THE PURIFICATION AND PROPERTIES OF PYRUVATE KINASE FROM BAKER'S YEAST

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James R. Hunsley

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ABSTRACT

THE PURIFICATION AND PROPERTIES
OF PYRUVATE KINASE FROM BAKER'S YEAST

by James R. Hunsley

Washio and Mano (1960) published a 17-fold purification of pyruvate kinase from yeast, but the preparation was stable only a few days. In order to study monovalent cation and substrate requirements for this enzyme, the primary goal of this work was, therefore, to purify and stabilize yeast pyruvate kinase.

Polyanions and polyhydroxy compounds were found to stabilize the enzyme. Using the techniques of toluene autolysis, ammonium sulfate fractionation, treatment with DEAE cellulose, and chromatography on cellulose phosphate in 50 per cent glycerol, the enzyme was purified 28-fold to about 95 per cent purity. The LDH linked assay of Bücher and Pfleiderer (1955) was adapted for this work.

The purified enzyme, as found by Washio and Mano (1960), had an absolute requirement for potassium ions which could be replaced by rubidium and ammonium ions to about 50 per cent of activity. The K_A's for potassium ion at constant and varying ionic strength were 0.173 and 0.029 M respectively.

The pH optimum of the reaction was shown to be 6.1 to 6.4 in cacodylate, imidazole, and maleate buffers with cacodylate giving the highest observable rates.

Linear kinetics were obtained with the substrate ADP yielding a K_m of 3.6×10^{-4} M. Kinetics toward PEP showed a cooperative effect with a large Hill slope of 4.2 and an apparent K_m of 1.1×10^{-3} M. FDP was shown to activate the enzyme at low concentrations of PEP. As previously predicted by Pye and Eddy (1965), Hommes (1964), and Hess and Brand (1965), the enzyme appears to be an important control point in yeast glycolysis.

The $s_{20,w}$ of the enzyme was calculated to be 8.23 S from sedimentation velocity data.

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BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
DEAE	diethylaminoethyl cellulose
FDP	fructose-1,6-diphosphate
LDH	lactic acid dehydrogenase
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
OD	optical density
PEP	phospho(enol)pyruvate
PFK	phosphofructokinase
PK	pyruvate kinase
TMA	tetramethylammonium
tris	tris(hydroxylmethyl)aminomethane

INTRODUCTION

The sole previously published preparation (Washio and Mano, 1960) of pyruvate kinase (EC 2.7.1.40) from yeast was unsuccessful although the enzyme was early shown to exist by several laboratories (Parnas, et al., 1935; Muntz, 1947; Seits, 1949). Washio and Mano (1960) were unable to obtain stability, high purity, or a kinetic assay, but did manage to accumulate useful kinetic data using an end point assay for pyruvate. The yeast enzyme was shown by them to have requirements for potassium and magnesium ions like the rabbit muscle enzyme (Kachmar and Boyer, 1953) but differed in requiring higher optimal concentrations and also in having a lower pH optimum. These workers also demonstrated that the stoichiometry of the reaction, shown below, was uniequivalent by chromatography of the reaction products.

$$\text{ADP} + \text{PEP} \xrightleftharpoons{K^+,\ Mg^{++}} \text{ATP} + \text{pyruvate}$$

Interest in this laboratory arose in the yeast enzyme as an alternative and comparative model to the rabbit muscle enzyme for monovalent cation activation. The possibility of obtaining mutant enzymes altered in catalytic properties toward monovalent cations is espe-

cially attractive in the yeast system.

In addition, the essentially irreversible reaction catalyzed by pyruvate kinase makes the enzyme a candidate for glycolytic control. Hommes (1964) applying the theory of crossover points (Chance, et al., 1958) to glycolyzing yeast extracts pointed out the inhibition and activation phenomena of glycolysis are between glucose-6-phosphate and fructose-6-phosphate, triose phosphate and 3-phosphoglycerate, and PEP and pyruvate. Pye and Eddy (1965) claimed the behavior of glycolytic intermediates in yeast could by explained by 4 control points: PK, 3-phosphoglycerate kinase, phosphofructokinase, and sugar entry. Hess and Brand (1965) suggested that the points of control were at the same three kinases. Hess (1965) earlier in the same colloquium gave the first data available for the possible control of yeast PK by FDP activation. Hommes (1966a) again identified the control point in the conversion of PEP to pyruvate but stated that exhaustive attempts to demonstrate allosteric effects in pyruvate kinase had failed. Hommes (1966b) also has shown that the PK content of yeast grown in media with glucose concentrations varying from 0.6 to 1.2 per cent increases about twenty fold while phosphofructokinase remains almost constant, suggesting the induction of pyruvate kinase and thus another level of control. No control has yet been found for phosphoglycerate kinase (Hess and Brand, 1965).

In this thesis the stabilization and purification of

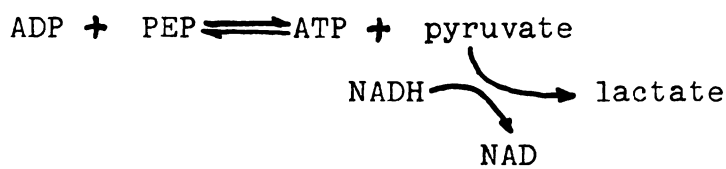
pyruvate kinase from yeast (*Saccharomyces cerevisiae*) is described, and an introduction given to the kinetic and allosteric properties of the enzyme.

MATERIALS AND METHODS

A. Enzymatic Activity and Protein Determinations

1. Assay for pyruvate kinase

Because of equilibrium considerations the pyruvate kinase reaction can only conveniently be measured in the direction of ATP synthesis. The pyruvate formed was continuously followed spectrophotometrically by employing the lactic acid dehydrogenase reaction as modified from the assay of Bücher and Pfleiderer (1955) for rabbit muscle pyruvate kinase. For these assays a modified Beckman DU ultraviolet spectrophotometer with a Gilford Model 2000 multiple sample absorbance recording attachment was used to record the decrease in optical density at 340 mμ, the absorption maximum of NADH, according to the following scheme:



The assay temperature was 30°C., maintained by a circulating water bath. The stoichiometry for the assay is one mole of NAD formed for each mole of ATP. Conditions for routine assays in this study are as follows:

Assay component	μmoles/ml
cacodylate (Na)	110
KCl	230
$MgCl_2$	24
NADH	0.15
ADP	0.80
PEP	3.0
LDH	(33 μg/ml)

The final volume of the assay mixture was 1.00 ml at pH 6.00, the reaction being initiated by addition of enzyme and recorded against a water blank. All dilutions of this enzyme for assay must be made in cold 50 per cent (v/v) aqueous glycerol solutions containing 0.010 M phosphate (Na), pH 6.50. The best commercial lactic dehydrogenase sometimes contains traces of PK, and small residual rates with this determination are due both to contaminant enzyme and hydrolysis of PEP to pyruvate at acidic (pH 6.00) assay conditions. Measured rates were corrected for the usual residual rate of about 0.0015 ΔOD/min.

2. Definition of a unit of activity and specific activity

A unit of pyruvate kinase activity is defined as that amount of enzyme which catalyzes the production of one μmole of pyruvate per minute under the conditions described in the assay procedure. The molar absorbance of NADH at 340 mμ was taken as 6.22×10^6 cm^2 per mole (Horecker and Kornberg, 1948); therefore, an optical density change corresponding to the production of one μmole of pyruvate is 6.22.

The specific activity is defined as units of enzy-

matic activity per mg of protein.

3. Determination of protein concentration

The spectrophotometric method of Warburg and Christian (1942) was used to estimate protein concentration.

4. Substrates and assay conponents

a. PEP. Phospho(enol)pyruvic acid, tricyclohexylammonium salt, was purchased from the Sigma Chemical Company. The reagent was assayed by two methods: 1) according to Bücher (1955), based on the molar absorbancy of PEP at pH 7.4 in the presence of 2.7×10^{-3} M $MgCl_2$, and 2) a determination based on addition of a limiting amount of this substrate to a standard assay system containing an excess of muscle pyruvate kinase (Tietz and Ochoa, 1958) and calculating from the absolute OD change of the reaction.

b. ADP. Adenosine diphosphate, sodium salt, was dissolved in water and adjusted to pH 7.0 with NaOH. ADP was estimated by two methods, 1) the procedure of Bock, et al. (1956), based on the absorption of the adenine moiety at 259 mμ, pH 7, and 2) the second method employed in assaying PEP.

c. NADH. Nicotinamide adenine dinucleotide (reduced), di-sodium salt obtained from Pabst Laboratories, was dissolved in 1.0×10^{-2} M tris (HCl) buffer, pH 7.5.

d. LDH. Lactic acid dehydrogenase, obtained from Sigma, was the Type II crystalline rabbit muscle enzyme substantially free of pyruvate kinase. A stock

solution was prepared by diluting the ammonium sulfate suspension (10 mg/ml) to 330 μg per ml in water.

