropic allosteric activator by lowering the Hill constant for PEP and both required metal ions and lowering the apparent K_m or K_A for each (Taylor and Bailey, 1967; Haeckel et al., 1968; Hunsley and Suelter, 1969b). There are at least two exceptions to this generalization and, for the first, a regulatory pyruvate kinase from E. coli (Maeba and Sanwal, 1968), FDP affected only the V_{max} of the reaction but not the sigmoidality of the PEP rate concentration curve. AMP was responsible for the PEP K_m change and converted PEP kinetics from sigmoid to hyperbolic. For the second, a preparation from rainbow trout muscle (Somero and Hochachka, 1969), the PEP saturation curves were stated to have remained hyperbolic under all conditions. The Brevibacterium flavum enzyme (Ozaki and Shiio, 1969) is also AMP activated with no effect on PEP sigmoidicity. In fish embryos, a previously unreported positive effector, 3',5'-cyclic AMP, has been found (Milman and Yurowitzki, 1967; Mil'man and Yurovitskii, 1969), but no kinetic mechanism stated. The very small stimulation of yeast pyruvate kinase activity by ATP (Haeckel et al., 1968) was probably due either to FDP contamination or the complexing of a divalent metal inhibitor.

4. Inhibitors

Divalent metal ions-An elegant rationale for predicting the likelihood of Ca^{2+} inhibition of divalent metal requiring enzymes which utilize nucleotides and other phosphate substrates has been presented by Cohn (1963): enzymes which do not require a metal atom to function as a bridge atom between enzyme and substrate, but only to bind the nucleotide, are Ca^{2+} activatable. Enzymes with a requisite for direct metal binding are inhibited by Ca^{2+}. Ternary complexes with a bridging divalent metal atom of the last class have been proven now for two pyruvate kinases, from rabbit muscle and bakers' yeast (Mildvan and Cohn, 1966; Mildvan et al., 1970).

The mechanism of the Ca^{2+} inhibition is complex. Kachmar and Boyer (1953) reported Ca^{2+} to give both competitive and non-competitive inhibition in relation to K^{+}. For the muscle enzyme, proton relaxation measurements demonstrating that Ca^{2+} strictly competed for Mn^{2+} in the metal-enzyme complex significantly were made in the presence of 0.10 M KCl (Mildvan and Cohn, 1965). Ca^{2+} has been implicated in glycolytic control at the pyruvate kinase site in two tissues, Ehrlich ascites tumor cells and guinea pig cerebral cortex slices (Bygrave, 1966; Takagaki, 1968).

Cu^{2+} at physiological concentrations has been

hypothesized as an FDP-reversible inhibitor for rat and mouse liver pyruvate kinase (Passeron et al., 1967; Carminatti et al., 1968). More recent work with rat liver has shown that the FDP mediated reversal of this inhibition was of little significance (Bailey et al., 1968; Rozengurt et al., 1969).

Metabolically derived inhibitors-Although ATP has generally been recognized as an inhibitor of pyruvate kinases from rabbit muscle (Reynard et al., 1961), rat liver (Tanaka et al., 1967; Weber et al., 1967a), brewers' yeast (Haeckel et al., 1968), A. xylinum (Benziman, 1969), and desert locust flight muscle (Bailey and Walker, 1969), no evidence has yet been presented that ATP may limit enzymatic activity in glycolyzing tissue or tissue extracts. In vitro, the mode of action of the nucleotide has been ascribed to the following three findings:

a. competition with PEP (Reynard et al., 1961; Boyer, 1969).

b. competition with ADP (Reynard et al., 1961; Weber et al., 1967a; Benziman, 1969; Holmsen and Storm, 1969).

c. complexing of Mg^{2+} (Wood, 1969).

Inhibition of brewers' yeast pyruvate kinase upon addition of citrate, NADP, AMP, 3',5'-cyclic AMP, and nucleotide triphosphates are likely to be due to the last listed

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mechanism (Haeckel et al., 1968). Release of ATP inhibition by FDP in cooperative systems has been reported (Tanaka et al., 1967; Haeckel et al., 1968; Bailey and Walker, 1969) but the mechanism is still obscure.

L-phenylalanine and diethylstilbestrol are also reversible inhibitors of unknown metabolic significance for pyruvate kinase preparations from rabbit muscle (Kimberg and Yielding, 1962) and rat prostate, seminal vesicle, and uterus (Vijayvargiya et al., 1969), respectively. For the rat tissues, L-alanine was seen to protect against L-phenylalanine antagonism.

A class of inhibitors requiring incubation with the enzyme prior to assay has also received attention. Physiological concentrations of long chain free fatty acids inhibit crude rat liver pyruvate kinase (Weber et al., 1967a) as well as acetyl-Coenzyme A (Weber et al., 1967b). The rat heart muscle enzyme is also inactivated in the presence of fatty acid salts (Tsutsumi and Takenaka, 1969). The criticism that there has been no direct information on enzymatic inhibition in situ by free fatty acids has been offered by Williamson (Weber et al., 1967a).

5. Kinetic cooperativity

Table 1 summarizes pyruvate kinase preparations which have been noted to yield cooperative steady-state kinetics

Table 1. Properties of cooperative pyruvate kinases.

Enzyme source	Homotropic ligand	Heterotropic effector	Reference
Brewers' yeast	PEP FDP		Hess et al. (1966).
	PEP K^+, NH_4^+	FDP FDP	Hess and Haeckel (1967).
	PEP ADP Mg^{2+} FDP citrate ATP	FDP Mg^{2+}	Haeckel et al. (1967).
Bakers' yeast	PEP Mg^{2+} K^+, NH_4^+ FDP	FDP FDP FDP	Hunsley and Suelter (1969b).
Rat liver	PEP	FDP	Tanaka et al. (1967).
	PEP	FDP	Taylor and Bailey (1967).
	PEP	FDP	Susor and Rutter (1968).
	PEP ATP	H^+ H^+	Rozengurt et al. (1969).

Table 1 (continued)

Rat adipose tissue	PEP	FDP	Pogson (1968).

Table 1 (continued)

Rat adipose tissue	PEP	FDP	Pogson (1968).
Human erythrocyte	PEP FDP		Koler and Vanbellinghen (1968).
Escherichia coli	PEP	AMP	Maeba and Sanwal (1968).
	PEP	FDP	Malcovati and Kornberg (1969).
Groundling embryo	PEP	FDP	Milman and Yurovitskii (1969).
Frog liver and eggs	PEP	FDP	Schloen *et al*. (1969).
Desert locust fat body	PEP	FDP	Bailey and Walker (1969).
King crab muscle	PEP	(decreasing temperature)	Somero (1969).
Euglena gracilis	PEP FDP	FDP	Ohmann (1969).
Acetobacter xylinum	PEP		Benziman (1969).
Brevibacterium flavum	PEP		Ozaki and Shiio (1969).

for at least one substrate, metallic cofactor, or inhibitor. Two attempts at fitting such data to the Monod-Wyman-Changeux model of allosteric interactions (Monod *et al.*, 1965) for rat liver L (Rozengurt *et al.*, 1969) and brewers' yeast (Wieker *et al.*, 1969) enzymes are limited in that only the PEP, FDP, and ATP variables were considered.

6. Temperature effects

Literature in this area is scanty. Kayne and Suelter (1965) observed a break in the Arrhenius plot for the muscle pyruvate kinase reaction at about 16^{o}, the metabolic significance of which, if any, is unknown. More recently, Somero and Hochachka (1968) noted temperature-dependent changes in the K_m for PEP for the rainbow trout and *Trematomus bernacchii* (antarctic fish) muscle enzymes. Enzyme-ATP, -ADP, and -FDP interactions were independent of temperature, but Ca^{2+} inhibition dependent. Minima in the PEP K_m's were the same as the fish habitat temperatures.

II. Molecular heterogeneity and regulation of enzyme levels

1. Nutritional and hormonal control

During a systematic study of the levels of enzymes related to gluconeogenesis in rat liver (Krebs and Eggleston, 1965), pyruvate kinase activities were found to vary over a 10 fold range. Highest levels were associated

with high carbohydrate diet, and lowest activities with starvation or low carbohydrate regimen when gluconeogenesis is favored. This relationship was correlated with the insulin level of the rat (Tanaka et al., 1965; Weber et al., 1965). Enzyme levels dropped in alloxan diabetic rats, peaked to normal upon administration of insulin, and hormonal induction was prevented by protein biosynthetic inhibitors. More importantly, the activity in liver extracts was electrophoretically resolved into 4 peaks, one identical to the muscle enzyme and the other 3 immunologically distinct and unique to liver (Tanaka et al., 1965), of which only the 3 liver types fluctuated with diet or insulin. The muscle type in liver is not invariant, however. Marked increases were noted in regenerating rat liver (Tanaka et al., 1967) and in livers of rats perfused with the blood or plasma from Walker tumor bearing donors (Suda et al., 1968). Actinomycin S and p-fluorophenylalanine inhibit the latter phenomenon.

The effect of antibiotics on enzyme levels resulting from mixed sequential high carbohydrate and high protein dietary studies (Szepesi and Freedland, 1968) illustrates the complexity of the induction process. Their data were consistent with translational level control of enzyme synthesis during carbohydrate to protein dietary transitions,

and transcriptional control during the opposite transition. No data were available on isozymic variations. Induction may be independent of insulin. Liver pyruvate kinase can be induced in alloxan diabetic rats by a fructose-glycerol diet (Sillero et al., 1969). Further dissection of glycolytic and gluconeogenic pathways led these workers to hypothesize secondary metabolite induction.

Uterine pyruvate kinase activity is increased by estradiol-17β injection of ovariectomized rats. The augmentation is blocked by actinomycin D, 5-fluorouracil, or cycloheximide (de Asúa et al., 1968). A peaking has also been reported for the rat brown fat enzyme during suckling (Hahn and Greenberg, 1968).

Induction is not limited to mammals. Activity is induced in several yeasts (Hommes, 1966; Gancedo et al., 1967; Fernández et al., 1967), E. coli K12 (Malcovati and Kornberg, 1969), and Euglena gracilis (Ohmann, 1969). The inducible activity in E. coli K12 was separated from a constituitive enzyme and shown to be kinetically distinct. Potassium deficiency increases pyruvate kinase from wheat seedlings (Sugiyami and Goto, 1966) by an unknown process.

2. Isozymes and multiple forms

Tissue-distinct forms of the enzyme have been recognized for many years (Table 2). Several of these forms, in

Table 2. Multiple forms of pyruvate kinase and presumptive isozymes.

Organism	Multiple forms seen	Heterogeneity[a]	Reference
Rabbit	Muscle[b]	3 electrophoretic bands	Reynard et al. (1961).
Rat	Liver	-	von Fellenberg et al. (1963).
	Muscle	-	
	Kidney	-	
Rat	Liver	4 electrophoretic bands, one muscle-like	Tanaka et al. (1965).
	Muscle	-	
Human	Erythrocyte[b]	2 genetic alleles[b]	Zuelzer et al. (1968).
Rainbow trout	Muscle	2 electrophoretic bands, one liver-like	Somero and Hochachka (1968).
	Liver	-	
Human	Liver	-	Bigley et al. (1968).
	Muscle	-	
	Kidney	-	
E. coli	2	Separated chromatographically	Malcovati and Kornberg (1969).
Rat	Hepatoma	4 to 5 bands on isoelectric focusing	Criss (1969).
	Muscle	-	
	Liver	4 bands on isoelectric focusing	

Rat Hepatoma isoelectric focusing Criss (1969).

Muscle -

Liver 4 bands on isoelectric focusing

Table 2 (continued)

Rabbit	Muscle	4 bands on isoelectric focusing	Susor et al. (1969).
	Liver	2 bands on isoelectric focusing (B type)	
Frog	Lung	2 electrophoretic bands	Schloen et al. (1969).
	Kidney	2 electrophoretic bands	
	Spleen	4 electrophoretic bands	
	Fertilized eggs	5 electrophoretic bands	
	Muscle	-	
	Liver	-	
	Heart	-	

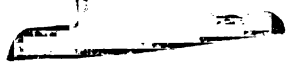
[a]Resulting from any situation, including presumptive isozymes.
[b]Only tissue studied.

turn, show isozymic heterogeniety, some of which may be related to other tissue-distinct enzymes. Regulation of individual presumptive isozymic variants has not been reported, though variable sensitivity of purified rat liver pyruvate kinases toward Cu^{2+} inactivation (Carminatti et al., 1968) and FDP activation (Susor and Rutter, 1968) might be explained in this way.

3. Conformers and interconvertibility of forms

A body of literature now provides evidence for the kinetic or physical identification of conformational states of pyruvate kinase preparations. The conversion processes are not well understood, not always reversible, and of uncertain in vivo importance.

Crystalline rabbit muscle pyruvate kinase changed in electrophoretic mobility and limiting viscosity on the reversible binding of diethylstilbestrol, a surface-active synthetic estrogen (Kimberg and Yielding, 1962). With the same enzyme, breaks in Arrhenius plots and temperature induced UV difference spectra were fitted to a two conformational equilibrium model (Kayne and Suelter, 1965). Fluorescence polarization and sedimentation parameters were consistent with the low temperature and cation-activated forms being more compact than the high temperature form (Kayne and Suelter, 1968).



Some preparations of the rat liver enzyme (Tanaka et al., 1967; Susor and Rutter, 1968) are variably sensitive to FDP activation and loss of this property could be induced through incubation at 37° in dilute solutions or storage at -20° with no change in immunological specificity in the first case. On the other hand, the purified enzyme may gain sensitivity toward PEP and FDP upon purification or incubation at 25° (Susor and Rutter, 1968; Bailey et al., 1968). The latter investigators found FDP and high pH incubations favored the formation of a cooperative, sensitive conformation. With a more stable preparation, the facile reversible transformation of this enzyme from a cooperative, FDP-sensitive form to a normal, FDP insensitive form was mediated by the pH of the reaction mixture (Rozengurt et al., 1969). This group discovered dithiothreitol and substrates to protect against inactivation during purification. A similar equilibrium for rat epididymal fat pyruvate kinase (Pogson, 1968) was mediated by citrate or EDTA and countered by FDP. In this case, the conformations were not only stable enough to be electrophoretically resolved but a difference in sedimentation value was also seen for the two types. Thus, the conversions may have been association-dissociation phenomena dependent on some divalent cation.

Several enzymes display complex pH optima with two or more peaks which may represent the activities of a mixture of conformers in the assay solution (Bailey et al., 1968; Haeckel et al., 1968; Hunsley and Suelter, 1969b; Bailey and Walker, 1969). In the last 3 cases (yeast and desert locust flight muscle enzymes) activity peaks in the high pH range are produced upon addition of PEP or FDP, respectively.

For Alaskan king crab muscle pyruvate kinase, lower temperatures mimic the heterotropic effect of FDP on PEP kinetics common to other cooperative systems (Somero, 1969).

The kinetics of the FDP-promoted dissociation of yeast pyruvate kinase are consistent with the existence in solution of two conformers (Kuczenski and Suelter, 1970).

METHODS AND MATERIALS

I. Enzymes

The pyruvate kinase in this study was purified from fresh commercial bakers' yeast, Saccharomyces cerevisiae ("Budweiser," Anheuser-Busch, Inc.), according to the method of Hunsley and Suelter (1969a) (Fraction VII), and stored at 4^{o} as a concentrated suspension in 3.6 M $(NH_4)_2SO_4$ containing 10 mM Na phosphate buffer, pH 6.5. The preparations were stable for months in this state with no known deterioration in either chemical or physical properties. The minimum specific activity of any preparation used, except in Mg^{2+} kinetic experiments (150 μmoles/min/mg minimum), was 200 μmoles of pyruvate formed/min/mg under maximal assay conditions. $(NH_4)_2SO_4$-free solutions of enzyme were prepared by Sephadex G-25 chromatography in appropriate buffers and tested for sulfate content with saturated $BaCl_2$. All manipulations of the enzyme were at room temperature to avoid cold denaturation.

For comparative purposes, phenylmethanesulfonyl fluoride, a potent protease inhibitor, was included in the isolation procedure at the cell lysis and first $(NH_4)_2SO_4$

precipitation steps (Hunsley and Suelter, 1969a). A final concentration of 2.0 mM in the extract was used which has been shown sufficient to inhibit esterolytic protease activity during the isolation of yeast hexokinase (Schulze and Colowick, 1969). No effect on pyruvate kinase activity in the extracts was noted upon the additions of inhibitor.

Lactic dehydrogenase, the assay coupling enzyme, was either Calbiochem grade A or the Sigma type II rabbit muscle enzyme substantially free of pyruvate kinase and was desalted free of $(NH_4)_2SO_4$ over medium Sephadex G-25 in 0.20 M Tris-HCl, pH 7.5, before use.

Rabbit muscle pyruvate kinase used in the assay of PEP and ADP was prepared by a modification (Kayne and Suelter, 1965) of the Tietz and Ochoa (1958) procedure.

The following enzymes and proteins were used as molecular weight standards: crystalline bovine serum albumin, crystalline rabbit muscle aldolase, 3 times recrystallized soy bean trypsin inhibitor, crystalline swine stomach pepsinogen, equine alcohol dehydrogenase, 5 times recrystallized bovine pancreas ribonuclease, crystalline pig heart malic dehydrogenase, type II α-chymotrypsinogen A (all Sigma products), and sperm whale myoglobin (Calbiochem A grade). Mixed crystals of rabbit muscle α-glycerophosphate dehydrogenase and triose phosphate isomerase were from

Calbiochem.

II. Chemicals

All water was either double glass distilled or glass distilled and deionized (Crystalab Deeminite). Pyruvic acid (Matheson, Coleman, and Bell), distilled at reduced pressure, was neutralized with $(CH_3)_4NOH$. TriCHA PEP, Tris ADP, Na ADP, tetraCHA FDP, Ba FDP, and Na NADH were Sigma Chemical Company products. Ammonium FDP-^{14}C, specific activity 69 mC/mM, was purchased from the Amersham-Searle Corporation. Ba FDP was converted to the $(CH_3)_4N^+$ salt with $\left[(CH_3)_4N\right]_2SO_4$ prepared from Eastman $(CH_3)_4NOH$. Na ADP routinely was chromatographed at pH 7 over Dowex 50W-X8, Tris cationic form, to yield the Tris salt.

Eastman $(CH_3)_4NCl$ was recrystallized from hot ethanol (Kayne, 1966). Sephadex G-25 (medium), G-200, and Blue Dextran were from Pharmacia. N, N, N', N'-tetramethylethylenediamine, N, N'-methylenebisacrylamide, and acrylamide were supplied by Canalco and used without further purification. Forty per cent solutions of Ampholine carrier ampholytes, pH ranges 3-10 and 5-8, were LKB products. All other chemicals and reagents were of the highest commercially obtainable purity.

III. Experimental methods

1. Assay of activity and determination of enzyme concentration

After desalting over Sephadex G-25 in H_2O, an extinction coefficient at 280 nm was determined for purified enzyme by plating approximately 5 mg aliquots of known optical density in tared vials. The samples were lyophilized and dried to constant weight over P_2O_5 at 110° (Hunsley and Suelter, 1969a). Standard assays were performed at 30° on dilutions of the enzyme in 50% (v/v) glycerol-10 mM Na phosphate buffer, pH 6.5, by employing the linked lactic dehydrogenase reaction modified from Bücher and Pfleiderer (1955). The reaction mixture contained, per ml: 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2; 24 μmoles $MgCl_2$; 100 μmoles KCl; 5.0 μmoles triCHA PEP; 10 μmoles Tris ADP, pH 7; 1.0 μmole tetraCHA FDP; 0.15 μmole Na NADH; and 33 μg $(NH_4)_2SO_4$-free lactic dehydrogenase (Hunsley and Suelter, 1969a). Routine assays in the absence of FDP were identical except for an additional 100 μmoles KCl per ml and omission of FDP. Aliquots of 10 μl or less of enzyme dilutions were added to the reaction mixture at 30° in a 1 cm silica cuvette and the change in optical density at 340 nm was recorded on a Gilford model 2000 modified Beckman DU ultraviolet spectrophotometer. The initial rate was converted to micromoles of

pyruvate formed per minute by dividing the change in optical density per minute by 6.22 (Horecker and Kornberg, 1948).

ADP and PEP concentrations were estimated by a modification of the Bücher and Pfleiderer (1955) pyruvate kinase assay in the presence of excess rabbit muscle enzyme. FDP was estimated in the presence of rabbit muscle α-glycerophosphate dehydrogenase, triose phosphate isomerase and excess aldolase as modified from the assay of Rutter et al. (1966).

2. Electrophoresis and chromatography

Disc gel electrophoresis-50 µg of enzyme was electrophoresed in 6.0% polyacrylamide gel at 25° in 5 X 75 mm columns (Davis, 1964), stacking at pH 8.3 and running at pH 9.5. No sample gel was used. Instead, the samples were applied in 50% (v/v) glycerol containing 15 mM Tris phosphate, pH 6.9. After staining with 0.55% Amido-Schwarz in 7.5% acetic acid, the gels were destained electrophoretically.

Gel chromatography-An analytical Sephadex G-200 column, 2.6 X 36 cm, was poured with a 1.8 cm overlay of G-25. A hydrostatic head of 10 cm was maintained for the 0.10 M Tris-HCl, pH 7.5, elution buffer. One mg samples of protein were added to the column in 1.0 ml of 0.50 M sucrose-0.10 M Tris-HCl, pH 7.5. Effluents were automatically monitored at

235 nm in a Beckman DB ultraviolet spectrophotometer fitted with a flow cell, and fractions collected with a calibrated drop counting fraction collector. The void volume was determined with 0.2% Blue Dextran added in Tris-sucrose buffer (Andrews, 1965).

3. Chemical properties

Metal content-$(NH_4)_2SO_4$-free solutions of the enzyme in 0.10 M Tris-HCl, pH 7.5, were analyzed for metal content. Samples were examined on either an Aztec or Perkin-Elmer atomic absorption spectrophotometer. In addition, approximately 11 mg aliquots were dried on Whatman No. 54 acid washed filter paper discs, 2.5 cm diameter, and examined in a General Electric XRD-6 x-ray fluorescence unit. Secondary standards of $CaCl_2$ and $MnCl_2$ were plated on identical filter discs.

Amino acid analysis-The enzyme was prepared for hydrolysis by desalting on a G-25 Sephadex column in H_2O. The samples were hydrolyzed in constant boiling HCl according to Bailey (1967). Amino acid analyses were performed by the method of Spackman et al (1958) on a Beckman-Spinco model 120B amino acid analyzer and by the Piez and Morris (1960) modification of this procedure. Combined cysteine and cystine were estimated after performic acid oxidation (Moore, 1963) and hydrolysis, and determined as cysteic acid.

Tryptophan was estimated on samples desalted over Sephadex G-25 in 0.10 M Tris-HCl, pH 7.5, according to Goodwin and Morton (1946) with precautions noted by Beaven and Holiday (1952). Spectra were obtained with a Beckman DB ultraviolet spectrophotometer (Hunsley and Suelter, 1969a).

