PURIFICATION AND CHARACTERIZATION OF A HIGHER MOLECULAR WEIGHT FORM OF YEAST PYRUVATE KINASE

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ANN ELIZABETH AUST
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This is to certify that the

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presented by

ANN E. AUST

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

R Barker.

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Date July 24, 1975

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF A HIGHER MOLECULAR WEIGHT FORM OF YEAST PYRUVATE KINASE

By

Ann Elizabeth Aust

The objectives of this research were: (1) to modify a previously developed purification procedure for bakers' yeast pyruvate kinase so that yeast cells could be mechanically ruptured in the presence of potent protease inhibitors; (2) to determine whether the enzyme purified in this manner was native to yeast; and (3) to characterize the pyruvate kinase obtained from this purification procedure. The modified purification procedure developed in this research utilized the Manton-Gaulin homogenizer to mechanically rupture yeast at neutral pH in the presence of the potent protease inhibitor diisopropylfluorophosphate (DFP). This homogenizing technique allowed the pyruvate kinase to be more rapidly removed from proteases in an environment containing DFP, which assured a low protease activity level. The Manton-Gaulin homogenization was found to release more pyruvate kinase, as well as other contaminant proteins per pound of yeast. Thus, further purification steps were needed to utilize previously

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developed procedures. To this end a calcium phosphate precipitation step immediately after lysis and an additional cellulose phosphate column, pH 6.5, were used. The enzyme purified by this means was found to have a protease activity associated with it. The protease could be inhibited by DFP only in 1% SDS solution indicating that it probably existed as a protease-inhibitor complex. The proteolytic activity could be separated from PK on a Sephadex G-100 column.

Characterization of the purified enzyme revealed no macro- or micro-heterogeneity, indicative of no proteolytic degradation. To determine whether the enzyme purified in this manner was native to yeast, an immunoprecipitation technique with antibody prepared to the pure enzyme was used. This technique removed "native" PK from the cell-free extract within 5 hours after lysis of the cells. The immunoprecipitate was washed to remove nonspecifically adsorbed proteins and dissolved in SDS for analytical SDS gel electrophoresis. The protein bands visualized were those of purified IgG, used to effect the precipitation, and "native" pyruvate kinase. The subunit molecular weight for the "native" enzyme, calculated on the basis of its mobility in SDS gels, was identical to that of pure pyruvate kinase. This is consistent with the enzymes being identical.

The purified enzyme was shown to have a molecular weight of 209,000 by equilibrium sedimentation. Using

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SDS gel electrophoresis, a subunit molecular weight of 57,500 was determined indicating that the enzyme was a tetramer. The enzyme was shown to exhibit cooperative kinetics for PEP, 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 curve for PEP to hyperbolic without affecting V_m. At subsaturating Mg^{2+} concentration, ADP was found to inhibit FDP activation, producing a time dependent increase in velocity (hysteresis). This hysteretic activation was shown to be a pseudo-first order process. The first order rate of activation appeared to increase linearly with increasing Mg^{2+} concentration, but remained constant with changing enzyme concentration. Incubation of the enzyme with FDP before assaying produced final velocities that were greater than those without preincubation for PK concentrations greater than 0.5 μg/ml.

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PURIFICATION AND CHARACTERIZATION OF A HIGHER MOLECULAR WEIGHT FORM OF YEAST PYRUVATE KINASE

By

Ann Elizabeth Aust

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1975

To my family, Brian, Terri, and Steve,
and my parents

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Manton-

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NADH

NADP

PCMB

ABBREVIATIONS AND DEFINITIONS

ADP	adenosine 5' diphosphate
AMP	adenosine 5' monophosphate
ATP	adenosine 5' triphosphate
βME	2-mercaptoethanol
CP	phosphocellulose or cellulose phosphate
DEAE	diethyl aminoethyl
DFP	diisopropylfluorophosphate
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bio (β-aminoethyl ether)-N,N'-tetraacetic acid
FDP	fructose-1,6-diphosphate or fructose-1,6-bisphosphate
IgG	immunoglobulin G
LDH	lactic dehydrogenase
Manton-Gaulin PK	pyruvate kinase purified after lysis of the yeast with the Manton-Gaulin homogenizer
MES	2[N-morpholino]ethane sulfonic acid
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PCMB	parachloromercuribenzoate

PEP

PK

PMSF

SDS

Tetra C

Toluoly

Tri CHA

Tris

PEP	phosphoenolpyruvate
PK	pyruvate kinase
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
Tetra CHA	tetracyclohexylammonium
Toluolysis PK	pyruvate kinase purified after lysis of the yeast with toluene
Tri CHA	tricyclohexylammonium
Tris	triethylaminoethane

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INTRODUCTION

Lysing of yeast for the purpose of purifying enzymes has proved to be a problem because of the rigid nature of the yeast cell wall. Through the years, several different techniques have been devised to rupture yeast, most of which are done at elevated temperatures to liberate intracellular proteases as well as other proteins. Recently investigators have become aware of the fact that the liberated proteases were partially degrading or contaminating the enzymes they were purifying. In order to avoid this problem, mechanical methods of lysing were developed which could be used at 4° C in the presence of potent protease inhibitors. In most cases, modifying the lysis procedure in this manner resulted in purified enzymes of larger molecular weight or reduced heterogeneity (previously reported as isozymes). However, the fact that the enzyme was larger or apparently homogeneous did not remove the question as to whether the purified enzyme was "native" to yeast, for conceivably limited proteolysis could produce an active, homogeneous protein. Investigators, in an attempt to answer this question, have analyzed the purified proteins by a variety of physical methods

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methods looking for microheterogeneity. This type of investigation will only reveal proteolytic degradation; it will not determine whether the purified enzyme is identical to the native enzyme.

Since the discovery that yeast PK purified by the method of Hunsley and Suelter (1969a) resulted in isolation of a proteolytically degraded form of the enzyme (Aust *et al.*, 1975), methods for comparing the purified enzyme to enzyme in cell-free extract have been developed. Because the proteolytic damage suffered by PK in the previous preparation did not result in significant changes in the kinetic parameters except in one case, it was thought that a comparison of K_m and K_a values for pure enzyme and enzyme in the cell-free extract would be of little value. Analytical disc gel analysis of the enzyme in cell-free extract suggested that the molecular weight, as determined by the Hedrick and Smith (1968) method, was identical to that of the purified enzyme (Yun *et al.*, 1975). This communication describes the immunoprecipitation method of comparing the subunit molecular weight of PK from cell-free extract with the purified enzyme. This method involved (1) immunoprecipitation of PK from the cell-free extract, (2) extensive washing to remove nonspecifically adsorbed proteins, (3) solubilization of the immunoprecipitate in 1% SDS solution, (4) electrophoretic analysis of the solubilized immunoprecipitate on SDS-polyacrylamide gels, and (5) determination and compar

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comparison of the molecular weight determined for PK from cell-free extract with that of pure enzyme. Similar techniques have previously been used to study immunoglobulins (Baur *et al.*, 1971), O-antigens (Atwell *et al.*, 1973), H-2 alloantigens (Schwartz and Nathenson, 1971; Cullen *et al.*, 1972) and rat liver microsomal electron transport proteins (Welton *et al.*, 1973a; Welton *et al.*, 1973b).

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LITERATURE REVIEW

Pyruvate kinase (ATP pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the following reaction:

$$H^+ + PEP + ADP \overset{Mg^{2+},\ K^+}{\rightleftharpoons} pyruvate + ATP.$$

This enzyme is ubiquitously distributed as a component of the glycolytic enzyme sequence in all living organisms from simple, single cell organisms to the most complex eucaryotes. Pyruvate kinase has been shown to be an important glycolytic control point in both intact yeast cells and lysates (Hommes, 1964; Pye and Eddy, 1965; Hess and Brand, 1965a). This would be expected since both products of the pyruvate kinase reaction, ATP and pyruvate, feed into a number of other metabolic pathways, and the substrate phosphoenolpyruvate is a very important controlling compound for carbohydrate catabolism (Kornberg, 1973). The enzyme can be induced in yeast (Barwell and Hess, 1971), and there are reports of probable regulation of the enzyme activity during gluconeogenesis in yeasts (Fernandez *et al.*, 1967). All of these facts strongly suggest that pyruvate kinase plays an important role in yeast metabolism.

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Since the earliest investigation of yeast PK by Washio and Mano (1960), there have been a flurry of reports on the purified yeast enzyme. Original preparations of the enzyme reporting high purity and stability from *S. carlsbergensis* (Haekel *et al.*, 1968) have since been shown to be contaminated by a protease activity which would degrade the pyruvate kinase under certain conditions (Roschlau and Hess, 1972). The original preparation from *S. cerevisiae* (Hunsley and Suelter, 1969a) has been shown to produce a degraded form of the enzyme (Fell *et al.*, 1974; Aust *et al.*, 1975) and new preparations were developed to purify the native enzyme.

In this review, literature regarding the purified yeast enzyme, literature regarding protease contamination of other enzymes purified from yeast and characteristics of proteases isolated from yeast will be discussed.

Yeast Pyruvate Kinase

A. Activators

1. Monovalent Cations

Pyruvate kinase of *S. cerevisiae* and of *S. carlsbergensis* requires the presence of a monovalent cation for activity (Washio and Mano, 1960; Hunsley and Suelter, 1969b; Hess and Haebel, 1967). PK experiences an allosteric activation in the presence of K^+; thus, K^+ could be referred to as a homotropic activator. Although

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K^+ is the most effective activator in the presence or absence of FDP, NH_4^+ also activates. Na^+ is much less effective and activates only in the presence of FDP. Addition of K^+ to PK causes a slight quenching of its intrinsic fluorescence while addition of TMA does not (Kuczenski, 1970). This suggests that PK undergoes a small conformational change as the result of interaction with K^+. This change is specific for K^+ as opposed to TMA. This would indicate that K^+ activation is due to its interaction with the enzyme directly as is thought to be the case in muscle PK (Suelter *et al.*, 1966; Wilson *et al.*, 1967). The binding of K^+ to PK has an antagonistic effect on the binding of FDP, as viewed with intrinsic fluorescence changes (Kuczenski, 1970). This is thought to be due both to an ionic strength effect and to a specific K^+ effect.

The mechanism by which monovalent cations activate enzymes is controversial. One school of thought maintains that monovalent cations are necessary to establish an enzyme conformation necessary for catalysis (Evans and Sorger, 1966). Another theorizes a specific role for the cations in the mechanism of the reaction:interaction with the enol-keto tautomers of pyruvate at the active site (Suelter, 1970). The latter theory is much more specific and requires that the cation actually bind at the active site. This has been confirmed in the case of muscle P

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muscle PK through the use of NMR spectroscopy (Kayne and Reuben, 1970), but it is not known for the yeast enzyme.

2. Hydrogen Ion

Since a proton is taken up in the pyruvate kinase reaction it is conceivable that H^+ could have an effect on the enzyme. Wieker and Hess (1971) investigated the pH dependencies of the kinetic parameters characterizing the interaction of *S. carlsbergensis* PK and PEP in the presence and absence of FDP. n_H for PEP in the presence of FDP was found to be independent of pH while the n_H of PEP in the absence of FDP was dependent upon pH going from 1 below pH 4.0 to a limiting maximal value of 2.95 at pH 7.0. The pH dependence of the ratio of the half-saturating substrate concentration in the absence of FDP to that in the presence of FDP produced a curve similar to that for the n_H. These data suggested that the equilibrium between a state of higher affinity and a state of lower affinity of the enzyme for PEP is shifted to a state of higher affinity as the pH approaches 5.35, thus suggesting a role for the H^+ as an allosteric activator of yeast PK. The question of whether the H^+-activated and FDP-activated states are identical in conformation was not answered.

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3. FDP

Hess and Brand (1965a) were the first to identify FDP as an allosteric activator of yeast pyruvate kinase. The enzyme from many other sources has also been shown to be FDP activated. In both strains of yeast FDP acts as a heterotropic allosteric activator by lowering the Hill constant for PEP and both required cations and lowering the apparent K_m or K_A for each (Haekel *et al.*, 1968; Hunsley and Suelter, 1969b). Therefore, PK would best be classified as a K system in the nomenclature of Monod, Wyman and Changeux (1965).

4. Mg^{2+}

Mg^{2+} has been shown to be a homotropic activator in both *S. cerevisiae* (Hunsley and Suelter, 1969b) and *S. carlsbergensis* (Haekel *et al.*, 1968). In addition Mg^{2+}, or another activating divalent metal, is required for PK activity in both yeast strains examined, and the allosteric kinetics of Mg^{2+} are transformed to hyperbolic by FDP. The actual form of Mg^{2+} which binds to the enzyme resulting in catalytic activity is not known. Since Mg^{2+} can be complexed by PEP, ADP, FDP, and ATP, the kinetic analysis of Mg^{2+} can be quite complex. MacFarlane and Ainsworth (1972) have developed a complex equilibrium expression for Mg^{2+} in its free and complexed forms taking into account all substrates and products and have conducted an involved kinetic analysis of PK.

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Their evaluation of the results indicated that Mg^{2+} binds the enzyme in the free form, as do ADP and PEP, and is released in the complexed form, Mg-ATP.

Mg^{2+} appears to be required for PK stability, as will be discussed later. The binding of Mg^{2+} to the enzyme can be detected by a quenching of fluorescence, but the quenching is minimal compared to that produced by the binding of FDP (Kuczenski, 1970).

Mg^{2+} appears to affect the affinity of *S. carlsbergensis* PK for PEP and FDP (Haekel *et al.*, 1968). The K_m for PEP increases with decreasing Mg.

5. PEP

PK from both *S. cerevisiae* (Hunsley and Suelter, 1969b) and *S. carlsbergensis* (Haekel *et al.*, 1968) showed cooperative kinetics (homotropic activation) for PEP. In the presence of FDP the sigmoid kinetics are converted to hyperbolic with no change in V_m.

B. ADP

Kinetic studies of PK from both *S. cerevisiae* (Hunsley and Suelter, 1969b) and *S. carlsbergensis* (Haekel *et al.*, 1968) indicated that at saturating levels of all other substrates and cations, ADP appeared to be only weakly controlled by FDP and was, therefore, not an allosteric effector for ADP interactions.

C. Inhibitors

Cu^{2+} as well as some other heavy metals were shown with early preparations of yeast PK to be potent inhibitors (Washio and Mano, 1960). Other inhibitory compounds noted for the brewers' yeast enzyme (*S. carlsbergensis*), such as citrate, NADP, AMP, 3'-5'-cyclic AMP, and nucleotide triphosphates, appeared to exhibit a complex allosteric inhibition which could be due to their ability to complex Mg^{2+} (Haekel *et al.*, 1968).

ATP inhibition has been observed for both the brewers' yeast PK (Haekel *et al.*, 1968) and the bakers' yeast PK (*S. cerevisiae*) (MacFarlane and Ainsworth, 1972). In the case of the brewers' yeast PK, the inhibition appeared to be allosteric, but since no attempt was made to control the Mg^{2+} concentration throughout the experiment, it would be very difficult to draw firm conclusions. In the case of bakers' yeast PK, the Mg^{2+} levels were carefully controlled throughout the experiments and Mg-ATP complex was shown to be a competitive inhibitor with PEP.

D. Reaction Mechanism

Mildvan *et al.* (1970) were the first to show inconsistencies in kinetic and binding data for bakers' yeast enzyme that might indicate that this enzyme has a preferred order of binding PEP. A kinetic study by MacFarlane and Ainsworth (1972) seemed to confirm this, for their results suggested that the mechanism is of the ordered

Tri Bi type with the substrates binding in the order PEP, ADP, and Mg^{2+}. An interesting conclusion from this study, apparent in the last statement, was that Mg^{2+} binds the enzyme in a free form, not complexed to ADP. However, after the phosphoryl transfer in the quaternary complex, pyruvate is released followed by Mg-ATP complex. This posulated mechanism would suggest that Mg^{2+} binds the enzyme, bridges the phosphate group of PEP and the terminal phosphate group of ADP, assists the phosphorylation of the latter, and is ultimately eliminated, bound between the β- and γ-phosphate groups of ATP.

E. Cold Lability and Fructose 1,6-diphosphate Induced Instability

An instability of yeast pyruvate kinase was first described by Washio and Mano (1960). Glycerol was found to stabilize the enzyme, thus making its purification much easier (Hunsley and Suelter, 1969a). Subsequently Kuczenski and Suelter (1970) reported that pyruvate kinase was susceptible to inactivation at low temperatures, or in other words, that the enzyme was cold labile. Inactivation at room temperature and at 0° C was enhanced by the addition of micromolar amounts of the allosteric activator FDP. Addition of Mg^{2+} or Mn^{2+} prevented inactivation. A mechanism for the inactivation involving binding of FDP, followed by dissociation of the enzyme into subunits was proposed. Enzyme purified from bakers' yeast by a slight modification of the procedure of Hunsley and Suelter (1969a), produced a cold labile PK, also (Fell *et al.*, 1974). However, PK purified from brewers' yeast (*S. carlsbergensis*) was reported not to be cold labile (Bischofberger *et al.*, 1971).

Protease Contamination or Degradation of Yeast Pyruvate Kinase

There have been several methods for purification of yeast PK that have been developed over the past several years. Table 1 shows a summary of some of the important aspects of these preparations and characteristics of the resulting PK.

Roschlau and Hess (1972) reported proteolytic contamination of purified PK. The activity of the protease was detected noting that free amino acids were released upon storage of the PK at room temperature. Further heterogeneities were seen when PK was subjected to ultracentrifugal analysis (Bischofberger *et al.*, 1971) and SDS gel electrophoresis. The purification was modified by adding ammonium sulfate fractionation and ion exchange chromatography. The PK obtained was reported free of protease contamination.

Fell *et al.* (1974), in an attempt to repeat the procedure of Ashton (1971), found that SDS gel electrophoretic analysis gave multiple bands, suggesting that the polypeptide chains had been degraded. Therefore, Fell devised

TABLE 1

Properties of Yeast Pyruvate Kinase
Purified by Different Methods

TECHNIQUE	*S. cerevisiae* prepared by the method of Hunsley and Suelter (1969a)[1]	*S. cerevisiae* prepared by the method of Ashton (1971)[2]	*S. cerevisiae* prepared by the method of Fell *et al.* (1974)[1]	*S. carlsbergensis* prepared by the method of Haekel *et al.* (1968) or Roschlau & Hess (1972)[3]
Molecular wt., calculated from Svedberg equation	167,000	162,000	217,000	191,000
$\overline{M}w$, subunits, sedimentation equilibrium	42,000	21,000	55,000	51,000
$\overline{M}w$, subunits, SDS gel	----	18,000-20,000	56,000	55,000-60,000
$E_{1\ cm}^{0.1\%}$ at 280 nm	0.65	0.65	0.66	0.76
Specific activity, 25° C	220 (30° C)	180-200	340-400	250

TABLE 1 (continued)

TABLE 1 (continued)

TECHNIQUE	*S. cervisiae* prepared by the method of Hunsley & Suelter (1969a)[1]	*S. cerevisiae* prepared by the method of Ashton (1971)[2]	*S. cerevisiae* prepared by the method of Fell *et al.* (1974)[1]	*S. carlsbergensis* prepared by the method of Haekel *et al.* (1968) or Roschlau & Hess (1972)[3]
Cold lability	yes	yes	yes	no
Method of lysing yeast	Toluolysis at 45° C	Toluolysis	Toluolysis at -10 by thawing (4° C) in buffer & serine protease inhibitor	Toluolysis at 37° C
Protease contamination	?	?	no	no
Protease degradation	yes	yes	no	no
N-terminal	----	----	none detected	acetylated
C-terminal	----	----	----	valine

TABLE 1 (continued)

TABLE 1 (continued)

TECHNIQUE	*S. cerevisiae* prepared by the method of Hunsley & Suelter (1969a)[1]	*S. cerevisiae* prepared by the method of Ashton (1971)[2]	*S. cerevisiae* prepared by the method of Fell *et al.* (1974)[1]	*S. carlsbergensis* prepared by the method of Haekel *et al.* (1968) or Roschlau & Hess (1972)[3]
Isoelectric point	6.2 & 6.4	----	----	6.08
Requires mono- and divalent cations	yes	yes	yes	yes
Activation by FDP	yes	yes	yes	yes

[1] Hunsley and Suelter, 1969b; Kuczenski and Suelter, 1970.

[2] Ashton and Peacock, 1971.

[3] Bischofberger *et al.*, 1971.

a modified preparation by lysing the yeast by a toluolysis, freeze-thawing method and PMSF, a serine protease inhibitor, was added before dialysis. The resulting enzyme properties are shown in Table 1 and can be compared to those of the PK purified by the method of Ashton (1971). Both the molecular weight and specific activity increase with little if any change in the other properties.

Yun *et al.* (1975) modified the preparation of Hunsley (1969a) such that all column steps were conducted at room temperature in buffer containing 5 mM EDTA, 5 mM βME and 25% glycerol instead of 50%. The resulting enzyme had an increased molecular weight and specific activity greater than that of Hunsley and Suelter (1969a). However, when the enzyme was analyzed on SDS gel electrophoresis, the protein was found in multiple bands with the majority near the tracking dye. This indicated that the enzyme was contaminated by a protease activity. Further modification of this preparation which made use of a mechanical means for lysing the yeast in the presence of DFP produced an enzyme which had the same molecular weight as that by the previous modification (Aust *et al.*, 1975). The PK was still contaminated by protease activity, although the contamination was less than before. This communication will describe in detail this final modification and the enzyme produced by it.

Every preparation of yeast PK that has been reported has been plagued by proteolytic degradation or contamination at some point in its development. The problem is not peculiar to PK, but has been seen in the preparation of many other yeast enzymes and enzymes from other microorganisms. The remainder of the literature review will discuss similar problems in other yeast enzymes, possible roles for these proteases, and finally properties of the yeast protease purified.