B. Other Reagents and Materials

"Budweiser" baker's yeast, Anheuser-Busch, Incorporated, was obtained fresh from Michigan State University Food Stores. Analytical reagent grade chemicals were used throughout whenever possible. All solutions were prepared from deionized distilled water with a conductivity reading (Crystalab Deeminizer) below 1.0 ppm.

Glycerol was the Fisher Certified Reagent, Fisher Scientific Company. Imidazole was recrystalized from chloroform. Fructose-1,6-diphosphate was the sodium salt from Sigma. Cacodylic acid, Sigma or Fisher, was titrated to the desired pH (Sargent pH Meter, Model LS) with NaOH or TMAOH. Tetramethylammonium hydroxide and chloride were purchased from Eastman Organic Chemicals and used without further purification. Special Enzyme Grade ammonium sulfate was obtained from Mann Research Laboratories, Incorporated and was used throughout these studies. Visking Corporation dialysis tubing was soaked at least one hour before using in several changes of water.

Conductivity readings were taken with an Industrial Instruments Corporation conductivity bridge Model RC 16B2 in a platinum-glass flow cell of unknown cell constant.

C. Preparation of Columns for Chromatography

1. DEAE cellulose (about 0.9 meq/g) was purchased either from Sigma or Gallard-Schlesinger Chemical Manu-

facturing Corporation. 150 g of medium mesh material was washed consecutively with two liters of the following solutions, filtering after each wash: 95 per cent ethanol, 0.5 N NaOH, water, 0.1 N HCl, and 0.1 N HCl again. The residue was then washed 5 to 7 times with water decanting fines and filtering until the wash was free of chloride ion by the silver nitrate test. The damp dry filtered material was then suspended in 50 per cent aqueous (v/v) glycerol containing 1.0×10^{-2} M NaH_2PO_4, adjusted directly to pH 7.50 with NaOH, and refiltered. The material was then suspended in phosphate buffered glycerol, pH 7.50, added to a glass column 8.0 cm in diameter with a sintered glass bottom, and gravity packed to a height of 18 cm. The column was then washed with one column volume (900 ml) of buffered glycerol, pH 7.50, and placed in the cold room at 4° C.

2. Cellulose phosphate, Sigma, (0.9 meq/g) was treated the same as the DEAE above except that the HCl and NaOH washes were reversed. The damp dry filtered derivative was equilibrated with 0.01 M phosphate buffered 50 per cent glycerol adjusted to pH 6.50. This slurry was added to a glass column 5.0 cm in diameter with a sintered glass bottom and packed by gravity to a height of 18 cm. After washing the column with an additional column volume (400 ml) of buffered glycerol, pH 6.50, the column was placed in the cold room.

D. Polyacrylamide Disc Electrophoresis

Disc electrophoresis patterns of enzyme fractions of the purification scheme given later in this thesis were obtained by subjecting appropriate samples to analysis by modified techniques of Ornstein (1962) and Canalco (1963). All reagents were obtained from Canalco. To 7.5 per cent polymerized standard gels in glass tubes 4.5 mm inside diameter by 75 mm containing a spacer gel were added appropriate samples of protein (less than 200 μg) in 50 per cent aqueous (v/v) glycerol containing 0.015 M phosphate buffer adjusted to pH 6.90 with tris. Samples were electrophoresed at room temperature in an apparatus constructed in this laboratory at 5 ma constant current per tube in standard tris-glycine buffer, pH 8.3 using brom phenyl blue tracking dye and a Heath Company regulated power supply, Model IP-32. The runs were concluded at the time the tracking dye came within about 5 mm of the anodic end. The gels were removed within 10 minutes of the end of the run, stained in 0.55 per cent Amido-Schwarz in 7.5 per cent acetic acid, then destained in 7.5 per cent acetic acid.

E. Sedimentation Velocity Determination in the Analytical Ultracentrifuge

The Spinco Model E Analytical Ultracentrifuge with phase plate schlieren optics was used to determine the sedimentation constant for the enzyme. Calculations were made according to Schachman (1957) and corrected to the viscosity of water at 20° C.

EXPERIMENTAL

A. Stability Studies of Yeast Pyruvate Kinase

The failure of the only previously published attempt to obtain a stable pyruvate kinase from yeast was due to the extraordinary instability of the enzyme both in the unpurified and purified states (Washio and Mano, 1960). Washio and Mano achieved a 17 fold purification with poor yields as can be seen in Table I but the preparation lost all activity "within a few days."

Extensive stability studies of various preliminary stages of the purification scheme were conducted in an attempt to find a stabilizing agent which would be effective both at low and high protein concentrations and which could be conveniently used throughout a protein purification procedure. Table II shows the partial results of these studies. Polyhydroxy compounds are very effective in the desired stabilization, and glycerol in particular was chosen because of lower viscosity and efficiency of stabilization both at low and high protein concentrations. A typical experiment consisted of incubating known amounts of fresh active enzyme in cold reagent solutions of wide concentration ranges, incubating at temperatures of 4 and 25° C. for periods of either 2 or 3 days, and assaying for activity after the incubation period.

B. Isolation of PK from Yeast

A protein estimated to be about 95 per cent pure

Table I

The Yeast Pyruvate Kinase Preparation of Washio and Mano (1960)

Fraction	Total Protein (mg)	*Total Activity (units)	Specific Activity (units/mg)	Yield, %	Fold Purification
1	1887	1510	0.8	100	-
2	447	786	1.8	52	2.2
3	89	314	3.5	21	4.4
4	69	269	3.9	18	-
5	12	165	14.1	11	17.6

*One unit is that amount of protein which forms one μmole of pyruvate per minute as measured by the 2,4-dinitrophenylhydrazine assay of Friedman and Haugen (1943). Fraction 1 is the crude extract, Fractions 2 and 3 ammonium sulfate precipitations, Fraction 4 a dialysis, and Fraction 5 the result of DEAE cellulose treatment.

Table II

Results of Stabilization Experiments

Reagent	Concentration	*% Effectiveness
glycerol	40-60% (v/v)	90-100
sucrose in 0.10 M Na phosphate buffer, pH 6.0	1.5-2.0 M	65-82
glucose	0.10 M	10
sorbitol	75%	90-100
ethylene glycol in 0.10 M Na phosphate buffer, pH 6.0	60%	68
tripolyphosphate, Na, pH 6.5	5.0×10^{-3} M	90-100
pyrophosphate, Na, pH 6.5	1.0×10^{-2} M	90-100
orthophosphate, Na, pH 6.0	0.25-1.0 M	90-100
arsenate, Na, pH 6.5	0.10 M	90
d-(+)-tartrate, pH 6.5	0.10 M	90-100
$MnCl_2$	1.0×10^{-2} M	16
$MgCl_2$	1.0×10^{-2} M	60

Table II (continued)

Reagent	Concentration	*% Effectiveness
$MgCl_2$ plus ATP	1.0×10^{-2}, 5.0×10^{-3} M	90-100
ADP	4.0×10^{-3} M	14
citrate, TMA, pH 6.5	2.5×10^{-2} M	27
NaF	1.0×10^{-3} M	50-65

*Per cent remaining activity after incubation period based on known amount added. Experimental details are described in the text. The standard assay was used as described in Materials and Methods.

Ineffective reagents and conditions included: serum albumin, 2-mercaptoethanol, PEP, iodoacetate, ethylenediaminetetraacetic acid, reduced glutathione, cysteine, lactate, FDP, glucose-1-phosphate, glucose-6-phosphate, sodium sulfite, AMP, and heat and cold treatments.

exhibiting PK activity was isolated from fresh baker's yeast by a procedure involving autolysis with toluene, ammonium sulfate fractionation, treatment with DEAE, and chromatography on cellulose phosphate.

1. Autolysis with toluene and filtration

Plasmolysis of yeast was accomplished by a method modified from that of Kunitz and McDonald (1946). Ten pounds of yeast was crumbled into a stainless steel pail and to it added 2.4 liters of reagent grade toluene at 45° C. The covered pail was incubated in a 45° C. water bath and stirred occasionally with a wooden paddle until the yeast reached about 37° C. and liquified. The mixture was allowed to stand at room temperature for one to 2 hours, then rapidly cooled to 10° C. in an ice bath. This mixture was added to 3400 ml of water at 4° C. in the cold room and stirred for one hour and allowed to stand overnight (about 16 hours) until two phases separated. All successive operations were carried out at 0 to 4° C. The aqueous bottom layer was carefully siphoned off and centrifuged at 8500 rpm for 20 minutes in a GSA head, Sorvall RC-2 refrigerated centrifuge, resulting in a three layer system. The relatively clear brownish middle layer was removed with a suction collecting apparatus. To this centrifugate was added 20 g per liter of Fisher infusorial earth and the mixture filtered over 2 sheets of Whatman #1 filter paper, yielding a crystal clear crude enzyme solution designated Fraction I.