4. Kinetic properties

Linked assays as described previously were conducted at 30° on enzyme dilutions in 50% (v/v) glycerol-10 mM Na phosphate, pH 6.5. Extraneous alkali and ammonium ions in kinetic experiments were estimated to be 300 μM in Na^+ from NADH additions and less than 100 μM in NH_4^+ from enzyme additions (Hunsley and Suelter, 1969b). The reaction was initiated in all experiments by addition of enzyme. In addition, all catalytic variables except the one or two under examination were at saturating or near saturating levels. All rates were corrected for any blank rate encountered arising from pyruvate kinase in the lactic dehydrogenase. In studies involving measurement of pH, the reaction mixtures were tested directly immediately after assay with a Sargent model LS pH meter fitted with a Sargent S-30070-10 unit electrode.

Where kinetic data are treated as Hill plots, total concentrations of added substrate or activator are found on the abscissa. Lines through the points were drawn by eye

and the values of the slopes do not indicate the limits of accuracy of the experiment. The apparent K_m or K_A is defined as that concentration of substrate or activator where v = ½ maximal observed velocity. The Hill slope, n_H, is defined as $\Delta \log (v/V-v)/\Delta \log [L]$ where v is the observed velocity, V the maximal observed velocity, and L the variable ligand (Atkinson, 1966).

5. Binding properties

The binding of Mn^{2+} to yeast pyruvate kinase was studied at 30° by electron paramagnetic resonance and proton relaxation rate measurements as previously described for the rabbit muscle enzyme (Mildvan and Cohn, 1965). The data were plotted according to Scatchard (1949). The interaction of substrates with the enzyme-Mn complex was studied by the proton relaxation rate method and the data were treated by procedures I and III of Mildvan and Cohn (1966).

Binding of FDP to the enzyme was tested by incubation of the enzyme with FDP-^{14}C, Sephadex G-25 chromatography of the mixture, and identification of effluent protein and radioactive peaks.

Radioactivity was monitored by scintillation counting in a Packard Tri-Carb model 3310 scintillation spectrophotometer with a window setting of 50-1000 at 15% gain. Known aliquots of FDP-^{14}C were used as standards. The

scintillation mixture (Gordon and Wolfe, 1960; Kinard, 1957) contained 4% Cab-O-Sil.

6. Isoelectric focusing

Preparative procedure-A 110 ml capacity LKB model 8101 electrofocusing column was loaded with a linear 20-60% (v/v) glycerol gradient containing 1% final concentration of pH range 5-8 carrier ampholytes titrated to pH 7.2 with $(CH_3)_4NOH$. The voltage was raised in 100 v steps to 700 v in 3 hours and maintained there at 20° for 72 hours, after which time the column volume was displaced with H_2O and fractions collected (Vesterberg and Svensson, 1966). Protein concentrations at the termination of the experiment were estimated by a nephlometric assay (Mejbaum-Katzenellenbogen and Dobryszycka, 1959) with yeast pyruvate kinase standards.

Microisoelectric focusing in polyacrylamide gels-Electrofocusing was performed at 25° (Catsimpoolas, 1968) in riboflavin-catalyzed 6.5% polyacrylamide containing 2% carrier ampholytes (pH range 3-10) titrated to pH 7.2 with $(CH_3)_4NOH$. 5 X 75 mm glass columns were coated with a solution of 5% Plexiglass in CH_2Cl_2 and dried prior to use to facilitate removal of gels from the columns (Loening, 1967). Riboflavin-catalyzed gels were polymerized for 30 minutes 3

inches from a fluorescent lamp. 4.5% polyacrylamide gels were polymerized 15 minutes with 0.054% ammonium persulfate in the light. Cathodic ends of gels were marked with powdered charcoal for easy identification. All columns were focused at 5 ma per tube initial current in a disc electrophoresis apparatus with 5% phosphoric acid in the anode bath and 5% ethanolamine as the cathode.

After focusing, gels were removed from columns with water from a long blunt No. 22 hypodermic needle, fixed one hour in 10% trichloroacetic acid, and then transferred to 7% acetic acid for storage. To quantitate precipitated enzyme bands, gels were placed in 0.5 X 1 X 10 cm silica boats under 7% acetic acid and scanned at 280 nm in the Gilford linear transport apparatus. Scan tracings were cut out and weighed.

Since carrier ampholytes interfere strongly with protein dyes and dialyze out only very slowly, visualization of the protein was accomplished by light scattering. Gels were placed in 7 X 125 mm glass tubes which were filled with 7% acetic acid, and the tubes corked. These were placed in a black-bottomed Plexiglass tray, covered with ethanol, and illuminated along the axis of the tubes by a 150 watt reflector flood bulb through two successive 5 X 100 mm slits. Photography was accomplished at 90^{o} to the incident light

in a darkened room.

RESULTS

I. Criteria for purity

1. Disc gel electrophoresis

One of the most sensitive techniques available to the enzymologist for physical separation of protein species is polyacrylamide disc gel electrophoresis which differentiates by both charge and size (Ornstein, 1964). To establish that the purified yeast pyruvate kinase under investigation was molecularly homogeneous, enzyme at all stages of purification was routinely electrophoresed by this method. The enzyme sample was placed directly on the spacer gel in glycerol-buffer solution which protects against cold-induced inactivation (Hunsley and Suelter, 1969a) and subsequent production of complex mixtures of dissociated enzyme (Kuczenski and Suelter, 1970).

Illustrated in Figure 1 are the results of a disc electrophoretic experiment which demonstrated coincidence of enzymatic activity and the major stained protein band. The minor constituents comprised less than 5% of the total protein in the final yeast pyruvate kinase product and can be eliminated by narrowing the pool width of the last

chromatographic purification step (Hunsley and Suelter, 1969a). The pyruvate kinase stained disc migrated as a singlet independent of the amount of protein, the gel concentration, or the addition to polyacrylamide gels of reagents which are known to stabilize enzymatic activity: either 1 mM tetraCHA FDP and 10 mM $MgCl_2$ or 12.5% glycerol.

2. Contaminating enzyme activities

Two enzymes which could, if present in significant amounts as contaminants in the pyruvate kinase preparations, interfere with kinetic and binding determinations are adenylate kinase ($2ADP \rightleftharpoons AMP + ATP$) and aldolase (FDP $\rightleftharpoons$ glyceraldehyde-3-phosphate + dihydroxyacetone phosphate). Adenylate kinase activity in the purified product, assayed with a hexokinase-glucose-6-phosphate dehydrogenase-linked reaction in the presence of ADP, glucose, and NADP, was less than 2×10^{-3} μmoles/min/mg, the lower limit of the determination. Since yeast aldolase has been shown to be extremely unstable in the absence of sulfhydryl-reducing agents, it may be ignored (Rutter et al., 1966).

II. Chemical properties

1. Extinction coefficient

The dry weight based extinction coefficient at 280 nm, $\epsilon^{0.1\%}_{1\ cm} = 0.653$, lower than that defined by the Warburg and

Figure 1. Disc gel electrophoresis of 50 µg of yeast pyruvate kinase. The enzyme was electrophoresed as outlined in Methods and Materials in 6.0% polyacrylamide gel. The gel was split longitudinally, one half stained, and the other sliced into two mm pieces and each piece dispersed in 0.10 ml of 50% glycerol-10 mM Na phosphate (v/v), pH 6.5. An aliquot of 10 µl of this suspension was tested for activity in the standard assay containing FDP. The enzymatic activity was coincident with the dark staining band near the left cathodic end. The band to the extreme left was the opaque spacer gel (Hunsley and Suelter, 1969a).

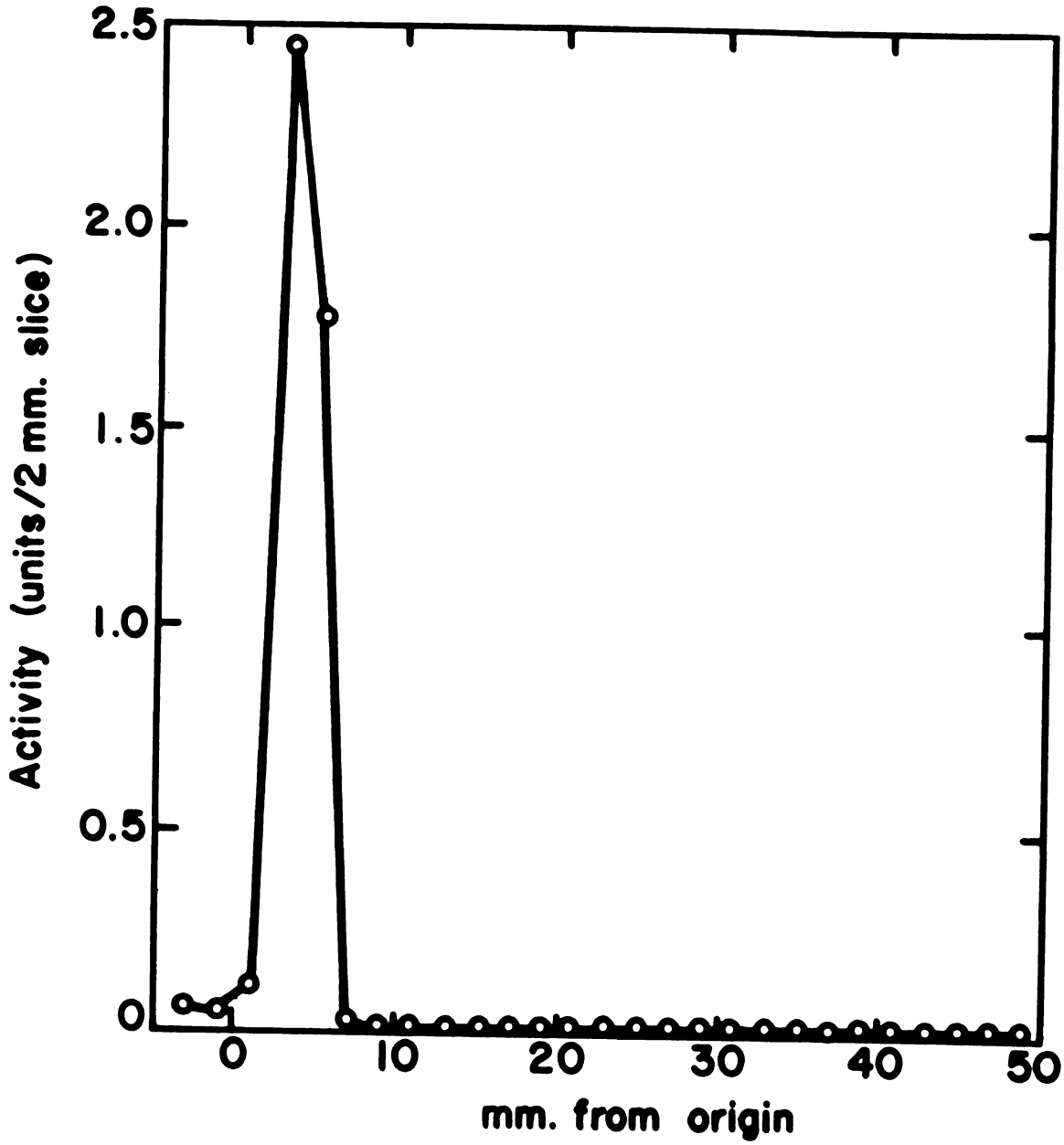

2.5
2.0
1.5
1.0
0.5
0
Activity (units/2 mm. slice)
0
10
20
30
40
50
mm. from origin

Figure 1

Christian (1942) assay, reflects the relatively low aromatic amino acid content of the enzyme (Hunsley and Suelter, 1969a).

2. Amino acid analysis

The amino acid composition (Table 3) is the average of two independent analyses. Uncertainties in extrapolation to zero time for threonine and serine residues gave rise to comparatively larger standard deviations. Determination of amide nitrogen of asparagine and glutamine was beyond the scope of this investigation and, therefore, aspartic and glutamic acid values represent combined acid and amide residues. The value found for NH_3 was 103 $\pm$ 1.2 moles per 165,800 g of enzyme. Alternate data for tyrosine are available from the alkaline ultraviolet absorption tryptophan estimation of Goodwin and Morton (1946). Forty-seven tyrosines per 165,800 g of enzyme (Hunsley and Suelter, 1969a) were estimated by this less reliable method.

3. Metal content

The NMR proton relaxation rate studies reported in this thesis are based on the observation that paramagnetic ions greatly increase the relaxation rate of nuclear spin states of water protons (Cohn and Leigh, 1962). During preliminary Mn^{2+} binding studies, relaxation rates of water protons in buffer containing only enzyme were unusually high.

Table 3. Amino acid analysis of yeast pyruvate kinase. Methods of analysis are described in Methods and Materials. The results are expressed as the mean ± standard deviation of two determinations (Hunsley and Suelter, 1969a).

Amino acid	Moles/165,800 g enzyme
Lysine	99.4 ± 2.0
Histidine	21.2 ± 1.9
Arginine	64.8 ± 1.2
Aspartic acid[a]	156.9 ± 7.8
Glutamic acid[a]	109.1 ± 2.2
Proline	68.6 ± 1.5
Glycine	94.4 ± 2.2
Alanine	122.5 ± 2.2
Valine	113.4 ± 5.3
Methionine	22.0 ± 3.4
Isoleucine	82.6 ± 5.2
Leucine	106.0 ± 1.3
Tyrosine	38.1 ± 1.1
Phenylalanine	43.2 ± 0.3
Cysteine[b]	14.4 ± 0.3
Threonine[c]	178 ± 15
Serine[c]	113.9 ± 8.3
Tryptophan	9.3 ± 0.2

[a]Acid and amide conbined.
[b]Determined as cysteic acid.
[c]Values extrapolated to zero time hydrolysis.

Contaminating metal ions such as Cu or Fe were suspected and the enzyme purification was modified so that the source of the metals, diatomaceous earth, was free of such ions. Examination of the modified preparation by atomic absorption spectrophotometry (Table 4) revealed Zn, Co, and Fe content to be reduced below the limit of detection for each. Cu content remained constant, regardless of whether enzyme solutions were treated with Chelex chelating resin or EDTA was incorporated in each step of the enzyme purification.

Table 4. Metal content of purified yeast pyruvate kinase. Purified enzyme was desalted in 0.10 M Tris-HCl, pH 7.5, over Sephadex G-25 and examined by atomic absorption spectrophotometry as described in Methods and Materials (Hunsley and Suelter, 1969a).

Metal	Moles metal/165,800 g enzyme
Zn	< 0.01
Co	< 0.01
Fe	< 0.05
Cu	0.14

No other unexpected elements in dried enzyme samples were found at levels greater than 0.01 moles per 165,800 g of enzyme through the use of an x-ray fluorescence unit, scanning from atomic numbers 13 through 30 (Hunsley and Suelter, 1969a).

4. Gel chromatography

Analytical gel chromatography proved not to be a useful tool for analysis of the molecular weight of pyruvate kinase. The proteins used as standards behaved as typical globular proteins with a molecular weight dependent elution volume (Andrews, 1965), but yeast pyruvate kinase eluted as skewed, non-Gaussian peaks corresponding to a molecular weight of up to 300,000. Activity was distributed throughout the peaks.

III. Kinetic properties

1. Requirement of monovalent cation

The presence of specific activating monovalent cation was required for optimum catalytic function of yeast pyruvate kinase (Table 5). $(CH_3)_4N^+$ ion could not replace alkali metal or ammonium ions demonstrating that the effect was not merely due to increased ionic strength. Sodium ion presented an intermediate case, that of functioning only in the presence of FDP, the allosteric activator. The very low rates associated with FDP addition alone and in combination with $(CH_3)_4NCl$ can be explained, then, through potentiation of low contaminant Na^+ levels in the assays.

Serial transfer of a clone of the original yeast was made in aerobic complex growth medium containing increasing concentrations of KCl up to 2.7 M. Growth at high salt

Table 5. Requirement of yeast pyruvate kinase activity for alkali metal or ammonium ions. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2, 24 μmoles $MgCl_2$, 5.0 μmoles triCHA PEP, 10 μmoles Tris ADP, 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH. FDP was added as the $(CH_3)_4N^+$ salt (Hunsley and Suelter, 1969b).

Reagents added	Final concentration (mM)	Initial velocities (μmoles/min/mg)
FDP	1.0	1.5
$(CH_3)_4NCl$	50	< 0.10
$(CH_3)_4NCl$	200	< 0.10
$(CH_3)_4NCl$, FDP	50, 1.0	1.0
$(CH_3)_4NCl$, FDP	200, 1.0	0.82
NaCl	50	< 0.10
NaCl	500	0.48
NaCl, FDP	170, 1.0	34
KCl	170	155
KCl, FDP	100, 1.0	154
NH_4Cl	30	72
NH_4Cl, FDP	30, 1.0	89

concentrations was poor and kinetic examination of cell lysates from high-salt cultures failed to show any changes in pyruvate kinase properties.

2. Kinetics of the Mg^{2+} activated system

Mn^{2+}, which is found in minute quantities in most biological tissue, can replace Mg^{2+} in almost every enzymatic reaction which requires the ion. Although Mn^{2+} presumably does not substitute for Mg^{2+} in the pyruvate kinase reaction in vivo, its paramagnetic properties can be exploited in binding studies to yield useful information about the mechanism of action of the divalent cation. To take advantage of the paramagnetic property of Mn^{2+}, the kinetic variables for substrates were examined in both the Mg^{2+} and Mn^{2+} systems. Variables in the Mg^{2+} system were first examined at optimal pH (Hunsley and Suelter, 1969b).

No effect on the conversion of pyruvate to lactate by the lactic dehydrogenase linking enzyme has been noted through the addition of the kinetic variables under investigation.

The effects of activating monovalent cations on initial velocity are given in Figure 2. The Hill slopes (n_H) for K^+ and NH_4^+, 2.85 and 3.80, respectively, were both reduced after addition of FDP to the assay system to 1.40 (K^+) and 1.30 (NH_4^+) and the apparent K_A for each significantly

Figure 2. Relationship between initial velocity of yeast pyruvate kinase and activating univalent cation concentration. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2, 24 μmoles $MgCl_2$, 5.0 μmoles triCHA PEP, 10 μmoles Tris ADP, 1.0 μmole $(CH_3)_4N$ FDP (when present), 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH. Hill slopes for the curves are shown in parentheses. Univalent cations were added as the chloride salts (Hunsley and Suelter, 1969b).

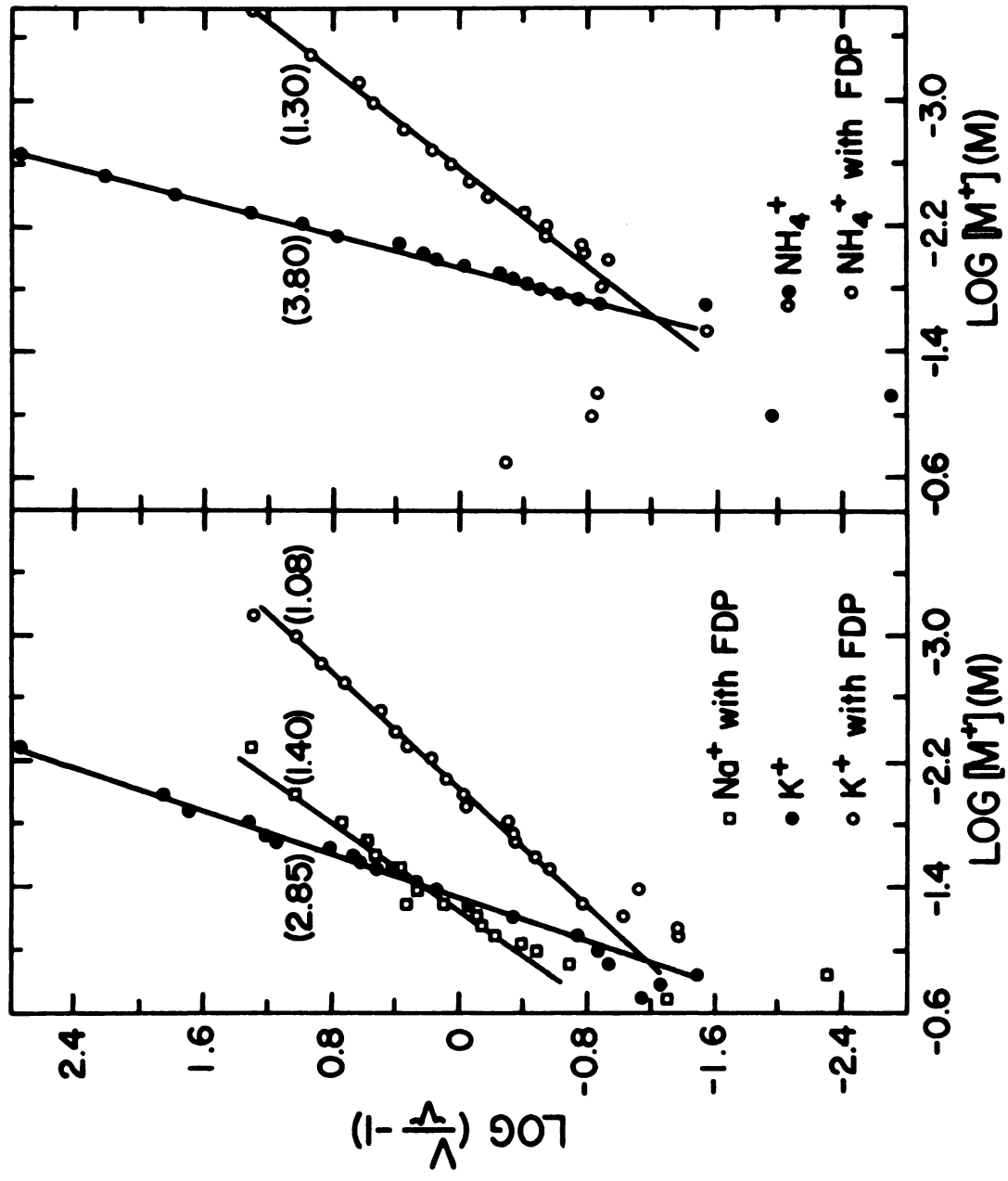

LOG (V/V∞ -1)
2.4
1.6
0.8
0
-0.8
-1.6
-2.4
(1.30)
(3.80)
(1.08)
(1.40)
(2.85)
∘ NH4+ with FDP
• NH4+
▫ Na+ with FDP
• K+
∘ K+ with FDP
-0.6
-1.4
-2.2
-3.0
LOG [M+] (M)

Figure 2

reduced. To summarize, FDP, in the case of monovalent cation, not only reduced cooperativity nearly to classical Michaelis-Menten kinetics (n_H = 1.00) but also decreased the apparent K_A. This pattern of FDP activation was seen for all remaining variables except ADP.

The activation by FDP in the presence of low concentrations of the required monovalent cations K^+ and NH_4^+, was also cooperative with n_H equal to 2.33 and 2.55, respectively. Na^+-dependent FDP activation gave a Hill slope near unity which was unaffected on lowering the Na^+ concentration (Figure 3).