Proteolytic Degradation or Contamination of Enzymes Purified from Yeast

Early procedures for isolating hexokinase from yeast involved drying of the yeast and autolysis of the yeast cells at 37° C to liberate the enzyme (Kunitz and McDonald, 1945; Berger *et al.*, 1946; Bailey and Webb, 1948; Darrow and Colowick, 1962; Lazarus *et al.*, 1966; Schulze *et al.*, 1967). Lazarus *et al.* (1966) discovered a common problem in these types of procedures; namely, the autolysis, which released an abundance of protease activity resulting in partial degradation of the enzyme either at that stage, or after isolation, due to adhering traces of protease (Ramel *et al.*, 1971). Since the cleavages were compatible with high retention of activity, the degradation was overlooked initially. Pringle (1970) showed that commercial preparations of hexokinase were contaminated with a trace of at least one protease. The contamination was discovered when the molecular weight in the presence of SDS was studied. It was shown that during the preincubation with SDS before electrophoresis, the enzyme was degraded to form multiple low molecular weight components. If the sample was treated with DFP or boiled immediately, there were only 2 minor low molecular weight components produced. When the preparative procedure was modified in such a way that mechanical disruption of the cells in the presence of DFP was used (Rustum *et al.*, 1971; Barnard, 1975), three isozymes of hexokinase, A, B, and C, could be purified free of protease activity as monitored by the high stability upon storage and lack of degradation in SDS.

Another extensively studied yeast enzyme, L-lactate dehydrogenase (cytochrome b_2) from yeast, was suspected of having proteolytic contamination when Nichols *et al.* (1966) reported that upon storage at 4° C the Michaelis constant for L-lactate increased. The mean mobility of the enzyme increased on polyacrylamide gels and multiple bands of L-lactate dehydrogenase activity were also found. Later, when Jacq and Lederer (1970) examined the amino acid composition of the crystallized enzyme, they reported that they felt it was necessary to show constant chemical composition of the enzyme because yield and specific activity varied from one preparation to the next in a not negligible way. There was some variability in the amino acid composition but no firm conclusions could be made. Lederer and Simon (1971) showed that the crystallized enzyme was composed of two types of subunits, 36,000

MW and 21,000 MW. After carefully determining the N and C terminals from both light and heavy chains, Lederer and Jacq (1971) concluded that the enzyme was being purified with an endopeptidase of the serine protease class. Jacq and Lederer (1972) subsequently modified the enzyme purification by using a mechanical means (Manton-Gaulin homogenizer) to rupture the cells and by including PMSF and found that crystallization never took place. Thus, the crystallized enzyme was an artifact produced by proteolytic degradation.

Diezel *et al.* (1972) reported that upon storage, phosphofructokinase purified in the presence of PMSF was converted from an enzyme which sedimented with 19S to one that sedimented as 17S. It was concluded that phosphofructokinase contained a proteolytic contaminant which did not degrade phosphofructokinase in the presence of its substrates, fructose-6-phosphate or ATP or in the presence of ammonium sulfate. In trying to find a protease inhibitor to use while isolating phosphofructokinase, Diezel *et al.* (1973) found DFP, PMSF, and ammonium sulfate (25%) to be good inhibitors. Thus, they developed a new purification procedure which made use of the ultrasonic disintegrator, affinity chromatography, ammonium sulfate precipitation, and gel filtration. This procedure considerably reduced the contamination but did not completely remove it. It was suggested that there might be an *in vivo* function for the protease attack on phosphofructokinase.

Yeast pyruvate decarboxylase catalyzes a two step reaction. It has been postulated that these two reactions occur at different locations on the enzymes' surface. In trying to determine whether this was really true or not, Juni and Heym (1968a) studied the enzyme from bakers' yeast and from a mutant derived from this organism. The enzyme from mutant yeast, upon aging, lost the ability to catalyze one of the two reactions. In studying this phenomenon it was discovered that this loss was due to selective proteolytic degradation of one portion of the protein. The reason this occurred in the mutant strain was that more proteases were released upon lysis (Juni and Heym, 1968b). When sonication was used to rapidly lyse the cells rather than the slow buffer extraction procedure previously used on dried yeast, it was found that activity ratios of pyruvate decarboxylase were as high as those of enzyme from bakers' yeast. This result demonstrated that proteases were not active in the intact cells but that their activity was released with time. Results from this study also indicated that the levels of proteases that were found in crude extracts depended on the strain of yeast used as well as the phase of growth of the harvested cells.

These are but a few examples of yeast enzymes which have been isolated in degraded or contaminated form and extensively studied. Due to the fact that in most cases enzyme activity was not completely destroyed in the degraded enzymes, the protease problem was not discovered except by accident while doing SDS gels or other physical characterization. In each case presented, the investigators switched from an autolytic procedure for rupturing the cells to a mechanical one (Manton-Gaulin homogenizer, french press, sonicator) and in several cases included serine protease inhibitors. These modifications produced preparations which were more satisfying to the investigators based on various criteria, but the question still lingers: are these enzymes further artifacts of preparation or are they native to yeast? Once there is doubt, can the question ever really be answered? This brings a new way of thinking into enzymology. Until recently it was generally accepted that purified enzymes were accurate representations of the native enzyme. They might behave slightly differently in their new environment, but they were physically the native enzyme. For many investigators working with microorganisms it has become painfully clear that this is not always the case. This might also be a problem with enzymes from more advanced organisms, but at this point it has not been recognized as one.

Possible *in vivo* Regulation by Yeast Proteases

Cabib and Bowers (1971), in studying budding in yeast, have shown that chitin is the specific component of the septum between mother and daughter cells. They have also shown that chitin formation takes place only during a limited portion of the cell cycle. Thus, formation of the chitin septum is initiated at a specific time in a specific location. Chitin synthetase, the enzyme responsible for mobilization of the chitin, was shown to normally exist as a zymogen bound to the cytoplasmic membrane (Keller and Cabib, 1971). The zymogen was shown to be activated by either an enzyme present in yeast or by trypsin (Cabib and Farkas, 1971). The *in vivo* activating enzyme for the zymogen was shown to be localized within the yeast vacuole and was therefore separated from the zymogen during most of the cell cycle (Cabib and Ulane, 1973). At the time of budding the vacuoles have been observed to migrate to the bud and finally coalesce into one or a few large bodies (Wiemken *et al.*, 1970). It has not been conclusively shown that the vacuoles at the bud positively contain the activating factor, but it is quite suggestive that this is the mechanism by which the activating factor comes in contact with the chitin synthetase zymogen thus causing bud formation. The activating factor has been shown to be a protease and can be inhibited by a soluble protein factor present in yeast (Cabib and Ulane, 1973). The evidence becomes quite convincing that an example has been found where proteolytic action on a yeast enzyme serves a distinct and necessary *in vivo* function but is under strict control through compartmentalization and inhibition.

Fructose 1,6-bisphosphatase is a key enzyme of the gluconeogenic pathway and is subject in yeast to a number of different control mechanisms. Not only are synthesis and activity regulated (Gancedo *et al.*, 1965), but an abrupt drop in activity is observed when glucose is added to a yeast culture with high levels of fructose 1,6-biphosphatase (Harris and Ferguson, 1967). The reappearance of the enzyme was shown to be dependent upon an energy source and was prevented by cycloheximide (Gancedo, 1971). Thus, it appeared that inactivation of the enzyme was irreversible and that reappearance required protein synthesis. On the basis of these observations Molano and Gancedo (1974) isolated a protein fraction from yeast which was capable of specifically inactivating the enzyme. When this inactivating protein factor was mixed with malate dehydrogenase, hexokinase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, or catalase from bakers' yeast or fructose 1,6-bisphosphatase from *Rhodotorula glutinis*, no such inactivation was seen. A fraction which could inhibit the inactivating factor was isolated from the yeast also. Based on this and the fact that the inactivating factor was a protein and that it followed the behavior of some yeast proteinases during extraction and initial purification steps, it was concluded that the inactivating protein was a protease. Though it was not shown conclusively that this protease had the *in vivo* function of inactivating fructose 1,6-bisphosphatase, it is tempting to suggest that this and other proteolytic enzymes display control of enzyme activity at the post-transcriptional level.

Yeast Proteases

Yeast is a eucaryotic organism which is predominantly found in a unicellular form (Rose and Harrison, 1969). It has a tough cell wall formed from chitin, glucan and protein. As an eucaryote, a yeast cell contains all the typical subcellular organelles present in higher plants and animals. One predominant structural component, the vacuole, is very conspicuous in yeast observed with a microscope. This vacuole appears to be the precursor of lysosomes in higher organisms. It is a single membrane vesicle which can be isolated free from other cellular components (Vitols *et al.*, 1961). In so doing, investigators have found that the vacuole contains many hydrolytic enzymes, including proteases, ribonucleases and esterases. Evidence has been presented which indicates that these hydrolases are localized exclusively in the vacuoles (Matile and Wiemken, 1967).

Three of these intracellular proteases which are contained within this vacuole (Cabib and Ulane, 1973) have been purified and characterized. For the purposes of this discussion, they will be referred to as A, B, and C. Protease A, an endopeptidase, is considered an acid protease since its pH optimum for hemoglobin substrate is pH 3 (Hata *et al.*, 1967a). There have been two molecular weights reported for A, 60,000 as determined in Model E ultracentrifuge by the Archibald method (Hata *et al.*, 1967b) and 42,000 as determined by Sephadex G-75 gel filtration (Saheki *et al.*, 1974). No chemical inhibitors have been reported, but 4 *in vivo* protein inhibitors have been identified and 2 have been purified (Saheki *et al.*, 1974). The two purified inhibitors have the same molecular weight as determined by SDS gel electrophoresis, 6100 ± 200. These inhibitors work very effectively at pH 5-7, but do very little to inhibit at pH 3.

Protease B, an endopeptidase, has a pH optimum of 6.7 (Lenney and Dalbec, 1967; Lenney, 1956). There has been controversy over the molecular weight of this protein; 32,000 was reported by Lenney and Dalbec (1967) using Sephadex G-75 gel filtration and 82,000 was reported by Schott and Holzer (1974) using the Model E ultracentrifuge and Yphantis analysis of the data. Two classes of chemical inhibitors have been found for protease B. The enzyme is inactivated by thiol binding compounds, such as PCMB, and by serine protease inhibitors, such as DFP (Lenney and Dalbec, 1967). One *in vivo* protein inhibitor has been isolated. The molecular weight determined by Sephadex G-75 was 10,000.

Protease C is a broad specificity carboxypeptidase (Hayashi *et al.*, 1970) which will proceed through proline residues and because of that has proved to be a useful reagent in protein chemistry (Hayashi *et al.*, 1973). The molecular weight of this enzyme is 61,000 as determined by the Archibald method (Hayashi *et al.*, 1969). This enzyme is inhibited by two classes of chemical inhibitors just as is protease B. Thiol reactive compounds, such as PCMB, and serine protease inhibitors, such as DFP, can completely inhibit its activity (Doi *et al.*, 1967). The DFP inhibition was not immediate, but occurred over a period of time. An *in vivo* protein inhibitor has also been isolated for protease C (Hayashi *et al.*, 1969). Its molecular weight is 20,000. The pH optimum of the enzyme for casein as substrate is 6.0 (Doi *et al.*, 1967).

Pro-protease C (protease-inhibitor complex) can be activated without dissociation of the inhibitor with protein denaturants, such as urea, guanidine hydrochloride, acids and solvents including dimethylformamide, 2-chloroethanol, dioxane, formamide, ethanol and n-propanol (Hayashi *et al.*, 1972). This activated, pro-protease would react with DFP, but pro-protease C before treatment with denaturants would not. From these results it was concluded that the denaturants rearranged the quaternary structure of the proenzyme and led to demasking of the active site allowing reaction.

Table 2 summarizes some of the properties of the three yeast proteases discussed.

TABLE 2

Properties of Three Purified Proteases from *S. cerevisiae*

Properties	A	B	C
pH optimum	3[1]	6.7[4,5]	6[7]
Chemical inhibitor	none	DFP, PCMB[4]	DFP, PCMB[7]
in vivo protein inhibitor	6,100 MW[3]	10,000 MW[4]	20,000 MW[8]
molecular weight	60,000[2] or 42,000[3]	32,000[4] or 82,000[6]	61,000[8]
in vivo activator	protease B[3]	protease A[11]	protease A[10]
Mechanism of action	endopeptidase[1]	endopeptidase[4]	endopeptidase[9]

[1] Hata *et al.*, 1967a; [2] Hata *et al.*, 1967b; [3] Saheki *et al.*, 1974; [4] Lenney and Dalbec, 1967; [5] Lenney, 1956; [6] Schott and Holzer, 1974; [7] Doi *et al.*, 1967; [8] Hayashi *et al.*, 1969; [9] Hayashi *et al.*, 1970; [10] Hayashi *et al.*, 1968; [11] Saheki and Holzer, 1975.

Besides being compartmentalized and inhibited by specific *in vivo* inhibitors, yeast proteases appear to undergo a complicated regulation among themselves. Hayashi *et al.* (1968) and Hayashi and Hata (1972) have shown that protease A can activate pro-protease C. Saheki *et al.* (1974) have shown that purified protease B can inactivate the two purified inhibitors of protease A. Neither protease A nor C has this capability. Saheki and Holzer (1975), in studies on crude yeast extracts, have shown that upon incubation at pH 5.1 and 25° C, an increase in protease B activity is paralleled with the disappearance of protease B inhibitor. Addition of purified protease A to fresh crude extracts accelerated the inactivation of the protease B inhibitor and the appearance of maximal activities of protease B and C. Pepstatin, a potent protease A inhibitor with no effect on B or C, markedly retarded the increase of protease B activity, by reducing the amount of protease B inhibitor destroyed. Thus, a direct role for activation of protease B by protease A has been shown in crude extracts. It has also been demonstrated that the protease C inhibitor can be destroyed by proteases A and B (Keiditsch and Strauch, 1970). It has also been shown that the appearance of protease A activity paralleled the activation of B and C (Saheki and Holzer, 1975). Thus, it appears that there could be inter-regulation among the proteases which would involve destruction of inhibitors so that further protease activity could be expressed.

Several interesting observations have been made about the appearance of proteases after yeast growth in different nutrient media. Yeast grown in complex medium show low protease activity in extracts, whereas yeast grown in minimal medium show high levels of protease activity (Manney, 1968). Extracts of yeast harvested in the exponential growth phase have very low protease activities while those harvested during late exponential or stationary phase have high protease activities (Katsunuma *et al.*, 1972). An explanation for the biological significance for this might be that in poor medium or stationary phase yeast cells need only a very low maintenance metabolism with very few biosynthetic enzyme activities. In this situation it would be beneficial for the cell to degrade unnecessary biosynthetic enzymes to make metabolic intermediates available for other purposes. A similar concept was proposed by Mandelstam (1962) in explaining the significance of proteolytic enzymes and protease inhibitors in *E. coli*.

The literature suggests that the yeast intracellular proteases may have self-regulatory mechanisms and regulatory mechanisms for other intracellular proteins. In most cases, however, the mechanism of this regulation is yet to be elucidated.

METHODS AND MATERIALS

Materials

Fresh Budweiser bakers' yeast, *Saccharomyces cerevisiae*, was obtained from Food Stores at Michigan State University. Lactic dehydrogenase, the coupling enzyme used in the pyruvate kinase assay, was Sigma type II rabbit muscle enzyme substantially free of pyruvate kinase. The LDH was desalted by passage over Sephadex G-25 in 0.2 M K-MES or $(CH_3)_4N$-MES, pH 7.5, or by dialysis against the same buffer. Yeast pyruvate kinase purified using toluolysis to lyse the yeast by the method of Yun *et al.* (1975) was a gift from Dr. S.-L. Yun.

The following proteins were used as molecular weight standards: type II rabbit muscle lactic dehydrogenase, grade I rabbit muscle aldolase, bovine serum albumin, bovine pancreas α-chymotrypsinogen A (all from Sigma Chemical Co., St. Louis, MO), bovine liver catalase, and rabbit muscle pyruvate kinase (both from Worthington Biochemical Corp., Freehold, NJ). Yeast hexokinase and mixed crystals of rabbit muscle α-glycerophosphate dehydrogenase and triose phosphate isomerase were obtained from Sigma Chemical Co., St. Louis, MO.

DFP, Na ADP (Fermentation grade and Grade I), tetracyclohexylammonium FDP, tricyclohexylammonium PEP, MES, cacodylic acid, βME, DTE, DTT, Na EDTA, Tris base Dowex chelating resin, DEAE, dansyl Cl, SDS, and Brilliant Blue R Coomassie (Coomassie Blue) were obtained from Sigma Chemical Co., St. Louis, MO. Na ADP, Na ATP, and Na NADH were obtained from P and L Biochemical Co., Milwaukee, WI. $(CH_3)_4NOH \cdot 5H_2O$ and Dowex 50-X8 were purchased from J. T. Baker Co., Cleveland, OH. $(CH_3)_4NCl$ was obtained from Eastman Organic Chemicals, Rochester, NY. N,N,N',N'-tetramethylenediamine, N,N'-methylenebisacrylamide, and acrylamide were purchased from Canalco, Rockville, MD. Ampholine carrier ampholytes pH range 5-8 were products of LKB, Sweden. Sephadex G-25 coarse and G-100 were obtained from Pharmacia, Uppsala, Sweden. Cellulose phosphate, 1.12 and 1.24 mEq/g, was a product of Brown Co., Berlin, NH. Polyamide sheets were obtained from Cheng Chin through Gallard-Schlesinger Chemicals, Carle Place, NJ. Pyronin B was purchased from Hartman-Leddon, Philadelphia, PA. Agar for Ouchterlony double diffusion analysis and immunoelectrophoresis was obtained from Difco, Detroit, MI. Freund's complete and incomplete adjuvant were purchased from Gibco, Grand Island, NY. Female New Zealand rabbits, weighing 5 pounds, were obtained from CLAR, Michigan State University, East Lansing, MI. Film used to photograph Ouchterlony and immunoelectrophoretic results was Kodak High Contrast Copy Panchromatic Film obtained from Eastman-Kodak, Rochester, NY. Ammonium sulfate was Mann Special Enzyme Grade, Schwarz-Mann, Orangeburg, NY. All other reagents were analytical grade.

Water for all experiments was distilled and further deionized by passage through a column packed with Amberlite MB-3 mixed bed resin from Mallinkrodt, St. Louis, MO.

Assay of Pyruvate Kinase

Pyruvate kinase was assayed using the linked lactic dehydrogenase reaction modified from Bücher and Pfleiderer (1955). Routine assays contained per ml: 100 μmoles $(CH_3)_4N$-cacodylate, pH 6.2; 24 μmoles $MgCl_2$; 100 μmoles KCl; 10 μmoles tri CHA PEP; 10 μmoles NaADP; 1 μmole tetraCHA FDP; 0.15 μmole Na NADH; and 33 μg lactic dehydrogenase. After the reaction mixture was allowed to equilibrate, a blank rate, arising from PK contamination of the LDH, was measured. The reaction was initiated by adding 5-10 μl of a PK solution containing 10-20 μg/ml. The initial rate of the reaction was corrected for the blank rate. For routine assays during purification, 1 μmole of tri CHA PEP was used instead of 10.

Protein Concentration

The extinction coefficient was determined for pure PK as described by Yun *et al.* (1975) and was found to be

$E_{280}^{0.1\%} = 0.51$. In the initial steps of the PK purification, the biuret method (Layne, 1957) was used to determine the protein concentration using bovine serum albumin as a standard.

Definition of Unit and Specific Activity

One unit of pyruvate kinase activity is defined as the amount of enzyme that catalyzes the transformation of 1 μmole of substrate per minute under the conditions specified. During the PK purification before the first cellulose phosphate column at pH 6.5, the specific activity was calculated using the protein concentration determined by the biuret method. After the CP column at 6.5 and whenever pure enzyme was used, the specific activity was calculated using the extinction coefficient for the pure enzyme.

Purification of Pyruvate Kinase

A. Buffers

Buffers of the following composition were used: pH 6.5 buffer contained 10 mM sodium phosphate, 5 mM sodium EDTA, 5 mM βME and 25% glycerol adjusted to pH 6.5 with 5 N NaOH. The pH 7.5 buffer was identical to the above except that it was adjusted to pH 7.5. The ammonium sulfate buffers were also identical in composition to the above except that they contained ammonium sulfate at the appropriate concentrations, and the pH was adjusted to 6.5. The final 90% saturated ammonium sulfate buffer contained 10 mM sodium phosphate, 5 mM sodium EDTA, 10 mM βME, 0.02% sodium azide, and was adjusted to pH 6.5 with 5 N NaOH. βME was added to all buffers just before use to avoid excessive oxidation of the thiol.

B. Preparation of DFP

One gram of DFP was dissolved in 49 ml of dried isopropanol (dried over Fisher Type 5A molecular sieve or sodium sulfate) yielding an approximate 0.1 M solution. The stock solution was stored as 10 ml aliquots at -20° C. Condensation of water vapor in the DFP solutions was avoided since the presence of water would result in hydrolysis of the DFP. Whenever DFP was added to protein-containing solutions, it was first diluted 1 to 10 with the appropriate solvent and then added slowly with rapid stirring to the protein solution.

Due to the extreme toxicity of DFP, all operations involving DFP at concentrations greater than 1 mM were conducted in a hood with good air flow. The user wore gloves and chemically resistant apron at all times. All contaminated glassware was placed in 0.5 N NaOH for a period of at least 24 hours for complete hydrolysis of the DFP (Saunders, 1957).

C. Preparation of Cellulose Ion Exchange Resins

Cellulose phosphate (CP) resin was washed as previously described (Hunsley and Suelter, 1969a). After completion of the washing procedure, the pH was adjusted to 7 with 1 N H_3PO_4 for storage at 4° C. The resin was never allowed to remain at a pH below 7 for prolonged periods due to the susceptibility of the phosphate ester to hydrolysis which would render the resin useless. For column preparation, the cellulose was suspended in the appropriate buffer and the pH adjusted directly, using a Sargent pH meter. The resin was suction filtered and resuspended in the same buffer and de-aerated. The columns were poured to the dimensions described in the Results section and prior to use washed with at least 1 column volume of buffer containing fresh βME. If the pH of the eluate was not that of the column buffer, the column was washed further until the proper pH was achieved. After use, the resin was stored at 4° C to be washed again. After four washings, sufficient phosphate had been removed from the column so that it would no longer bind PK. The resin was then discarded and new resin prepared for use.