Immediately to Fraction I was slowly added 242 g per liter of solid ammonium sulfate with rapid stirring to 1.62 M. Upon dissolving, the pH of the solution was adjusted to pH 6.2 by direct measurement with 0 to 5 ml of 3 N NH_4OH. After allowing to stand between 3 and 12 hours, the suspension was spun as before, discarding the precipitate. To the clear supernatant, Fraction II, was added 96 g per liter of ammonium sulfate to 2.22 M stirring rapidly until dissolved. This was centrifuged as before after standing 2 to 4 hours, the supernatant was discarded, and the precipitate was rapidly dissolved in a minimum (less than 100 ml) of 2.0×10^{-2} M phosphate buffer (Na), pH 7.5. To this was added an equal volume of glycerol, yielding Fraction III. Fraction III was then dialyzed 48 hours versus 2 changes of 10 volumes of 1.0×10^{-2} M phosphate (Na), pH 6.5, in 50 per cent glycerol. The conductivity of the resultant dialysate (Fraction IV) should be near that of the glycerol buffer alone. Fraction IV is stable for at least 3 months if stored at -20° C.

2. DEAE treatment

A volume of Fraction IV containing a maximum of 60,000 units was carefully adjusted to pH 7.50 with 3 N NH_4OH. This was added to the DEAE column described on page 7, washing after loading with at least one column volume (900 ml) of glycerol buffer, pH 7.50 and collecting 15 ml fractions about every 15 minutes at the maximum flow rate. After assaying the fractions, those with a specific activity

greater than 22 were pooled yielding Fraction V which was stored in a freezer at -20° C.

3. Cellulose phosphate chromatography

To a volume of the DEAE effluent (Fraction V) containing about 50,000 units was added 3 N acetic acid to pH 6.50. This was then added to the cellulose phosphate column described on page 8 and after loading was washed with one column volume (400 ml) of the phosphate buffered glycerol solution, pH 6.50. No activity washed through the column. The enzyme was eluted from the column with a 600 ml 0.010 to 0.20 M linear ammonium sulfate gradient in 50 per cent aqueous (v/v) glycerol. Conductivities of 15 ml fractions collected at the maximum flow rate of about one ml per minute were measured to insure linearity of the gradient. The active fraction eluted at about 0.07 M ammonium sulfate. Figure I shows the elution profile of the column.

Tubes were pooled that contained enzyme of specific activity above 120, yielding Fraction VIa, and above 60, yielding Fraction VIb. Both were stored at -20° C. The highest purity almost homogeneous Fraction VIa was used for all successive studies. A summary of the purification scheme is presented in Table III with calculated yields.

Fractions of the purification III through VIb were subjected to polyacrylamide disc electrophoresis to check for heterogeniety of protein species. Figure IX displays the patterns obtained.

Figure I

Elution Profile of Yeast PK from Cellulose Phosphate

Approximately 50,000 units of yeast PK in 50 per cent glycerol (v/v) containing 1.0 X 10^{-2} M phosphate buffer (Na), pH 6.50 was eluted with a linear 0.01 to 0.20 M ammonium sulfate gradient in 50 per cent glycerol according to the Experimental section of the text. The assay was standard as described in Materials and Methods.

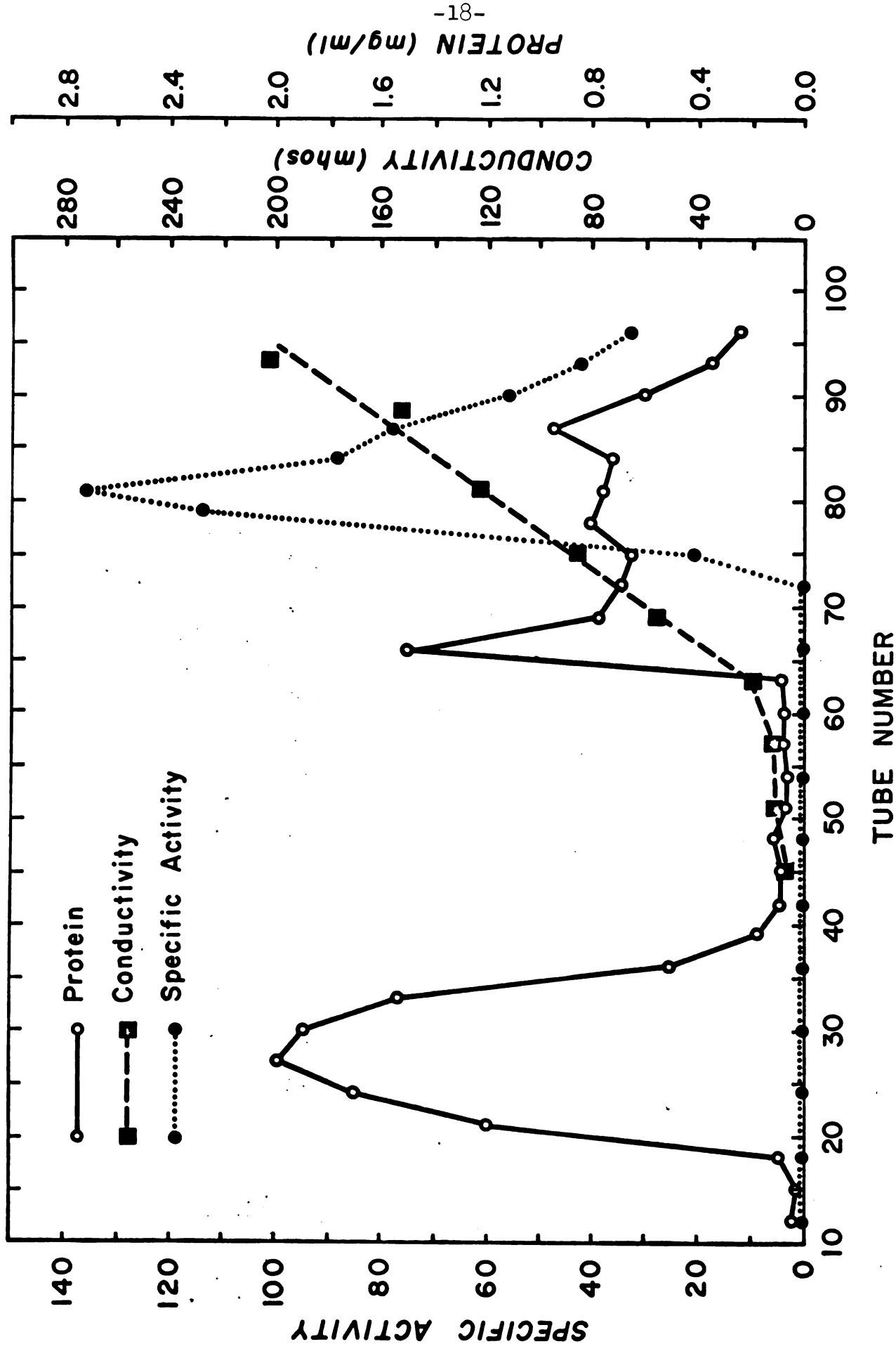

PROTEIN (mg/ml)
CONDUCTIVITY (mhos)
SPECIFIC ACTIVITY
TUBE NUMBER
Protein
Conductivity
Specific Activity

Figure I

Table III

Summary of Yeast Pyruvate Kinase Preparation

Fraction	Volume (ml)	Total Activity (units)	Specific Activity	% Yield	Fold Purification	Units/ml
I	4150	720,000	5.24	100	-	174
II	4550	555,000	4.50	64.2	-	122
III	464	525,000	15.1	73.3	2.88	1130
IV	542	633,000	18.8	86.7	3.59	1170
V	(329)	(45,400)	30.7	*67.2	5.86	138
VIa	(103)	(11,700)	149	*28.6	28.4	114
VIb	(103)	(9,430)	118	*22.9	22.5	91.5

*Per cent yield based on total units taken from the previous fraction. The standard assay was used as described in Materials and Methods.

The enzyme was concentrated by dialyzing aliquots of Fraction VIa against 10 volumes of 2.0 M ammonium sulfate for 12 hours, then adding 172 g per liter of solid ammonium sulfate with rapid stirring to 3.0 M. The precipitated enzyme was centrifuged and dissolved in appropriate buffers. Less than one per cent of the activity remains in the supernatant.