Similarly to monovalent cation kinetics, with PEP as a variable (Figure 4) the homotropic effect was effectively abolished by the addition of FDP and the apparent K_m's lowered by an order of magnitude or more. This same relationship held true for Mg^{2+} (Figure 5), although cooperative behavior was still evident with FDP present. The Hill slope for the assays containing Na^+ remained intermediate in this instance at 2.86.

Only a very small FDP heterotropic effect on ADP kinetics was seen (Figure 6). This substrate displayed Michaelis-Menten kinetic behavior regardless of the presence or absence of FDP at saturating levels of PEP and divalent and monovalent cations.

Figure 3. Relationship between initial velocity of yeast pyruvate kinase and total FDP concentration. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2; 24 μmoles $MgCl_2$; 5.0 μmoles triCHA PEP; 10 μmoles Tris ADP; 33 μg lactic dehydrogenase; 0.15 μmole Na NADH; and o—o, 140 μmoles NaCl, □—□, 10 μmoles KCl, and ●—●, 5.0 μmoles NH_4Cl. Hill slopes for the curves are shown in parentheses. FDP was added as the $(CH_3)_4N^+$ salt (Hunsley and Suelter, 1969b).

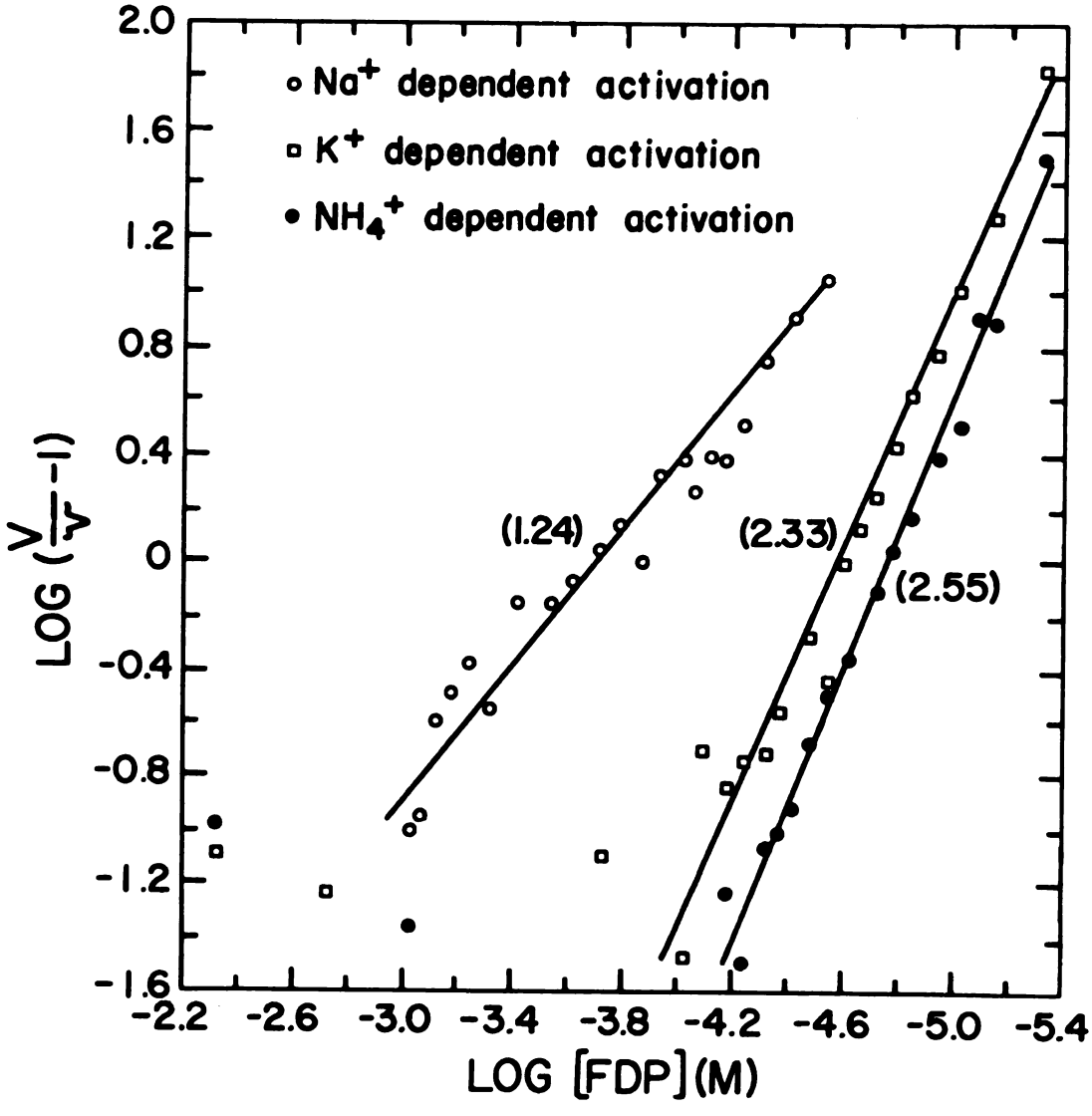

2.0
1.6
1.2
0.8
0.4
0
-0.4
-0.8
-1.2
-1.6
LOG ($\frac{V}{V}$ -1)
o Na+ dependent activation
□ K+ dependent activation
• NH4+ dependent activation
(1.24)
(2.33)
(2.55)
-2.2 -2.6 -3.0 -3.4 -3.8 -4.2 -4.6 -5.0 -5.4
LOG [FDP](M)

Figure 3

Figure 4. Relationship between initial velocity of yeast pyruvate kinase and total PEP concentration. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2; 24 μmoles $MgCl_2$; 10 μmoles Tris ADP; 1.0 μmole $(CH_3)_4N$ FDP (when present); 33 μg lactic dehydrogenase; 0.15 μmole Na NADH; left, ●—●, 180 μmoles KCl, o—o, 100 μmoles KCl, and □—□, 170 μmoles NaCl; and right, ●—●, 50 μmoles NH_4Cl and o—o, 30 μmoles NH_4Cl. Hill slopes for the curves are shown in parentheses. PEP was added as the triCHA salt (Hunsley and Suelter, 1969b).

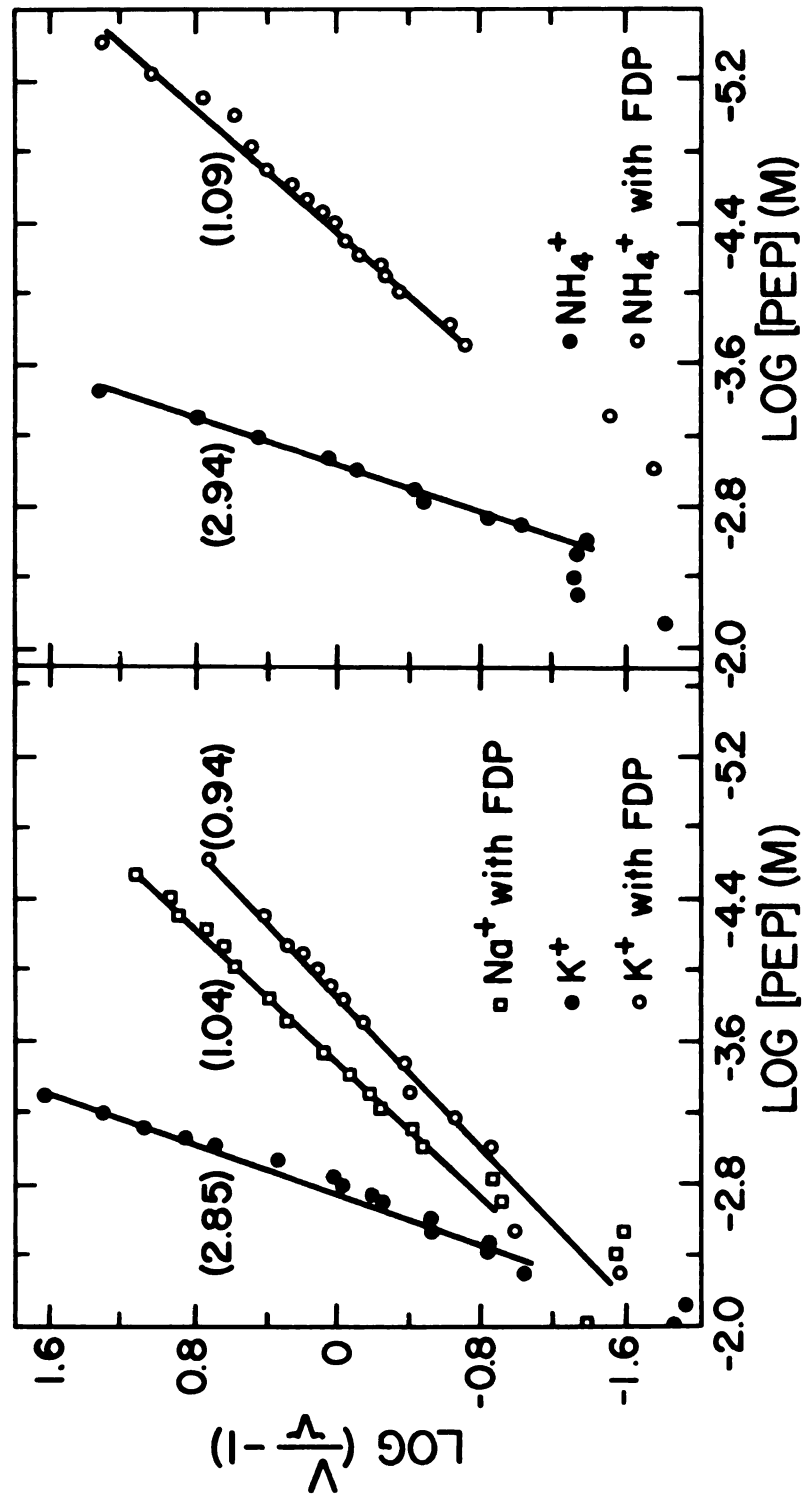

LOG (V/V - 1)
1.6
0.8
0
-0.8
-1.6
(2.85)
(1.04)
(0.94)
(2.94)
(1.09)
Na+ with FDP
K+
K+ with FDP
NH4+
NH4+ with FDP
-2.0
-2.8
-3.6
-4.4
-5.2
LOG [PEP] (M)

Figure 4

Figure 5. Relationship between initial velocity of yeast pyruvate kinase and total $MgCl_2$ concentration. The assay mixture (1.00 ml) contained 100 µmoles $(CH_3)_4N$ cacodylate, pH 6.2; 5.0 µmoles triCHA PEP; 10 µmoles Tris ADP; 1.0 µmole $(CH_3)_4N$ FDP (when present); 33 µg lactic dehydrogenase; 0.15 µmole of Na NADH; left, ●—●, 180 µmoles KCl, o—o, 100 µmoles KCl, and □—□, 170 µmoles NaCl; and right, ●—●, 50 µmoles NH_4Cl, and o—o, 30 µmoles NH_4Cl. Hill slopes for the curves are shown in parentheses (Hunsley and Suelter, 1969b).

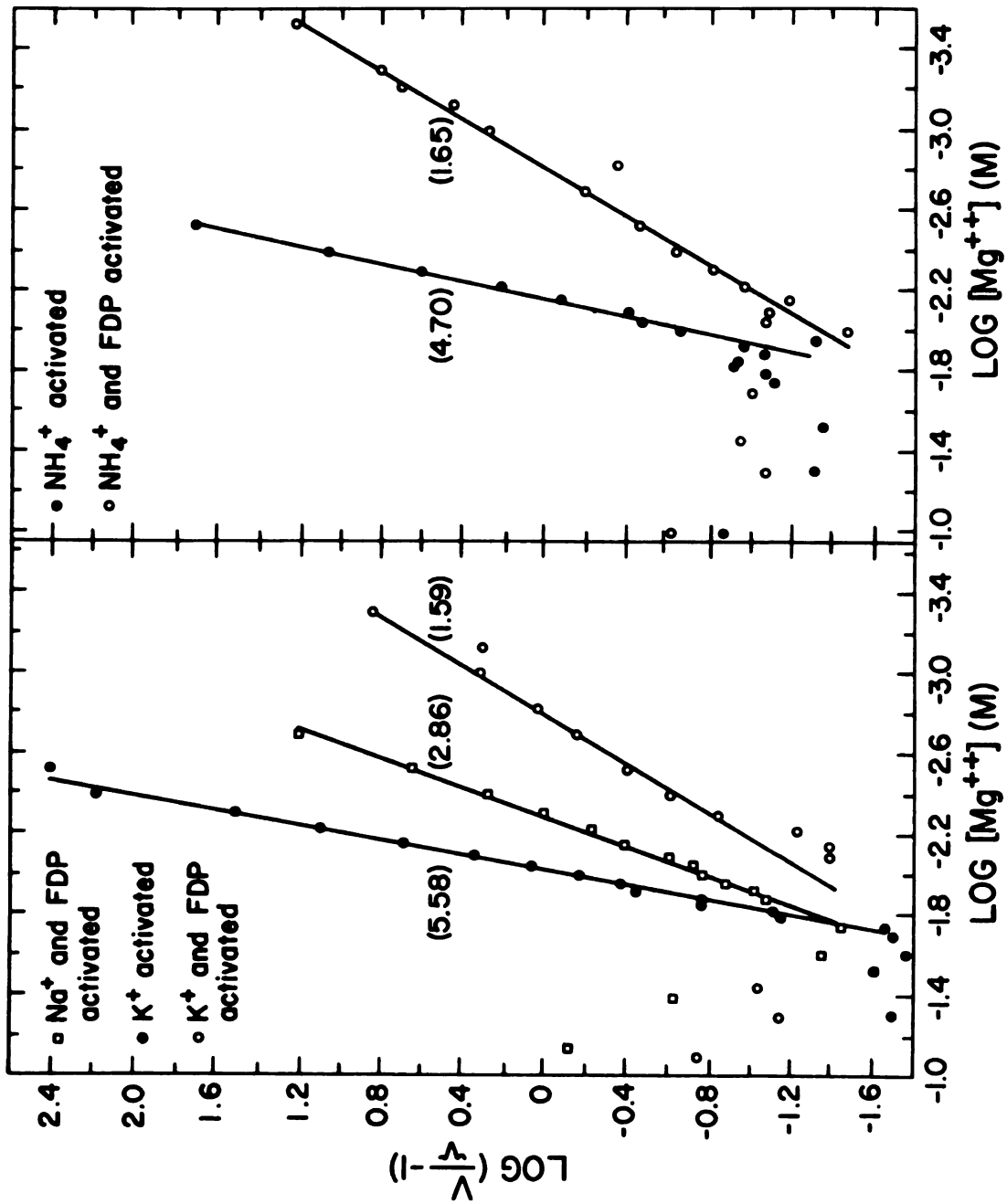

Na+ and FDP activated
K+ activated
K+ and FDP activated
(5.58)
(2.86)
(1.59)
NH4+ activated
NH4+ and FDP activated
(4.70)
(1.65)
LOG (ν/ν-1)
LOG [Mg++] (M)
LOG [Mg++] (M)

Figure 5

Figure 6. Relationship between initial velocity of yeast pyruvate kinase and total ADP concentration. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2; 24 μmoles $MgCl_2$; 5.0 μmoles triCHA PEP; 1.0 μmole $(CH_3)_4N$ FDP (when present); 33 μg lactic dehydrogenase; 0.15 μmole Na NADH; left, ●—●, 180 μmoles KCl, o—o, 100 μmoles KCl, and □—□, 170 μmoles NaCl; and right, ●—●, 50 μmoles NH_4Cl and o—o, 30 μmoles NH_4Cl. Hill slopes for the curves are shown in parentheses. ADP was added as the Tris salt (Hunsley and Suelter, 1969b).

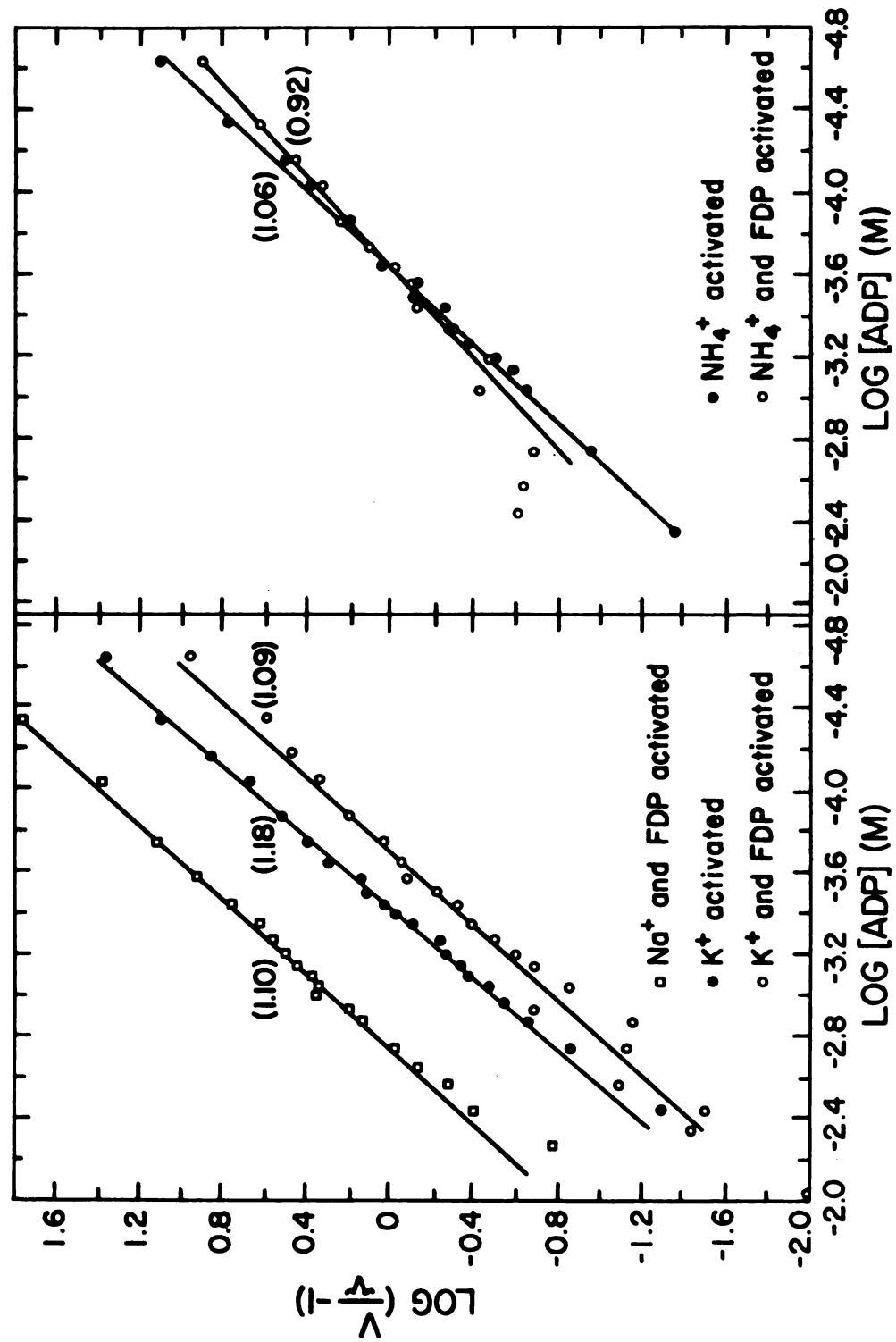

(1.06)
(0.92)
• NH_4^+ activated
○ NH_4^+ and FDP activated
LOG [ADP] (M)
(1.10)
(1.18)
(1.09)
▫ Na^+ and FDP activated
• K^+ activated
○ K^+ and FDP activated
LOG [ADP] (M)
LOG $(\frac{v}{v_f}-1)$

Figure 6

With all substrates saturating, FDP had no effect within experimental error on the observed maximal velocity of the reaction with K^+ or NH_4^+ as the activating monovalent cation (Figure 7). An analogous experiment was not possible for Na^+ since no activity with this ion is discernible in the absence of FDP (Table 5).

In Figure 8 are plotted the pH profiles of the K^+, K^+-FDP, and Na^+-FDP systems at variable PEP levels. Activity fell off rapidly on the acidic limb of the curves. The basic sides displayed complex profiles with discernible reproducible shoulders. In addition, the inclusion of FDP at low concentrations of PEP broadened the maxima in the basic pH range.

3. Kinetics of the Mn^{2+} activated system

pH 7.5 was chosen for the majority of these experiments because the salt-free enzyme had maximal stability at room temperature in this range. Concentrated solutions of the enzyme in 0.10 M Tris-HCl, pH 7.5, after 3 hours at 25° retained identical PEP and FDP kinetic parameters with a loss of total activity of about 10%. Binding experiments reported later in this thesis required such stability. At pH's below 7 the enzyme was very labile, even at room temperature.

Figure 9 illustrates the effect of varying Na^+ or K^+

Figure 7. Relationship between initial velocity of yeast pyruvate kinase and KCl or NH_4Cl concentrations. The assay mixture (1.00 ml) contained 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2, 24 μmoles $MgCl_2$, 12 μmoles triCHA PEP, 12 μmoles Tris ADP, 5.0 μmoles $(CH_3)_4N$ FDP (when present), 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH (Hunsley and Suelter, 1969b).