DEAE cellulose was washed as previously described (Hunsley and Suelter, 1969a). After completion of the washing procedure the resin was suspended in 0.02% sodium azide for storage at 4° C. For column preparation, the resin was washed several times with water to remove the sodium azide, then suspended in the appropriate buffer and the pH adjusted directly, using the Sargent pH meter. The resin was suction filtered and resuspended in the same buffer and de-aerated. The columns were poured to the dimensions described in the Results section and prior to use washed with at least 1 column volume of buffer containing fresh βME. If the pH of the eluate was not that of the column buffer, the column was washed further until the proper pH was achieved. Used DEAE was stored at 4° C for rewashing. The DEAE could be continually rewashed with no apparent effect on the resin capacity.

D. Preparation of Sephadex G-100

Sephadex G-100 was incubated at 100° C for 12 hours for complete swelling of the gel. The Sephadex was then poured into a Pharmacia column equilibrated by reverse flow. Samples were applied and also eluted by reverse flow.

Preparation of Substrates for Assay

Sodium salts of ADP and ATP were converted to Tris, $(CH_3)_4N$ or K salts by ion exchange chromatography using Dowex 50 W-X8 resin which had been equilibrated with the appropriate base (Tris, $(CH_3)_4NOH$, or KOH, 1 N) and washed exhaustively with H_2O until pH 7.0 was obtained.

These salt forms were more desirable for kinetic studies since sodium, in many cases, affects PK kinetics differently than potassium.

Determination of Substrate or Inhibitor Concentrations

ADP and PEP concentrations were determined using the same enzymatic assay as described previously for PK (Bucher and Pfleiderer, 1955) except that an excess of yeast PK was used. Concentrations of FDP were measured in the presence of excess rabbit muscle α-glycerophosphate dehydrogenase, triose phosphate isomerase and aldolase, as modified from the procedures of Rutter *et al.* (1966). ATP concentrations were determined using a modification of the method of Chou and Wilson (1972), using yeast hexokinase. The assay contained per ml: 0.0148 M $MgCl_2$, 0.027 M glucose, 0.5 mg NADP, 2 units glucose-6-phosphate dehydrogenase, and 0.04 M K-Hepes, pH 7.5.

Preparation of Dowex Chelating Resin

Dowex Chelating Resin was washed with 1 N HCl at 70° C three times. The resin was then washed excessively with H_2O, 1 N KOH, and H_2O again. Before use, it was equilibrated to the desired pH with 0.1 M K-MES, pH 6.2. Then ADP or ATP were passed over the column to remove contaminating divalent metal ions.

Kinetic Properties

The lactic dehydrogenase coupled assays were conducted as described previously except that the buffer used was $(CH_3)_4N$-MES, pH 6.2. Enzyme dilutions prior to assay were made in the $(CH_3)_4N$-MES buffer. The reaction was initiated by addition of enzyme. All assay components except those under examination were at saturating or near saturating concentrations.

Kinetic data treated as Lineweaver-Burke plots were analyzed using the Wilkin computer program which makes use of a weighted, least squares treatment of the data (Wilkinson, 1961). Kinetic data treated as Hill plots had lines drawn by eye through the data points. The apparent K_m or K_A is defined as that concentration of substrate or activator where v = 1/2 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).

Polyacrylamide Electrophoresis

Analytical disc gel electrophoresis was performed at room temperature using a current regulated power supply at 3 mamp per gel according to Davis (1964). Gels were routinely run at 6% acrylamide. Upon completion the gels were removed and stained overnight in 0.4% Coomassie Blue, 10% TCA, and 33% methanol (Johson *et al.*, 1971). Gels were then destained in 10% TCA-33% methanol for 2 hours at 45° C. The destaining solution was replaced by 10% TCA and the gels were incubated at 45° C for another hour. The 10% TCA was replaced with fresh 10% solution and the gels were stored at 4° C. In order to visualize the protein bands, gels were scanned at 550 nm using a Gilford gel scanner.

A modification of the sodium dodecyl sulfate acrylamide gel electrophoresis technique of Fairbanks *et al.* (1971) was used. The electrophoresis buffer and 5% acrylamide gels contained 1% SDS, 0.04 M Tris-acetate, pH 7.4, and 0.002 M EDTA. The protein samples at 1-2 mg/ml were preincubated 15 minutes at room temperature (unless stated otherwise) in the following mixture: 1% SDS, 10% glycerol, 1.4 M βME, 0.04 M Tris-acetate, pH 7.4, and 0.02 M EDTA. Pyronin B, the tracking dye, was added after the incubation. From 2 to 50 μl of this incubation mixture was applied to the 9.5 cm gels which had been pre-electrophoresed for 30 minutes. The proteins were electrophoresed at a constant voltage of 50 V (3-4 mA/gel). The electrophoresis normally required 3½ hours. Upon completion, the gels were removed and stained using the technique described above for the analytical disc gels. Gels were scanned as above and molecular weights calculated using the method of Weber and Osborn (1969). Molecular weight standards bovine serum albumin (68,000), bovine liver catalase (57,500), rabbit muscle aldolase (40,000), rabbit muscle lactic dehydrogenase (35,000), and bovine pancreas α-chymotrypsinogen A (26,000) were run in parallel and mixed with the proteins of unknown molecular weight.

Assay for Protease Activity in "Pure" Pyruvate Kinase

Slight protease contamination of pure PK was qualitatively detected by the sensitive SDS gel electrophoresis and Coomassie Blue staining technique previously described. The enzyme to be tested was mixed with the SDS mixture: 1% SDS, 10% glycerol, 1.4 M βME, 0.04 M Tris-acetate, pH 7.5, and 0.002 M EDTA and allowed to incubate at 37° C for 24 hours or 100° C for 10 minutes. Heavy protease contamination could be detected merely by incubating at room temperature for 15 minutes. The preincubated samples were then applied to SDS gels and electrophoresed as described. After staining with Coomassie Blue, a gel containing only one protein band was said to be free of protease. One containing multiple bands of decreasing molecular weight was more heavily contaminated, while one containing stained protein migrating with the tracking dye was said to be heavily contaminated.

Isoelectric Focusing

Isoelectric focusing was performed in a small column (10 x 150 mm) using pH 5 to 8 ampholytes with 0.15 mg enzyme according to the procedure of Behnke *et al.* (1975). Electrofocusing was accomplished at a constant voltage of 200 V for 5 hours at room temperature. Fractions of 0.2 ml were collected. The pH of each fraction was determined at room temperature with a Sargent pH meter equipped with a Sargent S-30070-10 combination glass electrode. Pyruvate kinase activity was determined for each fraction.

Amino Acid Analysis

Amino acid analysis was performed following the method of Moore and Stein (1963). Pure pyruvate kinase was desalted on Sephadex G-25 equilibrated with distilled H_2O. The absorption at 280 nm was used to determine the protein to be added to the hydrolysis vials. The H_2O was removed by evaporation at 90° C or by lyophilization. Five-tenths milliliter of 6 N HCl and about 10 mg of phenol were added to each sample. The tubes were vacuum sealed and allowed to incubate at 110° C ± 1° for 24, 48, and 72 hours. Tubes were removed from the oven at these times and stored at -20° C until analysis was performed.

N-Terminal Analysis

N-terminal analysis was performed using the method of Gray (1972). Pure pyruvate kinase was desalted on Sephadex G-25 equilibrated with distilled H_2O. The absorption at 280 nm was used to determine the protein (250 μg) to be used. After lyophilization, the dried protein was dissolved in 50 μl of 1% SDS and boiled for 5 minutes. Fifty microliters of N-ethylmorpholine and 75 μl of dansyl chloride (25 mg/ml in anhydrous dimethyl formamide) were added and allowed to react 5 hours. The protein was precipitated with acetone and washed with acetone several times. The protein was dried and finally hydrolyzed using the method described in the amino acid analysis section. After 16 hours at 110° C, the vial was opened and the HCl was removed by evaporation. The protein was dissolved in 10 μl acetone:acetic acid (3:2) and spotted on polyamide sheets (5 cm x 5 cm). The PK was run on one side and dansyl amino acid standards on the opposite side.

Analytical Ultracentrifugation

A Spinco Model E analytical ultracentrifuge equipped with an interference optical system was used for the equilibrium sedimentation of pure PK. The centrifugation was performed at 15,220 rpm, 20° C, using the meniscus depletion technique of Yphantis (1964). The enzyme at a concentration of 0.45 mg/ml was dialyzed against 0.1 M Tris-HCl, pH 7.5, containing 0.25 M KCl, 0.025 M $MgCl_2$, 10 mM PEP, 2 mM FDP, 5 mM DTT, according to Kuczenski and Suelter (1970), before centrifugation. Interference patterns were photographed on Type II G emulsion spectroscopic plates. After development, the patterns on the plates were measured with a Boeckeler two-dimensional comparator. The partial specific volume, $\bar{v}$, of PK was calculated to be 0.743 from its amino acid composition, according to Cohn and Edsall (1943).

Immunization of Animals

Adult female rabbits were immunized with pure pyruvate kinase by three weekly subcutaneous injections along the back. The first injection contained 0.3 mg of PK in 1 ml of 50% Freund's complete adjuvant. The second and third injections also contained 0.3 mg of PK but in 1 ml of 50% Freund's incomplete adjuvant. Ten days after the last injection, and thereafter every seven days, 30-50 ml of blood were collected from the marginal ear vein. The serum was separated from the whole blood and either used immediately for isolation of IgG or stored at -20° C.

Preparation of IgG

IgG was prepared using the method of Masters *et al.* (1971). Serum was made 1.75 M (26 g/100 ml) in ammonium sulfate and stirred on ice for 30 minutes. The precipitated IgG was removed by centrifuging at 27,000 x g for 10 minutes. The pellet was resuspended in a volume of 0.015 M sodium phosphate buffer, pH 7.85, equal to the original volume of serum and the 1.75 M ammonium sulfate precipitation was repeated. The pellet obtained by centrifuging at 27,000 x g for 10 minutes was resuspended in the pH 7.85 buffer described above and the solution dialyzed for 24 hours against 2 changes of 1000 ml of the same buffer.

A DEAE column was prepared (1.88 cc DEAE/ml serum) and washed exhaustively with 0.015 M sodium phosphate buffer, pH 7.85. The dialyzed IgG was applied to the column and eluted with the pH 7.85 sodium phosphate buffer. Fractions containing protein were pooled and dialyzed against distilled-deionized H_2O. The IgG was lyophilized and stored at -20° C. When IgG was needed, it was dissolved in 0.015 M sodium phosphate, pH 8.0, containing 0.02% sodium azide, and its concentration was determined using $E_{280}^{0.1\%} = 1.35$.

Ouchterlony Double Diffusion Analysis

Ouchterlony (1950) double diffusion analysis was performed in disposable petri plates (100 x 15 mm) containing 1% agar, 0.015 M sodium phosphate, pH 8.0, and 0.02% sodium azide. The plates were poured by melting a stock solution of the agar mix and pipetting 10 ml of the solution into a plate, allowing it to cool and solidify. Holes were punched in the gel by using a Grafar Auto-gel punch. The agar was removed from the holes by suction using a pipette attached to a water aspirator. IgG was placed in the center well and antigen in the outer wells. The plates were developed at room temperature from 12-24 hours.

Immunoprecipitation of "Native" Pyruvate Kinase from Cell-Free Yeast Extract

A cell-free extract of the yeast was prepared just as in the Manton-Gaulin preparation. The cells were lysed using the Manton-Gaulin homogenizer in the presence of 1 mM DFP and 5 mM βME. The pH of the lysate was adjusted to 7.0 with 4 N KOH and treated with 40 mM $CaCl_2$ and 0.1 M potassium phosphate, pH 7.0. The mixture was allowed to remain for one hour and then centrifuged at 14,000 x g for 30 minutes. The calcium phosphate supernatant was assayed for PK activity. A volume of this supernatant equivalent to 17 units of PK activity was added to 10 mg of purified IgG in 0.015 M sodium phosphate, 0.02% sodium azide, pH 8.0. Glycerol, or buffer containing glycerol, was added to a final concentration of 8.5% in order to stabilize PK for enzymatic assay. A control containing pre-immune IgG in the same ratio of PK to IgG protein was run but no precipitate formed. The resulting pellets were washed once with 0.015 M sodium phosphate, pH 8.0, buffer containing 0.02% sodium azide, once with 0.25 M NaBr in the same buffer, and once with 0.1% DOC in the same buffer. The precipitates were then prepared for SDS gel electrophoresis by dissolving in 40 μl of a mixture containing 2% SDS, 20% glycerol, 2.8 M βME, and

0.08 M Tris-acetate with 0.004 M EDTA, pH 7.4. Half of the solution was allowed to incubate at room temperature for five minutes while the other half was boiled for the same period of time. Pyronin B tracking dye was added and the samples were applied to 1% SDS polyacrylamide gels for electrophoresis. Upon completion of the electrophoresis the gels were stained with Coomassie Blue and scanned at 550 nm using a Gilford spectrophotometer.

Immunoelectrophoresis

Agar for immunoelectrophoresis was prepared by dissolving 1 g in 100 ml of 0.05 M sodium barbital buffer, pH 8.6. The hot solution was applied to a 3 x 1 inch glass slide and allowed to cool. The trough for antibody and the wells for antigen were cut using a flat plastic pattern, a metal punch, and a razor blade. The agar was removed from the trough and wells by sunction. One microliter of 0.15% bromphenol blue and 2 µl of the protein sample, 1 mg/ml, were applied in the well. The slides were placed on the buffer compartments and filter paper wicks moistened with buffer were laid on each end of the slide, the other end of the wick being placed in the buffer. The samples were electrophoresed at a constant 125 V (10 mA) for 1.5 hours. After electrophoresis the slides were removed and 80 µl immune serum or IgG was placed in the center trough. The slides were allowed to develop 12 hours.

Photography of Results

Ouchterlony plates and immunoelectrophoretic slides were photographed with Kodak High Contrast Copy Film. The plates or slides were placed over a hole in a black metal box with indirect fluorescent illumination. To record the results, a Nikormat Camera was used at ASA 64, f 3.5 with a 0.5 second exposure.

RESULTS

Purification of Pyruvate Kinase

All buffers and columns were prepared in advance and stored at 4° C until needed. This was necessary in order to complete the purification as quickly as possible to avoid proteolytic degradation. All operations after the lysis of the yeast were conducted at room temperature due to the cold lability of the PK. Table 3 shows a summary of a typical purification.

A. Lysis of Yeast

One pound of fresh bakers' yeast was crumbled into 800 ml of distilled-deionized water at 4° C and stirred for 10 minutes. Ten milliliters of 0.1 M DFP, prepared as described in the Methods section, was added to 100 ml of distilled-deionized water containing 50 mM βME and let stir for 5 minutes. This was thought to be a necessary precaution due to reports concerning an inhibitor present as a contaminant in some lots of DFP (Gould *et al.*, 1965). This inhibitor, if not preincubated with βME or another sulfhydryl reducing compound, can inhibit enzymes with essential sulfhydryls (Murachi *et al.*, 1965). After stirring, the DFP mixture was added slowly

TABLE 3

Purification Summary for Pyruvate Kinase from *S. cerevisiae*

FRACTION	Volume	Total protein	Total units	Specific activity	Yield
	ml	*mg*		*units/mg*	*%*
$CaCl_2$ supernatant	1240	24,000	250,000	10.4	100
$(NH_4)_2SO_4$ fraction (40-55%)	200	12,200	226,000	18.5	90
Cellulose phosphate chromatography, pH 6.5	400	2,500	180,000	72	72
DEAE-cellulose phosphate chromatography, pH 7.5	530	504	130,000	258	52
Cellulose phosphate chromatography, pH 6.5	130	286	95,000	336	38
Sephadex G-100	61	100	35,000	350	14

to the stirring yeast suspension. The yeast mixture was passed twice through a precooled Manton-Gaulin homogenizer at 8000-8500 psi. The homogenized suspension was adjusted directly to pH 7.0 with 4 N KOH using a Sargent pH meter. This mechanical method of lysing the yeast was found to release more PK as well as other contaminating proteins per pound of yeast than the toluolysis procedure previously used (Hunsley and Suelter, 1969a). Therefore, further purification steps were needed in order to utilize the remainder of the previously developed technique. To this end, a calcium phosphate precipitation step was utilized in the following manner: 4.4 g/liter $CaCl_2$ (40 mM) was added to the yeast mixture and stirred thoroughly. After dissolution of the $CaCl_2$, 1.0 M potassium phosphate, pH 7.0, containing 10 mM DFP and 50 mM βME was added to a final concentration of 0.1 M potassium phosphate. The calcium phosphate was allowed to precipitate for 1 hour at room temperature. The brownish-yellow supernatant obtained after centrifugation at 14,000 x g for 30 minutes was Fraction I. All remaining steps were conducted at room temperature.

B. Ammonium Sulfate Fractionation

Solid ammonium sulfate, 243 g/l, was slowly added to Fraction I with constant stirring. After dissolution of ammonium sulfate, the mixture was adjusted to pH 6.2 with 4 N KOH and left stirring for 1 hour. The supernatant obtained after centrifugation at 14,000 x g for 30 minutes was Fraction II.

Solid ammonium sulfate, 97 g/l, was slowly added to Fraction II with constant stirring. After dissolution the mixture was adjusted to pH 6.2 with 4 N KOH, allowed to stir for 1 hour, and centrifuged at 14,000 x g for 30 minutes. The precipitate containing the PK was suspended in pH 6.5 buffer to give a final volume of near 200 ml and dialyzed at least 12 hours, preferably 15, against 2 liters of pH 6.5 buffer containing 1 mM DFP. After the dialysate was centrifuged at 27,000 x g for 15 minutes to remove a white precipitate, the supernatant was diluted with pH 6.5 buffer to a final volume of 1500 ml to give Fraction III. This dilution reduces the ammonium sulfate concentration to near 10 mM and protein concentration to near 10 mg/ml and is required to achieve adsorption of enzyme to the CP pH 6.5 column described in the next step.

Occasionally, Fraction III had a hazy white appearance and upon standing began to precipitate. The nature of the precipitate is unknown but it was usually removed in the calcium phosphate step. When Fraction III was hazy, it was applied rapidly to the CP pH 6.5 column before precipitation occurred.

C. Cellulose Phosphate Column, pH 6.5

This column step was added to the previously established procedure (Hunsley and Suelter, 1969a) in order, as stated previously, to remove the additional protein released from the yeast by the mechanical rupturing procedure. CP of a high capacity, 1.24 mEq/g, was washed, equilibrated with pH 6.5 buffer and poured in a 4 cm column to a height of 30 cm. After further equilibration with buffer, Fraction III was applied to the column and the column was washed with pH 6.5 buffer to remove non-adsorbed proteins. PK, which was bound, was removed with 0.15 M ammonium sulfate, pH 6.5, buffer. All fractions containing eluted protein with O.D. greater than 0.5 were pooled to give Fraction IV and dialyzed for 24 hours against 2 changes of pH 6.5 buffer at 10 times the volume of Fraction IV.

D. DEAE-CP Column, pH 7.5

CP, 1.12 mEq/g, was prepared in pH 7.5 buffer as was described in the Methods section, poured to a height of 20 cm in a 4 cm column, and equilibrated. DEAE, 0.9 mEq/g, was prepared in pH 7.5 buffer, as was described in the Methods section, poured to a height of 20 cm in a 4 cm column, and equilibrated. The effluent tube of the DEAE column was then connected to the top of the CP column and Fraction IV was applied to the DEAE column. PK did not bind to these columns and thus the eluate was collected fractionally until all protein was eluted. Those fractions containing PK at a specific activity of 220 or greater were pooled to give Fraction V.

E. CP Column, pH 6.5

CP resin, 1.12 mEq/g, was prepared in pH 6.5 buffer poured to make a column 2.5 x 28 cm, as described in the Methods section, and equilibrated. Fraction V, after adjusting to pH 6.5 with 3 N acetic acid, was applied to this column and the column was washed with pH 6.5 buffers. PK was eluted with a 600 ml linear ammonium sulfate gradient (0.01 M to 0.2 M) in pH 6.5 buffer and fractions were collected. Those fractions having PK with specific activities greater than 340 were pooled to form Fraction VI. The pooled fractions were dialyzed against 90% saturated ammonium sulfate prepared to pH 6.5 as described in the Methods section.

The enzyme at this stage migrated as a single band during analytical disc gel electrophoresis. However, if the enzyme was incubated in 1% SDS containing βME for 24 hours at 37° C, before electrophoresis on SDS-polyacrylamide gels, several bands consistent with extensive proteolytic degradation can be seen (Figure 1A). This observation required the addition of an extra chromatographic step in the purification.

FIGURE 1: COMPARISON OF SDS ELECTROPHORETIC MOBILITY OF YEAST PYRUVATE KINASE BEFORE AND AFTER SEPHADEX G-100 CHROMATOGRAPHY.

Fifty micrograms of PK before (A) and after (B) Sephadex G-100 chromatography was incubated with 1% SDS, 0.04 M Tris-acetate, pH 7.4, 1.4 M βME, and 0.002 M EDTA for 24 hours at 37° C. The incubation mixture was electrophoresed on 0.1% SDS gels as described in the Methods section.