B. Catalytic Properties of the Enzyme

1. Univalent cation requirement of PK

Yeast pyruvate kinase like the muscle enzyme (Kachmar and Boyer, 1953) has an absolute requirement for univalent cations as shown by Washio and Mano (1960) with partially purified enzyme (see Table IV). The univalent cation activation kinetics of this enzyme was investigated further by assaying the enzyme in the presence of varying concentrations of KCl, NH_4Cl, RbCl, LiCl, NaCl, and TMACl, replacing the usual sodium cacodylate buffer with TMA cacodylate. Assays with each cation were performed at the concentration optimum for KCl, 0.23 M. Table V shows the relative activities given by these cations.

The potassium requirement was pursued further in 2 experiments, varying the KCl concentration in each, ionic strength kept constant with added TMACl in one, and increasing ionic strength (no TMACl) in the other. A/v versus A plots (see Figures II and III) of the activation in both experiments lead to estimated K_A's of 0.17 and 0.029 M and V_{max}'s of 216 and 145 units per mg

Table IV

Univalent Cation Requirement of Yeast PK
(Washio and Mano, 1960)

Cation	Concentration (M)	*Pyruvic Acid Formed (μmoles)
K^+	2.25×10^{-1}	1.05
NH_4^+	"	0.51
Li^+	"	0.06
Na^+	"	0

*2,4-dinitrophenylhydrazine assay of Friedman and Haugen (1943).

Table V

Relative Univalent Cation
Requirement of Yeast PK

Cation	% Activity, Based on K^+
K^+	100
NH_4^+	50.4
Rb^+	49.5
TMA^+	1.6
Na^+	0.05
Li^+	0.005

The standard assay was used as described in Materials and Methods substituting given cations as chloride salts at 0.23 M for KCl as described in the Experimental section. TMA cacodylate was used as the buffer, pH 6.00. The reaction was initiated with 0.19 μg of enzyme.

Figure II

A/v versus A Plot of Potassium Activation
of Yeast PK at Constant Ionic Strength

The standard assay was used as described in Materials and Methods substituting sodium cacodylate buffer with TMA cacodylate at pH 6.00. TMACl was added so that the KCl plus TMACl concentration was constant at 0.23 M. The reaction was initiated with 0.19 µg of enzyme.

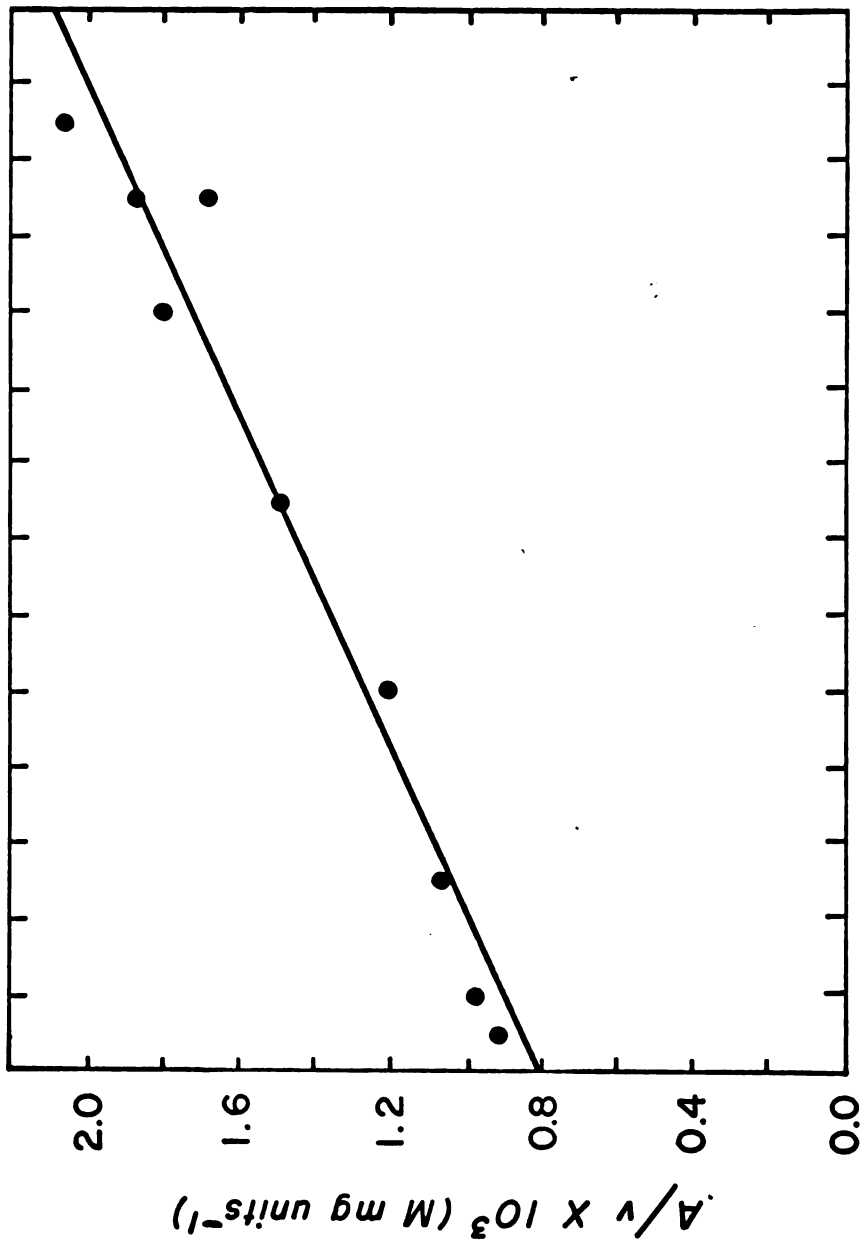

A/ν X 10³ (M mg units⁻¹)
2.0
1.6
1.2
0.8
0.4
0.0
0.00
0.04
0.08
0.12
0.16
0.20
0.24
A(M)

Figure II

Figure III

A/v versus A Plot of Potassium Activation
of Yeast PK at Increasing Ionic Strength

The standard assay was used as described in Materials and Methods substituting sodium cacodylate buffer with TMA cacodylate at pH 6.00. The ionic strength increased with added KCl. The reaction was initiated with 0.19 μg of enzyme.