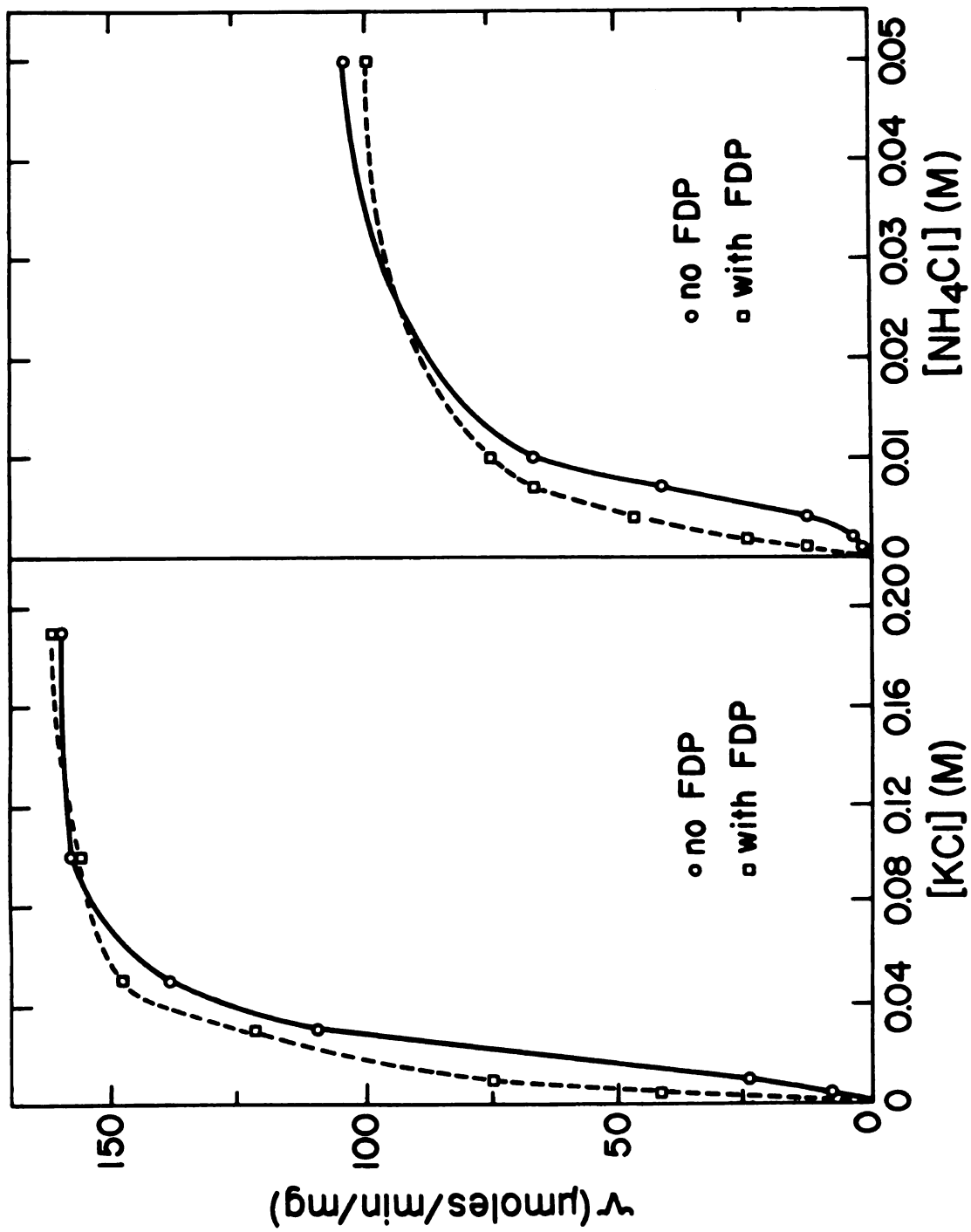

v (μmoles/min/mg)
150
100
50
0
0.04
0.08
0.12
0.16
0.200
[KCl] (M)
no FDP
with FDP
0.01
0.02
0.03
0.04
0.05
[NH4Cl] (M)
no FDP
with FDP

Figure 7

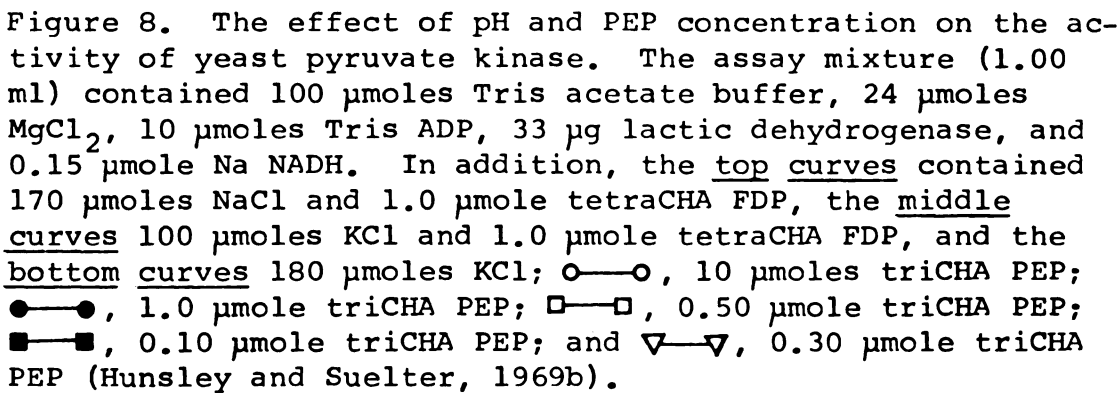


Figure 8. The effect of pH and PEP concentration on the activity of yeast pyruvate kinase. The assay mixture (1.00 ml) contained 100 µmoles Tris acetate buffer, 24 µmoles $MgCl_2$, 10 µmoles Tris ADP, 33 µg lactic dehydrogenase, and 0.15 µmole Na NADH. In addition, the top curves contained 170 µmoles NaCl and 1.0 µmole tetraCHA FDP, the middle curves 100 µmoles KCl and 1.0 µmole tetraCHA FDP, and the bottom curves 180 µmoles KCl; o—o, 10 µmoles triCHA PEP; ●—●, 1.0 µmole triCHA PEP; □—□, 0.50 µmole triCHA PEP; ■—■, 0.10 µmole triCHA PEP; and ▽—▽, 0.30 µmole triCHA PEP (Hunsley and Suelter, 1969b).

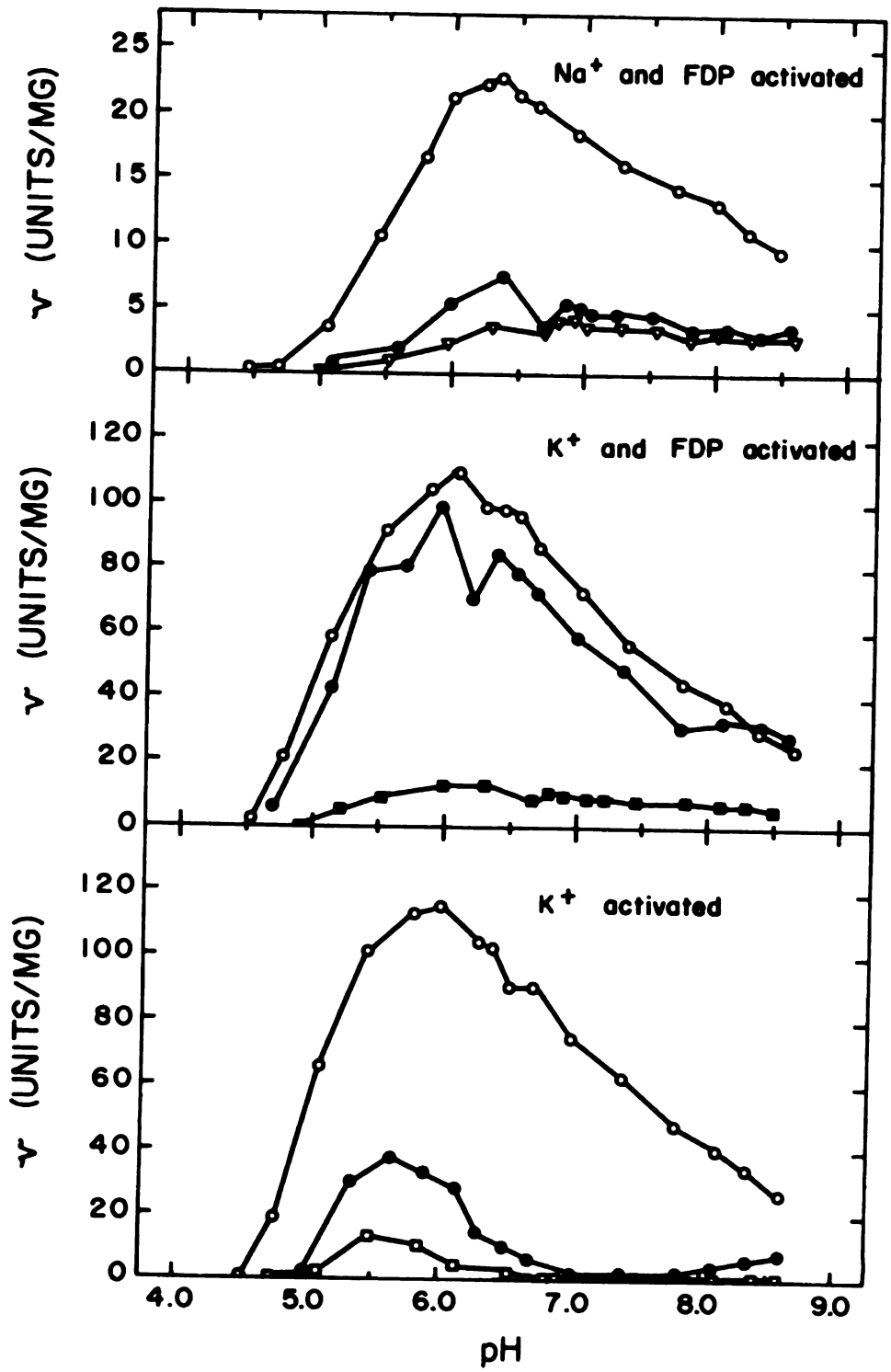

Na+ and FDP activated
K+ and FDP activated
K+ activated
v (UNITS/MG)
v (UNITS/MG)
v (UNITS/MG)
25
20
15
10
5
0
120
100
80
60
40
20
0
120
100
80
60
40
20
0
4.0
5.0
6.0
7.0
8.0
9.0
pH

Figure 8

Figure 9. Relationship between initial velocity of yeast pyruvate kinase and NaCl or KCl concentration. The assay (1.00 ml) contained 100 µmoles Tris-HCl, pH 7.5, 1.0 µmole $MnCl_2$, 1.0 µmole triCHA PEP, 1.0 µmole Tris ADP, 1.0 µmole tetraCHA FDP (when present), 33 µg lactic dehydrogenase, and 0.15 µmole Na NADH. Hill slopes for the curves are shown in parentheses.

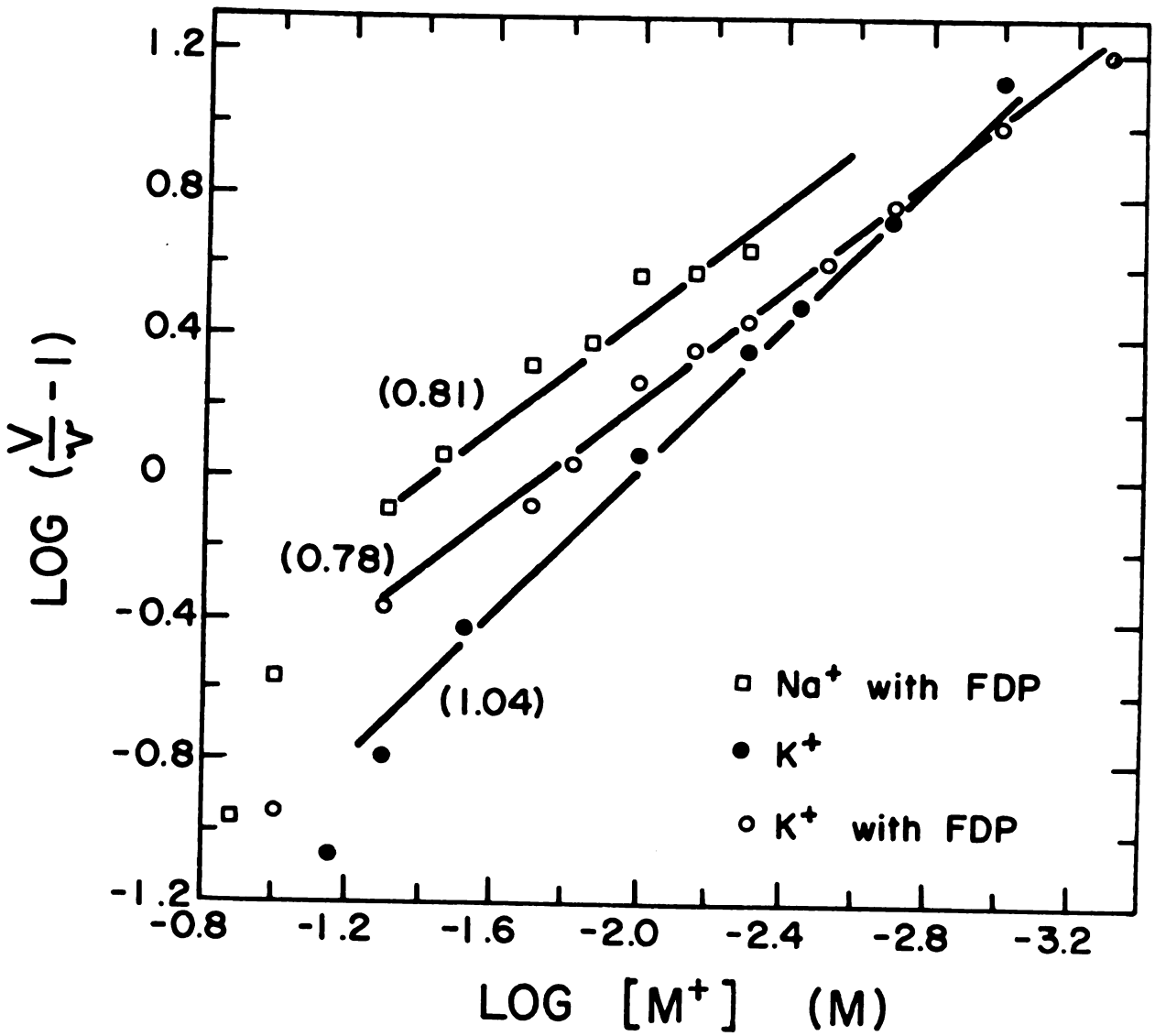

1.2
0.8
0.4
0
-0.4
-0.8
-1.2
LOG (V/V - 1)
(0.81)
(0.78)
(1.04)
Na+ with FDP
K+
K+ with FDP
-0.8
-1.2
-1.6
-2.0
-2.4
-2.8
-3.2
LOG [M+] (M)

Figure 9

concentration on pyruvate kinase catalysis. Here, in contrast to the Mg^{2+} system (Figure 2), FDP neither greatly decreased the cooperativity of K^+ activation nor affected binding. Hill slopes of less than 1.00, observed for Na^+ (0.81) and K^+ (0.78) in the presence of FDP, are indicative of negative cooperative phenomena (Levitzki and Koshland, 1969).

Since very small velocity changes could be noted upon addition of FDP to the kinetic system containing K^+, an experiment similar to that of Figure 3 could be performed only with Na^+ (Figure 10). In this case the FDP Hill slope was 1.20 indicating nearly linear kinetics.

The familiar heterotropic FDP activation of PEP and Mg^{2+} held true for the Mn^{2+} system (Figures 11-13). The response of the enzyme towards Mn^{2+} at pH 7.5 and 6.2 (Figures 12 and 13) closely resembled that towards Mg^{2+} at pH 6.2 (Figure 5), with the exception of the Na^+-dependent kinetics (Figure 13) which suggest a pH dependent enzyme-Mn interaction.

Again, under saturating conditions, little effect of FDP on ADP kinetics was observed (Figure 14).

4. Na-FDP kinetic interactions

Since, without FDP incorporation, no activity was observed with Na^+ as the activating monovalent cation, the

Figure 10. Relationship between initial velocity of yeast pyruvate kinase and total FDP concentration. The assay (1.00 ml) contained 100 μmoles Tris-HCl, pH 7.5, 1.0 μmole $MnCl_2$, 200 μmoles NaCl, 1.0 μmole triCHA PEP, 1.0 μmole Tris ADP, 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH. The Hill slope for the curve is shown in parentheses. FDP was added as the tetraCHA salt.

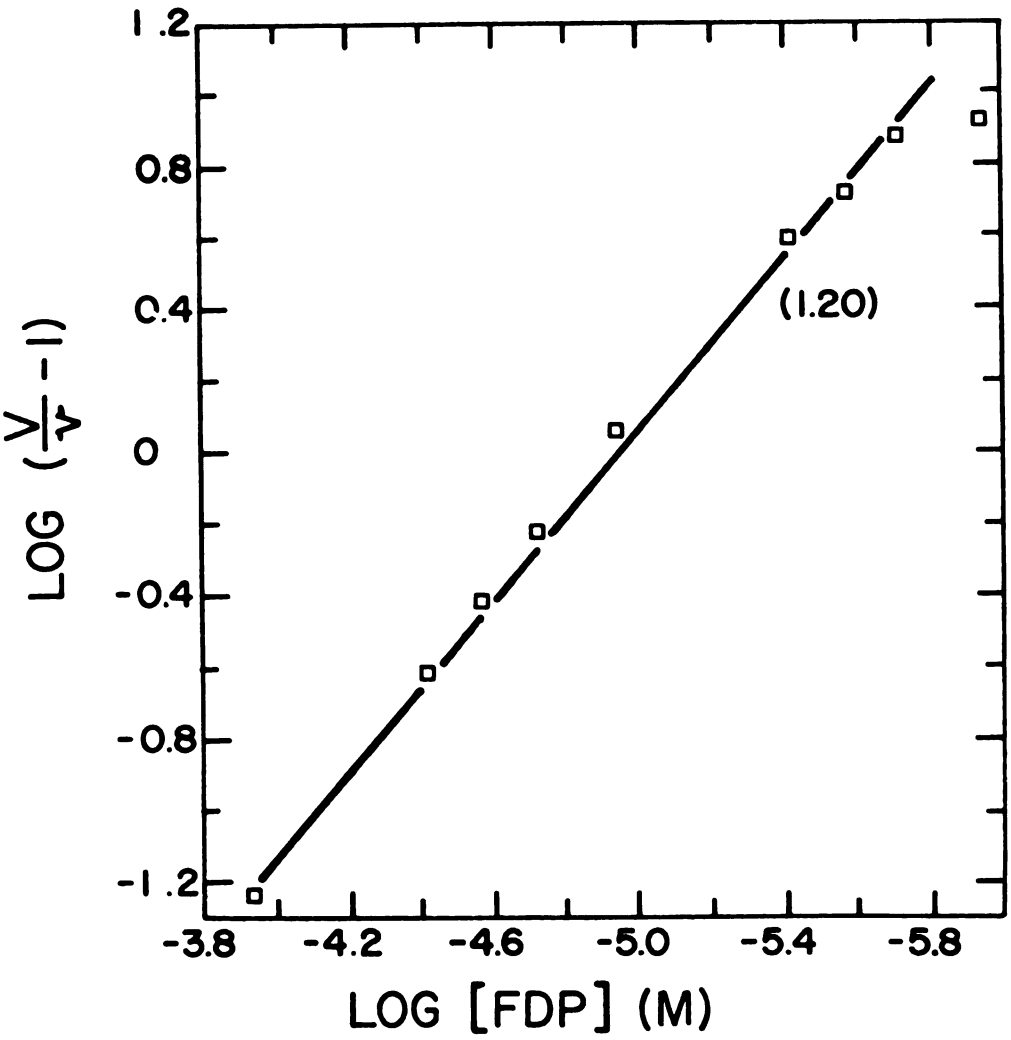


Figure 10

Figure 11. Relationship between initial velocity of yeast pyruvate kinase and total PEP concentration. The assay (1.00 ml) contained 100 μmoles Tris-HCl, pH 7.5, 1.0 μmole $MnCl_2$, 200 μmoles NaCl or KCl, 1.0 μmole Tris ADP, 1.0 μmole tetraCHA FDP (when present), 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH. Hill slopes for the curves are shown in parentheses. PEP was added as the triCHA salt.

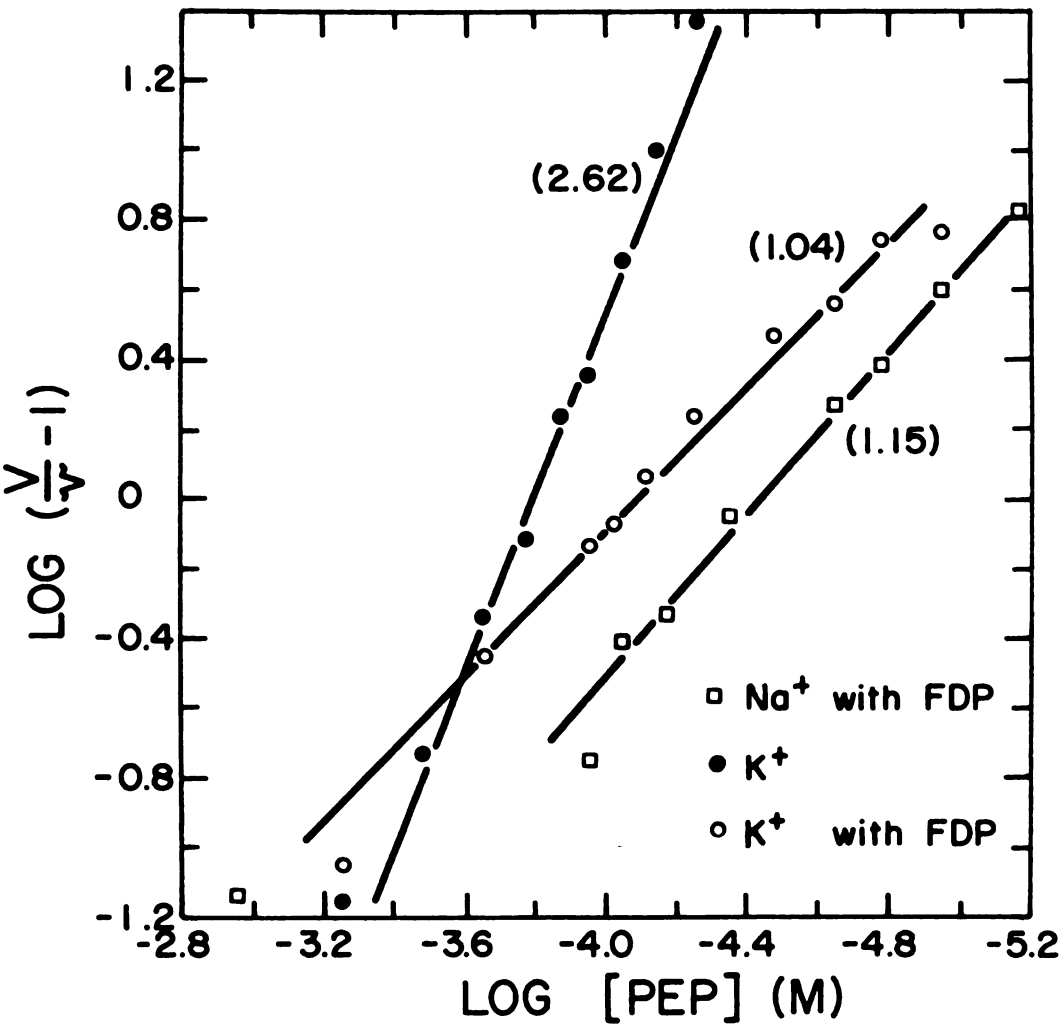

1.2
0.8
0.4
0
-0.4
-0.8
-1.2
LOG ($\frac{V}{V}$ - 1)
(2.62)
(1.04)
(1.15)
Na+ with FDP
K+
K+ with FDP
-2.8
-3.2
-3.6
-4.0
-4.4
-4.8
-5.2
LOG [PEP] (M)

Figure 11

Figure 12. Relationship between initial velocity of yeast pyruvate kinase and total $MnCl_2$ concentration. The assay (1.00 ml) contained 100 µmoles Tris-HCl, pH 7.5, 200 µmoles NaCl or KCl, 1.0 µmole triCHA PEP, 1.0 µmole Tris ADP, 1.0 µmole tetraCHA FDP (when used), 33 µg lactic dehydrogenase, and 0.15 µmole Na NADH. Hill slopes for the curves are shown in parentheses.