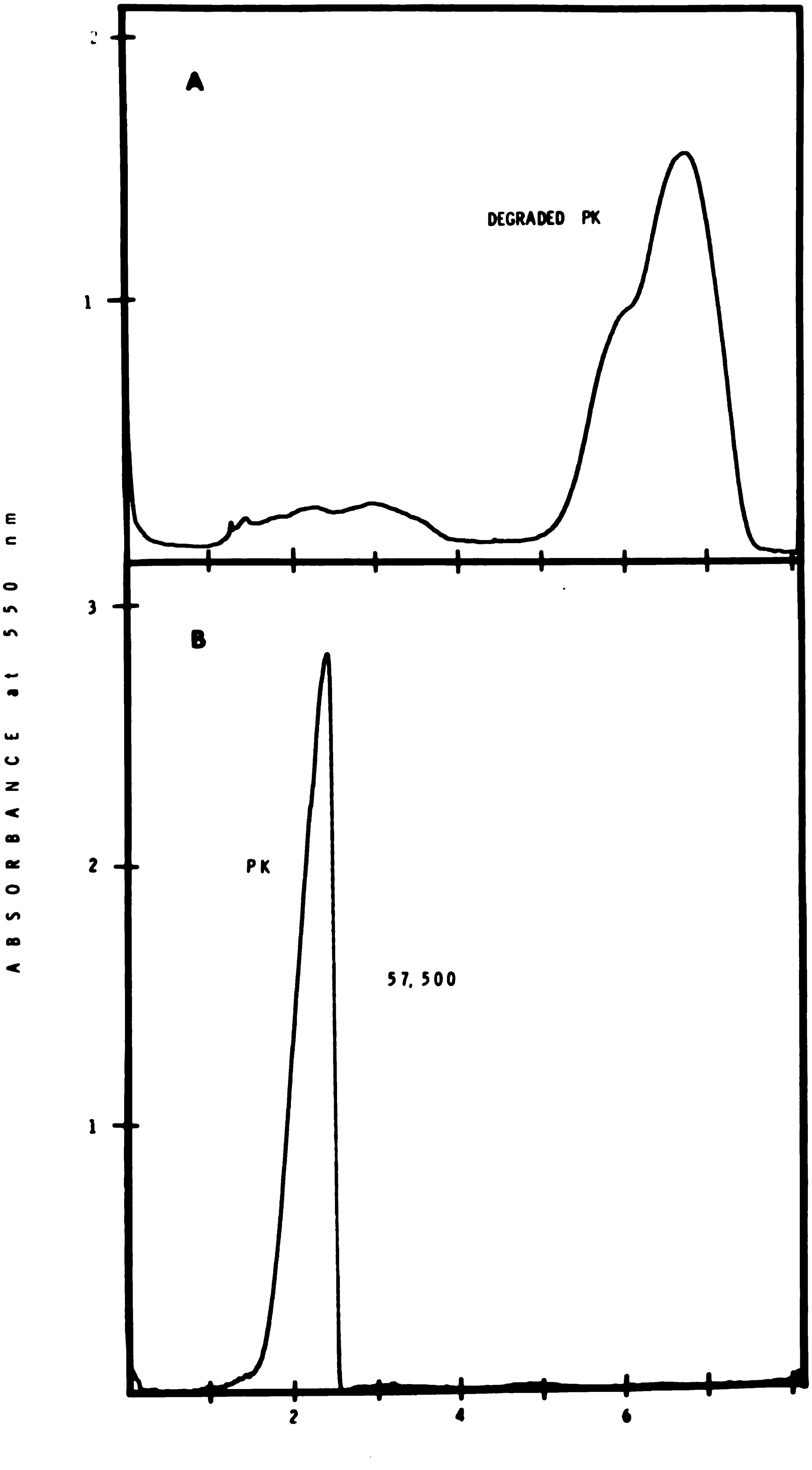
A
DEGRADED PK
B
PK
57,500
1
2
3
2
4
6
ABSORBANCE at 550 nm
MIGRATION DISTANCE CM

Figure 1

F. Sephadex G-100 Column

The precipitated enzyme from Fraction VI was removed by centrifugation at 27,000 x g for 20 minutes, dissolved in a minimum volume of pH 6.5 buffer. Ten milliliters was applied to a G-100 column, 5 x 90 cm, and eluted with the pH 6.5 buffer. Fractions with PK activity greater than 340 were examined for protease contamination by using the SDS incubation with subsequent SDS gel electrophoresis previously described. Those fractions with specific activity greater than 340, that were free of protease contamination as examined in this way, were pooled and PK precipitated by dialysis against 90% ammonium sulfate at pH 6.5 producing Fraction VII.

Enzyme after this column step migrated as a single band in SDS gel electrophoresis after 24-hour incubation in 1% SDS containing βME (Figure 1B). This indicates that the Sephadex G-100 column separated the proteolytic activity from the PK producing enzyme free of protease contamination. This also indicates that the protease has a smaller molecular weight than PK, which allows it to be separated on the G-100 column. However, substantial quantities of PK are sacrificed due to residual contamination. Therefore, a more effective means of separating the protease from the PK at this point would effect a greater yield of uncontaminated PK.

G. Storage of Pyruvate Kinase

Precipitated PK was removed from Fraction VII by centrifugation at 27,000 x g for 15 minutes. The PK was resuspended with the following buffer: 10 mM sodium phosphate, 6 mM EDTA, 30 mM DTE, 50% glycerol, and 0.2% sodium azide, pH 7.0. The top of the solution was blown with nitrogen to exclude oxygen that would react with the DTE. The vial was securely capped and stored at room temperature. Since this storage method has only recently been used, the maximum period of time which PK is stable is not known; however, it is stable at least a month. It is best if every 2 to 3 weeks the enzyme is precipitated by dialysis against 90% saturated ammonium sulfate at pH 6.5 and resuspended in the 10 mM sodium phosphate buffer, pH 7.0, described above with fresh DTE. This maintains a reducing atmosphere for the labile sulfhydryls to prevent loss of PK activity. The pure enzyme is handled entirely at room temperature to avoid cold inactivation. Enzyme purified by this technique will be referred to as Manton-Gaulin PK.

H. Criteria of Purity

Using the sensitive analytical method of SDS gel electrophoresis with Coomassie Blue staining, PK, after the final Sephadex G-100 column, was examined for contaminating proteins. Figure 1B shows the results of scanning an SDS gel containing 50 μg of protein. As can be seen, only one protein band is present with no trace of contaminating protein present. Since this technique is sensitive to below 1 μg of protein, the contamination would have to be less than 2%.

Ouchterlony Double Diffusion Analysis of Purified PK and Cell-Free Extract

Ouchterlony double diffusion analysis was used to determine whether antibody produced in response to purified PK could be used to precipitate PK from crude extract. As can be seen in Figure 2, the antibody precipitates a single component from crude extract, and the preciptin line fuses with that of the purified PK indicating that the crude extract component was antigenically similar to pure PK and was most likely "native" PK.

Immunoelectrophoresis of Purified PK and Cell-Free Extract

A more sensitive technique for determining the number of antigenic components in a multiprotein system is immunoelectrophoresis. This technique separates antigenic components, first on the basis of charge during electrophoresis and finally on the basis of size during diffusion of the antigen and antibody. Since it was important to demonstrate that the antibody being used would precipitate one and only one antigenic component from the cell-free extract, this more sensitive technique was used.

FIGURE 2: OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF MANTON-GAULIN PK AND YEAST CELL-FREE EXTRACT.

The center well contained 35 μl of immune serum. Wells 1 and 3 contained 35 μg Manton-Gaulin PK in the sodium phosphate, pH 6.5, buffer described in Methods. Wells 2 and 4 contained 35 μl cell-free yeast extract. Well 5 contained the sodium phosphate, pH 6.5, buffer.

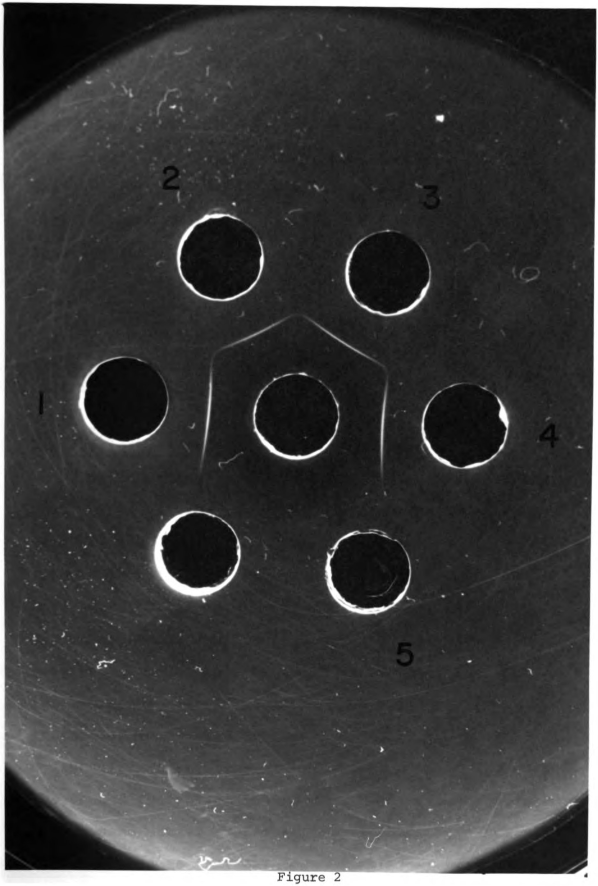

Figure 2

Cell-free extract was added to one well in the immunoelectrophoretic agar and pure Manton-Gaulin PK to the opposite well. The samples were electrophoresed and immune serum added to the trough and allowed to diffuse. The results in Figure 3 show that cell-free extract has one antigenic component which has similar, if not identical, electrophoretic mobility as that of pure PK, consistent again with the conclusion that the component precipitated from crude extract was indeed "native" pyruvate kinase.

Immunoprecipitation of PK from Cell-Free Extract

A. Enzymatic Characterization of the Immunoprecipitate and Resulting Supernatant

One additional method was used to determine whether "native" PK was the antigenic component being precipitated by the immune IgG. Remaining activity of PK was assayed in cell-free extract to which immune IgG had been added. This experiment was conducted using the method of immunoprecipitation described in the Methods section. Two controls were run in parallel with the immunoprecipitation. One contained preimmune IgG instead of immune IgG so that any inhibition due to nonspecific interaction with the IgG could be eliminated. The second control contained only crude extract diluted in the same manner as the fractions containing IgG. This was done so that any loss of activity suffered by

FIGURE 3: IMMUNOELECTROPHORETIC ANALYSIS OF MANTON-GAULIN PK AND YEAST CELL-FREE EXTRACT.

Two micrograms of Manton-Gaulin PK was placed in well A and 3 μl of yeast cell-free extract in Well B. The proteins were electrophoresed for 1.5 hours. Eighty microliters of immune serum was placed in the center trough and allowed to diffuse.

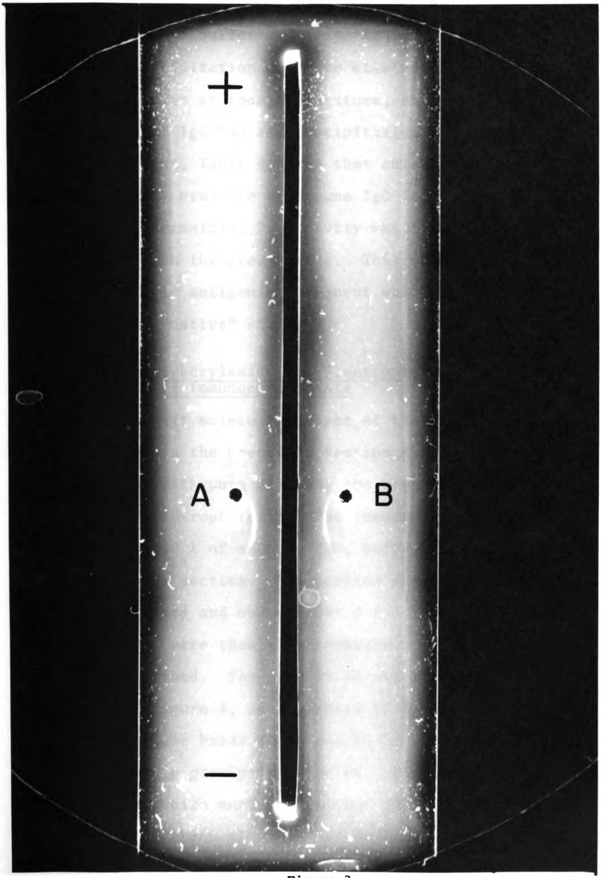

Figure 3

the PK under the buffer and temperature conditions of the immunoprecipitation could be eliminated. After 2½ hours incubation at room temperature, only the tube containing immune IgG had any precipitation. Results of this experiment, Table 4, show that only the crude extract in the presence of immune IgG lost PK activity and that the remaining 2% activity was found to be associated with the precipitate. This shows conclusively that the single antigenic component which the IgG precipitates is "native" PK.

B. 1% SDS Polyacrylamide Gel Electrophoretic Analysis of Immunoprecipitate

The subunit molecular weight of the PK precipitated as described in the preceding section A was determined and compared with pure PK using the sensitive technique of SDS gel electrophoresis. The immunoprecipitate was dissolved in 40 λ of an SDS, βME, buffer mixture described in the Methods section. One portion was incubated at room temperature and one at 100° C for 15 minutes. The two solutions were then electrophoresed on 5% acrylamide gels as described. The results of the room temperature incubation, Figure 4, show several protein bands. All but one of these bands are shown to be attributable to the IgG used to precipitate the PK. The one remaining protein band which must be PK since PK is the only protein which has been shown to be precipitated from the crude extract by the immune IgG has the identical molecular

TABLE 4

Enzymatic Characterization of the Immunoprecipitate and Resulting Supernatant

The mixtures described below were incubated at room temperature for 2½ hours. Samples were withdrawn at 0 and 2½ hours and assayed for PK activity. An immunoprecipitate, which formed only in sample 3, was removed by centrifugation at 16,000 x g for 1 minute. The immunoprecipitate (a), resuspended in the buffer described in the text, and the resulting supernatant (b) were assayed for PK activity.

Incubation mixture	PK Activity (units/ml) at Time 0	PK Activity (units/ml) at Time 2½ hrs	Percent Remaining activity
1. Control IgG cell-free extract	338	335	99
2. Cell-free extract	338	338	100
3. Immune IgG cell-free extract	338	6.6	2
a. Immunoprecipitate	--	6.6	2
b. Supernatant	--	0.0	0

FIGURE 4: A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROFILES OF PURIFIED IMMUNE IgG AND THE IMMUNOPRECIPITATE FORMED BETWEEN YEAST CELL-FREE EXTRACT PROTEINS AND ANTIBODY TO MANTON-GAULIN PK.

Scan A is of purified immune IgG while Scan B is of the immunoprecipitate formed between yeast cell-free extract proteins and antibody to Manton-Gaulin PK (the same immune IgG used in Scan A). IgG refers to the unreduced immune IgG. L chain refers to the light chain of IgG (MW = 25,000).

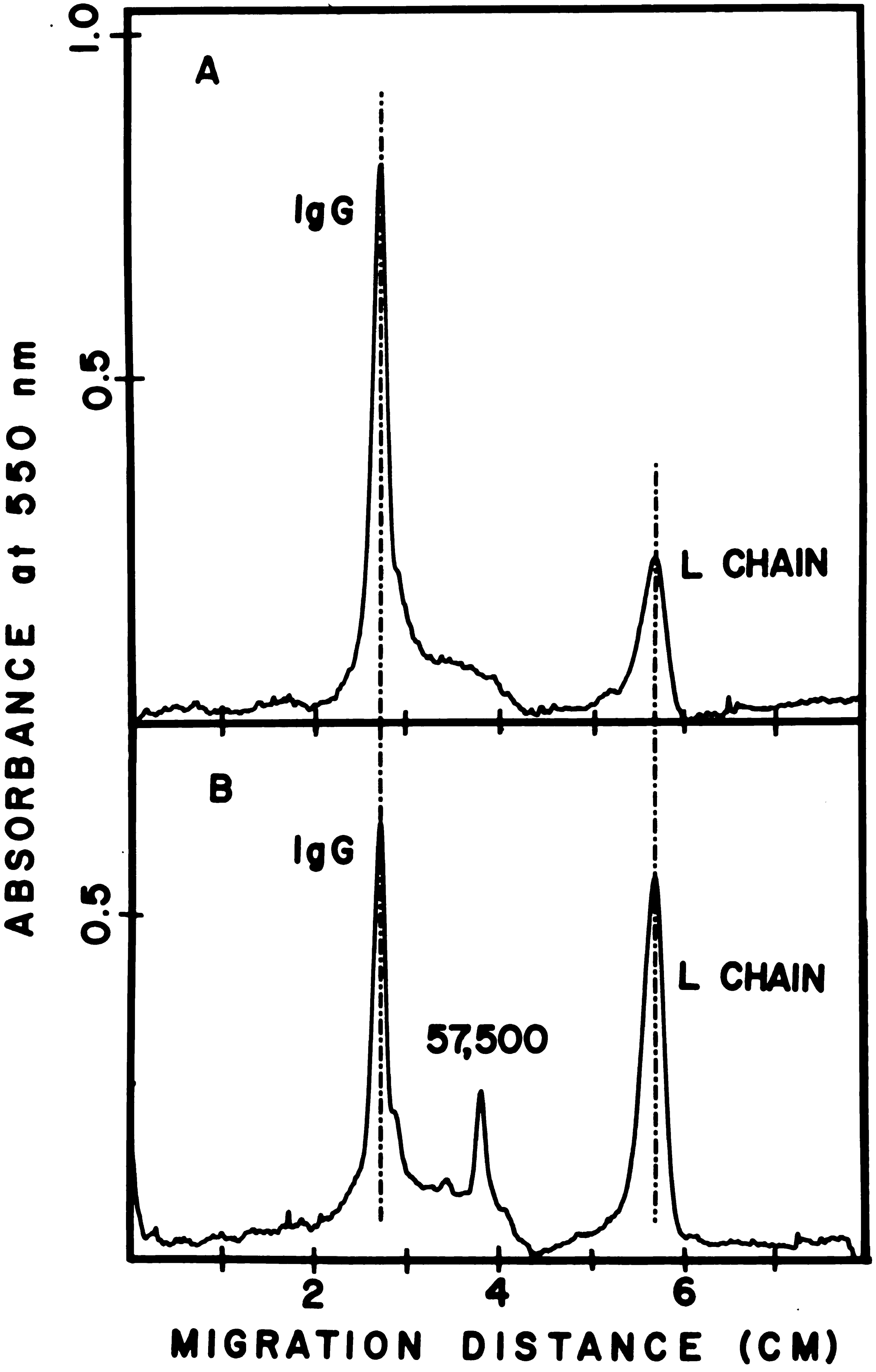
ABSORBANCE at 550 nm
1.0
0.5
0.5
A
IgG
L CHAIN
B
IgG
57,500
L CHAIN
2
4
6
MIGRATION DISTANCE (CM)

Figure 4

weight of purified PK and comigrates when mixed with the pure enzyme (Figure 5).

The portion of the SDS mixture which was incubated at 100° C shows an identical molecular weight for PK. The incubation at elevated temperature was done to demonstrate that there was no proteolytic enzyme present in the immunoprecipitate which could have degraded the "native" PK. Therefore the nondegraded, "native" enzyme has the same molecular weight as the pure PK, within the limits of determination of the analytical SDS gel technique.

Ouchterlony Double Diffusion Analysis of PK Purified from Yeast Two Different Ways

Pyruvate kinase purified from yeast lysed with a Manton-Gaulin homogenizer and PK from yeast lysed with toluolysis were immunologically compared using antibody prepared against Manton-Gaulin enzyme. The results of the Ouchterlony double diffusion analysis, Figure 6, show that the 2 enzymes have similar antigenic determinants and form fused precipitin lines. Rabbit muscle PK, which was also included in the Ouchterlony, does not form a precipitin line, suggesting that it is antigenically dissimilar from the yeast enzyme.

Subunit Molecular Weight

The subunit molecular weight of PK was determined using the SDS gel electrophoresis method developed by Weber and Osborn (1970). The mobility of PK was compared

FIGURE 5: A COMPARISON OF THE SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROFILES OF MANTON-GAULIN PK AND THE IMMUNOPRECIPITATE FORMED BETWEEN YEAST CELL-FREE EXTRACT PROTEINS AND ANTIBODY TO MANTON-GAULIN PK.

Scan A is of the purified Manton-Gaulin PK, Scan B is of the immunoprecipitate formed between yeast cell-free extract proteins and antibody to Manton-Gaulin PK, and Scan C is of a mixture of the two samples.