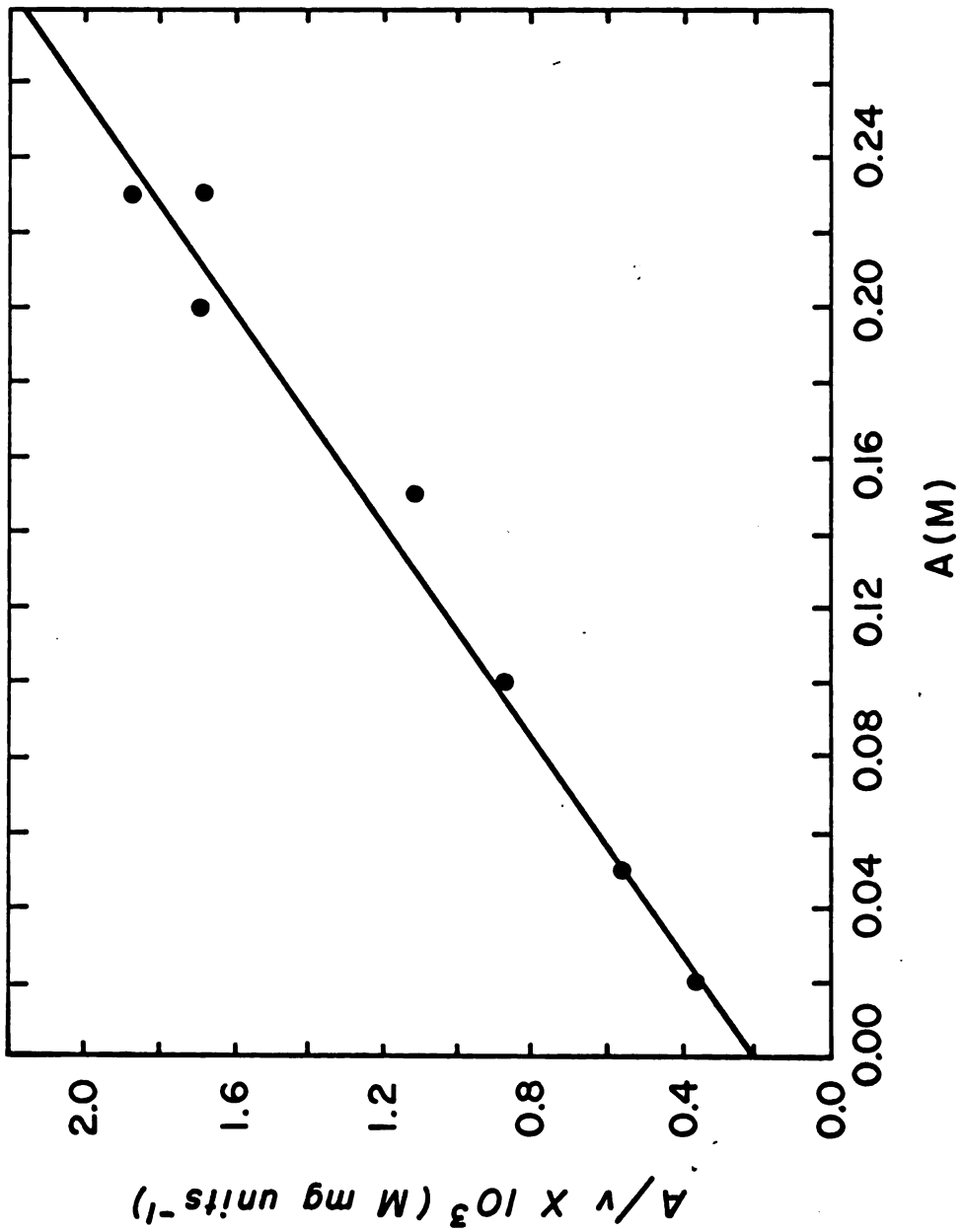

A/ν X 10³ (M mg units⁻¹)
0.0
0.4
0.8
1.2
1.6
2.0
0.00
0.04
0.08
0.12
0.16
0.20
0.24
A(M)

Figure III

respectively.

2. pH activity profile

The pH activity profile of the enzyme was determined with the standard assay substituting equimolar amounts of imidazole (HCl), cacodylate (TMA), or maleate (TMA) buffers. The pH of duplicate or triplicate reactions was measured directly. The results seen in Figure IV show that the enzyme has more absolute activity in cacodylate than either imidazole or maleate buffers in the ratios of 1.0 to 0.65 to 0.73 respectively at the optimal pH.

3. Kinetics of ADP and PEP

Kinetics for ADP and PEP were obtained under standard assay conditions using varying amounts of ADP or PEP. If nonlinear rates were observed only initial rates were measured. A v versus v/S plot of ADP kinetics is shown in Figure V yielding an estimated K_m of 3.6×10^{-4} M. PEP kinetics are nonlinear; a Lineweaver-Burke plot and a Hill plot of the data can be seen in Figures VI and VII.

4. Effect of FDP on the kinetics of the enzyme

Hess (1965) in a terse note containing unpublished data and no methodology pointed out the FDP stimulation of PK in presumably crude yeast extracts. Under conditions of low catalytic rates due to the allosteric effect of PEP, low concentrations of FDP can fully activate the enzyme as can be seen in Table VI.

Figure IV

Optimum pH for Yeast Pyruvic Kinase Reaction

The standard assay was used as described in Materials and Methods substituting sodium cacodylate with equal amounts of cacodylate (TMA), maleate (TMA), or imidazole (HCl) at varying pH's. The pH of the actual reaction mix is plotted. The reaction was initiated with 0.076 μg of enzyme.

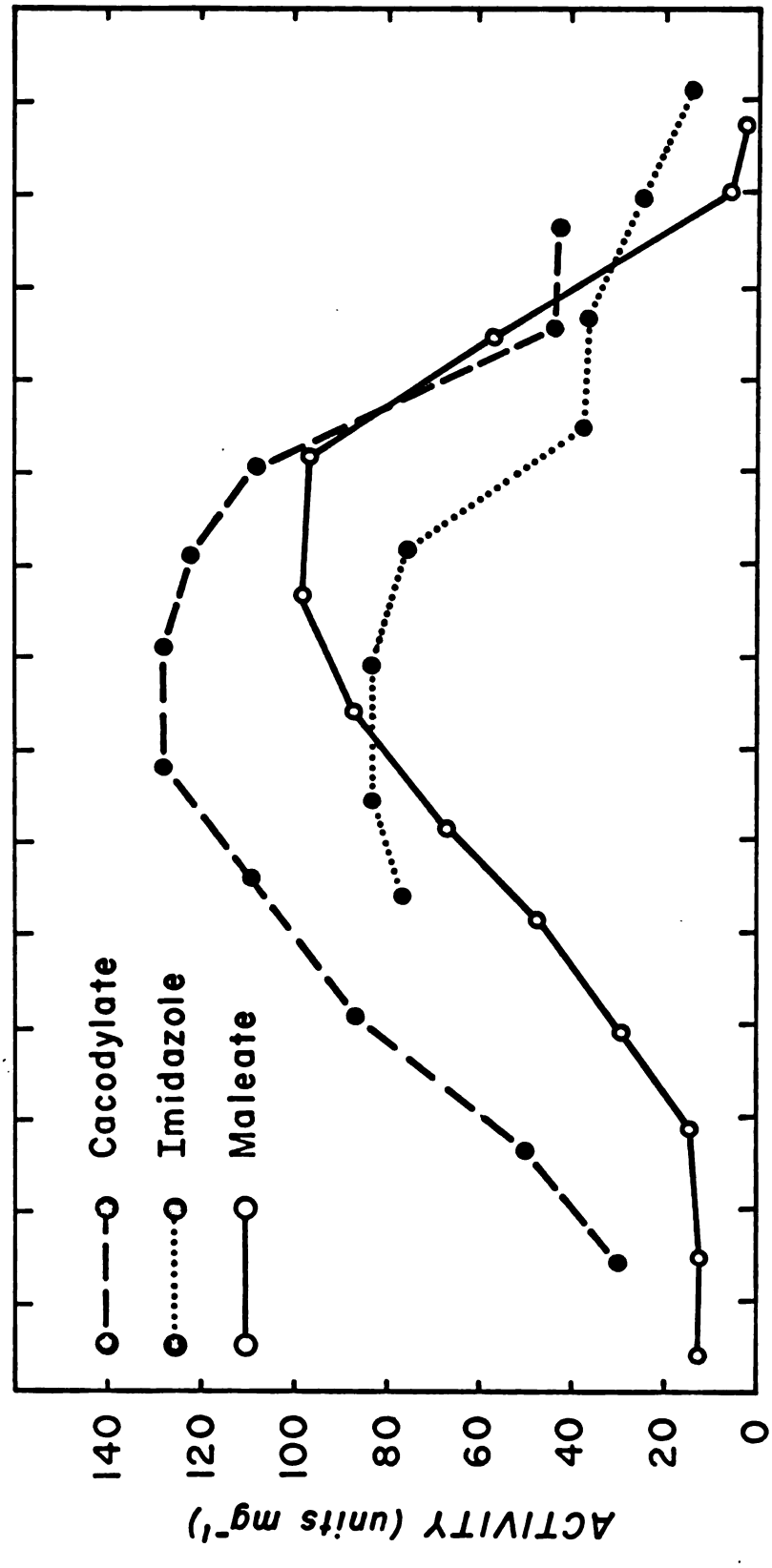

ACTIVITY (units mg⁻¹)
140
120
100
80
60
40
20
0
4.9
5.3
5.7
6.1
6.5
6.9
7.3
pH
Cacodylate
Imidazole
Maleate

Figure IV

Figure V

v versus v/S Plot of ADP Kinetics of Yeast PK

The standard assay was used as described in Materials and Methods varying the amounts of ADP added. The reaction was initiated by addition of 0.19 μg of enzyme.