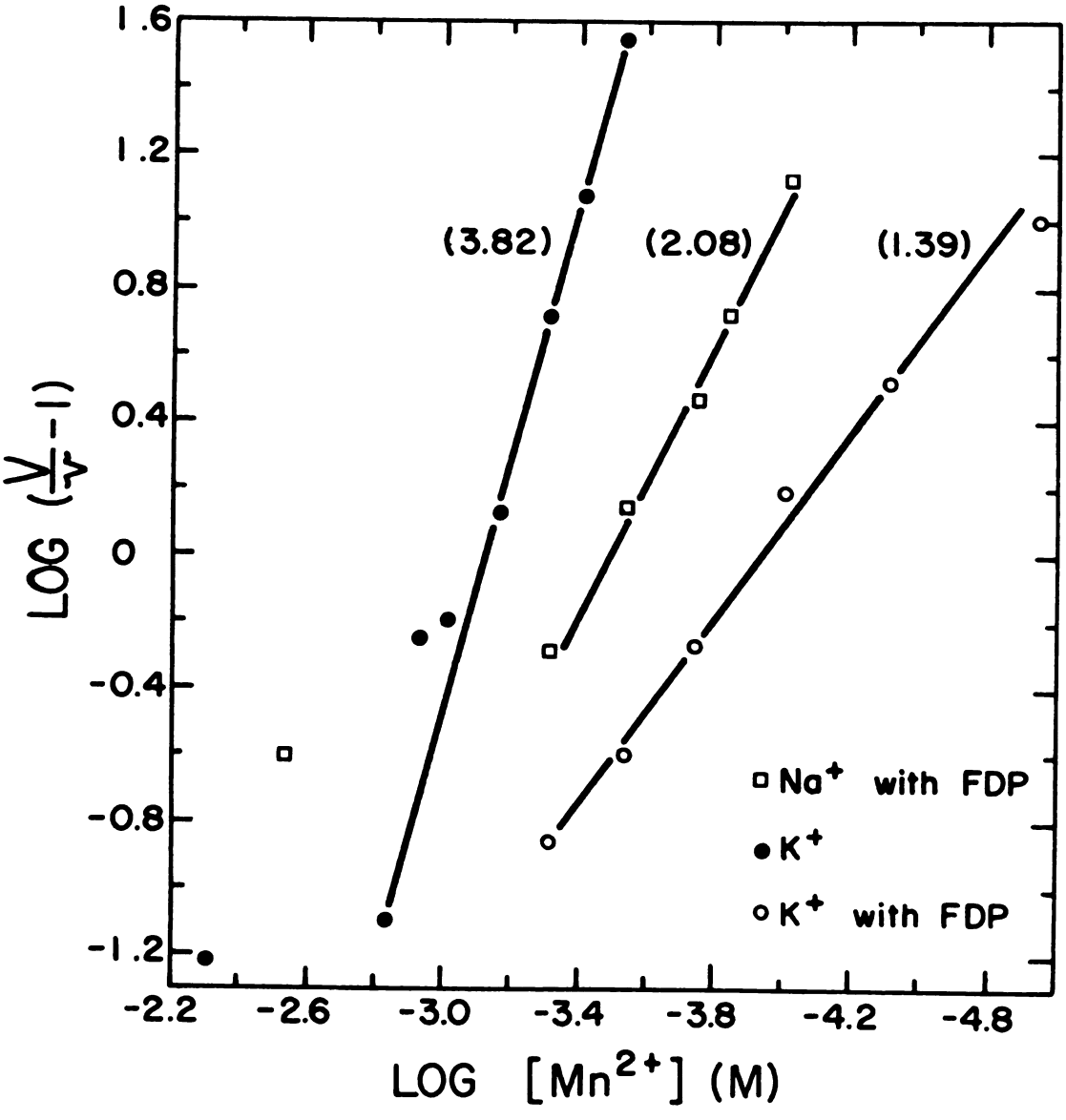

1.6
1.2
0.8
0.4
0
-0.4
-0.8
-1.2
LOG (V/v - 1)
(3.82)
(2.08)
(1.39)
Na+ with FDP
K+
K+ with FDP
-2.2
-2.6
-3.0
-3.4
-3.8
-4.2
-4.8
LOG [Mn2+] (M)

Figure 12

Figure 13. Relationship between initial velocity of yeast pyruvate kinase and total $MnCl_2$ concentration. The assay (1.00 ml) contained 100 µmoles $(CH_3)_4N$ cacodylate, pH 6.2; 5.0 µmoles triCHA PEP; 5.0 µmoles Tris ADP; 1.0 µmole tetraCHA FDP (when present); 33 µg lactic dehydrogenase; 0.15 µmole Na NADH; and □—□, 170 µmoles NaCl, ●—●, 180 µmoles KCl, and ○—○, 100 µmoles KCl. Hill slopes for the curves are shown in parentheses.

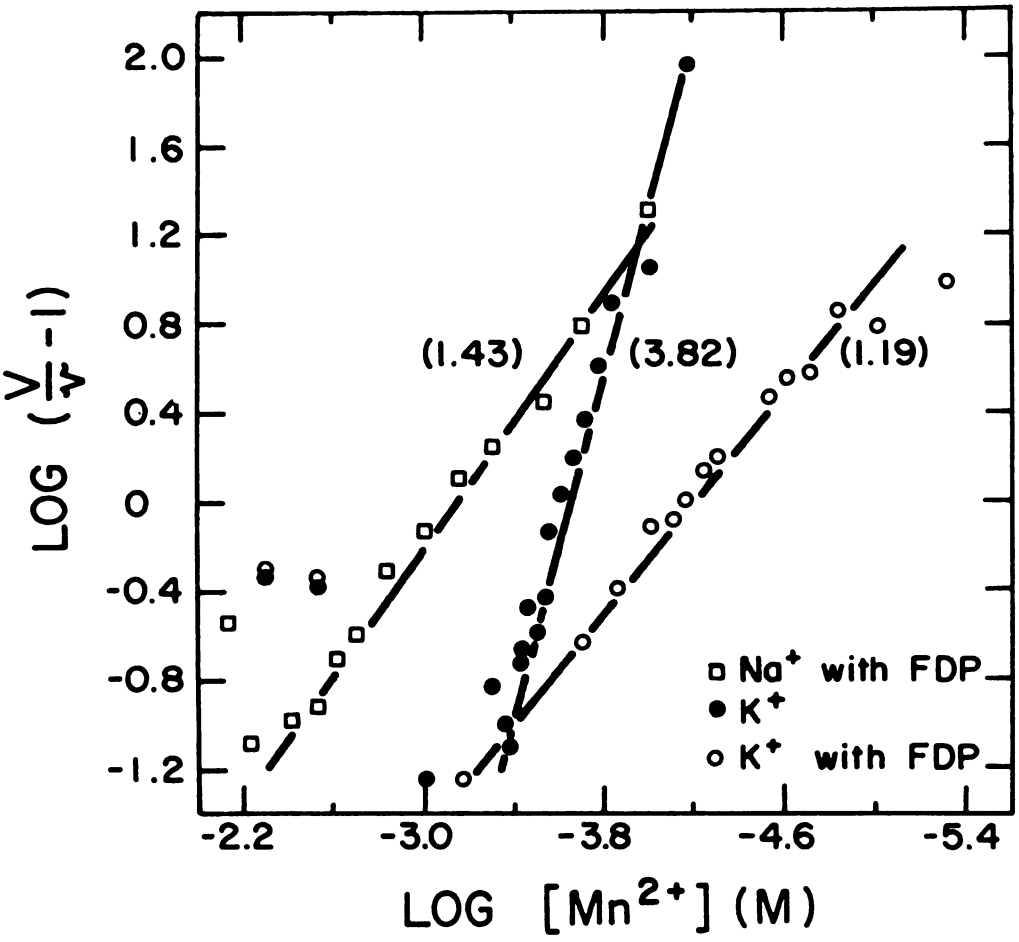

2.0
1.6
1.2
0.8
0.4
0
-0.4
-0.8
-1.2
LOG (V/V-1)
(1.43)
(3.82)
(1.19)
Na+ with FDP
K+
K+ with FDP
-2.2
-3.0
-3.8
-4.6
-5.4
LOG [Mn2+] (M)

Figure 13

Figure 14. Relationship between initial velocity of yeast pyruvate kinase and total ADP concentration. The assay mixture (1.00 ml) contained 100 μmoles Tris-HCl, pH 7.5, 1.0 μmole $MnCl_2$, 1.0 μmole triCHA PEP, 1.0 μmole tetraCHA FDP (when present), 200 μmoles NaCl or KCl, 33 μg lactic dehydrogenase, and 0.15 μmole Na NADH. Hill slopes for the curves are shown in parentheses. ADP was added as the Tris salt.

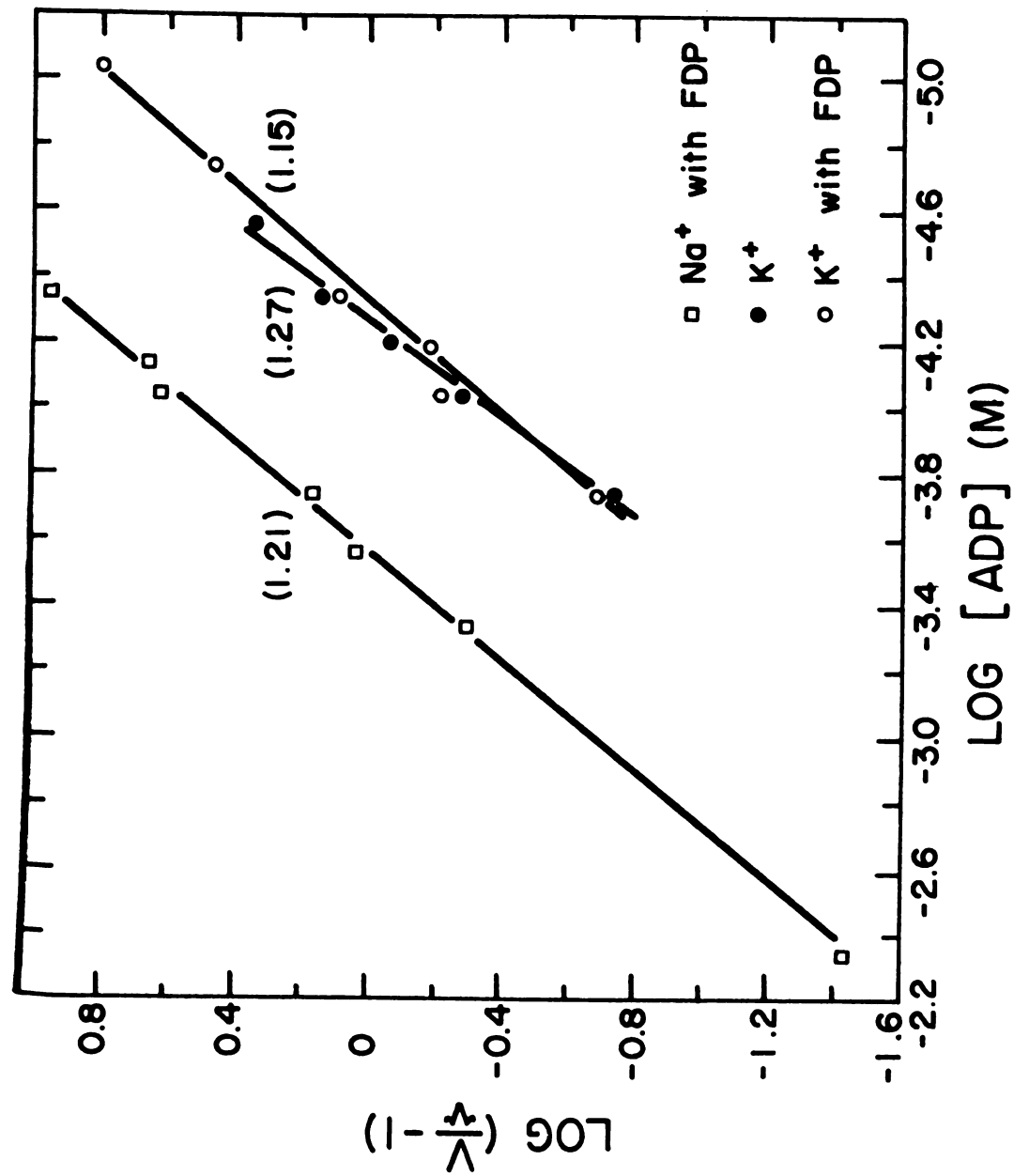

LOG (V/V* -1)
0.8
0.4
0
-0.4
-0.8
-1.2
-1.6
LOG [ADP] (M)
-2.2
-2.6
-3.0
-3.4
-3.8
-4.2
-4.6
-5.0
(1.15)
(1.27)
(1.21)
□ Na+ with FDP
● K+
○ K+ with FDP

Figure 14

study of the mutual kinetic dependence of these ligands was facilitated. Increasing concentrations of FDP proved to decrease the Hill slope for Na^+, but increase the apparent K_A. In Figure 15 are plotted the V_m's for a series of kinetic determinations at 5 different NaCl concentrations, each extrapolated to infinite FDP concentration. The K_A for NaCl at infinite FDP concentration was 100 mM. In contrast, changing the NaCl concentration had little effect either on n_H or the apparent K_A for FDP (Figure 16). The variation may be due to a nonspecific ionic strength effect since no attempt was made to control ionic strength in these cases.

5. Mn-substrate interactions

Results of studying the kinetic interrelationships of Mn^{2+} and ADP or Mn^{2+} and PEP and the effect of FDP on these interactions are given in Figures 17-24. For convenience the scales of each set of these two series of figures were identically drawn. Generally the following can be said about the results:

a. FDP increased the affinity of the enzyme towards Mn^{2+} at low concentrations of either substrate.

b. FDP reduced n_H for Mn^{2+} at high concentrations of either substrate.

c. FDP reduced n_H for PEP at high Mn^{2+} concentrations.

Figure 15. Lineweaver-Burk plot of effect of NaCl on V_m of yeast pyruvate kinase at infinite FDP concentration. Values of V_m including error were estimated from Hofstee plots (Hofstee, 1959) of enzyme velocity and variable FDP concentration at constant levels of NaCl. The assays (1.00 ml) contained 100 µmoles Tris-HCl, pH 7.5, 1.0 µmole $MnCl_2$, 1.0 µmole triCHA PEP, 1.0 µmole Tris ADP, 33 µg of lactic dehydrogenase, and 0.15 µmole Na NADH. FDP was added as the tetraCHA salt.

Figure 16. Relationship between NaCl concentration and K_A of yeast pyruvate kinase for FDP. Values of apparent K_A for FDP were estimated, including error, from Hill plots at constant NaCl concentrations. The plot is derived from Dixon (1953). Assay conditions were identical to those described for Figure 15.

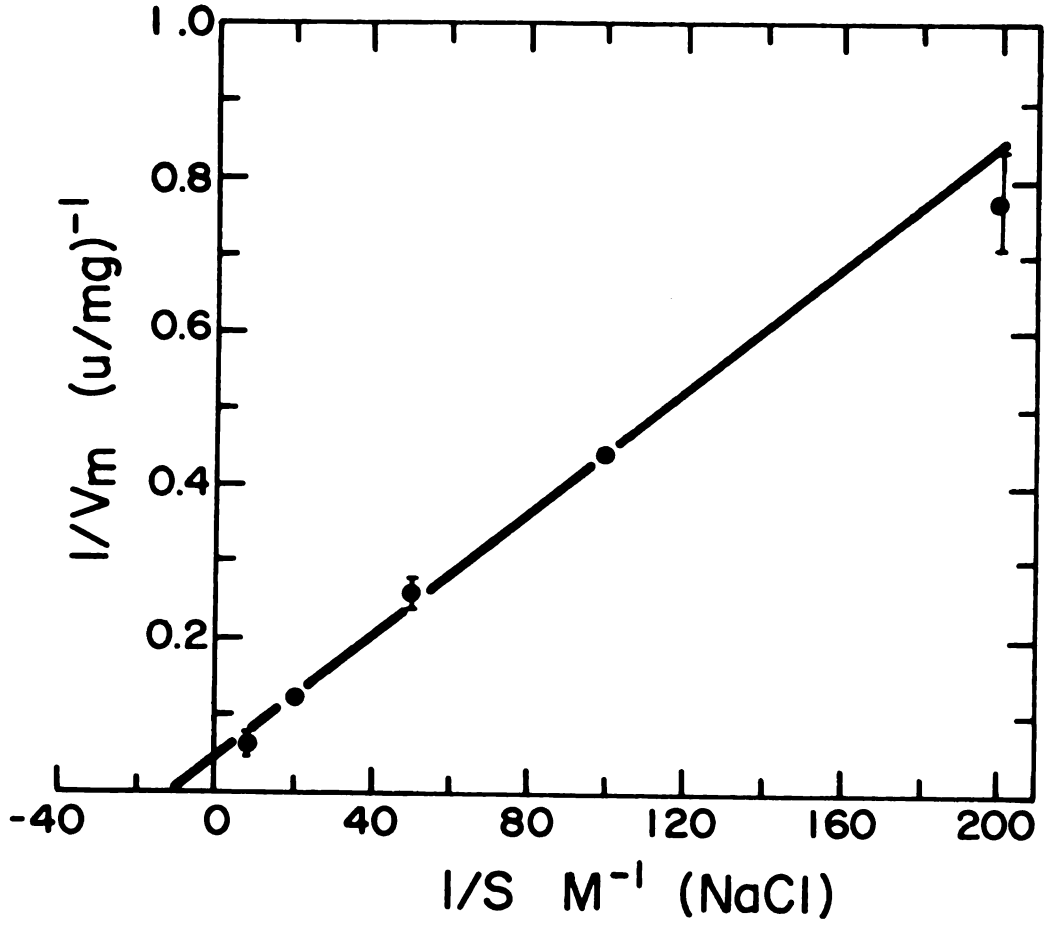


Figure 15

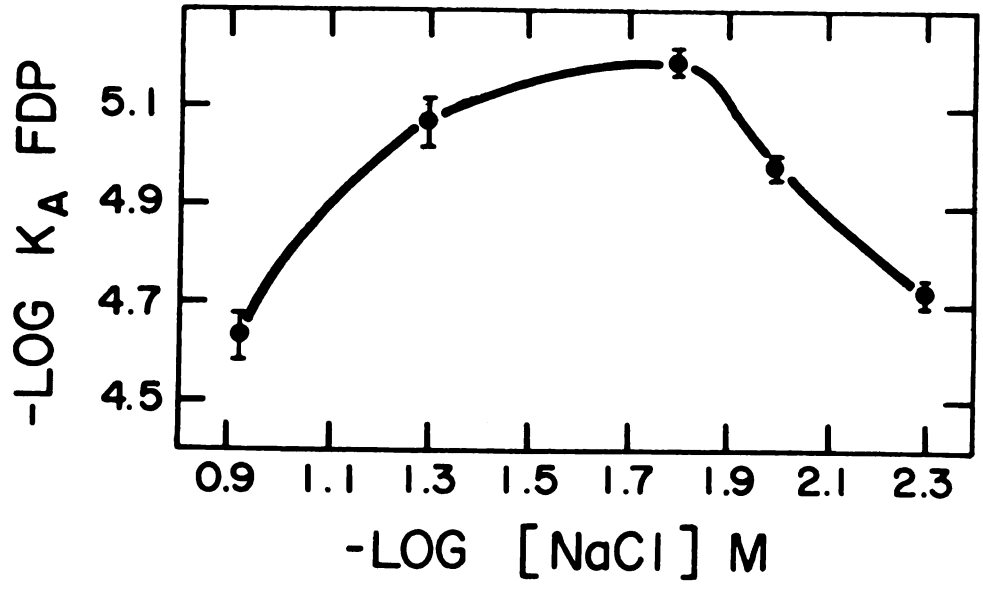


Figure 16

Figure 17. Relationship between Hill slope for Mn and total ADP concentration for yeast pyruvate kinase. Values of n_H were estimated from Hill plots, varying $MnCl_2$ concentration at constant levels of ADP. The assays (1.00 ml) contained 100 µmoles Tris-HCl, pH 7.5, 200 µmoles KCl, 1.0 µmole tri-CHA PEP, 1.0 µmole tetraCHA FDP (when present), 33 µg lactic dehydrogenase, and 0.15 µmole Na NADH. ADP was added as the Tris salt.

Figure 18. Relationship between total ADP concentration and apparent K_A of yeast pyruvate kinase for $MnCl_2$. Values of apparent K_A were estimated from Hill plots, varying $MnCl_2$ concentration at constant levels of ADP. Assay conditions were identical to those described for Figure 17. The plot is derived from Dixon (1953).