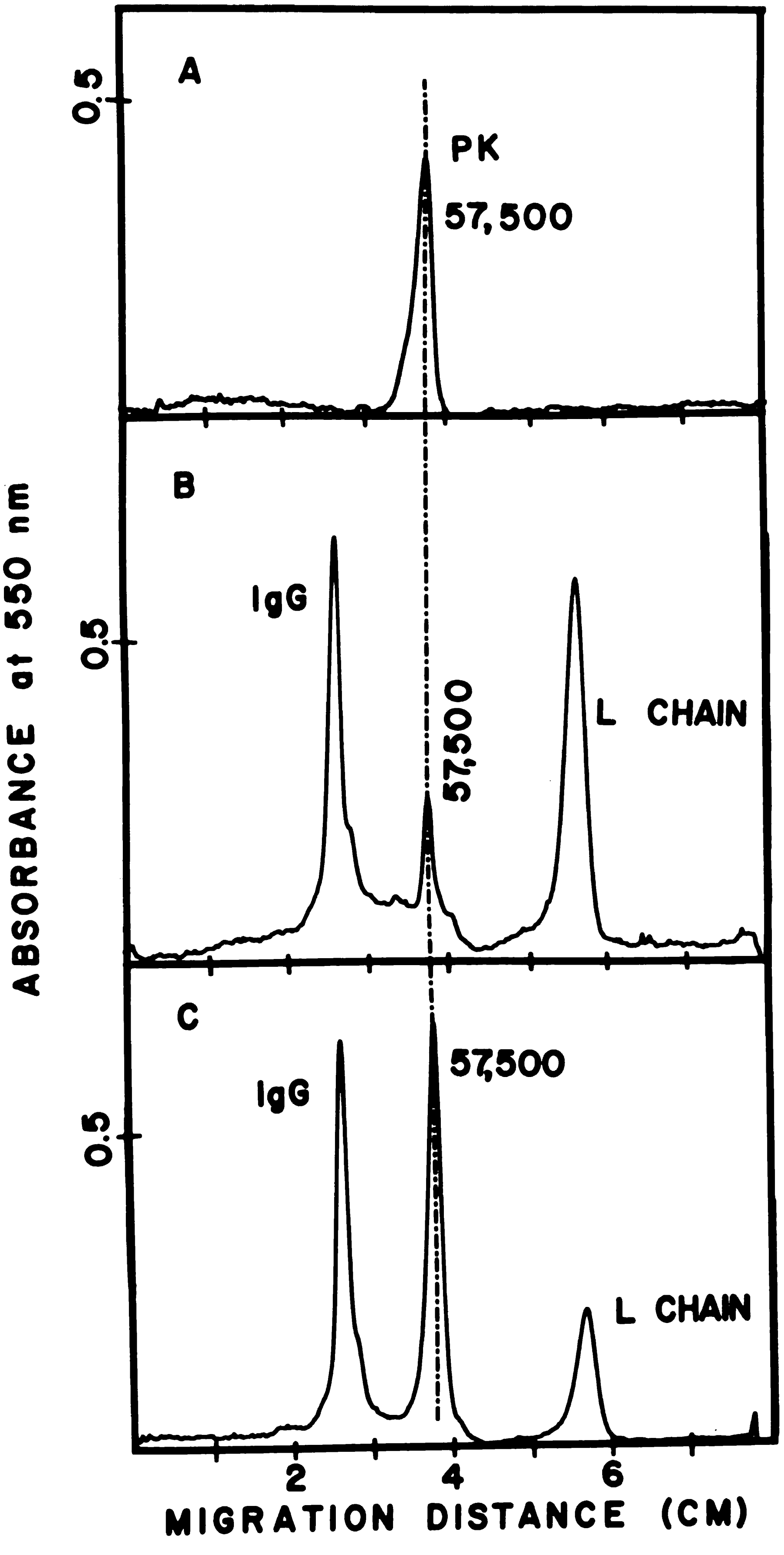
A
0.5
PK
57,500
B
0.5
IgG
57,500
L CHAIN
C
0.5
IgG
57,500
L CHAIN
ABSORBANCE at 550 nm
2
4
6
MIGRATION DISTANCE (CM)

Figure 5

FIGURE 6: OUCHTERLONY DOUBLE DIFFUSION ANALYSIS OF MANTON-GAULIN PK, TOLUOLYSIS PK, AND RABBIT MUSCLE PK.

The center well contained 35 μl of immune serum prepared to Manton-Gaulin PK. Wells 1 and 4 contained 35 μg of Manton-Gaulin PK. Wells 2 and 5 contained 35 μg of toluolysis PK. Wells 3 and 6 contained 35 μg of rabbit muscle PK.

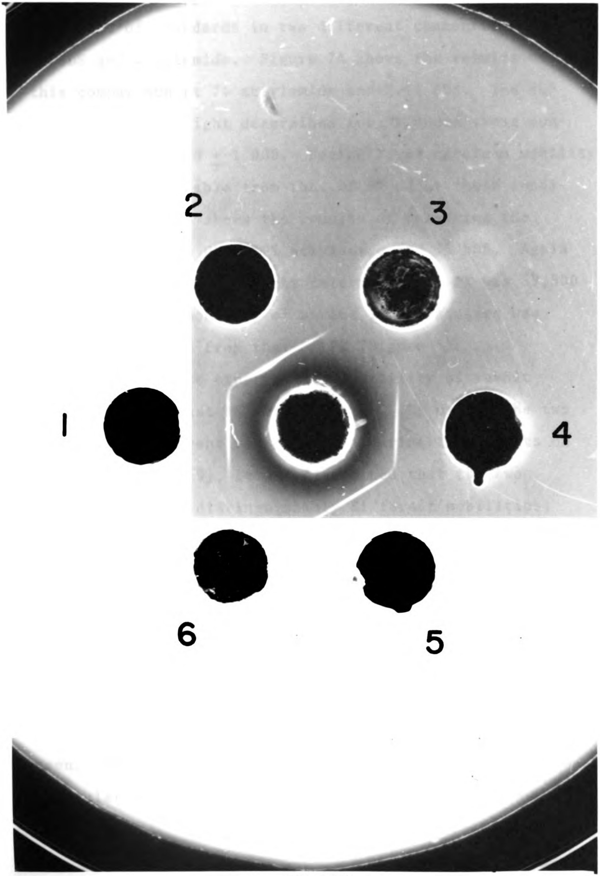

Figure 6

with that of standards in two different concentrations of SDS and acrylamide. Figure 7A shows the results of this comparison at 7% acrylamide and 0.1% SDS. The subunit molecular weight determined for PK under these conditions was 57,500 ± 1,000. Bovine liver catalase mobility was indistinguishable from that of PK under these conditions. Figure 7B shows the results of comparing the same standards to PK in 5% acrylamide and 1% SDS. Again the subunit molecular weight determined for PK was 57,500 ± 1,000 and the mobility of bovine liver catalase was indistinguishable from that of PK (Figure 8).

In a separate experiment the mobility of rabbit muscle PK and yeast PK were compared. By mixing the two enzymes in different ratios and comparing the results on SDS gels (Figure 9), it was determined that the two enzymes did have distinguishably different mobilities, the muscle enzyme moving slower, which would be consistent with its having a higher molecular weight than yeast PK.

The mobility of toluolysis PK was also compared with that of Manton-Gaulin PK, using SDS gel electrophoresis. Figure 10 shows the results of scanning a gel containing a mixture of the two proteins. The enzymes were mixed in different ratios, but no detectable difference was ever seen. This suggests that the two proteins have identical molecular weights, within the limits of this technique.

FIGURE 7: DETERMINATION OF THE SUBUNIT MOLECULAR WEIGHT OF MANTON-GAULIN PK USING SDS-POLYACRYLAMIDE ELECTROPHORESIS.

This plot represents a comparison of relative mobilities of the standard proteins BSA, bovine serum albumin (68,000); CAT, catalase (57,500); ALD, aldolase (40,000); LDH, lactic dehydrogenase (35,000); and CHY, α-chymotrypsinogen A (25,000) to Manton-Gaulin PK on 7% polyacrylamide gels containing 0.1% SDS (A) and 5% polyacrylamide gels containing 1% SDS (B).

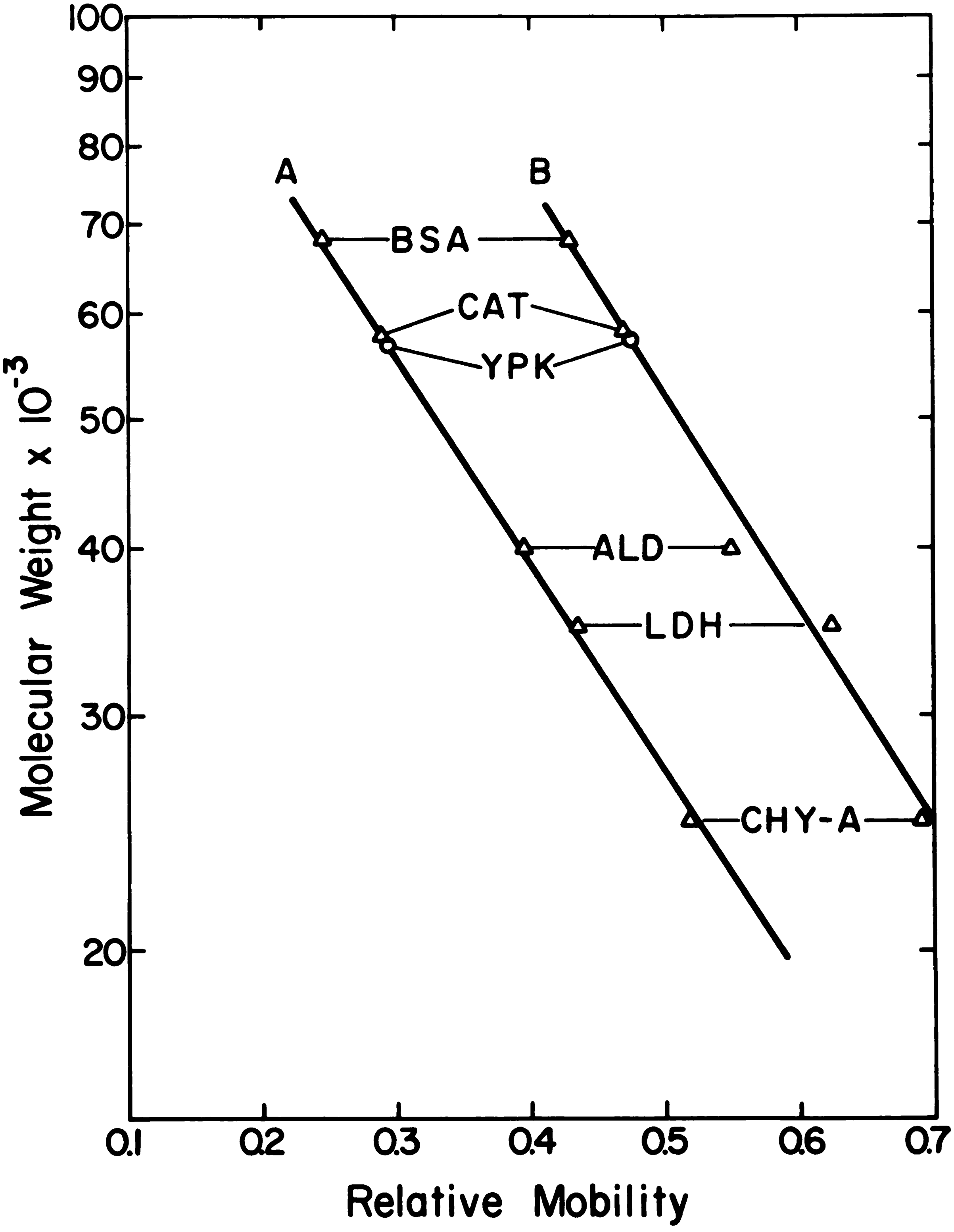
A
B
BSA
CAT
YPK
ALD
LDH
CHY-A
100
90
80
70
60
50
40
30
20
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Molecular Weight x 10^{-3}
Relative Mobility

Figure 7

FIGURE 8: COMPARISON OF CATALASE AND MANTON-GAULIN PK ON SDS POLYACRYLAMIDE GELS.

This scan is of a mixture of 2 μg catalase and 2 μg Manton-Gaulin PK.

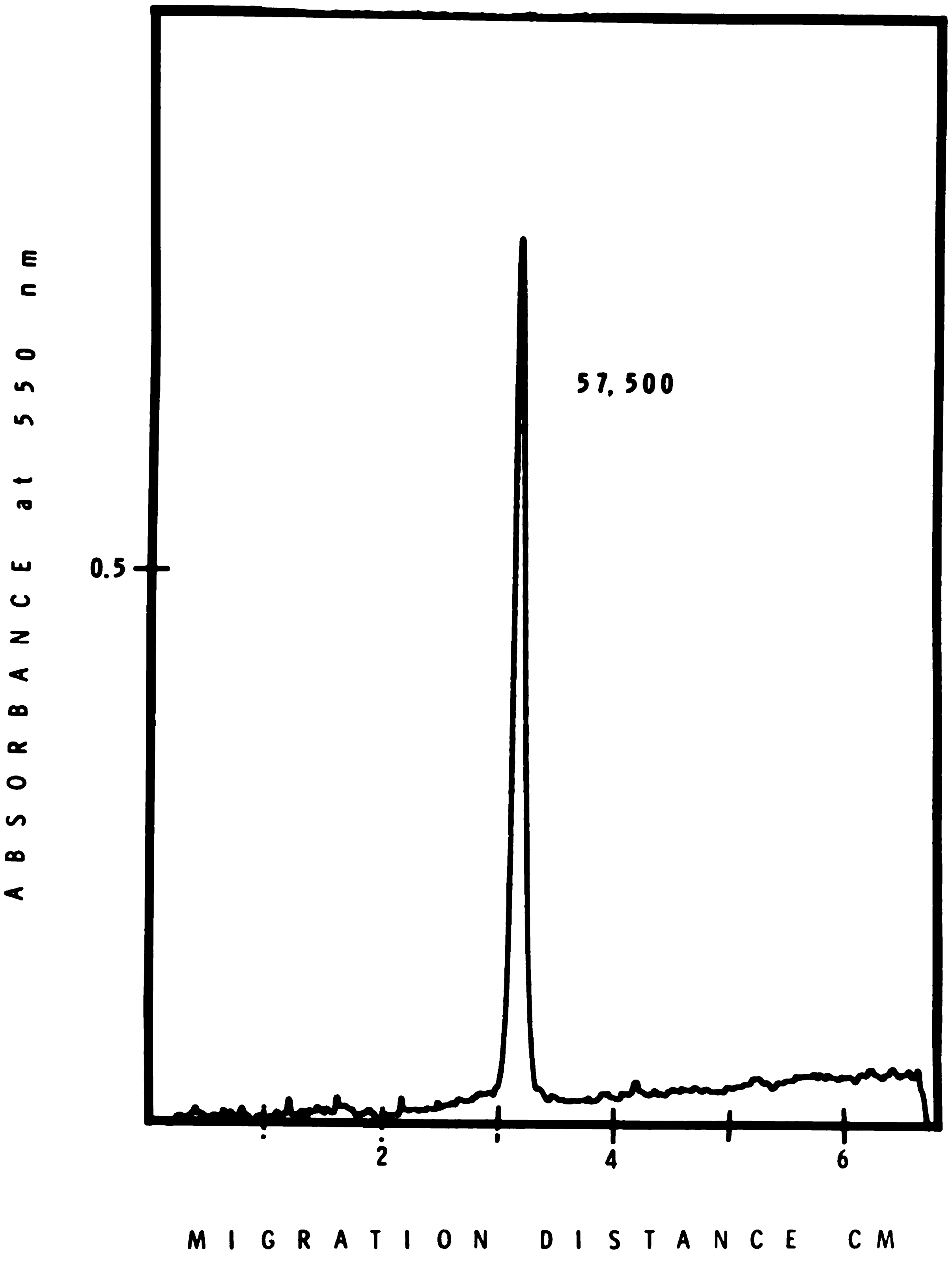

Figure 8

FIGURE 9: COMPARISON OF RABBIT MUSCLE PK AND MANTON-GAULIN PK BY SDS-POLYACRYLAMIDE ELECTROPHORESIS.

Gel A represents a mixture of 5 μg rabbit muscle PK and 2 μg Manton-Gaulin PK. Gel B represents a mixture of 2 μg rabbit muscle PK and 2 μg Manton-Gaulin PK. Gel C represents 2 μg rabbit muscle PK and 5 μg Manton-Gaulin PK.

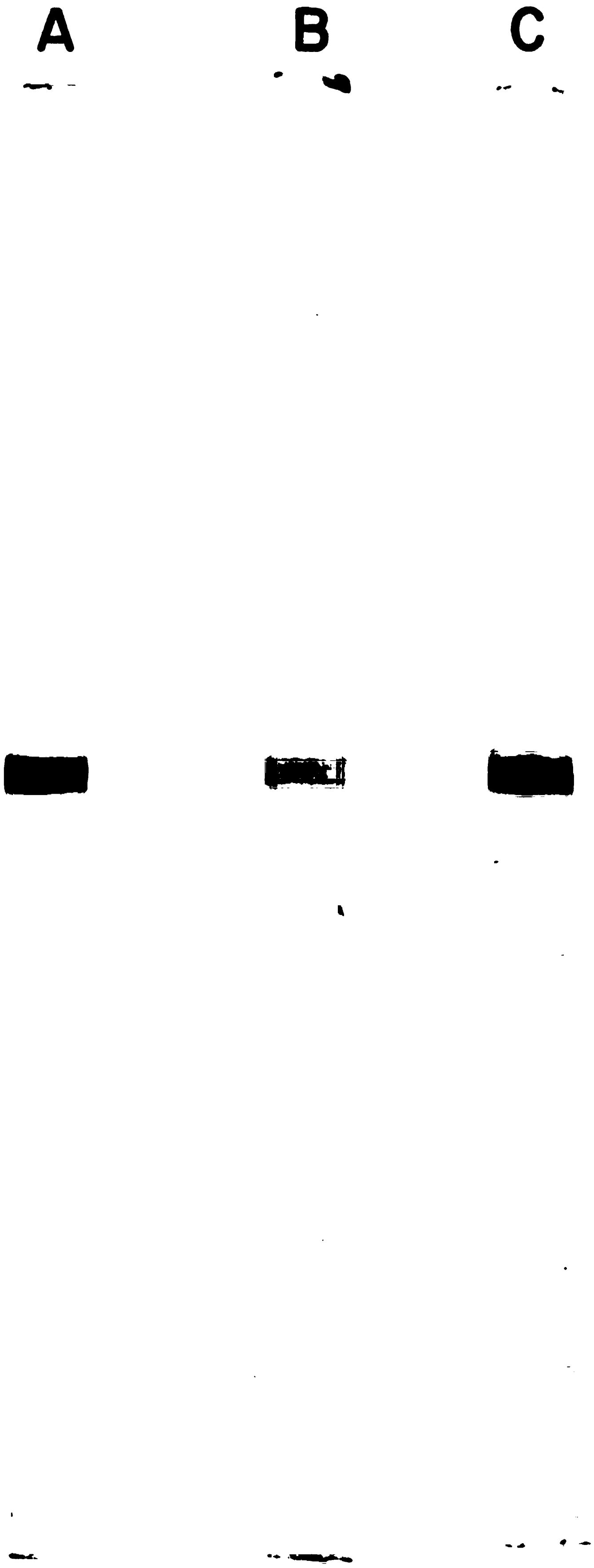

Figure 9

FIGURE 10: COMPARISON OF TOLUOLYSIS PK AND MANTON-GAULIN PK BY SDS-POLYACRYLAMIDE ELECTROPHORESIS.

The scan represents a mixture of 2 μg of toluolysis PK and 2 μg of Manton-Gaulin PK.

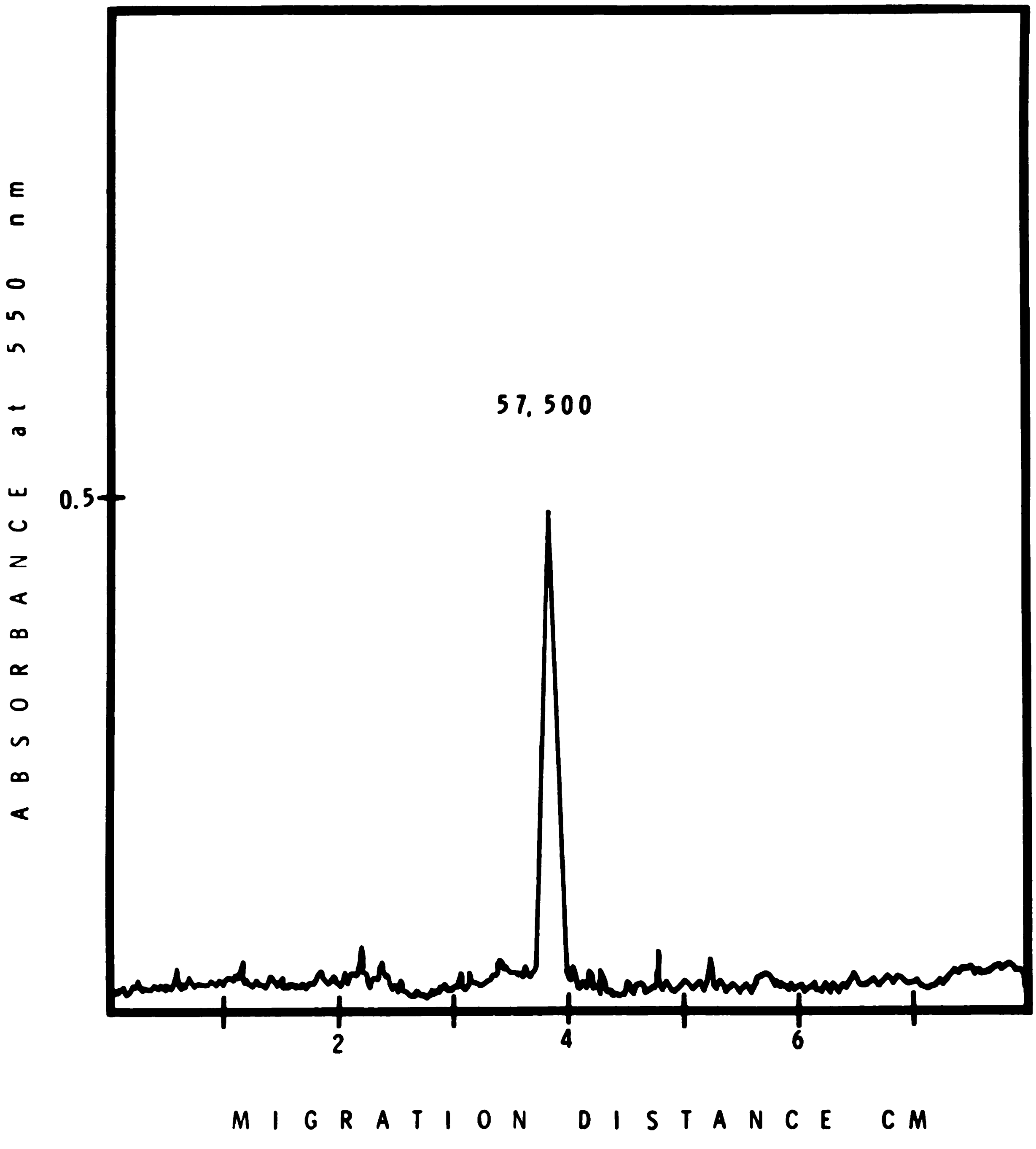

Figure 10

Isoelectric Focusing

The isoelectric point of pure PK was determined for two different preparations. Figure 11 shows the results of one of the experiments. The isoelectric point determined from the two experiments was identical, pH 6.7. All fractions collected were assayed for activity; only one peak of activity was found. This result is also consistent with PK being a nondegraded form, since multiple peaks of activity were not found.