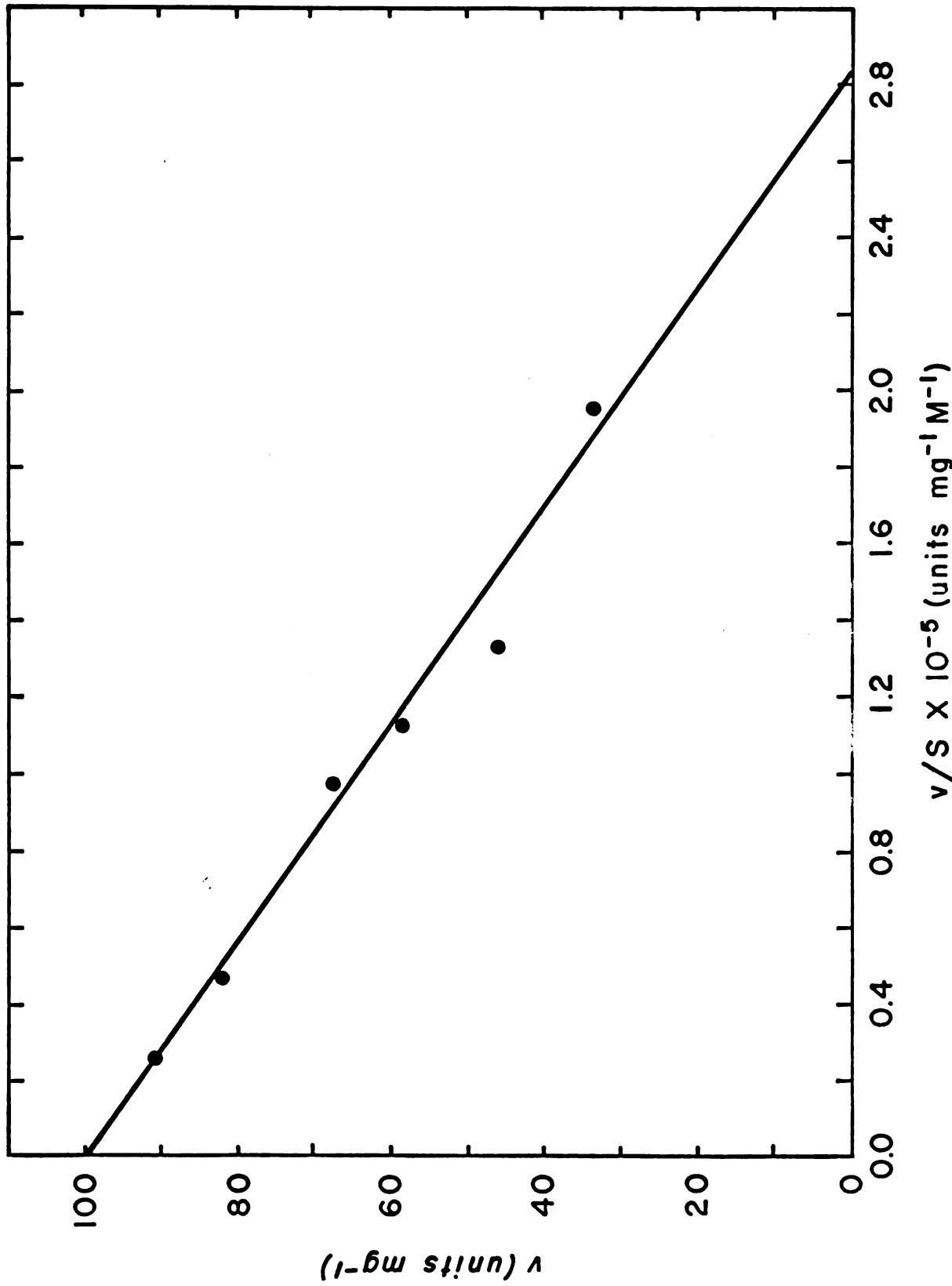

v (units mg⁻¹)
0
20
40
60
80
100
v/S X 10⁻⁵ (units mg⁻¹M⁻¹)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8

Figure V

Figure VI

Lineweaver-Burke Plot of PEP Kinetics of Yeast PK

The standard assay was used as described in Materials and Methods varying the amounts of PEP added. The reaction was initiated by addition of 0.19 μg of enzyme.

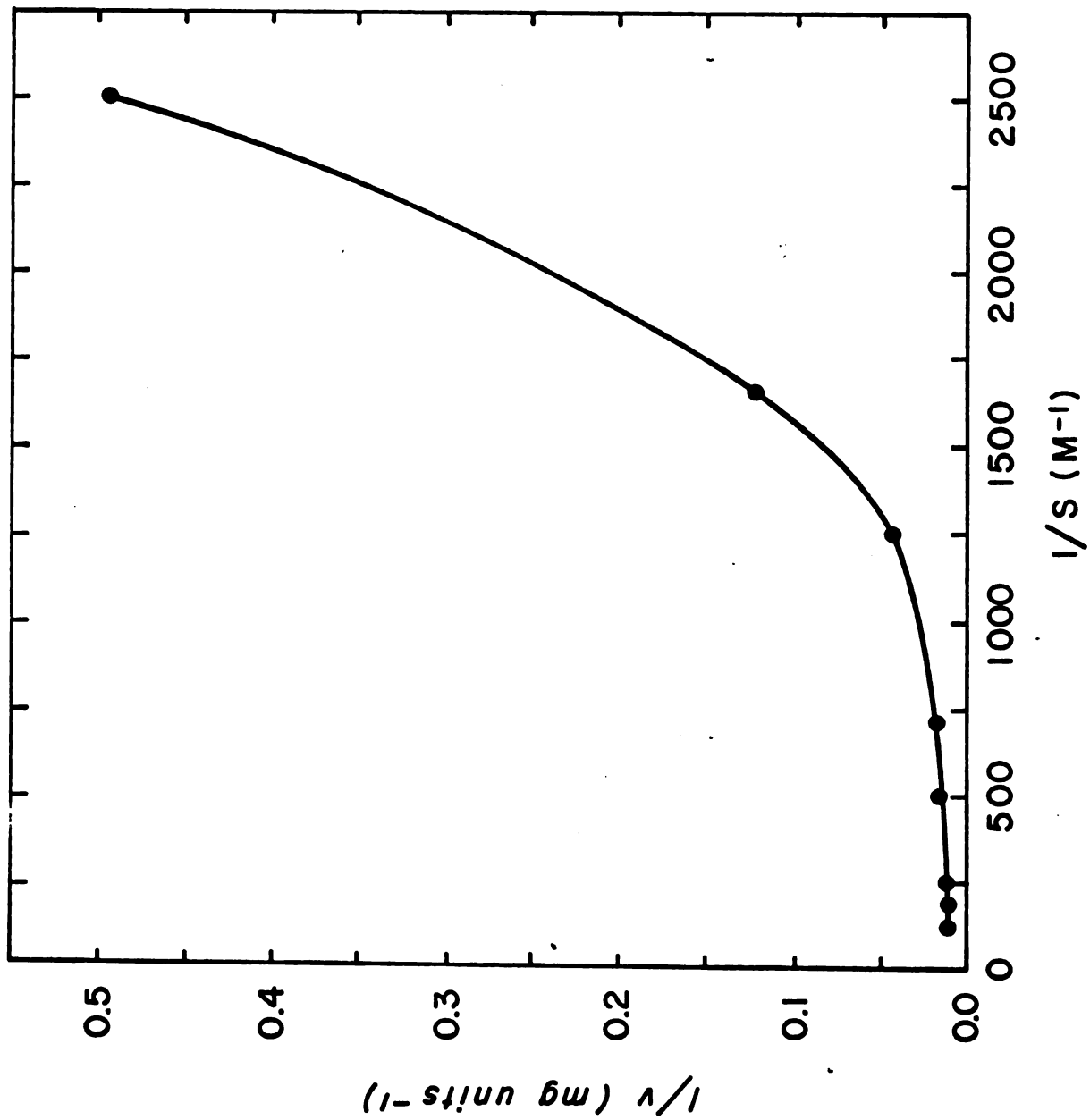


Figure VI

Figure VII

Hill Plot of PEP Kinetics of Yeast PK

The standard assay was used as described in Materials and Methods varying the amounts of PEP added. The reaction was initiated by addition of 0.19 μg of enzyme.