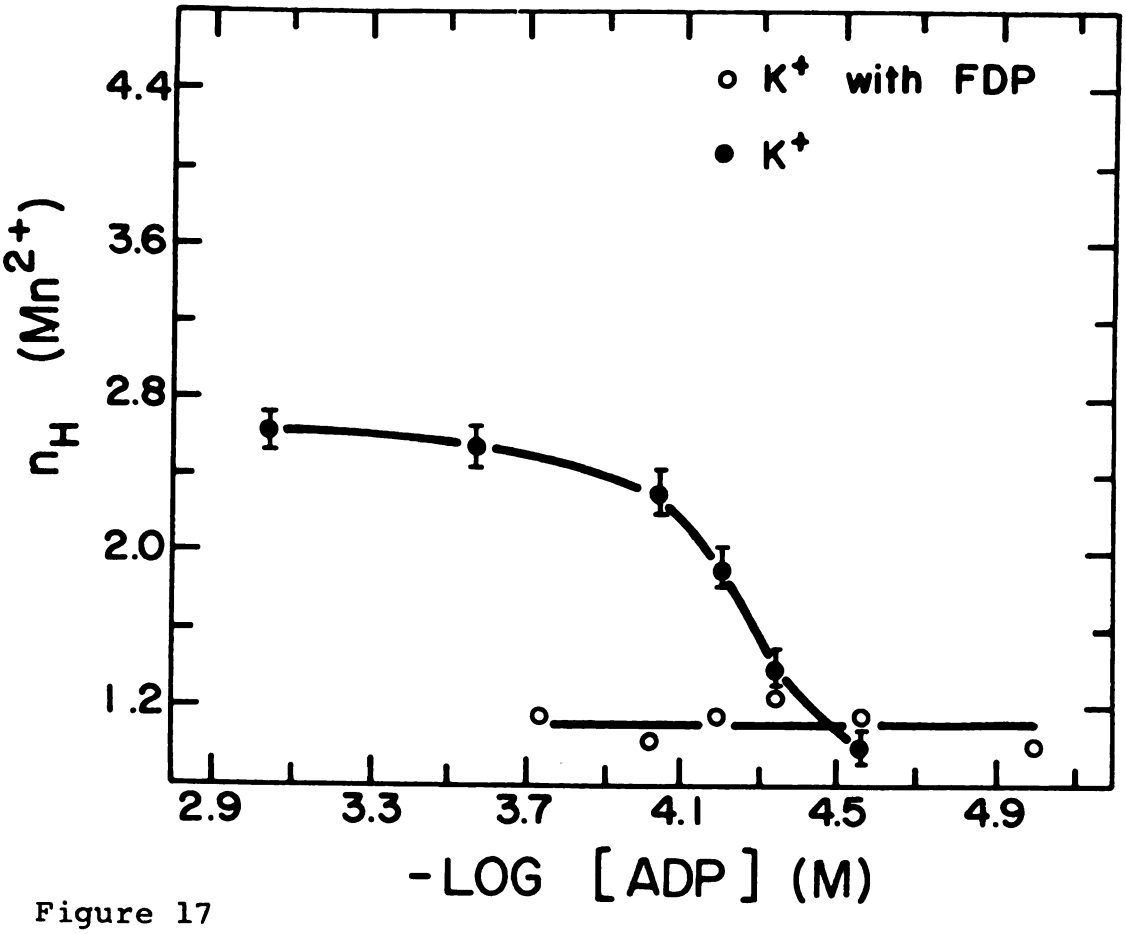


Figure 17

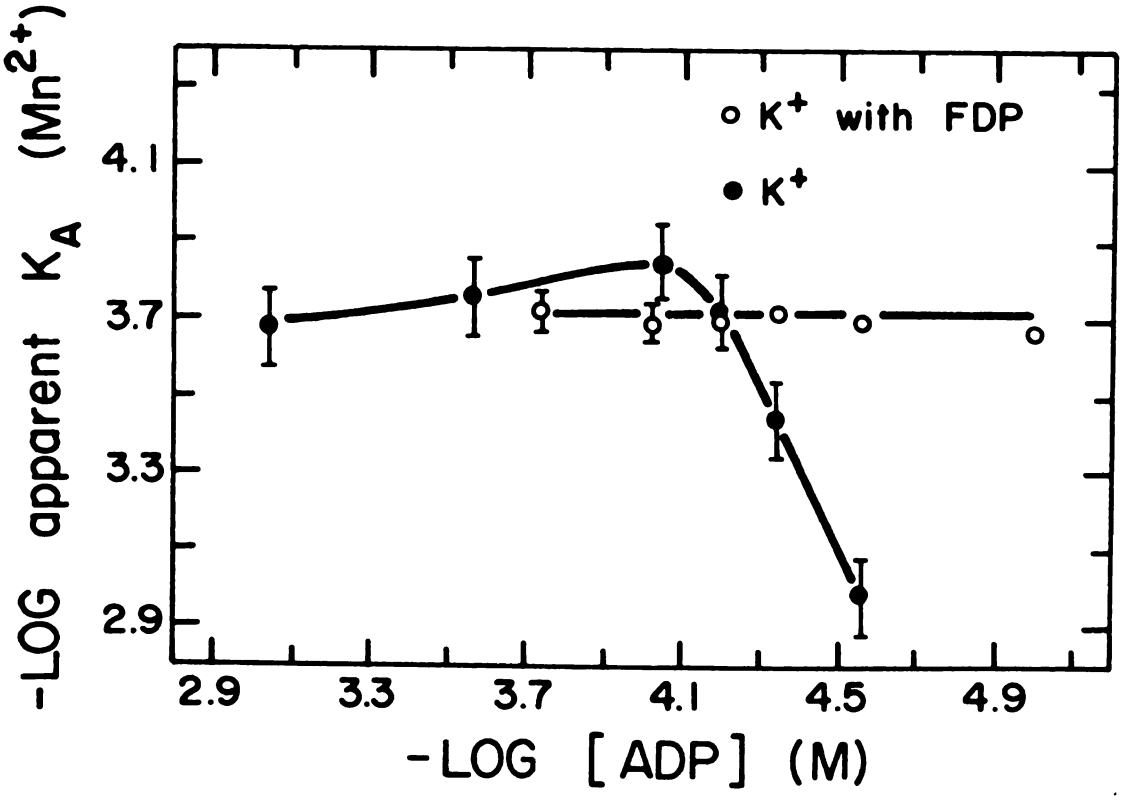


Figure 18

Figure 19. Relationship between Hill slope for ADP and total Mn concentration for yeast pyruvate kinase. Values of n_H were estimated from Hill plots, varying ADP concentration at constant levels of $MnCl_2$. Assay conditions were identical to those described for Figure 17.

Figure 20. Relationship between total Mn concentration and apparent K_m of yeast pyruvate kinase for ADP. Values of apparent K_m were estimated from Hill plots, varying total ADP concentration at constant levels of $MnCl_2$. Assay conditions were identical to those described for Figure 17. The plot is derived from Dixon (1953).

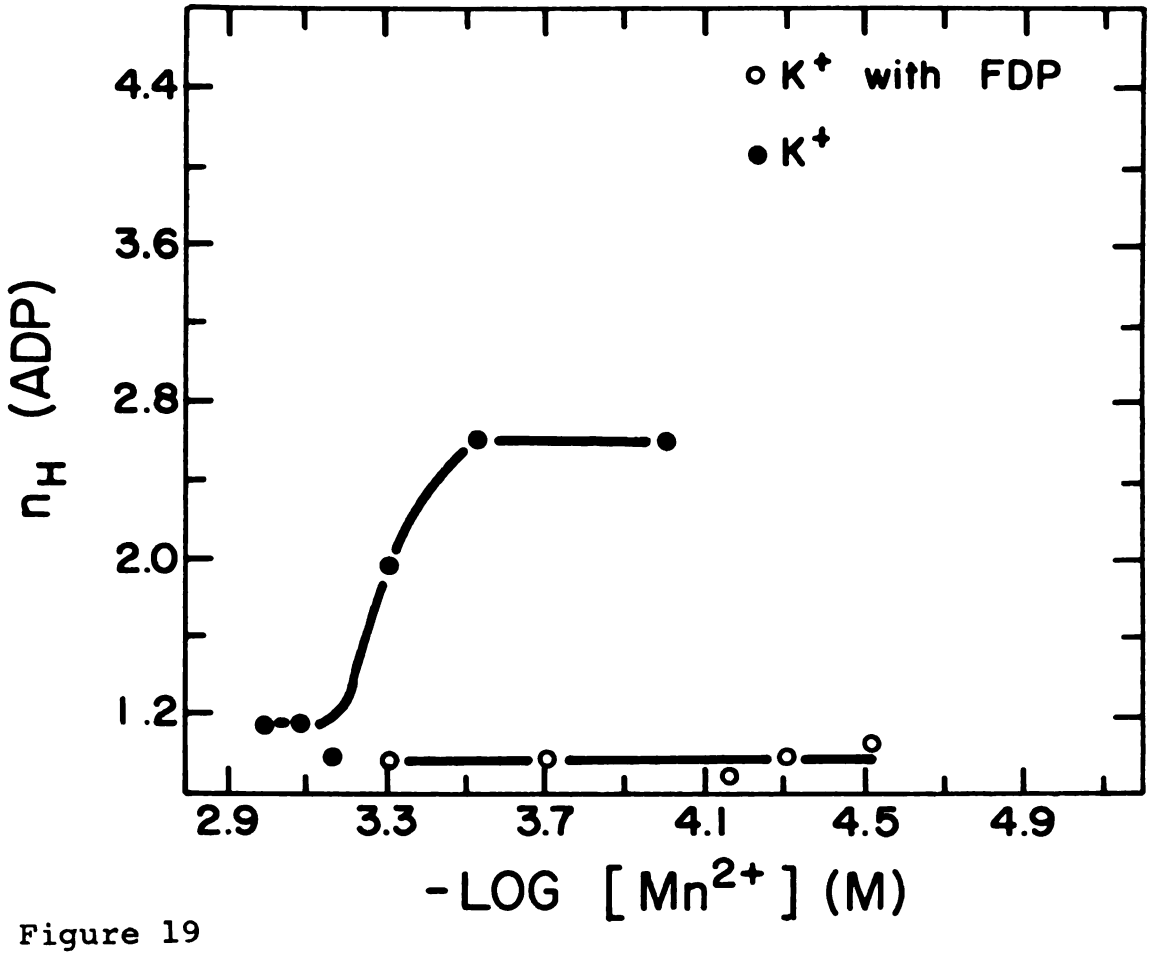


Figure 19

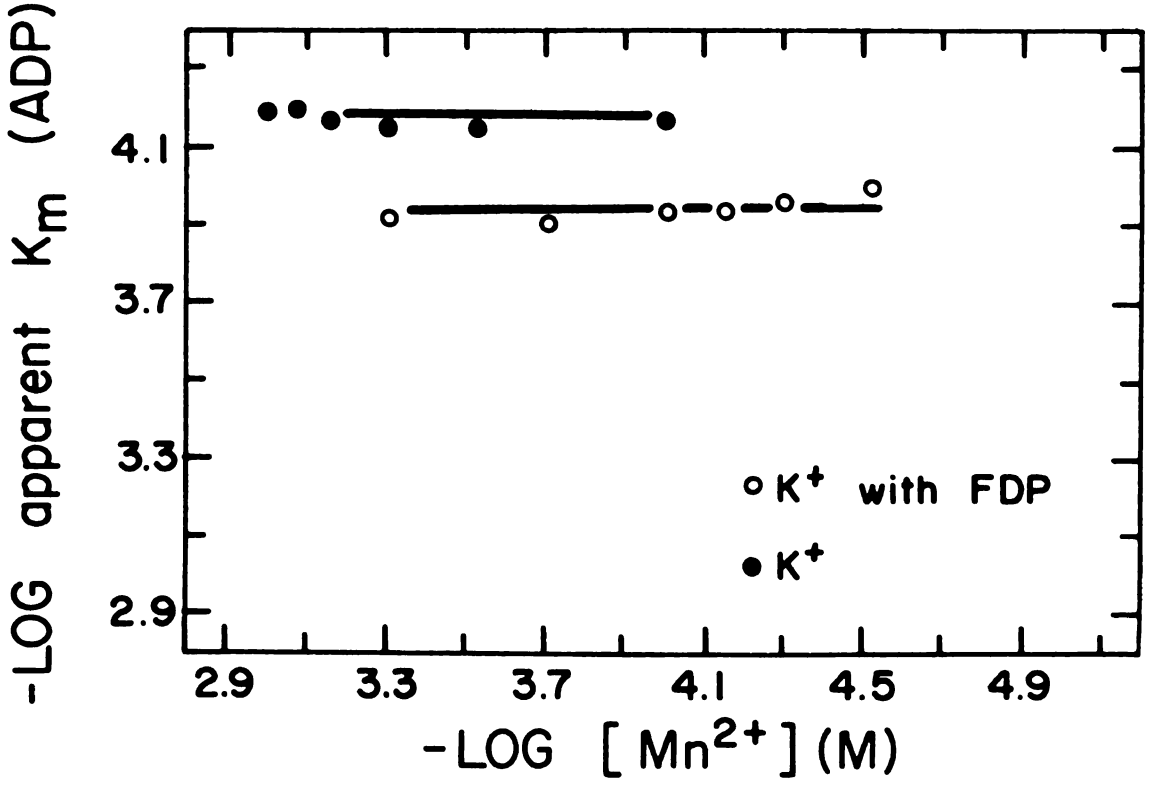


Figure 20

Figure 21. Relationship between Hill slope for Mn and total PEP concentration for yeast pyruvate kinase. Values of n_H were estimated from Hill plots, varying $MnCl_2$ concentration at constant levels of PEP. The assays (1.00 ml) contained 100 µmoles Tris-HCl, pH 7.5, 200 µmoles KCl, 1.0 µmole Tris ADP, 1.0 µmole tetraCHA FDP (when present), 33 µg lactic dehydrogenase, and 0.15 µmole Na NADH. PEP was added as the triCHA salt.

Figure 22. Relationship between total PEP concentration and apparent K_A of yeast pyruvate kinase for $MnCl_2$. Values of apparent K_A were estimated from Hill plots, varying $MnCl_2$ concentration at constant levels of PEP. Assay conditions were identical to those described for Figure 21. The plot is derived from Dixon (1953).

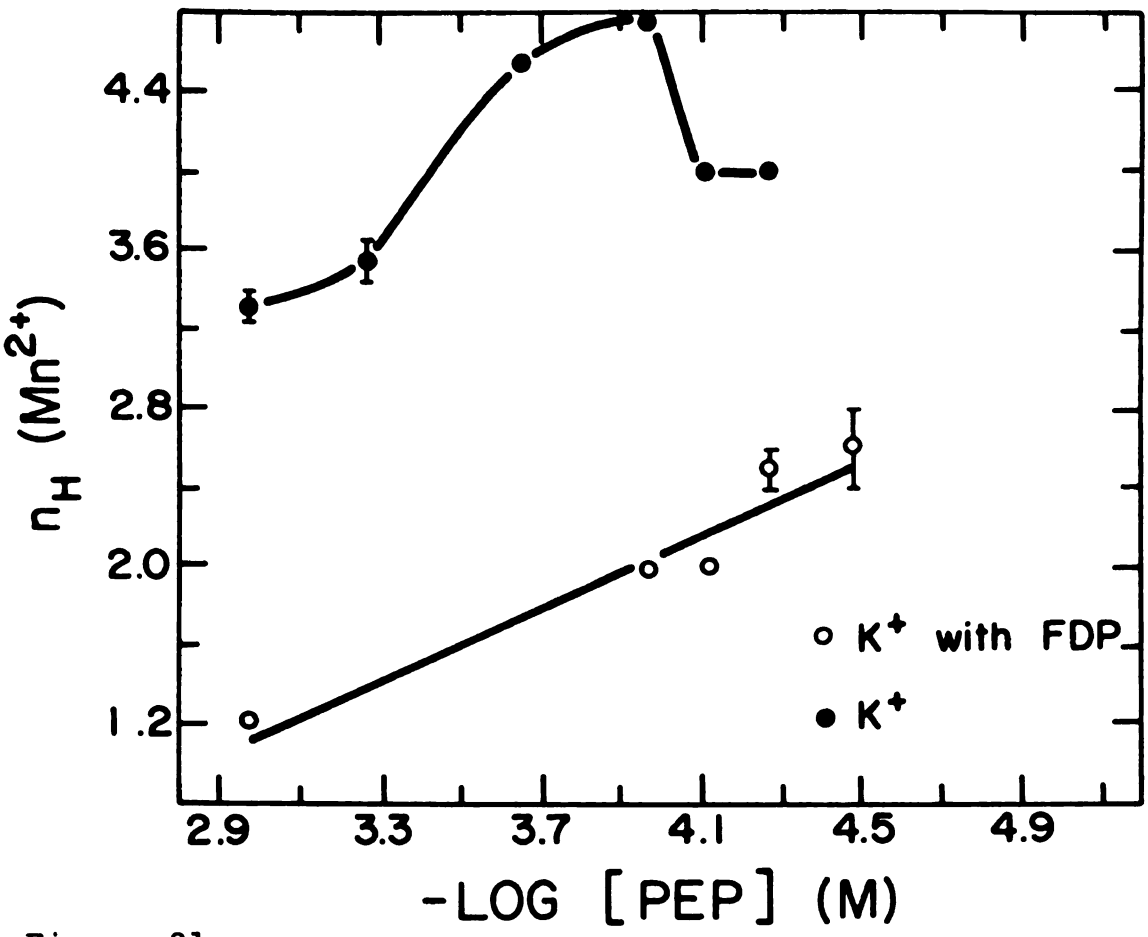


Figure 21

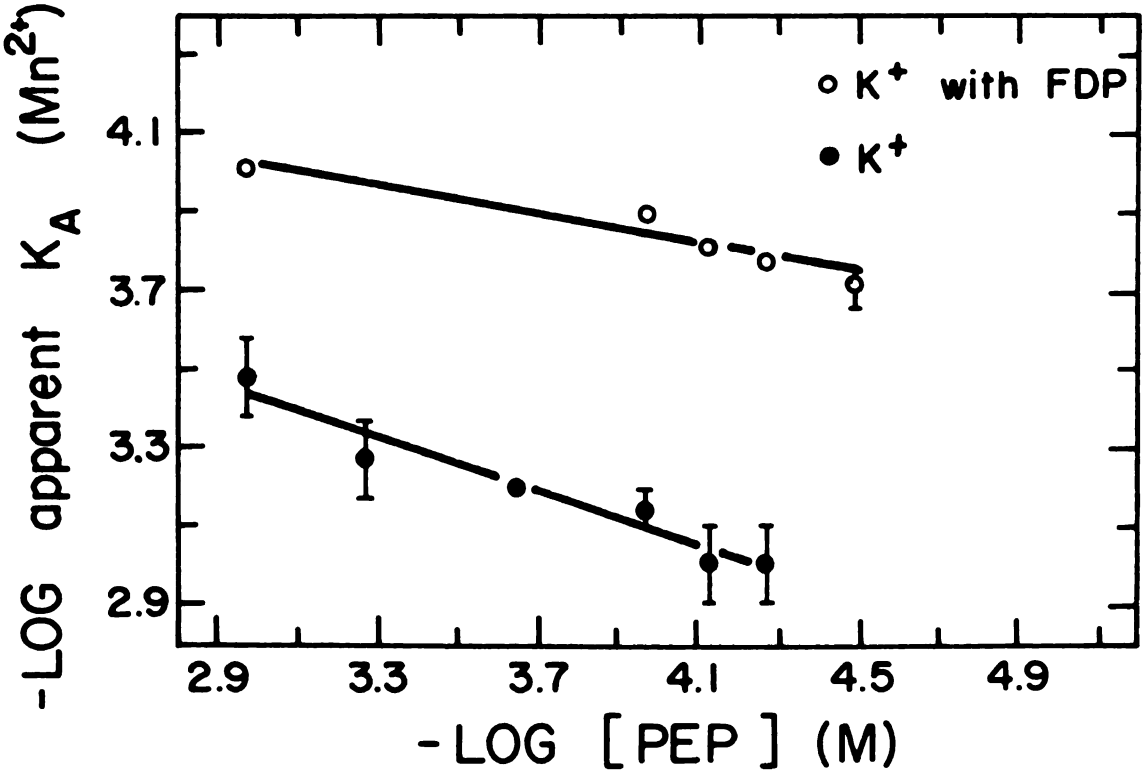


Figure 22

Figure 23. Relationship between Hill slope for PEP and total Mn concentration for yeast pyruvate kinase. Values of n_H were estimated from Hill plots, varying PEP concentration at constant levels of $MnCl_2$. Assay conditions were identical to those described for Figure 21.

Figure 24. Relationship between total Mn concentration and apparent K_m of yeast pyruvate kinase for PEP. Values of apparent K_m were estimated from Hill plots, varying total PEP concentration at constant levels of $MnCl_2$. Assay conditions were identical to those described for Figure 21. The plot is derived from Dixon (1953).

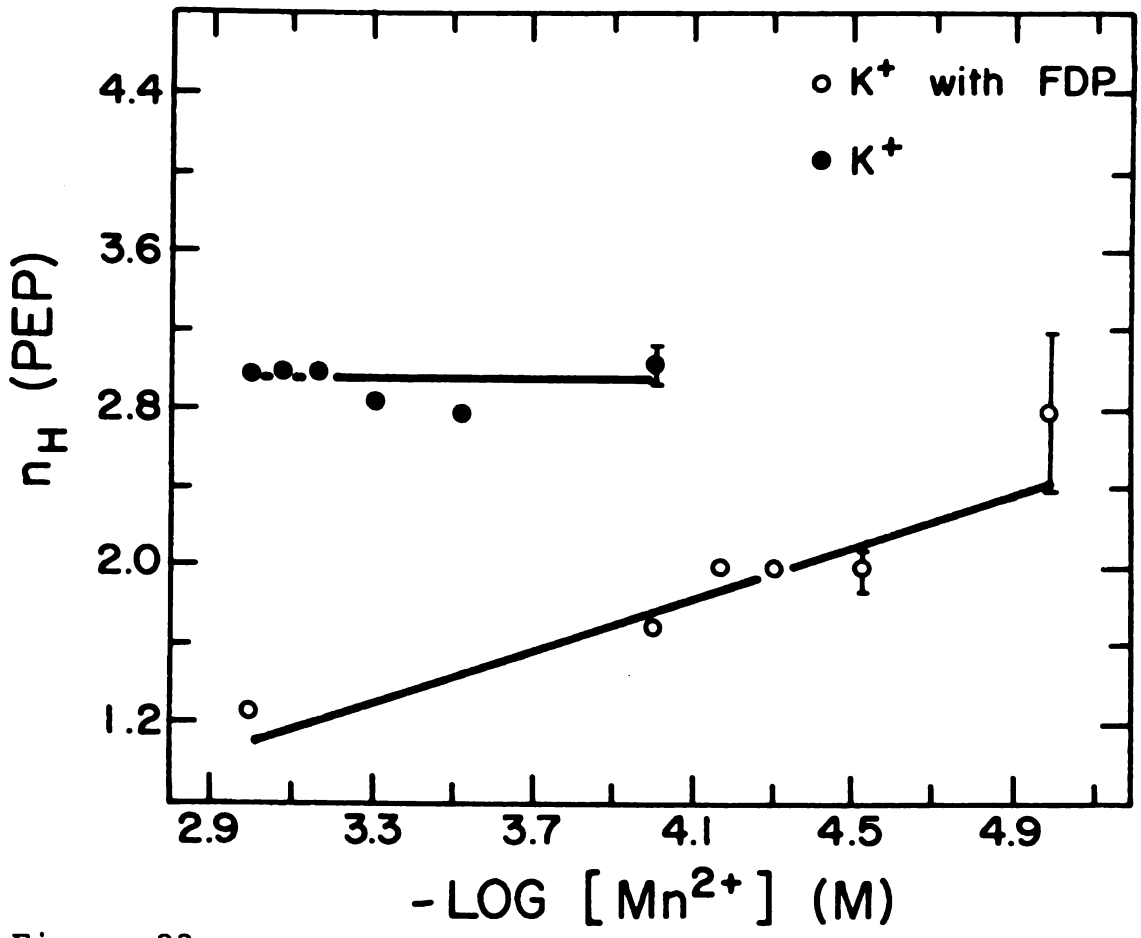


Figure 23

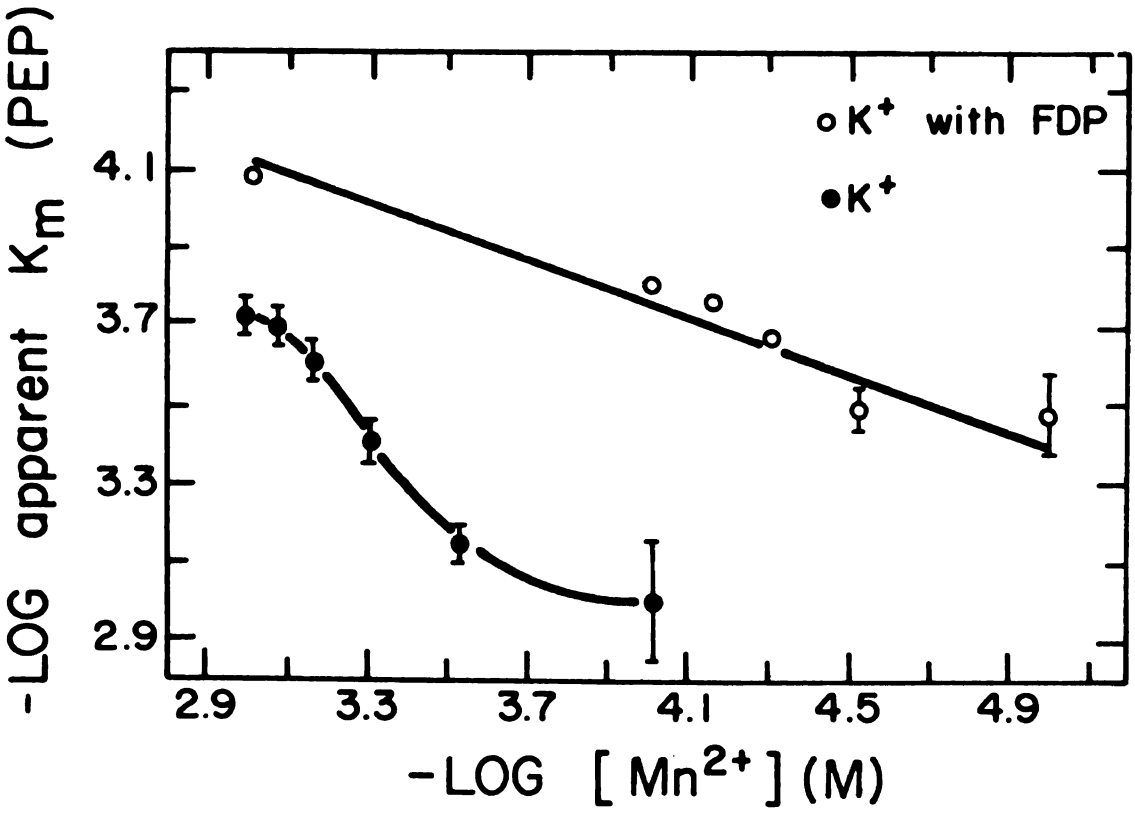


Figure 24

d. FDP reduced n_H for ADP at low Mn^{2+} concentrations.