Molecular Weight of Pyruvate Kinase

The molecular weight of purified PK was determined by the high speed sedimentation equilibrium method of Yphantis (1964), by Dr. S.-L. Yun, Biochemistry Department, Michigan State University, East Lansing, MI. The plot of log meniscus depletion versus radius squared is shown in Figure 12. The plot is linear except at the extreme low end. Although this curving upwards could be due to slight contamination by a lower molecular weight protein (a different protein or perhaps some dissociated PK) or to convection, it is most likely due to experimental error in reading the fringes of the shorter radial distances. This problem has been discussed in detail (Yphantis, 1964). The molecular weight calculated from this equilibrium experiment was 209,000 ± 6,000. This was the result of one determination.

FIGURE 11: ISOELECTRIC FOCUSING OF MANTON-GAULIN PK.

One hundred fifty micrograms of Manton-Gaulin PK was focused in pH 5-8 ampholytes. Two-tenth milliliter fractions were collected. The plot represents the pH (o) and PK activity (Δ) in each of these fractions.

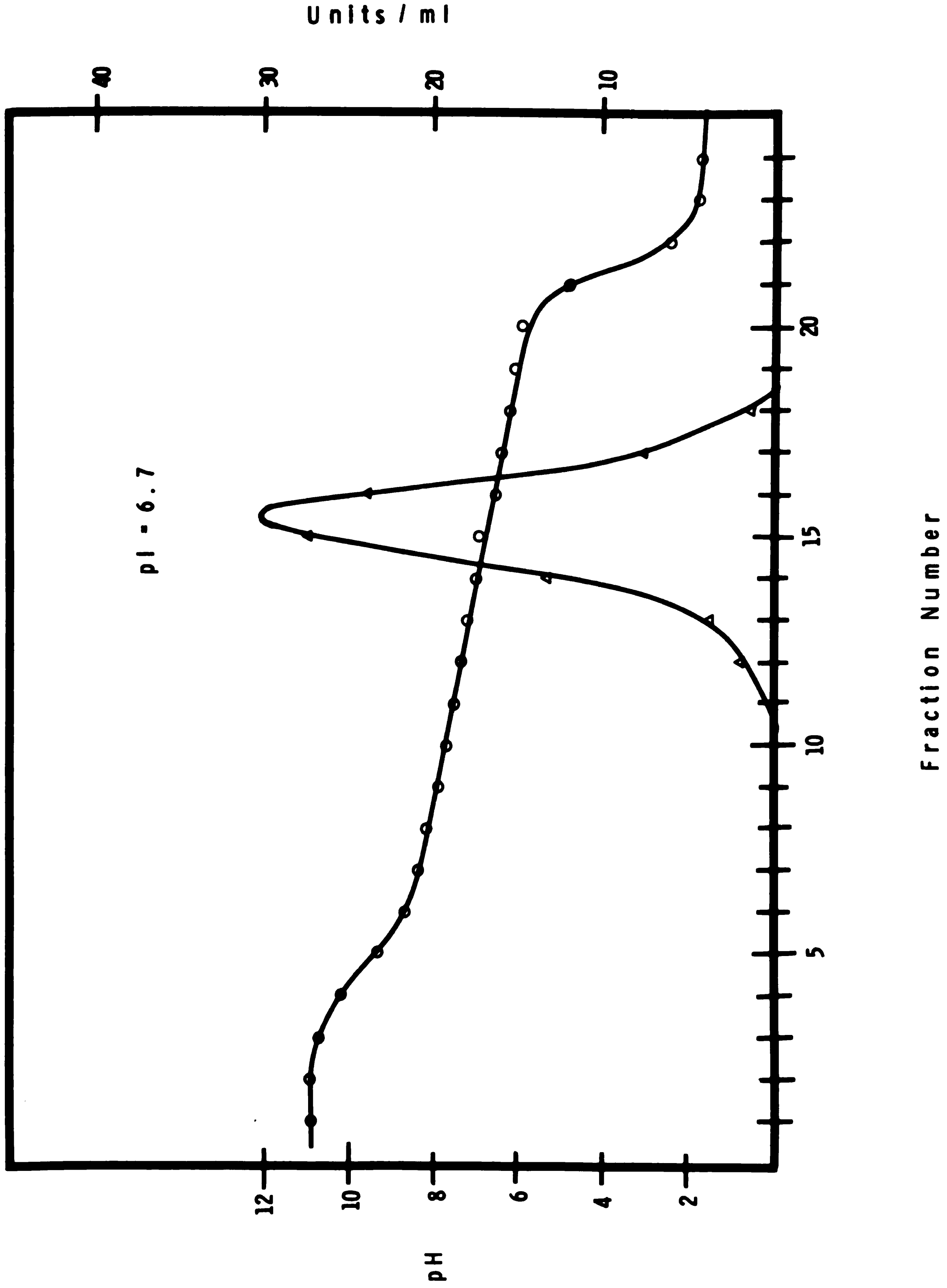
Units / ml
40
30
20
10
pI = 6.7
pH
12
10
8
6
4
2
5
10
15
20
Fraction Number

Figure 11

FIGURE 12: MOLECULAR WEIGHT DETERMINATION OF MANTON-GAULIN PK BY HIGH SPEED SEDIMENTATION EQUILIBRIUM.

The plot represents the molecular weight analysis of a high speed sedimentation equilibrium experiment on 0.45 mg of Manton-Gaulin PK.

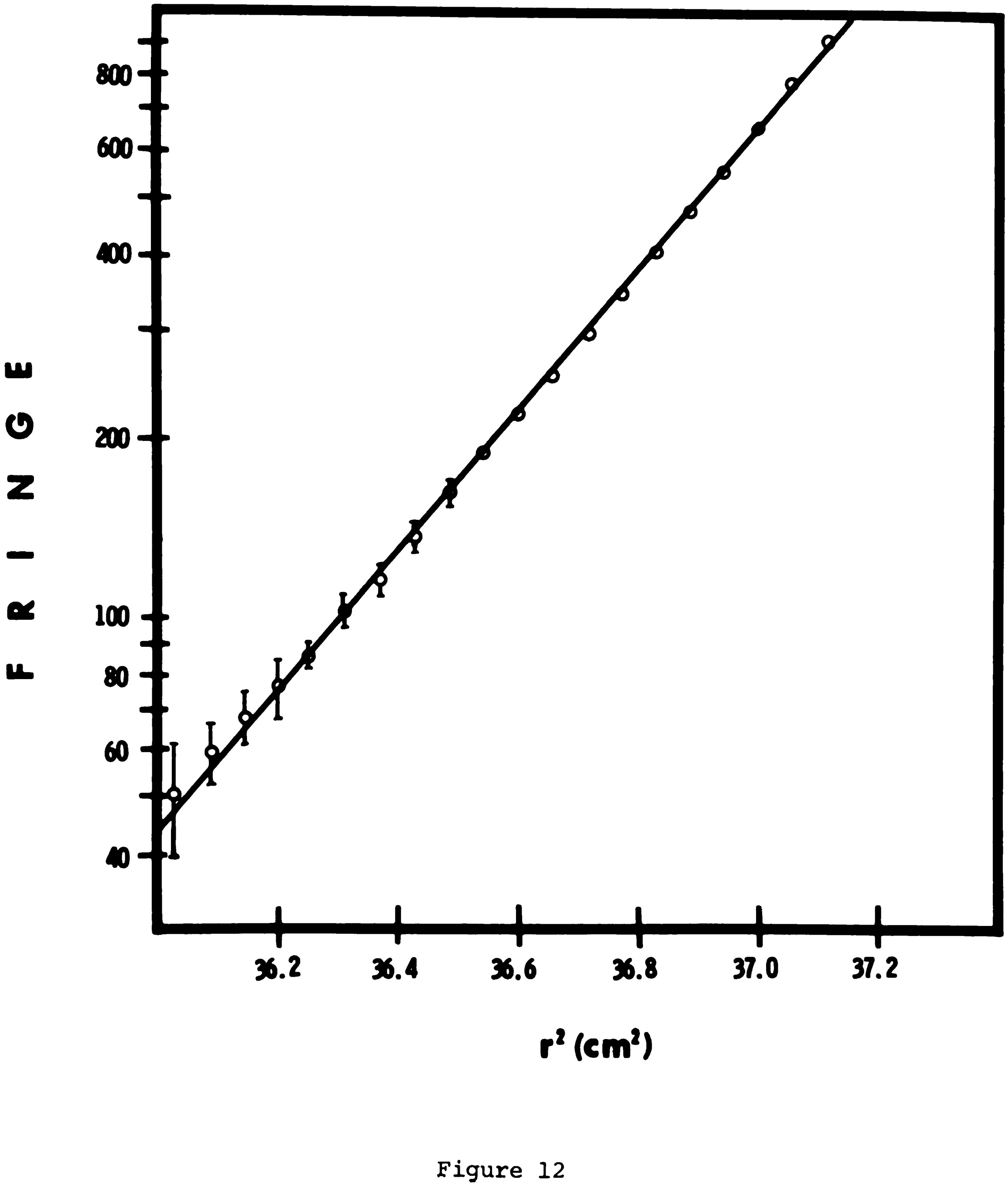
FRINGE
800
600
400
200
100
80
60
40
36.2
36.4
36.6
36.8
37.0
37.2
r² (cm²)

Figure 12

N-Terminal Analysis

The N-terminal amino acids of Manton-Gaulin PK and whale myoglobin were examined to determine whether there were degradative products present. The procedure used was that of Gray (1972). The chromatogram of yeast PK showed no spots at all. That of myoglobin showed a predominant spot for valine, which is the reported N-terminal, and a faint spot at the methionine position. An analysis of the supernatant and washes of yeast PK showed no detectable amino acids. These results suggest that pyruvate kinase has no free N-terminal and are consistent with the hypothesis that the enzyme is not a degraded form of "native" PK, since multiple N-terminals were not found.

Amino Acid Composition

The amino acid composition was determined for two different preparations of yeast PK. The first analysis represents a 24-, 48- and 72-hour hydrolysis (Table 5) performed by Worthington Biochemical Corp., Freehold, NJ. The second is 24- and 72-hour hydrolyses performed in Dr. Wood's laboratory in the Biochemistry Department, Michigan State University, East Lansing, MI (Table 6). Table 7 displays the averages of the two different determinations and an average of all the results with standard deviations. There is good agreement between all except serine, proline, and methionine. There is reason to

TABLE 5

Amino Acid Composition of Manton-Gaulin PK: Determination I

The values represent moles amino acid per 210,000 g PK.

AMINO ACID	24 hour	48 hour	72 hour
Lysine	126.1	130.5	135.3
Arginine	83.7	86.7	92.7
Histidine	24.7	24.9	23.4
Aspartic acid	210.3	210.2	219.3
Threonine	130.2	118.0	123.5
Serine	88.9	84.5	72.0
Glutamic acid	138.3	140.0	143.1
Proline	90.9	81.0	89.3
Glycine	121.0	118.0	125.7
Alanine	155.2	162.1	162.8
Valine	161.6	176.2	181.4
Methionine	40.1	41.4	44.2
Isoleucine	108.6	119.6	129.9
Leucine	127.1	133.0	135.2
Tyrosine	54.1	55.0	56.0
Phenylalanine	53.4	55.0	53.5

TABLE 6

Amino Acid Composition of Manton-Gaulin PK: Determination II

The values represent moles amino acid per 210,000 g PK.

AMINO ACID	24 hour	72 hour
Lysine	125.0	128.3
Arginine	84.8	---
Histidine	24.8	22.9
Aspartic acid	215.2	206.4
Threonine	131.0	128.2
Serine	81.8	80.4
Glutamic acid	136.3	141.7
Proline	---	19.8
Glycine	122.7	121.2
Alanine	161.5	161.9
Valine	154.5	174.9
Methionine	29.8	33.3
Isoleucine	114.6	124.8
Leucine	124.5	119.3
Tyrosine	50.9	51.5
Phenylalanine	53.2	53.3

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AM
Lys
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TABLE 7

Amino Acid Composition of Manton-Gaulin PK:
Average of Two Determinations

Column 1 represents the average of the 24-, 48- and 72-hour hydrolysis of the first amino acid determination (Table 5). Column 2 represents the average of the 24- and 72-hour hydrolysis of the second amino acid determination (Table 6). The final column represents the average of columns 1 and 2. The values represent moles of amino acid per mole of PK (molecular weight equals 210,000).

AMINO ACID	1	2	3
Lysine	135.3[b]	128.3[b]	131.8±3.5
Arginine	92.7[b]	84.8	88.7±4.0
Histidine	24.3±0.7	23.9±1.0	24.1±0.9
Aspartic acid	213.3±4.3	210.8±4.4	212.1±5.0
Threonine	133.7[a]	133.0[a]	133.4±0.4
Serine	98.8[a]	83.0[a]	
Glutamic acid	140.5±2.0	139.0±2.7	139.8±2.7
Proline	87.1±4.3	19.8	
Glycine	121.6±3.2	122.0±0.8	121.8±2.8
Alanine	160.0±3.4	161.7±0.2	160.9±3.0
Half cysteine			20[c]
Valine	181.4[b]	174.9[b]	178.2±3.7
Methionine	41.9±1.7	31.6±1.8	
Isoleucine	129.9[b]	124.8[b]	127.4±2.6

TABLE 7 (continued)

AMINO ACID	1	2	3
Leucine	135.2[b]	121.9±2.6	128.6±6.5
Tyrosine	55.0±0.8	51.2±2.1	53.1±2.2
Phenylalanine	54.0±0.7	53.3±0.1	53.7±0.7
Tryptophan[d]	---	---	---

[a] Calculated by first order decay to zero time (Moore and Stein, 1963).

[b] Value from 72-hour hydrolysis.

[c] Determined by DTNB titration.

[d] Not yet determined.

believe the values for serine, proline, and methionine for the second determination are inaccurate (Wood, 1975). A value for tryptophan has not been determined but, due to the low extinction coefficient, low tryptophan content would be expected.

Cold Lability and FDP Inactivation Studies on Pyruvate Kinase

Experiments were conducted to determine the stability of PK, as monitored by enzymatic activity, under a variety of conditions at two different temperatures, 0° C and 23° C. In the first experiment, PK was diluted to 10 μg/ml in (1) 0.1 M $(CH_3)_4N$-cacodylate, pH 6.2; (2) 0.1 M

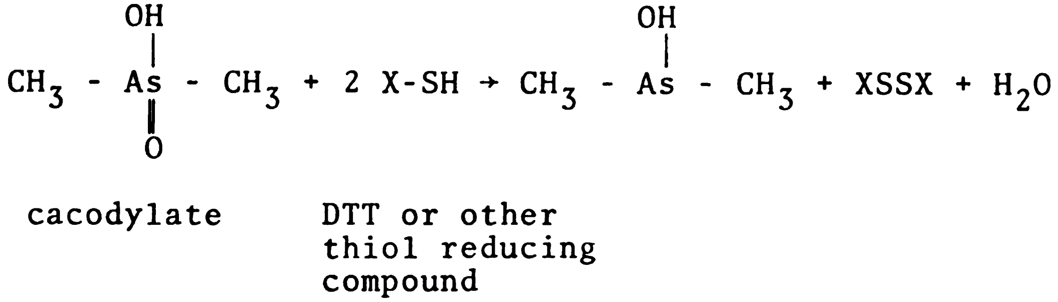
$(CH_3)_4N$-cacodylate, pH 6.2, containing 0.024 M $MgCl_2$; and (3) 0.1 M KCl, and 0.1 M $(CH_3)_4N$-cacodylate, pH 6.2 containing 25% glycerol. In a second set of three incubation mixtures, the same conditions as described above were used except that 5 mM DTT was included in each. One set of these six fractions was incubated at 0° C and a duplicate set at 23° C. After 24 hours the fractions were assayed and the activities determined and compared with the original activity. The results shown in Table 8 suggest that PK at 0° C is more unstable than at 23° C under any of the conditions used. Glycerol does protect the enzyme from inactivation to a certain degree at 0° C. Glycerol helps retain almost full activity at 23° C as compared to buffer or buffer plus cations. One of the most striking results of this experiment was that all the fractions containing DTT were nearly completely inactivated. This is quite unusual, since normally sulfhydryl reducing compounds help retain activity, not destroy it. It was thought that there was some type of reaction between DTT and the buffer cacodylate which resulted in inactivation of PK. A similar inactivation in the presence of cacodylate and DTT has been reported by Jacobson and Murphy (1962).

The reaction taking place is proposed below:

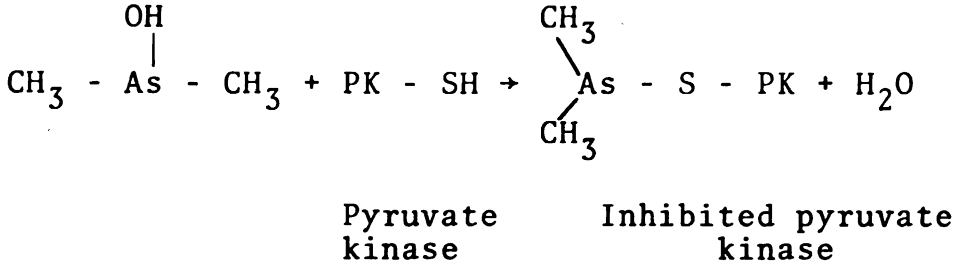
TABLE 8

Cold Lability and Inactivation of Manton-Gaulin PK

INCUBATION MIXTURE	% ORIGINAL ACTIVITY
23°	
1. Buffer	6.0
2. Buffer + 0.024 M $MgCl_2$ + 0.01 M KCl	9.0
3. Buffer + 25% glycerol	97.0
4. Buffer + 5 mM DTT	0.8
5. Buffer + 0.024 M $MgCl_2$ + 0.01 M KCl + 5 mM DTT	0.8
6. Buffer + 25% glycerol + 5 mM DTT	0.8
0°	
1. Buffer	1.2
2. Buffer + 0.024 M $MgCl_2$ + 0.01 M KCl	0.7
3. Buffer + 25% glycerol	16.0
4. Buffer + 5 mM DTT	0.3
5. Buffer + 0.024 M $MgCl_2$ + 0.01 M KCl + 5 mM DTT	0.3
6. Buffer + 25% glycerol + 5 mM DTT	0.4



$$CH_3-\underset{\underset{O}{\|}}{\overset{\overset{OH}{|}}{As}}-CH_3 + 2\ X\text{-}SH \rightarrow CH_3-\overset{\overset{OH}{|}}{As}-CH_3 + XSSX + H_2O$$



$$CH_3-\overset{\overset{OH}{|}}{As}-CH_3 + PK-SH \rightarrow (CH_3)_2As-S-PK + H_2O$$

Because of this inactivation, future stability studies were done in a different buffer.

The next experiment was conducted to determine whether FDP, the allosteric activator, had any effect on PK stability at 23° C or 0° C. The buffer selected for this experiment was 0.1 M Tris-MES, pH 7.5, containing 10 mM βME. Four samples were incubated at 23° C: buffer, 100 μg PK; buffer, 100 μg PK, 3 mM FDP; buffer, 100 μg PK, 0.024 M $MgCl_2$, 0.1 M KCl; buffer, 100 μg PK, 0.024 M $MgCl_2$, 0.1 M KCl, 3 mM FDP. Five samples were incubated at 0° C; the first 4 were identical to those at 23° C, the fifth contained buffer, 100 μg PK and 25% glycerol. The samples were incubated for 1 hour at the given temperatures and aliquots withdrawn and assayed every 15 minutes. The results again indicated that PK is more stable at 23° C than 0° C. Thus the enzyme must be cold labile. The enzyme is inactivated rapidly by FDP at either temperature, 0° C being more rapid than 23° C. The cations Mg^{2+} and K^{+} offer considerable protection against this inactivation by FDP. Glycerol offers good protection against the cold inactivation. All of the results are consistent with those of Kuczenski (1970).

Studies on Protease Contamination

Since PK was still slightly contaminated with protease activity after its purification (before Sephadex G-100 chromatography), this meant that protease activity was present with the enzyme throughout the purification. Because of this constant threat of possible proteolytic degradation, it became of interest to determine whether the protease present before the G-100 column could be inhibited by DFP and under what conditions, if any, this inhibition would take place. It has previously been discussed that three proteases from yeast have been purified and their properties studied. Two (B and C) of the three proteases are inhibited by DFP. These same two proteases exist as protease inhibitor complexes *in vivo*. These protease inhibitor complexes having been isolated and studied were found to dissociate in denaturing solvents, such as SDS, thus releasing the free protease for full activity or inhibition by DFP. Therefore, in order to determine whether the protease could be inhibited by DFP and whether it was free or as a protease inhibitor complex, the SDS qualitative assay was used.

Three samples were studied. The first was preincubated with 10 mM DFP for 15 minutes at room temperature. The DFP was removed by passage over a Sephadex G-25 column and the sample concentrated by dialysis against Sephadex G-25. If the protease could be inhibited by DFP and was not in a complexed form, it would be inhibited by this treatment. This sample was incubated in the regular 1% SDS mixture for 15 minutes at room temperature and subjected to SDS gel electrophoresis.

The second and third samples of PK were incubated in the regular 1% SDS mixture with 10 mM DFP added to one of the samples. The incubation mixture without DFP was a control to determine how much proteolytic degradation occurred in the SDS solution after being incubated for 15 minutes. After the incubations, the two samples were applied to SDS gels and electrophoresed. Figure 13 shows scans of the gels containing the three samples. The sample preincubated in DFP shows no protein bands at all, just degraded protein near marker dye (Figure 13A). The control is degraded to peptides which had mobilities nearly equal to that of the marker dye (Figure 13C). The third sample, which was incubated in DFP and SDS, shows PK quite distinctly with a few other bands (Figure 13B). These results suggest that at least part of the protease contamination which copurifies with PK is

FIGURE 13: COMPARISON OF ACTIVE AND INHIBITED PROTEOLYTIC ACTIVITY ON MANTON-GAULIN PK USING SDS-POLYACRYLAMIDE ELECTROPHORESIS.

Scan A is of Manton-Gaulin PK preincubated with 10 mM DFP, passed over Sephadex G-25 and electrophoresed on 1% SDS-polyacrylamide gels. Scan B is of Manton-Gaulin PK preincubated in 1% SDS and 10 mM DFP and electrophoresed on 1% SDS-polyacrylamide gels. Scan C is of Manton-Gaulin PK electrophoresed on polyacrylamide gels.