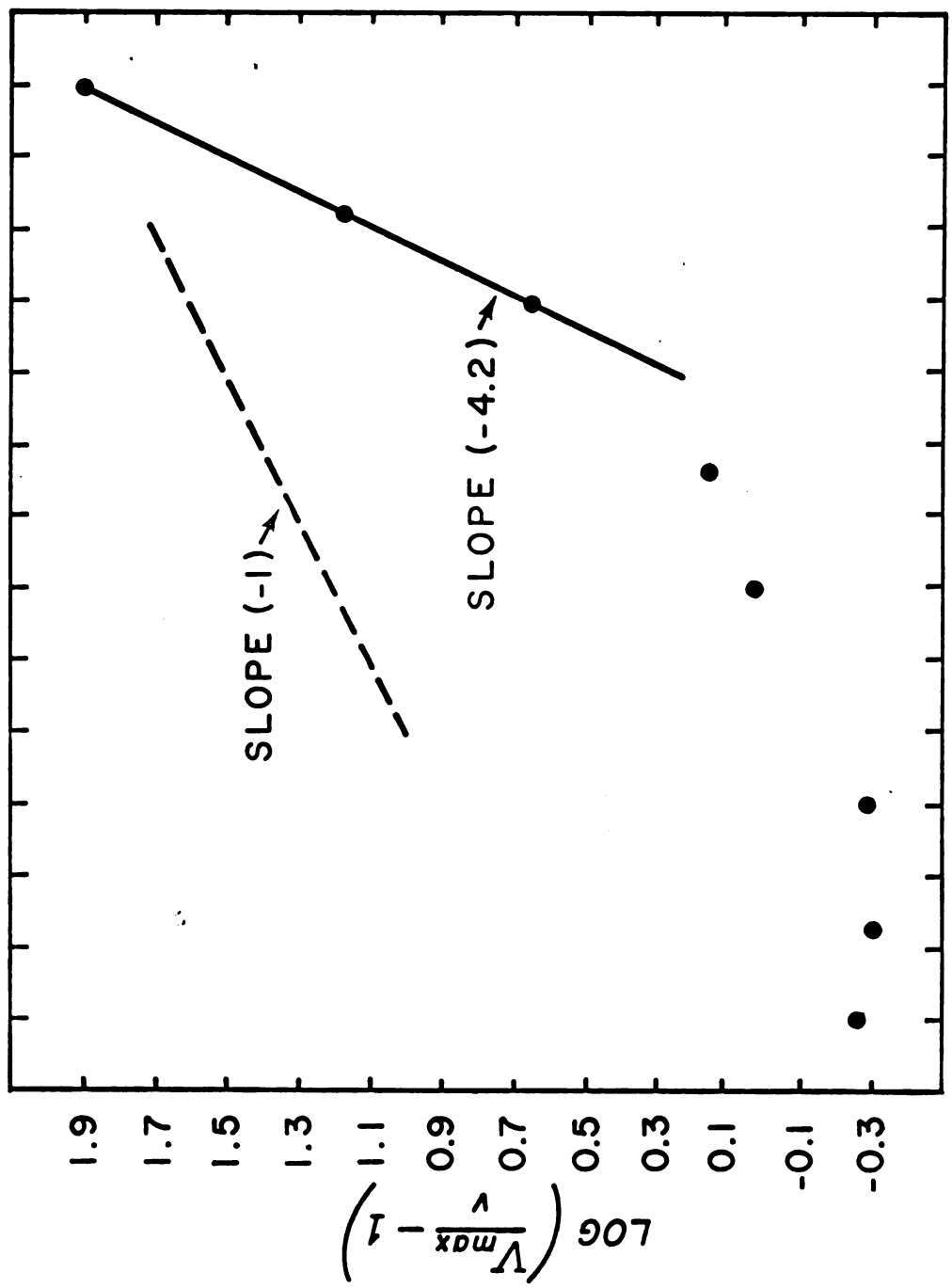

SLOPE (-1)
SLOPE (-4.2)
LOG $\left(\frac{V_{max}}{v} - 1\right)$
LOG S (M)
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-2.1
-2.3
-2.5
-2.7
-2.9
-3.1
-3.3

Figure VII

Table VI

FDP Activation of Yeast PK

pH	PEP (M)	FDP (M)	Activity (units/mg)
6.5	6.0×10^{-4}	-	0
6.5	6.0×10^{-4}	2.0×10^{-5}	36
6.5	6.0×10^{-4}	1.0×10^{-4}	106
6.0	6.0×10^{-4}	-	17
6.0	6.0×10^{-4}	2.0×10^{-5}	83
6.0	6.0×10^{-4}	1.0×10^{-4}	143
6.5	2.4×10^{-3}	-	62
6.5	2.4×10^{-3}	2.0×10^{-5}	108
6.5	2.4×10^{-3}	1.0×10^{-4}	139

Reaction mix contained cacodylate (Na) 110 μmoles, KCl 230 μmoles $MgCl_2$ 24 μmoles, NADH 0.15 μmoles, ADP 0.80 μmoles, LDH 33 μg, and FDP and PEP in amounts shown. Reaction volume was 1.00 ml.

The reaction was initiated by addition of 0.19 μg of enzyme.

C. Physical Properties of the Enzyme

One ml of concentrated enzyme in ammonium sulfate solution prepared according to the method on page 20 and containing 4.8 mg of protein was dialyzed against one liter of 0.050 M phosphate buffer (Na), pH 6.0, overnight. 0.50 ml of the resultant dialysate (4.5 mg/ml) was subjected to sedimentation analysis in the Model E analytical ultracentrifuge at 4.35° C. as described in the methods section. A frame from the resultant plate (Figure VIII) shows a single unskewed peak with a small low molecular weight contaminant of unknown origin. The specific activity of the enzyme used in this experiment was reduced by about 75 per cent during dialysis.

The calculated s value corrected to water at 20° C. is 8.23 S.

Figure VIII

Sedimentation Velocity Frame of Yeast PK

Sedimentation is proceding from left to right in this picture. The conditions for the experiment are given in the Experimental section.

Figure IX

Polyacrylamide Gel Disc Electrophoresis of Yeast PK Purification Fractions

See Materials and Methods for experimental procedures. Anodic ends are shown down. Fractions III, IV, V, VIa, and VIb as shown in order contained 200, 100, 100, 20, and 100 μg respectively.

Figure VIII

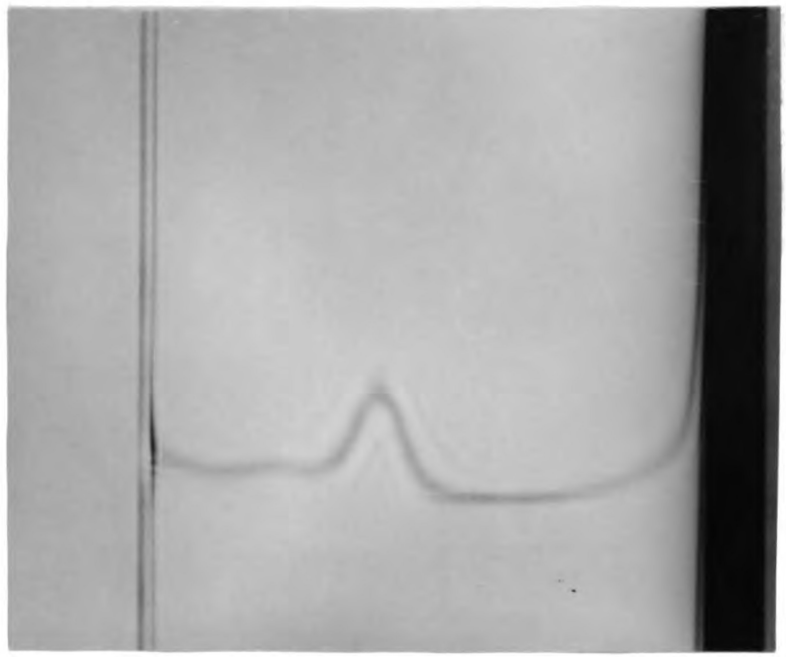
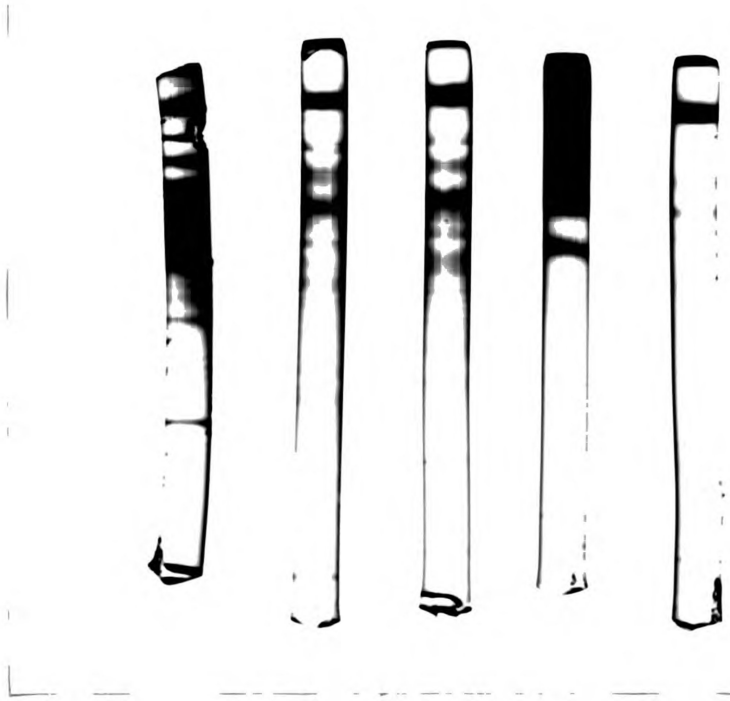


Figure IX



DISCUSSION

A. Pyruvate Kinase from Yeast

The per cent yield column in Table III summarizing the enzyme purification demonstrates that somewhat variable results are obtained in the assay of crude enzyme. Due to the allosteric nature of this enzyme a measured rate is highly dependent on the concentration of PEP in the assay. Early Fractions (I, II, III) can be shown to contain an interfering enzyme which hydrolyzes PEP to pyruvate and does not require ADP. This tends to lower the effective PEP concentration and the rate, resulting in misleading lower activities.