6. Miscellaneous kinetic observations

Effect of glycerol-High concentrations of glycerol (20-50%) protect yeast pyruvate kinase from cold-induced denaturation (Hunsley and Suelter, 1969a). Inclusion of 20% (v/v) glycerol (2.7 M) in kinetic studies identical to those described in Figures 2, 4, and 5 eliminated heterotropic kinetics for K^+, PEP, and Mg^{2+}, mimicking the action of FDP.

Ca^{2+} activation-Activity with Ca^{2+} as the sole divalent cation was about 1% of the rates with optimum Mg^{2+} concentrations in the presence of saturating concentrations of FDP and KCl.

IV. Binding properties

1. Proton relaxation rate and electron paramagnetic resonance studies

Binary complex-Data for Mn^{2+} binding of yeast pyruvate kinase are given in a Scatchard plot (Figure 25). The points lie on a smooth anomalous curve and the enzyme binds up to 6 Mn^{2+} with apparent affinities which vary by 3 orders of magnitude.

The tightest binding site titrated atypically, giving a dissociation constant, K_D = 3 μM for the first third of the site and a K_D = 72 μM for the remaining two thirds. The

Figure 25. Scatchard plot of the binding of manganese to yeast pyruvate kinase. Solutions of the enzyme at 30° were in 50 mM Tris-HCl, pH 7.5, containing 200 mM KCl. The free and bound Mn were determined by the proton relaxation rate of water using a value of ϵ_b = 15.1 obtained by EPR and proton relaxation measurements as outlined in Methods and Materials (Mildvan et al., 1970). The curve is arbitrarily fit by four straight line segments. MPK is the titration curve which was obtained for rabbit muscle pyruvate kinase (Mildvan and Cohn, 1965).

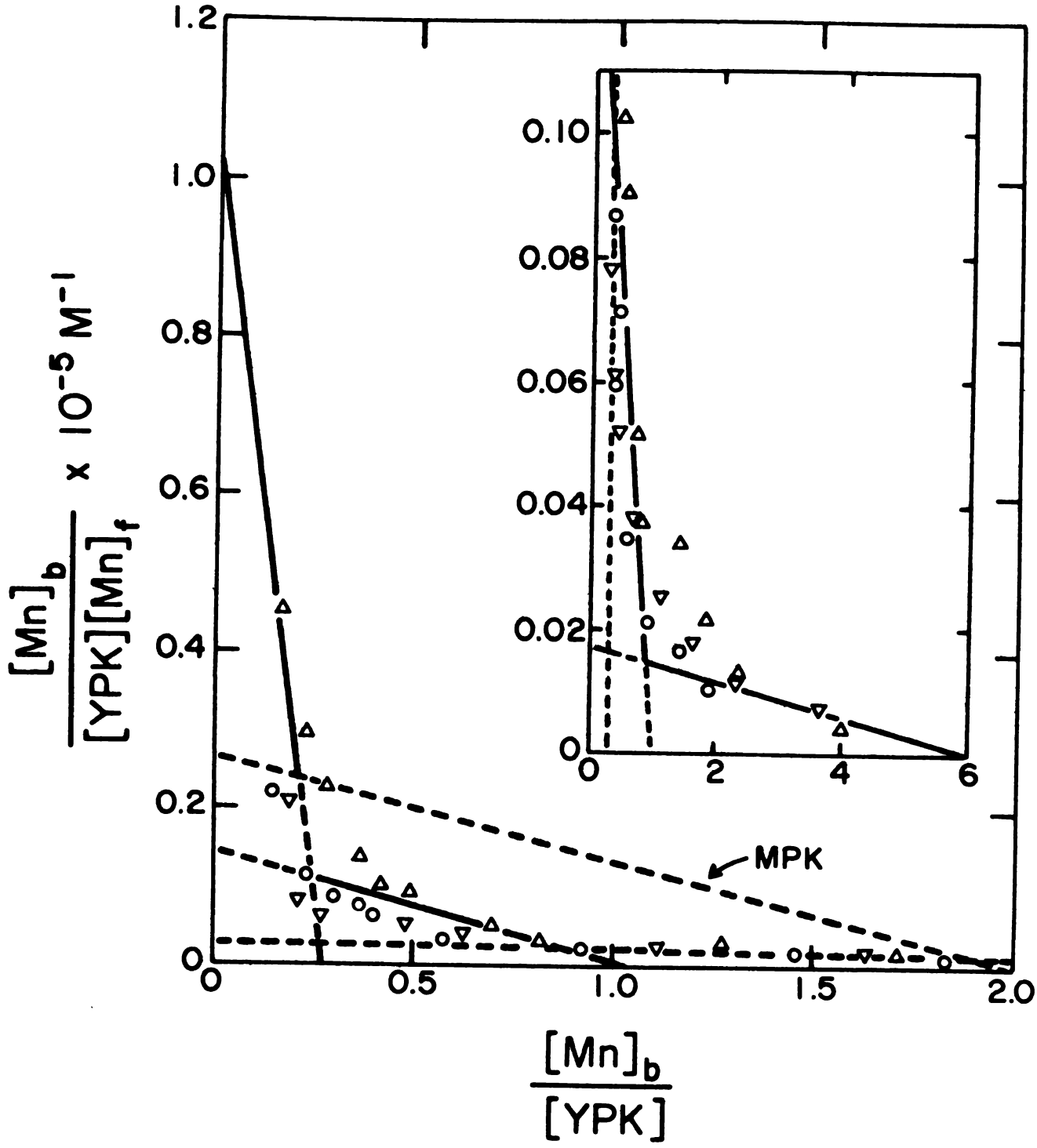

1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
1.5
2.0
0.10
0.08
0.06
0.04
0.02
0
0
2
4
6
MPK
$\frac{[Mn]_b}{[YPK][Mn]_f} \times 10^{-5}\ M^{-1}$
$\frac{[Mn]_b}{[YPK]}$

Figure 25

next two sites were weaker (K_D = 740 μM), and the last 3 sites were weakest (K_D = 1.8 mM) (Mildvan et al., 1970).

The site-site interaction detected in the Mn binding studies manifested itself only in the affinity but not in the enhancement (ϵ_b) of the bound manganese, which remains at 15.1 ± 1.8 (Table 6) as the occupancy of the binding sites increases from 0 to 6. The corresponding value of ϵ_b for the rabbit muscle enzyme is 32.7 ± 3.2 (Mildvan and Cohn, 1965).

Ternary complexes-The addition of all substrates to the yeast pyruvate kinase-Mn complex decreased the enhanced effect of Mn^{2+} on the proton relaxation rate of water (Table 6), suggesting the formation of enzyme-Mn-substrate bridge complexes as had been found for the rabbit muscle enzyme (Mildvan and Cohn, 1966). The addition of FDP lowered the ϵ value of the enzyme-Mn complex indicating an alteration of the environment of enzyme bound manganese.

Substrate titrations, measuring the relaxation rate of water protons, were carried out at low [Mn]/[E] (0.5 to 0.9) and at high [Mn]/[E] (3.5). The resulting values of K_3 and ϵ are given in Table 6 as E(Mn)S and $E(Mn)_3S$ complexes, respectively. Table 6 also gives the apparent K_m values and Hill coefficients obtained kinetically with the Mn^{2+} activated enzyme.

Table 6. Binary and ternary complexes of yeast pyruvate kinase with manganese and substrates. Dissociation constants K_D and K_3 and ϵ are derived from proton relaxation rate experiments as described in Methods and Materials. n_H and apparent K_m data are determined from kinetic experiments (Mildvan et al., 1970).

Complex	Activator[a]	Dissociation constant (μM) Binding K_D or K_3	Dissociation constant (μM) Kinetics $K_{0.5}$[b]	Hill Slope n_H	ϵ
$E(Mn)_6$	-	Variable (4-7200)	-	-	17.0
	K^+	Variable (3-1800)	200-400[c]	2.6-3.3	15.1
	FDP	Variable (10-1870)	100-200[c]	1.1	10.6
E(Mn)ADP	K^+	200	60[d]	2.6	13.0
	K^+ + FDP	260	130[d]	1.0	> 5.5
$E(Mn)_3$ADP	-	1270	-	-	<17.0
	K^+	332	60[e]	2.6	<15.1
	FDP	<1210	130[e]	1.0	<10.6
E(Mn)PEP	K^+	21	1000[f]	3.0	12.5
	K^+ + FDP	460	200[f]	1.9	5.2
$E(Mn)_3$PEP	-	376	-	-	<17.0
	K^+	324	800[g]	3.0	<15.1
	FDP	72	120[g]	1.5	<10.6

Table 6 (continued)

E(Mn)ATP	K^+	32	-	-	<15.1
E(Mn)pyruvate	K^+	765	-	-	<15.1

[a]The activators were 0.10-0.20 M KCl or 1.1 mM tetraCHA FDP or both. In absence of activator, 0.10 M tetramethylammonium chloride was used. Other components present were 50 mM Tris-HCl, pH 7.5. T = 30 ± 1° C.

[b]$K_{0.5}$ (apparent K_m, apparent K_A) is the concentration of the variable required for half maximal observed velocity.

[c]In presence of 1 mM Tris ADP and 1 mM triCHA PEP.

[d]Expressed in terms of total Tris ADP in presence of 63 μM Mn and 1mM triCHA PEP.

[e]Expressed in terms of total Tris ADP in presence of 190 μM Mn and 1 mM triCHA PEP.

[f]Expressed in terms of total triCHA PEP in presence of 63 μM Mn and 1 mM Tris ADP.

[g]Expressed in terms of total triCHA PEP in presence of 190 μM Mn and 1 mM Tris ADP.

No significant effect of FDP on the binding behavior of ADP was detected at low or high concentrations of Mn^{2+}. The dissociation constants of ADP measured in the binding studies are significantly greater than those estimated from kinetics.

At low concentrations of Mn^{2+}, FDP weakened the binding of PEP contrary to the behavior of the kinetic data. At high concentrations, FDP tightened the binding of PEP in qualitative agreement with the kinetic observations. Hence $E(Mn)_3$ shows a similar response to the allosteric activator, FDP, in both the binding and kinetic studies while E(Mn) does not. The monovalent activator K^+ appeared to raise the affinity of the $E(Mn)_3$ complex for ADP but had no effect on the binding of PEP (Mildvan et al., 1970).

2. Lack of tight FDP binding

In order to demonstrate the absence of tightly bound FDP to yeast pyruvate kinase, enzyme was incubated with FDP-^{14}C in the presence of $MgCl_2$ (to preserve stability) and chromatographed over Sephadex G-25 (Figure 26). The stoichiometry of FDP binding in the protein peak ranged from 0.01 to 0.1 moles FDP per 165,800 g of enzyme, indicating a lack of tight FDP binding ($K_D < \sim 10^{-8}$ M).

In addition, borohydride reduction of a mixture of enzyme, FDP, and $MgCl_2$ failed to alter the kinetic response of

Figure 26. Lack of binding of FDP to yeast pyruvate kinase. A mixture (0.60 ml total volume) of 6.07 mg of enzyme, 6.0 µmoles $MgCl_2$, 0.60 µmole tetraCHA FDP, 7.0×10^{-5} mC ammonium FDP-^{14}C (specific activity 69 mC/mmole) and 60 µmoles Tris-HCl, pH 7.5, was incubated 15 minutes at 25° and 0.50 ml chromatographed at the same temperature over medium G-25 Sephadex, eluting with 0.10 M Tris-HCl, pH 7.5. Aliquots of collected fractions were assayed for protein concentration and radioactivity measured by scintillation counting as described in Methods and Materials.

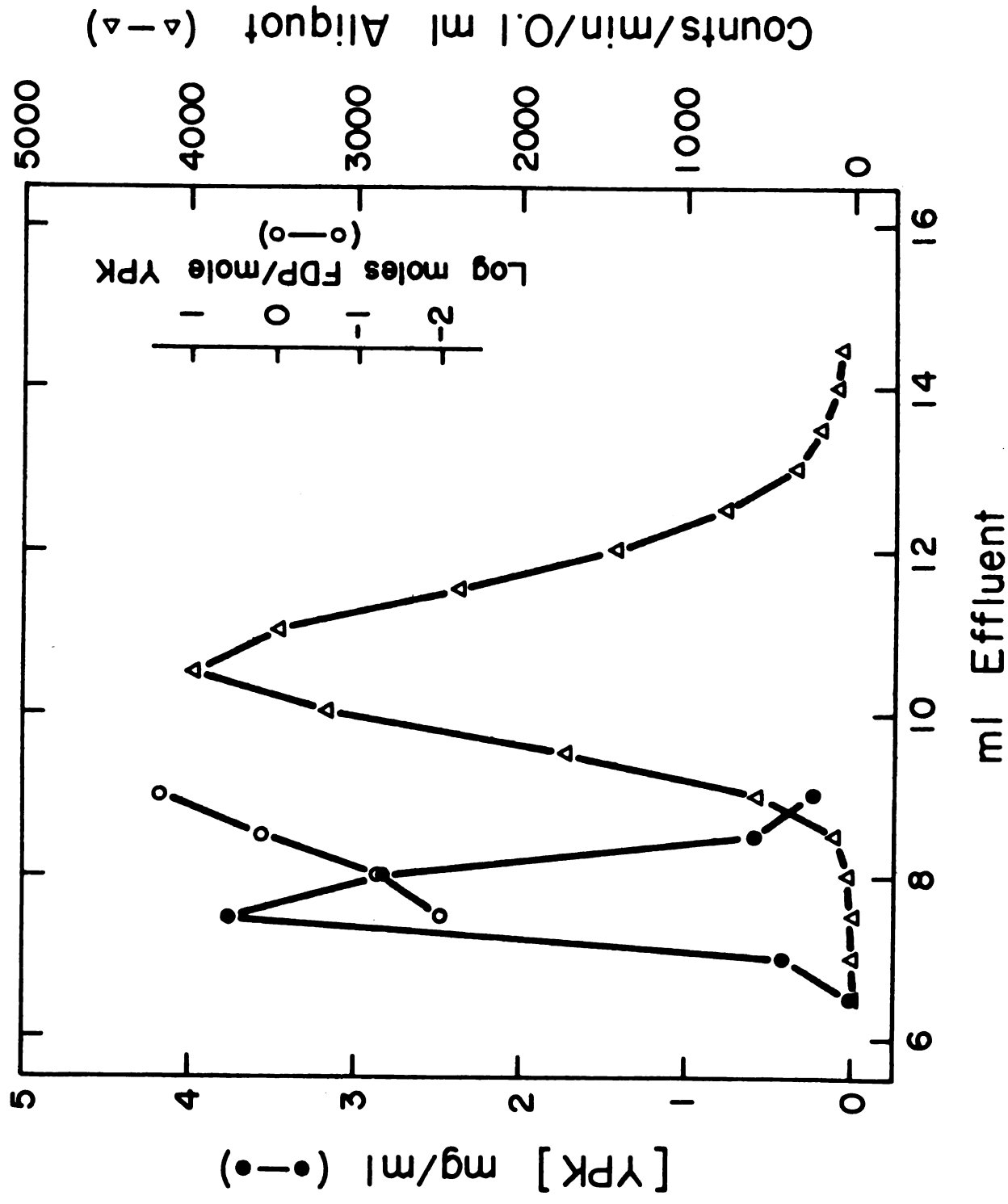

Counts/min/0.1 ml Aliquot (▵—▵)
5000
4000
3000
2000
1000
0
Log moles FDP/mole YPK
(o—o)
-2
-1
0
[YPK] mg/ml (•—•)
5
4
3
2
1
0
6
8
10
12
14
16
ml Effluent

Figure 26

the enzyme toward FDP. A Schiff base FDP binding mechanism could be dismissed (Horecker et al., 1963).

V. Isoelectric focusing

1. Preparative focusing

Two peaks of approximately equal distribution result from preparative isoelectric focusing of purified yeast pyruvate kinase (Figure 27) as monitored by maximal activity assays (±FDP). The isoelectric points of the peaks were about 6.2 and 6.4 and specific activities of the peaks ranged in the 450 to 500 μmoles/min per mg range by a nephlometric protein assay (Methods and Materials). Examination of the kinetic properties of these two peaks revealed no differences by two sensitive diagnostic tests: the PEP saturation curve in the presence of KCl at pH 6.2 (similar to Figure 4) and the extent of FDP activation at pH 6.2 in the presence of 10 mM K^+ (similar to Figure 2).

2. Microisoelectric focusing

Summarized in Figures 28 and 29 are an extensive series of microisoelectric focusing runs of yeast pyruvate kinase in polyacrylamide gel columns. Focusing of 200 μg each of $(NH_4)_2SO_4$ enzyme suspension (A), salt-free enzyme (B), $(NH_4)_2SO_4$ enzyme suspension in persulfate catalyzed gels (C), and phenylmethanesulfonyl fluoride treated enzyme (D) yielded identical patterns: two bands of equal intensity.

Figure 27. Preparative isoelectric focusing of yeast pyruvate kinase. 5.0 mg of $(NH_4)_2SO_4$-free enzyme was focused at 20^{o} for 72 hours at 700 volts in a linear 20% to 60% (v/v) glycerol gradient. pH range 5-8 ampholytes titrated to pH 7.2 with $(CH_3)_4NOH$ were used at a concentration of 1%. 0.75 ml fractions were collected at the end of the experiment, the pH measured, and enzymatic activity determined by both standard assays as described in Methods and Materials.

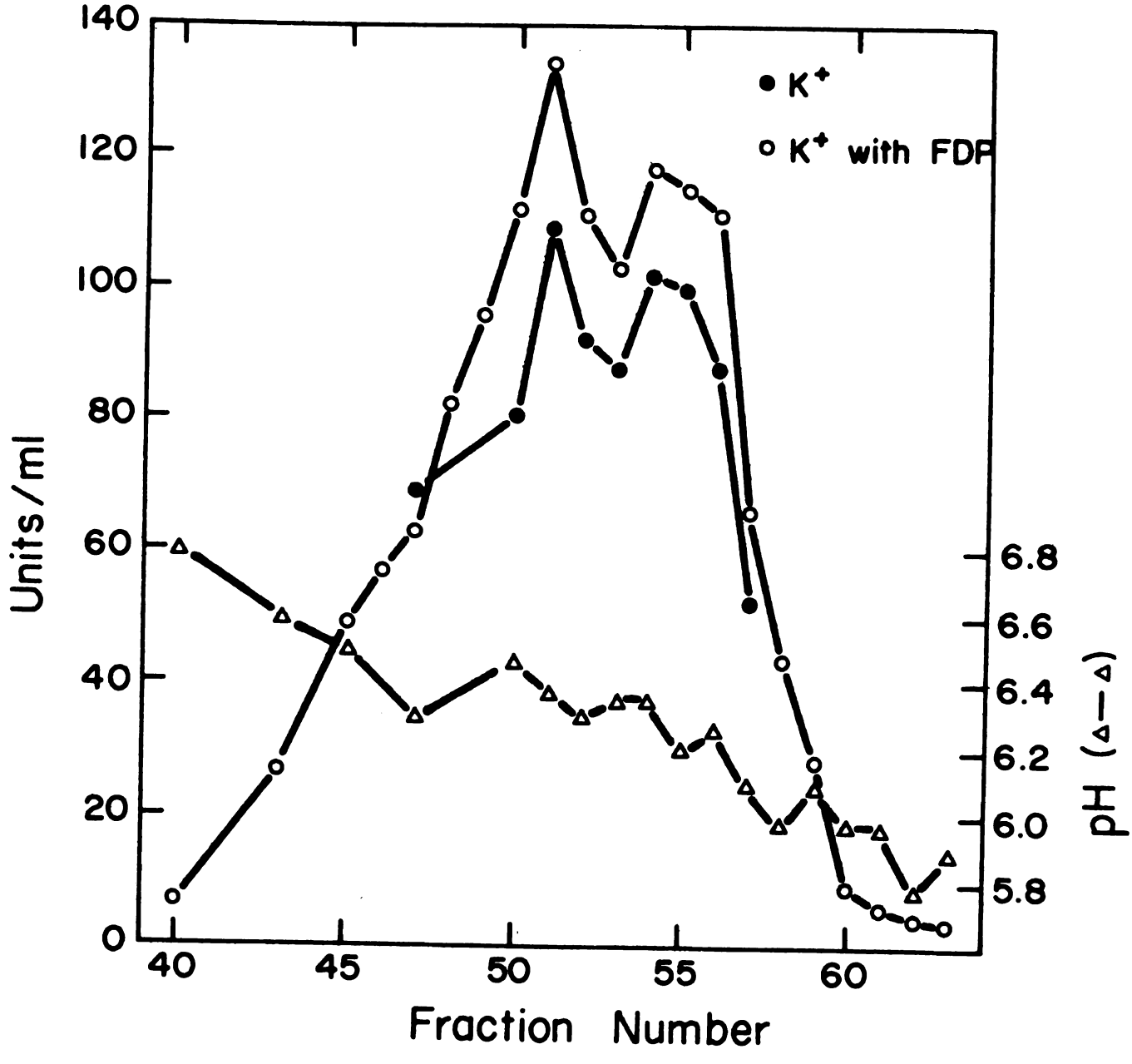

140
120
100
80
60
40
20
0
Units/ml
40
45
50
55
60
Fraction Number
● K+
○ K+ with FDP
6.8
6.6
6.4
6.2
6.0
5.8
pH (△—△)

Figure 27

Figure 28a. Microisoelectric focusing of yeast pyruvate kinase in polyacrylamide gel columns. Samples of enzyme, polyacrylamide reagents, and substrates or effectors were polymerized and subjected to focusing at 25°, then fixed in trichloroacetic acid and photographed. Details of the procedure are outlined in Methods and Materials. Special conditions are noted below. The cathodes are shown at the top of the photograph (high pH).