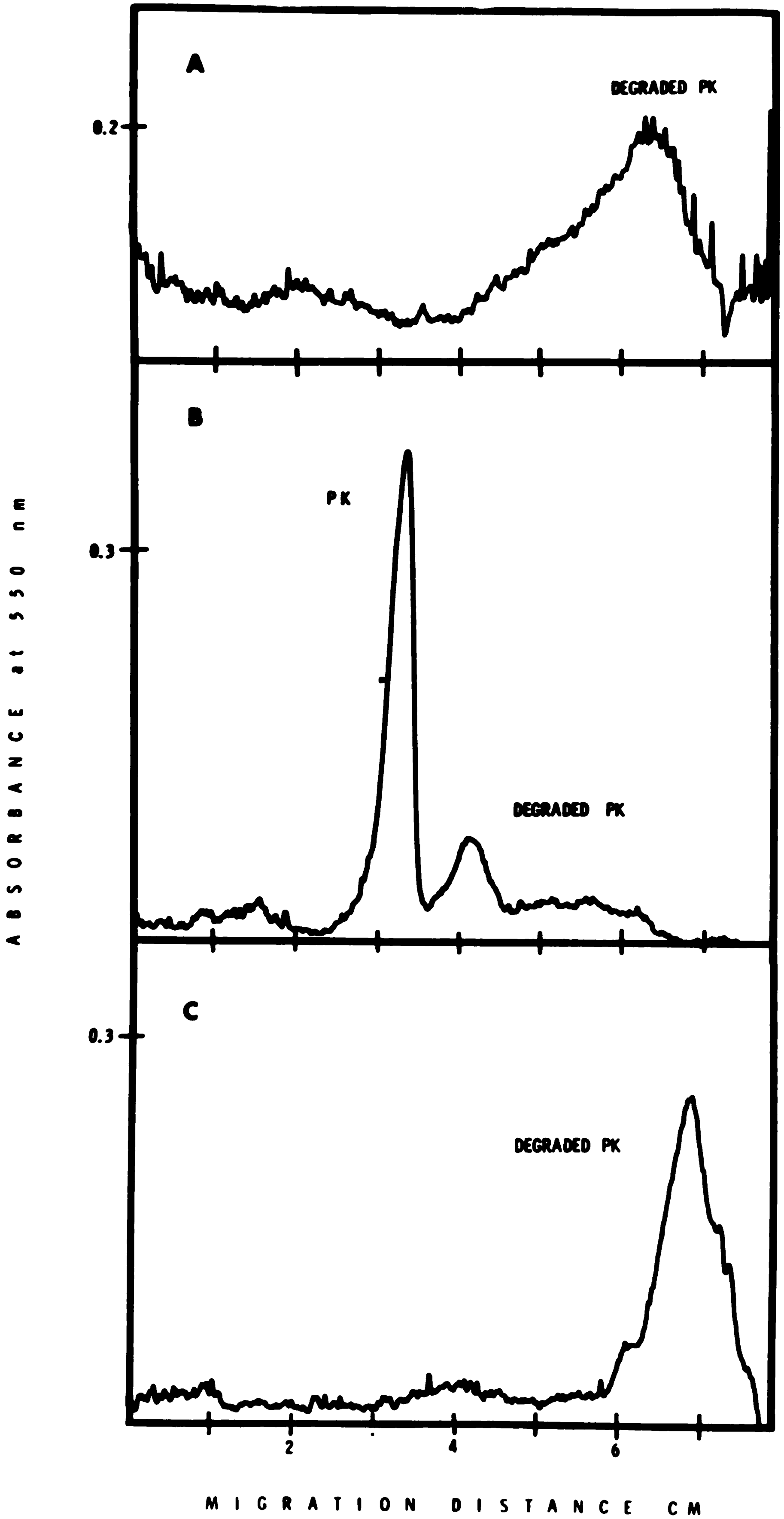

A
DEGRADED PK
0.2
B
PK
0.3
DEGRADED PK
C
0.3
DEGRADED PK
2
4
6
ABSORBANCE at 550 nm
MIGRATION DISTANCE CM

Figure 13

inhibited by DFP but only in the presence of a protein denaturing solvent; thus it probably exists as a protease-inhibitor complex.

Kinetic Parameters

Since PK purified by the method described in this communication produced an enzyme with molecular weight greater than that of the previous preparation (Hunsley and Suelter, 1969a), the kinetic studies for the substrates and cations were repeated to determine whether there was any change in these. Table 9 shows the results of the K_m determinations for this preparation. These results indicate that FDP is a heterotropic activator and that it

TABLE 9

Kinetic Parameters of Manton-Gaulin PK for Substrates and Cations

Substrate or activator	K_m	n_H
PEP	2.69×10^{-3}	2.7
PEP with FDP	1.01×10^{-4}	1
ADP	5.00×10^{-4}	1.1
ADP with FDP	3.25×10^{-4}	1
K with FDP	1.04×10^{-2}	1

transforms the sigmoid kinetics (n=2.7) of PEP to hyperbolic (n=1). In addition, the enzyme was shown to have an absolute requirement for the cations K^+ and Mg^{2+}. The kinetics of Mg^{2+} activation in the presence of FDP were considerably different than those observed for earlier preparations of the enzyme. The velocity experienced a considerable lag before reaching its maximum value. This will be discussed in the following section.

Hysteresis

Using the LDH coupled reaction method for assaying PK, the rate of the reaction at 5.28×10^{-4} M Mg^{2+} was observed to increase with time, finally approaching a constant rate. As the concentration of Mg^{2+} was increased, the time it took for the reaction to reach a constant rate appeared to decrease. Finally, at saturating Mg^{2+} the reaction rate was constant throughout the time periods observed. Figure 14 shows the phenomenon just described using the LDH coupled assay system. Thus, it appeared that PK was undergoing a hysteretic, time dependent, activation at Mg^{2+} concentrations below saturating levels.

In order to determine whether the hysteresis observed was due to some change in the enzyme produced during the purification process, the enzyme in the cell-free extract was examined. The results of this experiment, Figure 15, show that PK in the cell-free extract also experiences a hysteretic activation at subsaturating Mg^{2+} concentrations

FIGURE 14: TIME DEPENDENT ACTIVATION OF MANTON-GAULIN PK AT VARIOUS Mg^{2+} CONCENTRATIONS.

This plot represents the activity assay of Manton-Gaulin PK with all substrates and activators saturating except Mg^{2+}. The Mg^{2+} concentrations represented are (A) 5.28×10^{-4} M, (B) 1.06×10^{-3} M, (C) 24×10^{-3} M.

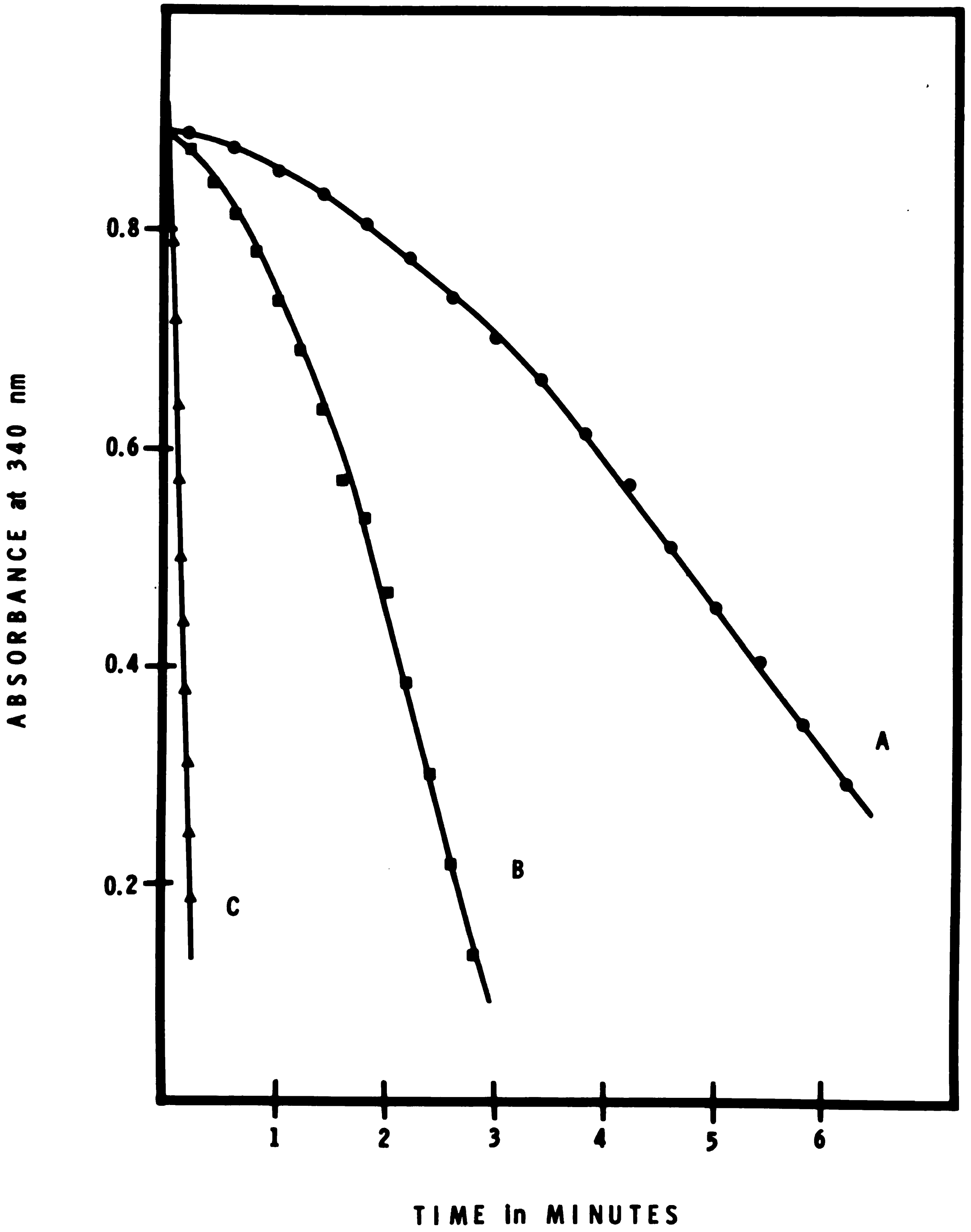


Figure 14

FIGURE 15: TIME DEPENDENT ACTIVATION OF PK IN CELL-FREE EXTRACT AT SUBSATURATING Mg^{2+} CONCENTRATIONS.

This plot represents the activity assay of PK in the cell-free extract with all substrates and activators saturating except Mg^{2+}. The Mg^{2+} concentration is 1.58×10^{-3} M. Plot A represents the activity of PK when cell-free extract was diluted in 0.049 M KOH, 0.1 M MES, pH 6.2, before addition to the assay. Plot B represents the activity of PK when cell-free extract was diluted in the same buffer + 1 mM FDP before addition to the assay.

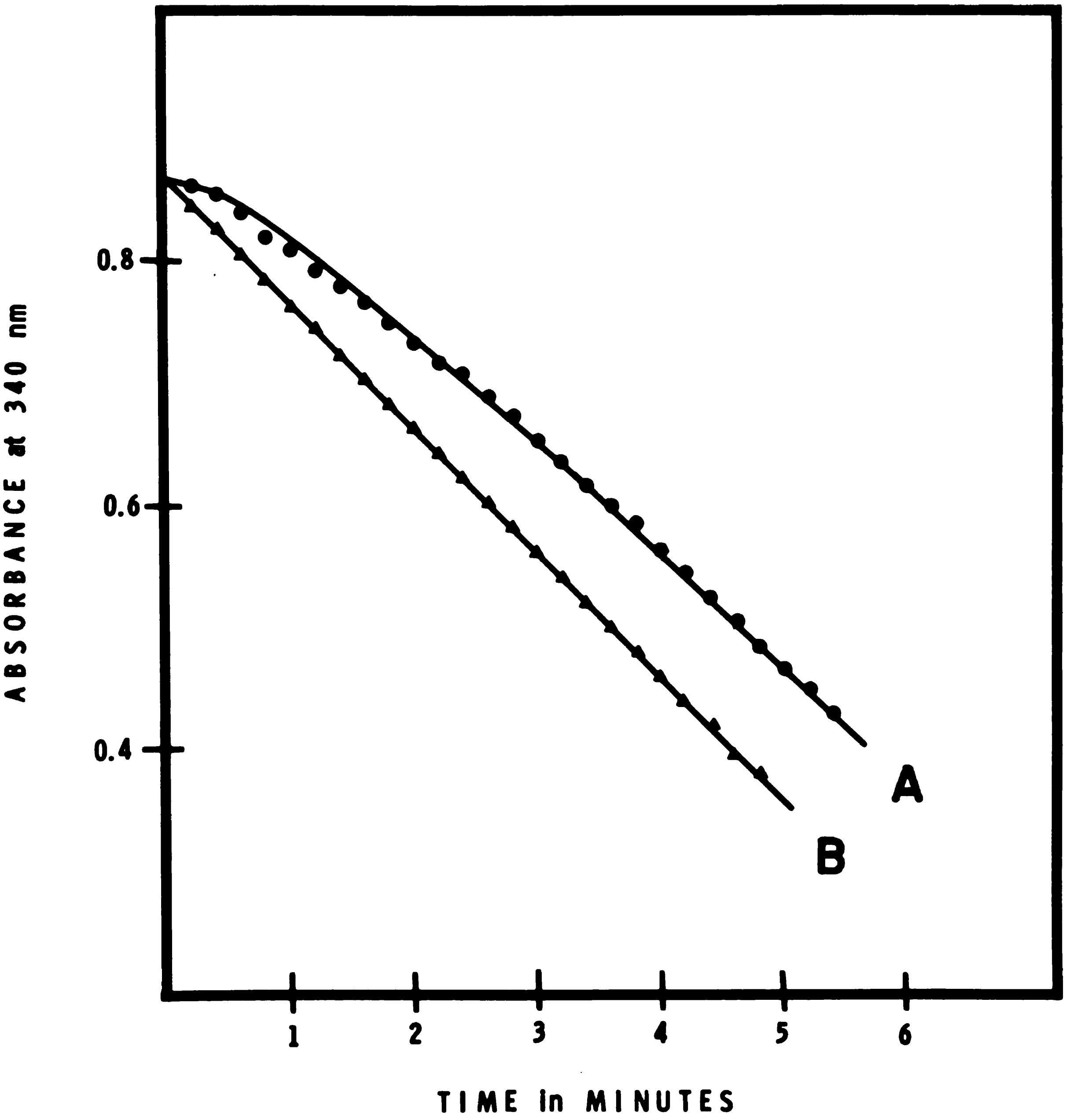


Figure 15

(1.58×10^{-3} M). Therefore, this was a property of the "native" enzyme.

Cause of Hysteresis

In an effort to determine what was producing the hysteresis, a series of preincubations were examined to see if the hysteresis could be removed by one or more components of the assay. PK was diluted into buffer (.049 M K^+, 0.1 M MES, pH 6.2) plus one or more components, listed in Table 10 at a final concentration of 50 µg/ml and incubated for 15 seconds before addition to the PK coupled assay containing 1.58×10^{-3} M $MgCl_2$. FDP, 1 mM, was the only component which could eliminate the hysteresis. On the other hand, ADP, 10 mM, appeared to be the only component which could prevent FDP from eliminating the hysteresis. Thus it would seem that the hysteresis observed at subsaturating Mg^{2+} concentrations was due to ADP inhibition of FDP activation.

Investigation of Inhibitor

A series of preincubation experiments were conducted to determine whether ADP or some contaminant or degradative product of ADP was responsible for its inhibition of FDP activation at subsaturating levels of Mg^{2+}. PK was diluted to a final concentration of 50 µg/ml into buffer, 0.049 M K^+ and 0.1 M MES, pH 6.2, plus one or more of the components listed in Table 11 and incubated for 15 seconds before addition to the PK coupled assay. The

TABLE 10

Comparison of Manton-Gaulin PK Activity after Preincubation of the Enzyme with Reaction Components

PK at a concentration of 50 μg/ml was incubated in 0.049 M KOH, 0.1 M MES, pH 6.2, with the additional components described here. In the column labeled "Hysteresis" a "+" indicates that the PK activity was time dependent, while a "-" indicates that the PK activity was linear.

		COMPOUND	HYSTERESIS
1.	0.024 M	$\mathrm{MgCl_2}$	+
2.	0.01 M	PEP	+
3.	0.01 M	ADP	+
4.	0.001 M	FDP	-
5.	0.100 M	KCl	+
6.	0.15 μM	NADH	+
7.	0.001 M 0.024 M	FDP Mg	-
8.	0.001 M 0.01 M	FDP PEP	-
9.	0.001 M 0.01 M	FDP ADP	+
10.	0.001 M 0.1 M	FDP KCl	-
11.	0.001 M 0.15 μM	FDP NADH	-
12.	0.001 M 0.010 M	FDP EDTA	-

TABLE 11

Comparison of the Ability of ADP Contaminants or Degradative Products to Inhibit FDP Activation at Subsaturating Levels of Mg^{2+}

Each of the following was incubated with 1 mM FDP, 0.049 M KOH, 0.1 M MES, pH 6.2, and 50 µg PK for 15 seconds at room temperature. A "+" indicates that the PK activity was time dependent, while a "-" indicates that the PK activity was linear.

PREINCUBATION	HYSTERESIS
10 mM ATP	slight
10 mM ADP	+
1 mM adenosine	-
1 mM adenine	-
10 mM PPi	+
10 mM PPi + 10 mM EDTA	+
10 mM NaH_2PO_4	-
1 mM AMP	-
10 mM EDTA	-
10 mM ADP + 10 mM EDTA	+

final concentration of ATP in the ATP and ADP stock solutions was determined using the hexokinase assay. The final concentration of ADP in the ATP and ADP stock solutions was determined using the PK assay. The 0.1 M ADP stock solution contained 1.53×10^{-3} M ATP. The 0.1 M ATP stock solution contained 1.69×10^{-3} M ADP. For these experiments the ADP and PPi were passed over Dowex chelating resin to remove trace metal contamination. This was felt necessary since the final rate in the hysteretic activation was dependent on the source of ADP. The two incubations, which include 1 mM FDP, 10 mM EDTA, and 10 mM ADP or 10 mM PPi, were done to determine if a metal not removed by the Dowex chelating resin was responsible for the hysteresis. Since these incubations still produce hysteresis, it is concluded that a trace metal contamination was not responsible for this problem. Table 11 shows the results of the preincubation experiment. The only compounds tested which had any inhibitory properties at all were ADP, PPi and ATP. ADP and PPi had nearly equal inhibitory ability while that of ATP was very slight. In fact, the inhibition attributed to ATP could actually be due to ADP contamination.

Because PPi is a possible contaminant of ADP, the next series of experiments was designed to determine whether ADP or the possible contaminant PPi was responsible for ADP inhibition of FDP activation at subsaturating Mg^{2+}. Since inorganic pyrophosphatase hydrolyzes PPi selectively in the presence of Mg^{2+} (Schlesinger and Coon, 1960), ADP and PPi were incubated independently with inorganic pyrophosphatase for 30 minutes at 30° C in a mixture which was a modification of the reaction mixture used by Moe and Butler (1972). The preincubation contained at a final concentration: 27 mM $MgCl_2$, 0.2 M KCl, 2 x 10^{-5} M EGTA, 5 units of yeast inorganic pyrophosphatase, 50 mM Tris-HCl, pH 7.4, and 25 mM of either PPi or ADP. A duplicate was run for both ADP and PPi which did not contain any inorganic pyrophosphatase so that any loss in ADP or PPi under the conditions of the preincubation could be accounted for. Aliquots were withdrawn at 0 time and 30 minutes from the preincubation mixtures and mixed to a final concentration of ADP or PPi equal to 5 mM with 1 mM FDP, 50 µg/ml PK, 0.049 M KOH and 0.1 M MES, pH 6.2 for 15 seconds, then the PK assay was initiated by adding enzyme from the mixture. Results shown in Table 12 indicate that only the preincubation mixture containing PPi and inorganic pyrophosphatase lost the ability to inhibit FDP activation. This would strongly suggest that it is ADP, not PPi, contamination which produces inhibition in the PK assay.

One more experiment was conducted to rule out any possible doubt that ADP was the source of the inhibition. Twenty-five millimoles ADP was preincubated for 30 minutes at 30° C with 1.8 units PK in the following mixture: 24 mM $MgCl_2$, 50 mM KCl, 10 mM ^{tri} CHA PEP, 1 mM tetra

TABLE 12

Comparison of the Ability of Inorganic Pyrophosphatase to Degrade the ADP or PPi Inhibitor of FDP Activation at Subsaturating Levels of Mg^{2+}

The following mixtures were incubated for 30 minutes at 30° C under the conditions described in the text. Aliquots were removed at 0 and 30 minutes and were tested for their ability to inhibit 1 mM of FDP activation in a 15 second preincubation with 50 µg/ml PK. A "+" indicates that the aliquot inhibited FDP activation while a "-" indicates that the aliquot was unable to inhibit FDP activation of PK.

	Hysteresis at time	
	0	30 min.
1. Inorganic pyrophosphatase + ADP	+	+
2. Inorganic pyrophosphatase + PPi	+	-
3. ADP	+	+
4. PPi	+	+

CHA FDP, 100 mM MES-49 mM K^+, pH 6.2. Aliquots were withdrawn from the incubation mixture at 0 time and 30 minutes and their ability to inhibit FDP activation tested, just as above. The results in Table 13 indicate complete destruction of the ability to inhibit FDP activation. This conclusively shows that ADP was involved in the inhibition of FDP activation at subsaturating Mg^{2+} and that PPi was independently involved.