The highest purity enzyme was obtained in high yield, about 29 per cent. Figure IX shows the polyacrylamide disc electrophoresis patterns of Fractions III through VIb. The almost homogeneous Fraction VIa contains a 4 to 6 per cent impurity which can be removed if desired by rechromatography on cellulose phosphate. This contaminant was demonstrated to arise from the higher ionic strength side of the active peak by disc electrophoresis.

The highest specific activity obtained of about 150 in the absence of FDP compares favorably to the crystalline muscle PK specific activity of from 130 to 250 (Kayne and Suelter, 1965). It is not known whether yeast PK acts as a fluorokinase or hydroxylamine kinase as described for the muscle enzyme (Tietz and Ochoa,

1958; Kupiecki and Coon, 1960).

While the stabilization of enzymes through the use of polyhydroxy compounds has been increasingly used, a common mechanism explaining the increased stability has as yet not been elucidated (Jarabek, et al., 1966). Of interest to this problem are the findings of Chilson, et al. (1965) who showed that high concentrations of glycerol, sucrose, ethylene glycol, glucose, and propylene glycol protected LDH from hybridization during freezing and thawing. Jarabek, et al. (1966) discovered that 20 per cent glycerol solutions as well as high concentrations of phosphate protected human placental 17β-hydroxysteroid dehydrogenase from an inactivating aggregation phenomenon due to cold. These workers also pointed out the accumulated evidence that polyanions and glycerol might have a common protective mechanism. Macromolecular polyanions, as suggested by Bernfield, et al. (1965), have a tendency to support the dissociation of several enzymes at high dilution. The catalytic properties of mammary glucose-6-phosphate dehydrogenase could be explained, Levy, et al. (1966) found, by an $X \rightleftharpoons Y$ equilibrium in which formation of Y was promoted by NADP or NADPH and X by 40 per cent glycerol and NAD. Allosteric kinetics were seen in aqueous solutions but not in 40 per cent glycerol. These data all suggest that polyanions and glycerol in particular prevent protein-protein interaction.

Table II lists some stabilizing reagents discovered for yeast pyruvate kinase. The most effective agents are polyhydroxy compounds and polyanions. The action of Mg^{++}, Mn^{++}, and ADP can probably be explained through substrate or cofactor protection, with fluoride and ATP plus Mg^{++} effects remaining unaccounted for. The gross effects of these stabilizing agents on molecular configuration are as yet unknown but undoubtedly some changes occur.

Polyanions were not used in the purification as stabilizers because of the high concentrations required and because of a tendency to ineffectiveness at high protein concentrations. Cellulose derivative chromatography is also sensitive to high ionic strength.

B. Yeast Catalytic and Physical Properties

The indication of Washio and Mano (1960) (see Table IV) that the optimal concentrations of activating monovalent cation is 0.225 M has been substantiated as shown in Table V. In addition, rubidium ion is as effective an activator as ammonium ion. The 1.6 per cent remaining activity with TMA ion, a nonactivating and noninhibitory species, is probably due to contaminating ammonium ion from the LDH suspension used in the assay.

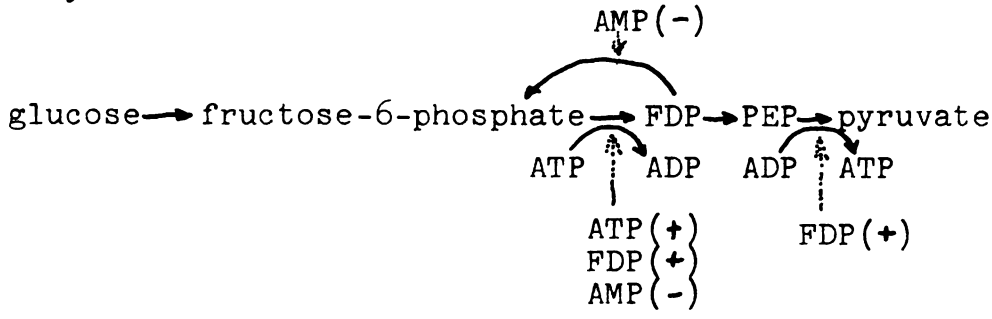
The K_A for KCl (see Figures II and III) activation is higher in the presence of TMA ion at constant ionic strength than in the absence (o.17 and 0.029 M, respectively). A possible explanation is that TMA^+ competes for Mg^{++} but

this seems unlikely. The higher V_{max} observed in the first case possibly points to an ionic strength effect. The A/v versus A plots under both conditions were linear and the K_A's obtained were both higher than the K_A of 1.15×10^{-2} M found for the rabbit muscle enzyme (Kachmar and Boyer, 1953).

As mentioned before, yeast PK has a significantly lower pH optimum of 6.1 to 6.4 (see Figure IV) than the muscle enzyme of 7.5 (Boyer, 1962) with a shift upwards of about 0.2 pH units observed with maleate buffer. This is considerably higher than the optimum of pH 5.7 found by Washio and Mano (1960). The kinetics of yeast PK toward ADP are self-explanatory (Figure V) yielding a K_m of 3.6×10^{-4} M. The PEP kinetics, however, in contrast to the muscle enzyme, are nonlinear (Figure VI) and yield a limiting Hill slope of 4.2 with an apparent K_m of 1.1×10^{-3} M. For comparison, a line with slope equal to 1 is drawn on the graph which would correspond to normal linear kinetics. Of particular interest is the nonlinearity of the Hill plot. At very high PEP concentrations (greater than 5×10^{-3} M) substrate inactivation appears to occur, but may be due to the companion salt of cyclohexylamine. Intermediate PEP concentrations show kinetics with normal slope. The muscle enzyme in contrast exhibits normal kinetics toward ADP and PEP in both crude and purified states (Dr. Karl Smiley, Jr.,

unpublished results). Hommes' (1966a) failure to obtain cooperative effects with this enzyme cannot be accounted for at this time. The accumulated evidence reviewed in the introduction that pyruvate kinase in yeast is a control point in glycolysis can now be explained not only by the PEP cooperative effect but by the effect of FDP as shown in Table VI. PK exhibiting low catalytic rates with low concentrations of PEP can be fully activated by FDP. The rates observed at pH 6.5 with PEP concentrations of 6.0×10^{-4} M are particularly striking; the enzyme was observed to be completely inactive in the absence of FDP but in the presence of 1.0×10^{-4} M FDP gave a rate of 106 units per mg.

The combined work of Viñuela, et al. (1963, 1964) and Ramaiah, et al. (1964) indicated that PFK of yeast is inhibited by ATP and stimulated by AMP, and Moore, et al. (1965) showed that the PFK inhibition by ATP can be decreased by FDP. Gancedo, et al. (1965) demonstrated fructose diphosphatase inhibition in yeast by AMP. These data added to the PK data give a fine control for glycolysis as shown in the scheme below:



As previously mentioned, no control has been found for the other likely regulatory point, 3-phosphoglycerate kinase. Other effectors have as yet not been found for yeast PK.

A rough estimate of the molecular weight of the enzyme can be made from the sedimentation velocity run (Figure VIII), assuming a spherical molecule of 0.73ρ, of about 110,000 to 140,000. A probably asymetric configuration would raise this estimate considerably. The small contaminant seen in the sedimentation velocity plate is possibly the 4 to 6 per cent contaminant of the best purity Fraction VIa used for the experiment, but this remains to be proven. The molecular weight in the presence of cofactors and substrates is not known, and determinations in the presence of FDP are especially needed.

SUMMARY

1. Pyruvate kinase from yeast was shown to be stabilized by a variety of reagents including polyanions and polyhydroxy compounds at high concentrations.

2. The enzyme was purified almost to homogeniety at high yields by toluene autolysis, ammonium sulfate fractionation, dialysis versus 50 per cent glycerol, treatment with DEAE cellulose, and chromatography on cellulose phosphate.

3. The purified enzyme was shown to have an absolute requirement for monovalent cations. Rubidium ion and ammonium ion could replace potassium ion with about one half efficiency. The K_A for potassium ion was measured and was found to be higher in the presence of TMA ion than in the absence, yielding K_A's of 0.17 and 0.029 respectively.

4. The pH activity profile was shown to have a maximum in the range of 6.1 to 6.4 in maleate, cacodylate, and imidazole buffers.

5. The kinetics of the enzyme toward ADP were shown to be linear with a K_m of 3.6×10^{-4} M. PEP kinetics showed a cooperative effect with a limiting Hill shope of 4.2 and an apparent K_m of 1.1×10^{-3} M. The allosteric nature of the enzyme was shown to be in agreement with the previous predictions that pyruvate kinase is a control

point in yeast glycolysis.

6. Full activation of the enzyme at low PEP concentrations could be brought about by low concentrations of FDP.

7. The $s_{20,\,w}$ of the enzyme by sedimentation velocity analysis was demonstrated to be 8.23 S.

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