Gel	% Acrylamide	Enzyme source	Enzyme (µg/gel)	Gel additions	[Addition] (mM)	Focusing time (minutes)
A	6.5	Normal	200	None	-	60
B	6.5	Normal, salt-free[a]	200	None	-	60
C	4.5[b]	Normal	200	None	-	60
D	6.5	PMSF treated[c]	200	None	-	45

[a]Desalted free of $(NH_4)SO_4$ over G-25 Sephadex in 0.10 M Tris-HCl, pH 7.5.
[b]Polymerized with persulfate.
[c]Phenylmethanesulfonyl fluoride included in original preparation of enzyme.

Figure 28b. Microisoelectric focusing of yeast pyruvate kinase in polyacrylamide gel columns. See note for Figure 28a. All gels were 6.5 % acrylamide and riboflavin catalyzed.

Gel	Enzyme source	Enzyme (µg/gel)	Gel additions	[Addition] (mM)	Focusing time (minutes)
A	Normal	200	tetraCHA FDP	1.0	45
B	Normal	200	tetraCHA FDP, $MgCl_2$	1.0, 10.0	45
C	Normal, salt-free[a]	200	tetraCHA FDP	1.0	45
D	Normal, salt-free[a]	200	tetraCHA FDP, $MgCl_2$	1.0, 10.0	45
E	Control	200	None	-	60

[a]Desalted free of $(NH_4)_2SO_4$ over G-25 Sephadex in 0.10 M Tris-HCl, pH 7.5.

Figure 28c. Microisoelectric focusing of yeast pyruvate kinase in polyacrylamide gel columns. See note for Figure 28a. All gels were 6.5 % acrylamide and riboflavin catalyzed.

Gel	Enzyme source	Enzyme (µg/gel)	Gel additions	[Additions] (mM)	Focusing time (minutes)
A	Prefocused, low pH fraction[a]	100	None	-	45
B	Prefocused, mixture of fractions[b]	100	None	-	45
C	Prefocused, high pH fraction[c]	100	None	-	45
D	Preincubated[d]	200	None	-	45
E	Preincubated[d]	200	tetraCHA FDP, $MgCl_2$	1.0, 10.0	45
F	Prefocused, low pH fraction[a]	100	tetraCHA FDP	1.0	45
G	Control	200	None	-	60

[a]10 mg of enzyme was preparatively prefocused (Figure 27), and the "low pH" fraction used in this experiment.
[b]10 mg of enzyme was preparatively prefocused (Figure 27), and a mixture of "low and high pH" fractions used in this experiment.
[c]10 mg of enzyme was preparatively prefocused (Figure 27), and the "high pH" fraction used in this experiment.
[d]Enzyme was preincubated 15 minutes at 25° in a mixture of 1.0 mM tetraCHA FDP and 10.0 mM $MgCl_2$ in 0.10 M Tris-HCl, pH 7.5, desalted over G-25 Sephadex in 0.10 M Tris-HCl, pH 7.5, and used in this experiment.

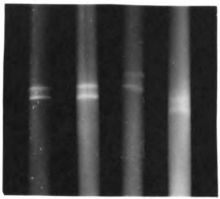


Figure 28a

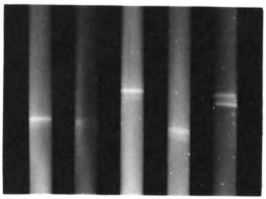


Figure 28b

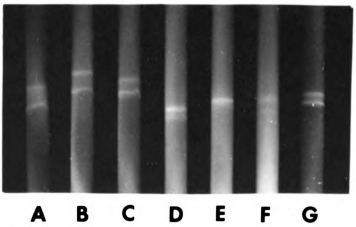


Figure 28c

Figure 29a. Microisoelectric focusing of yeast pyruvate kinase in polyacrylamide gel columns. See note for Figure 28a. All gels were 6.5 % acrylamide and riboflavin catalyzed.

Gel	Enzyme source	Enzyme (µg/gel)	Gel additions	[Addition] (mM)	Focusing time (minutes)
A	Normal	200	$MgCl_2$	10.0	60
B	Normal	200	triCHA PEP	1.0	45
C	Normal	200	triCHA PEP, $MgCl_2$	1.0, 10.0	45
D	Normal	200	Tris ADP	1.0	45
E	Normal	200	Tris ADP, $MgCl_2$	1.0, 10.0	45
F	Control	200	None	-	60

Figure 29b. Microisoelectric focusing of yeast pyruvate kinase in polyacrylamide gel columns. See note for Figure 28a. All gels were 6.5 % acrylamide and riboflavin catalyzed.

Gel	Enzyme source	Enzyme (µg/gel)	Gel additions	[Addition] (mM)	Focusing time (minutes)
A	Normal	200	$(CH_3)_4N$ pyruvate	1.0	45
B	Normal	200	$(CH_3)_4N$ pyruvate, $MgCl_2$	1.0, 10.0	45
C	Normal	200	Tris ATP	1.0	45
D	Normal	200	Tris ATP, $MgCl_2$	1.0, 10.0	45
E	Normal, salt-free[a]	200	KCl	30.0	45
F	Control	200	None	-	60

[a]Desalted free of $(NH_4)_2SO_4$ over G-25 Sephadex in 0.10 M Tris-HCl, pH 7.5.

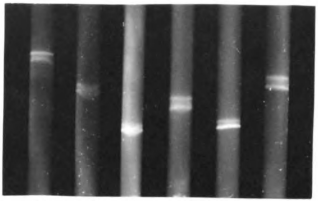


Figure 29a

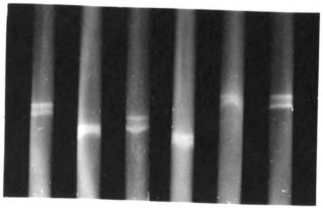


Figure 29b

Opaque gels such as 28a-D were the result of a small amount of protein denaturation. The variation in one gel of the relative position of the two bands to those in another gel resulted from the ionic composition of the gel and had no effect on band intensity or distribution.

An attempt was made to separate the two protein bands by slicing an unfixed gel similar to A shown in Figure 28a into 1 mm pieces. Each piece was homogenized in 0.50 ml of 50% (v/v) glycerol-10 mM Na phosphate, pH 6.5, and assayed by the maximal FDP standard assay. Two activity peaks were found corresponding in position to those made visible by acid fixation.

Addition of FDP to the gels (Figure 28b) with or without $MgCl_2$ shifted a large portion of the lower fraction (lower pH form) to the upper fraction (high pH form) for both the normal and salt-free enzyme.

This conversion was quantitated by scanning gel A from Figure 28a and gel A from Figure 28b at 280 nm in a linear transport device. Distribution of the enzyme in the control gel with no FDP was 55 $\pm$ 1% high pH form and 45 $\pm$ 1% low pH form. In the gel containing FDP the high pH form was 81 $\pm$ 1% of the total and the low pH form 19 $\pm$ 1%. The sum of the absorptions from each gel agreed within 8.7%. Therefore, selective destruction of one form cannot account for

the change in distribution.

Preparatively prefocused enzyme was refocused in the micro system (Figure 28c). The low pH fraction, high pH fraction, and a mixture of each gave identical distributions on refocusing. The majority of prefocused low pH fraction (F) in the presence of FDP was converted to the high pH form. Enzyme preincubated (D) with FDP (and $MgCl_2$ to protect against denaturation), but chromatographed free of FDP just prior to focusing, was skewed toward the high pH form. Similar FDP preincubated enzyme, focused in the presence of FDP and $MgCl_2$ (E), gave a predominantly high pH band.

Addition of activators and substrates of yeast pyruvate kinase other than FDP (Figures 29a and b) had little or no effect on the distribution of high or low pH forms in polyacrylamide gel columns.

DISCUSSION

The establishment that the yeast pyruvate kinase preparation utilized in these studies consists of a single protein species is of fundamental importance for the interpretation of the data presented. Disc electrophoretic experiments under a variety of conditions revealed no heterogeneity, providing enzyme prior to electrophoresis was protected against cold or ligand induced inactivation. The enzyme sedimented in the analytical ultracentrifuge as a single symmetrical peak of extrapolated molecular weight 162,000 to 168,000 in 0.10 M $(CH_3)_4N$ cacodylate, pH 6.2, containing 100 mM KCl, 26 mM $MgCl_2$, and 1.0 mM FDP and in 0.10 M Tris-HCl, pH 7.5. Both solvents were similar to those used for kinetic and binding work reported in this thesis (R. Kuczenski and C. H. Suelter, unpublished data).

A molecular weight of 165,000 was confirmed with the Yphantis (1964) high speed equilibrium technique and strong evidence given for a tetrametic subunit structure (R. Kuczenski and C. H. Suelter, unpublished observations). Rabbit muscle pyruvate kinase has a molecular weight of 237,000 (Warner, 1958) and consists of 4 subunits (Steinmetz

and Deal, 1966).

Yeast pyruvate kinase, like the rabbit muscle enzyme, has a low extinction coefficient at 280 nm which reflects a low aromatic amino acid complement. Comparison of amino acid mole fractions of the two enzymes reveals, however, marked differences in content (Kayne, 1966), the yeast enzyme containing about half as many histidine, methionine, and half-cystine residues and about twice as many tyrosine and threonine residues. The value for tyrosine residues per mole enzyme of 38.1 ± 1.1 obtained by chemical analysis is more reliable than the value of 47 ± 1 obtained by the spectrophotometric method which tends to exaggerate tyrosine values and to underestimate tryptophan (Beaven and Holiday, 1952). Thus, the tryptophan content of the yeast enzyme may be higher than estimated here.

Partially purified yeast pyruvate kinase has been shown to be completely inhibited by low concentrations of cupric ion (Washio and Mano, 1960). Because of this observation and because of the low molar ratio of bound copper to enzyme (0.14), an enzymological function of the metal ion is suspect. Efforts to reduce heavy metal contamination lowered Fe levels to below the limits of detectibility, but were unsuccessful in reducing Cu. The enzyme may simply function, either <u>in vitro</u> or <u>in vivo</u>, as an efficient Cu

scavenger.

The unexpected skewed elution patterns of the enzyme during analytical gel filtration are consistent with the 200,000 molecular weight estimated by Haeckel et al. (1968) with the same technique. Glycoproteins tend to have an expanded structure and chromatograph atypically during gel filtration (Andrews, 1965). However, both, because of the abnormal distribution and the fact that there is no evidence that the enzyme contains carbohydrate support the hypothesis that the Sephadex matrix induces aggregation of the enzyme (R. Kuczenski and C. H. Suelter, unpublished data).

The monovalent cation requirement (Table 5) of the yeast enzyme, like that from rabbit muscle, was both stringent and specific. The substantial activation by Na^+ in the presence of FDP may represent a survival mechanism in yeast unessential for muscle, where Na^+ only weakly activates pyruvate kinase (Kachmar and Boyer, 1953). Conway and Moore (1954) have described serial bakers' yeast fermentations in the presence of Na citrate and glucose in which 98% of the K^+ content of the cell is gradually replaced by Na^+. Oxygen consumption of resting cultures of these "sodium" yeasts is about two thirds, and the fermentation rate about one half, of normal. Unfortunately, no data are available on the levels of rate-limiting glycolytic enzymes in these

yeasts under these conditions. Wyatt (1964) has hypothesized a theory of metabolic control, in which he pointed out that in "systems with several metal activated enzymes and in systems with enzymes with multiple metal activations and inhibitions, many choices of pathways are available." Pertinently, yeast pyruvate kinase was able to utilize Ca^{2+} as the required divalent cation in the presence of FDP. It, therefore, may not be coincidental that yeast pyruvate kinase, whose substrates lie in the reaction sequence of many pathways, has a plastic specificity for activator cations.

Kinetically, the yeast pyruvate kinase reaction was complex. As studied in the pH 6.2, Mg^{2+}-dependent system at limiting K^+ or NH_4^+ but at near saturating concentrations of $MgCl_2$ and substrates (Figure 3), FDP gave a cooperative activation curve. In addition, FDP acted as a positive heterotropic effector toward K^+ and NH_4^+, Mg^{2+}, or PEP at saturating levels of all other variables (Figures 2, 4, and 5). Since FDP had no effect on the V_{max} of the reaction with either K^+ or NH_4^+ as the required monovalent cation (Figure 7), the enzyme may be classified in the nomenclature of Monod et al. (1965) as a K system, that is, the presence of FDP modifies only the apparent affinities of the protein for substrates or metal ions. For the Mg^{2+} activated system (Haeckel et al., 1968), the appearance of sigmoid ADP

kinetics at low concentrations of PEP or K^+ were indicative of additional kinetic interactions in the system.

Other similar kinetic interactions were apparent for Mn^{2+} activated kinetics. Lowering of the pH tends to decrease sigmoidicity of the Mn^{2+} saturation curve in the presence of Na^+ (Figures 12 and 13). Varying Na^+ concentration seemed to have little effect on FDP activation for either n_H or apparent K_A (Figure 16). In the opposite case, however, FDP acted as a weak negative heterotropic effector toward Na^+ and K^+ (Figure 9) by decreasing the apparent affinity. This is in contrast to the action of FDP on K^+ or NH_4^+ in the Mg^{2+}-dependent system where FDP acted as a positive heterotropic effector (Figure 2).

More importantly, in the presence of K^+, strong mutual kinetic dependences of n_H, apparent K_m, or apparent K_A were uncovered between Mn^{2+} and ADP or PEP (Figures 17-24). FDP abolished all kinetic interactions between Mn^{2+} and ADP (Figures 17-20), but Mn^{2+} and PEP were still able to mutually influence both the affinity or Hill slope of the other (Figures 21-24) under similar conditions. These data do not reduce to a simple model (Monod et al., 1965) partly because in Figures 17-20 resolution of the ADP and Mn^{2+} variables in quantitative terms of free ADP, free Mn^{2+}, and ADP-Mn complex (dissociation constant = 1.0×10^{-4} M, Mildvan and

Cohn, 1966) is dependent on the availability of computer programs to solve polynomials of greater than 3 degrees. For a kinetic system in which n complexes are formed between available ligands (substrates, activators, proteins) and divalent cation, the solution of a polynomial function of (n + 1) degrees is required to determine the free ion concentration (Kerson et al., 1967). Therefore, the interactions are best discussed as resultant change of enzymatic activity in terms of substrate flux, assuming Mn^{2+} behaves similarly to Mg^{2+} and the enzyme at high dilutions (Srere, 1967) mimics the action in vivo.

It is unlikely that the substrates, metal cofactors, or activators of pyruvate kinase in situ are always at saturating levels or at the optimum pH. Consider the effects of varying the total substrate and Mn^{2+} concentrations around the intermediate level of about 100 μM for each. At this concentration of Mn^{2+}, the only effect of FDP on ADP is to eliminate cooperative kinetics (Figures 19 and 20); for PEP at 100 μM Mn^{2+}, FDP greatly reduces both the apparent K_m and cooperativity (Figures 23 and 24). Inversely, at both intermediate and high levels of ADP (Figures 17 and 18), the only effect of FDP is to reduce n_H for Mn^{2+}. The apparent K_m for Mn^{2+} at 100 μM PEP is reduced 5 fold upon addition of FDP and cooperativity decreased but not abolished

(Figures 21 and 22).

In the glycolytic scheme, after a sudden increase in glucose concentration, the rise in FDP concentration would precede that of the PEP concentration. Applying this temporal difference to the kinetic data presented in Figures 17-24 demonstrates that the increase in FDP concentration would first uncouple all ADP-Mn cooperative interactions. Subsequently, the remaining PEP-Mn interactions would be released by the additional increase in PEP, relieving enzymatic cooperative control (Figures 21-24).

The variable stoichiometry of Mn^{2+} binding in the binary complex (Figure 25) might be explained in 3 ways:

a. Aggregation of the protein by Mn.

b. Heterogeneity of the yeast pyruvate kinase preparation.

c. Site-site interaction among Mn binding sites.

Aggregation of the enzyme under identical experimental conditions as the binding studies was not detected in the analytical ultracentrifuge. Microheterogeneity was indeed found by isoelectric focusing for this enzyme, which was shown to consist of an approximately equal mixture of two species. Although FDP had a profound effect on the distribution of the bands, no large changes were induced by FDP in the atypical Mn binding curves. The observed

conformational heterogeneity does not, then, account for variable stoichiometry of binding. The third alternative of site-site interaction is therefore left. In contrast, the rabbit muscle enzyme binds either 2 or 4 (depending on the monovalent cation present) in either case with an invariant K_D of 63 to 75 μM (Mildvan et al., 1970).

Comparison of the effects of FDP on the binding parameters of the PEP ternary complexes suggests that Mn^{2+} must fill at least one binding site before FDP may lower the affinity for PEP (Table 6). The agreement of the binding data with kinetically determined apparent K_m values for PEP, and the lack of agreement of the corresponding parameters for ADP suggest the yeast enzyme-metal complex may bind PEP prior to the binding of ADP, in a preferred order.

The results from both preparative and microisoelectric focusing experiments are consistent with the existence in solution of two yeast pyruvate kinase conformers of differing isoelectric point which are resolvable through differences in net charge. It is proposed that the rate for conversion of the forms in the absence of any ligand is slow, but that the allosteric effector, FDP, upon binding the conformer of low isoelectric point, rapidly converts it into the high pH form.

Data which support this model are as follows:

a. Both bands off preparative focusing columns were kinetically identical in both sensitivity to FDP activation and homotropic response to PEP.

b. Variations in distribution of bands in microisoelectric focusing columns were independent of $(NH_4)_2SO_4$ concentrations, gel polymerization catalyst, source of enzyme (whether prepared in the presence or absence of phenylmethanesulfonyl fluoride at concentrations sufficient to inhibit yeast proteases), or addition of any other substrate or cofactor of the pyruvate kinase reaction other than FDP.

c. The affinity of the enzyme for FDP was not great enough to form a stable or slowly dissociating complex which might account for net charge differences in the two bands. Besides, binding of this anion would create a complex of lower isoelectric point, not higher, as observed.

d. The process is not related to a dissociation-association phenomenon. Identical conversion patterns were obtained in the presence of FDP or FDP and $MgCl_2$. FDP is known to induce dissociation of the enzyme (Kuczenski and Suelter, 1970), which the addition of $MgCl_2$ prevents. The two band pattern is inconsistent with any scheme involving dissociation (or reassociation) of a tetramer consisting of identical or nonidentical subunits.

e. The conversion process was quantitative. Scans of

the gels at 280 nm demonstrate that loss of absorption of the low pH form induced by FDP was countered by a gain of absorption of the high pH form.

f. The conversion was reversible. Incubation of enzyme with FDP and $MgCl_2$ and gel filtration to remove these ions followed by focusing in gels with or without FDP and $MgCl_2$ showed that a significant fraction had reverted to the low pH form only in the absence of FDP in the duration of the experiment (about one hour).

g. The predominantly low pH form, high form, and a mixture of the two resulting from preparative isoelectric focusing reequilibrated to equal distributions of both forms upon refocusing in microcolumns. The low pH form collected from the preparative column still retained its sensitivity to FDP-induced conversion on refocusing in microcolumns.

The microisoelectric focusing experiments provide additional evidence that the preparation of yeast pyruvate kinase used in this study was molecularly homogeneous by one of the most sensitive separation techniques now known to protein chemists. For instance, the preparation of rabbit muscle pyruvate kinase almost universally used for experimentation was recently shown to consist of at least 4 subspecies, reminiscent of a mixture of isozymes (Susor *et al.*,

1969) by this same technique.

Independent evidence for the existence of two conformers of this enzyme in solution has been provided by Wiecker et al. (1969) who found that data from brewers' yeast pyruvate kinase kinetic experiments fitted a simplified allosteric model (Monod et al., 1965) which provides for the two conformer state, and Kuczenski and Suelter (1970) whose data explaining the kinetics of the FDP-induced inactivation of the enzyme were consistent with an equilibrium mixture of two or more tetrameric forms in solution. In addition, increased activity induced in the basic pH range by FDP may be the result of conformational transitions. There is as of yet no basis for concluding, however, that the hypothesized conformers resulting from kinetic analyses and the double band electrofocusing patterns arise from an identical conformational mechanism. Further fast reaction kinetic studies may provide clues toward the solution of this problem.

Physical separation of "conformers" of a pyruvate kinase preparation from rat epididymal fat pads has previously been accomplished, but the conformational change probably involved dissociation and reassociation mediated by a divalent cation. In the case of yeast pyruvate kinase, the indications are that both conformers remain intact tetramers.

SUMMARY

The chemical, steady-state kinetic, binding, and conformational properties of a homogeneous preparation of bakers' yeast pyruvate kinase have been studied. The enzyme contained about 0.14 moles of tightly bound Cu^{2+} per 166,000 g protein and deviated significantly in amino acid content from the rabbit muscle enzyme.

Activating monovalent cations were shown to be essential for enzymatic activity. Cooperative kinetics were seen for K^+ and NH_4^+, Mg^{2+}, and phosphoenolpyruvate, but not for ADP at saturating concentrations of all other substrates and metal ions, pH 6.2. Fructose diphosphate heterotropically transformed the cooperative kinetic variables, yielding near hyperbolic saturation curves for each. The enzyme was shown to possess a less stringent specificity for both divalent and monovalent cations in the presence of FDP. Upon addition of PEP or FDP, the activity profile broadened in the range above pH 6.5.

For Mn^{2+}-dependent kinetics at pH 7.5, strong interactions were noted between ADP or PEP and Mn^{2+}, and the effect of FDP on these was investigated.

Proton relaxation rate and electron paramagnetic resonance studies of the enzyme binary and ternary complexes revealed an atypical Mn^{2+} binding curve suggestive of site-site interaction. The qualitative and quantitative agreement of the kinetic and binding data support the view that the enzyme-Mn-PEP ternary complexes containing at least one Mn are the kinetically active ones and that there may be a preferred order of binding PEP.

It was proposed that the double peaked enzyme distribution on preparative or microisoelectric focusing was the result of separating two slowly equilibrating conformations of the tetrametic protein which differ in net charge. FDP was shown to specifically and rapidly convert the conformer of low isoelectric point to the high pH form which, upon removal of the effector, reverted again to a mixture of both conformers.

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