TABLE 13

The Ability of Manton-Gaulin PK to Degrade the ADP Inhibitor of FDP Activation at Subsaturating Levels of Mg^{2+}

	Hysteresis at time	
	0	30 min
1. PK + buffer + ADP	+	-
2. Buffer + ADP	+	+

Investigation of Coupling Assay

In order to determine whether the hysteretic phenomenon observed at subsaturating Mg^{2+} was due to PK or the coupling enzyme LDH, the direct assay method of Pon and Bondar (1967) was used. This reaction proved to be far more insensitive than the coupled assay system. Since this reaction requires measuring change in O.D. at 230 nm, the concentrations of PEP and ADP, both of which absorb at 230 nm, had to be decreased to 2 mM so that the total absorption would not be so great that the small changes produced by enzyme activity would be insignificant compared to the total O.D. of the solution. Lowering the ADP concentration in the coupled assay system produces less of a hysteretic transition, therefore lowering the ADP in the uncoupled system made the hysteretic change nearly impossible to detect. Since PPi has been shown to produce a hysteretic effect similar to that of ADP, 20 mM PPi was included in the assay. This produced a detectable hysteretic effect which was eliminated with FDP preincubation. Therefore, the hysteresis was due to PK, not LDH, in the coupled assay system. All subsequent experiments were done using the coupled assay system due to its greater sensitivity.

Analysis of Hysteretic Curves

The approach to steady state rate was examined in the following manner to determine whether it was a first order process.

The observed velocity (v) is related to enzyme concentration (E) in the following manner:

$$v = K\ [E],$$

where K is a function of substrate concentrations and kinetic constants. The time dependent activation process can be described as

$$[E] = [E_o]e^{-kt}$$

where $[E_o]$ represents the initial concentration of inhibitable enzyme. Then,

$$v_t = K[E_o] \int_o^t e^{-kt}$$

or

$$v_t = v_f \ (1 - e^{-kt})$$

$$v_t - v_f = (v_o - v_f)e^{-kt} \qquad \text{or}$$

$$\ln \ (v_t - v_f) = \ln \ (v_o - v_f) - kt$$

If the time dependent approach to steady state is a first order process, then a plot of ln $(v_t - v_f)$ vs t will produce a straight line with slope -k and intercept ln $(v_o - v_f)$. In order to determine v_t at many different points along the hysteretic curve, the O.D. at different times obtained from the recorder tracing was analyzed using a curvfitting computer program which makes use of Chebyshev polynomials to find the best fitting curve to the data (O.D. vs t) and then to calculate the slope at each of the times read into the program. This program was written by Mr. G. Ho, Chemistry Department, Michigan State University, East Lansing, MI. The final velocity was defined as the velocity at which the slope no longer changes and v_t as the slope at changing times. $(v_t - v_f)$ was then calculated at the different times and plotted vs time.

Relationship of Velocity and Rate of Activation to Protein Concentration

To determine whether the time dependent approach to steady state was related to protein concentration, the analysis method previously described was used to compare data for different protein concentrations. The coupled assay system was used at 1.58×10^{-3} M Mg^{2+} (50% saturating). The comparison was made in two ways. First, the enzyme was diluted in buffer (0.049 M K^+ - 0.1 M MES, pH 6.2), then an aliquot added to initiate the assay and the hysteretic rate recorded. Secondly, the enzyme was diluted in buffer (0.049 M K^+ - 0.1 M MES, pH 6.2) plus 1 mM FDP, then an aliquot was added to initiate the assay and the linear rate recorded. The data from the first set were analyzed in two ways: (1) first order plots to determine rates of activation, and (2) determination and comparison of v_f. The second set of data was used to determine initial velocity only. Figures 16, A-E, show the first order plots of the time dependent activation. In all cases the graphs are reasonably linear, indicating a first order activation of the enzyme. Figure 17 shows a plot of velocity vs protein concentration for the Case I and Case II. The velocity values for Case I are final velocities (v_f). The velocities for Case II

FIGURE 16: EFFECT OF DIFFERENT PROTEIN CONCENTRATIONS ON HYSTERESIS OF MANTON-GAULIN PK.

The activity of PK at the following protein concentrations was recorded and log $(v_f - v_t)$ was plotted. The total Mg^{2+} concentration in the assay was 1.58×10^{-3} M.

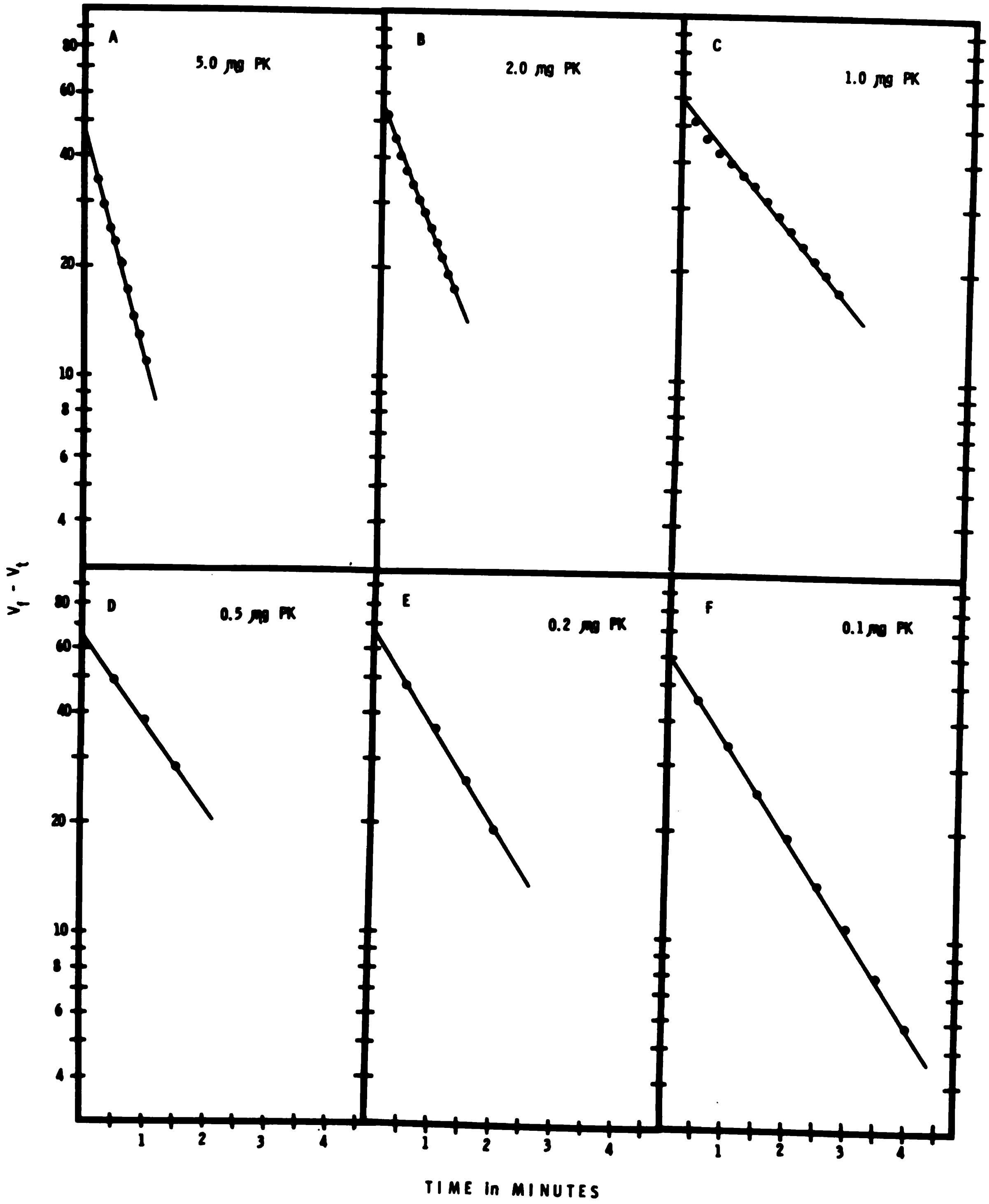

A
5.0 µg PK
B
2.0 µg PK
C
1.0 µg PK
D
0.5 µg PK
E
0.2 µg PK
F
0.1 µg PK
80
60
40
20
10
8
6
4
$V_f - V_t$
1
2
3
4
TIME in MINUTES

Figure 16

FIGURE 17: EFFECT OF DIFFERENT PROTEIN CONCENTRATIONS ON THE VELOCITY OF THE REACTION FOR ENZYME WITH AND WITHOUT FDP PREINCUBATION.

Plot A represents the velocities for enzyme at different concentrations which has been preincubated with FDP. Plot B represents the final velocities for enzyme at different concentrations which has not been preincubated with FDP. The total Mg^{2+} concentration in the assay was 1.58×10^{-3} M.

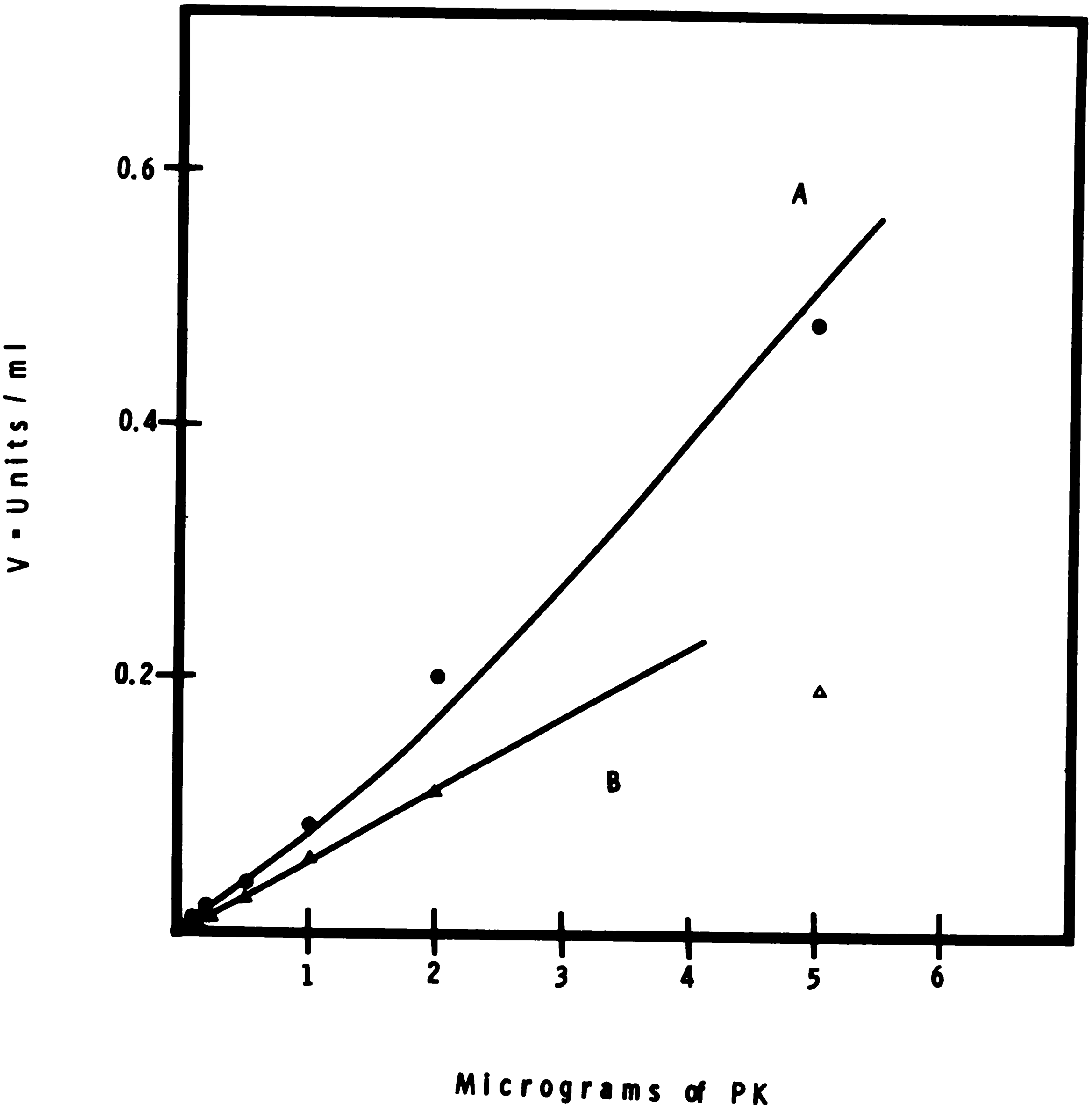


Figure 17

samples preincubated in FDP before assay show higher velocities than those of Case I. Figure 18 shows a plot of specific activity vs protein for both cases. For Case I the results are nearly linear except for 5 μg protein, at which point specific activity drops off. The specific activity for Case II appears to increase hyperbolically with increasing protein, perhaps indicating a dissociation mechanism in the FDP preincubation. Figure 19 shows a plot of k of activation for Case I vs protein. The k values are nearly constant for the region from 0.1 μg through 2 μg of protein. At 5 μg the k value is much greater. This is very similar to what is seen in Figure 18, where the specific activity is fairly constant from .1 to 2 μg.

Relationship of Mg^{2+} Concentration to Rate of PK Activation

Earlier it was mentioned that when the concentration of Mg^{2+} was increased, it appeared that the activation process took less time. In an effort to determine whether there was a relationship between rate of activation and Mg^{2+} concentration, the rate of activation at different Mg^{2+} concentrations was analyzed using the method discussed previously. Figure 20, A-E, shows linear first order plots for the different Mg^{2+} concentrations. Figure 21 is a plot of k of activation determined from the first order plots vs Mg^{2+} concentration (total). The k values for 1.06×10^{-3} M through

FIGURE 18: EFFECT OF DIFFERENT PROTEIN CONCENTRATIONS ON THE SPECIFIC ACTIVITY FOR ENZYME WITH AND WITHOUT FDP PREINCUBATION.

Plot A represents the specific activities for enzyme at different concentrations which has been preincubated with FDP. Plot B represents the specific activities for enzyme at different concentrations which has not been preincubated with FDP. The total Mg^{2+} concentration was 1.58×10^{-3} M.

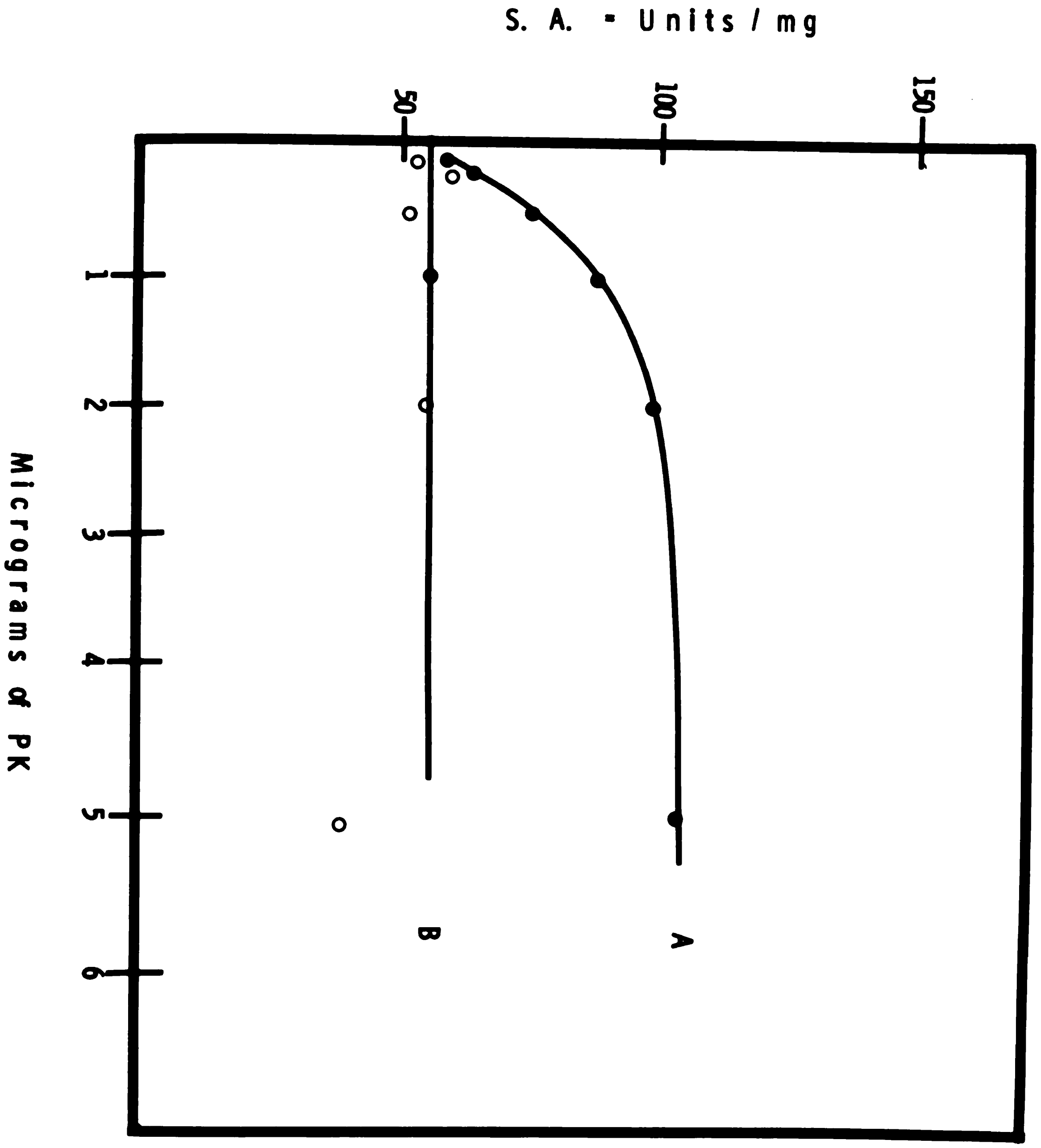


Figure 18

FIGURE 19: EFFECT OF DIFFERENT PROTEIN CONCENTRATIONS ON THE FIRST ORDER RATE CONSTANT FOR ENZYME NOT PREINCUBATED WITH FDP.

The plot represents the relationship of the first order rate constant (k) at different protein concentrations. The total Mg^{2+} concentration was 1.58×10^{-3} M.

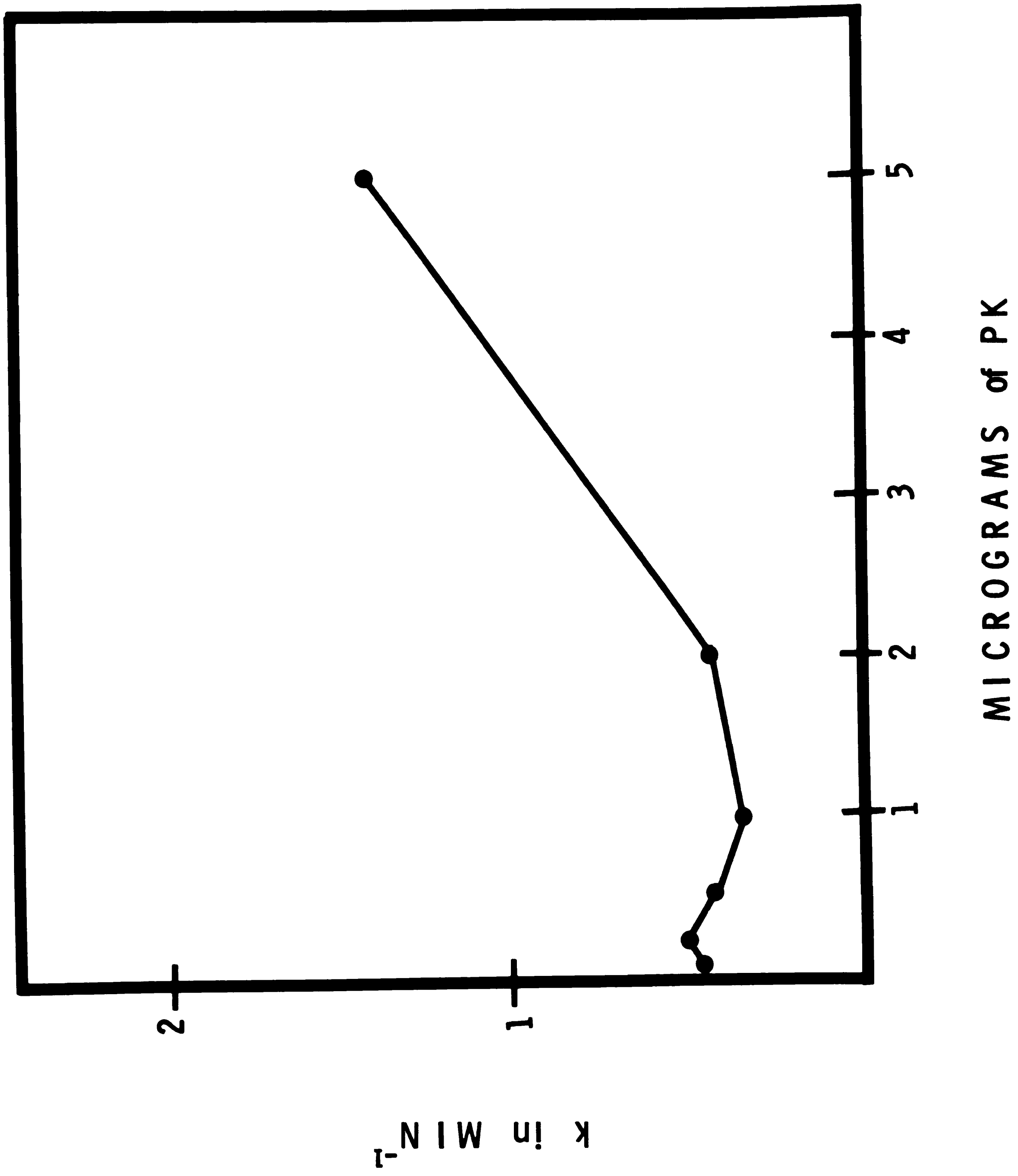


Figure 19

FIGURE 20: EFFECT OF DIFFERENT Mg^{2+} CONCENTRATIONS ON THE HYSTERESIS OF MANTON-GAULIN PK.

The activity of PK at the following $MgCl_2$ concentrations was recorded and log $(v_f - v_t)$ was plotted.

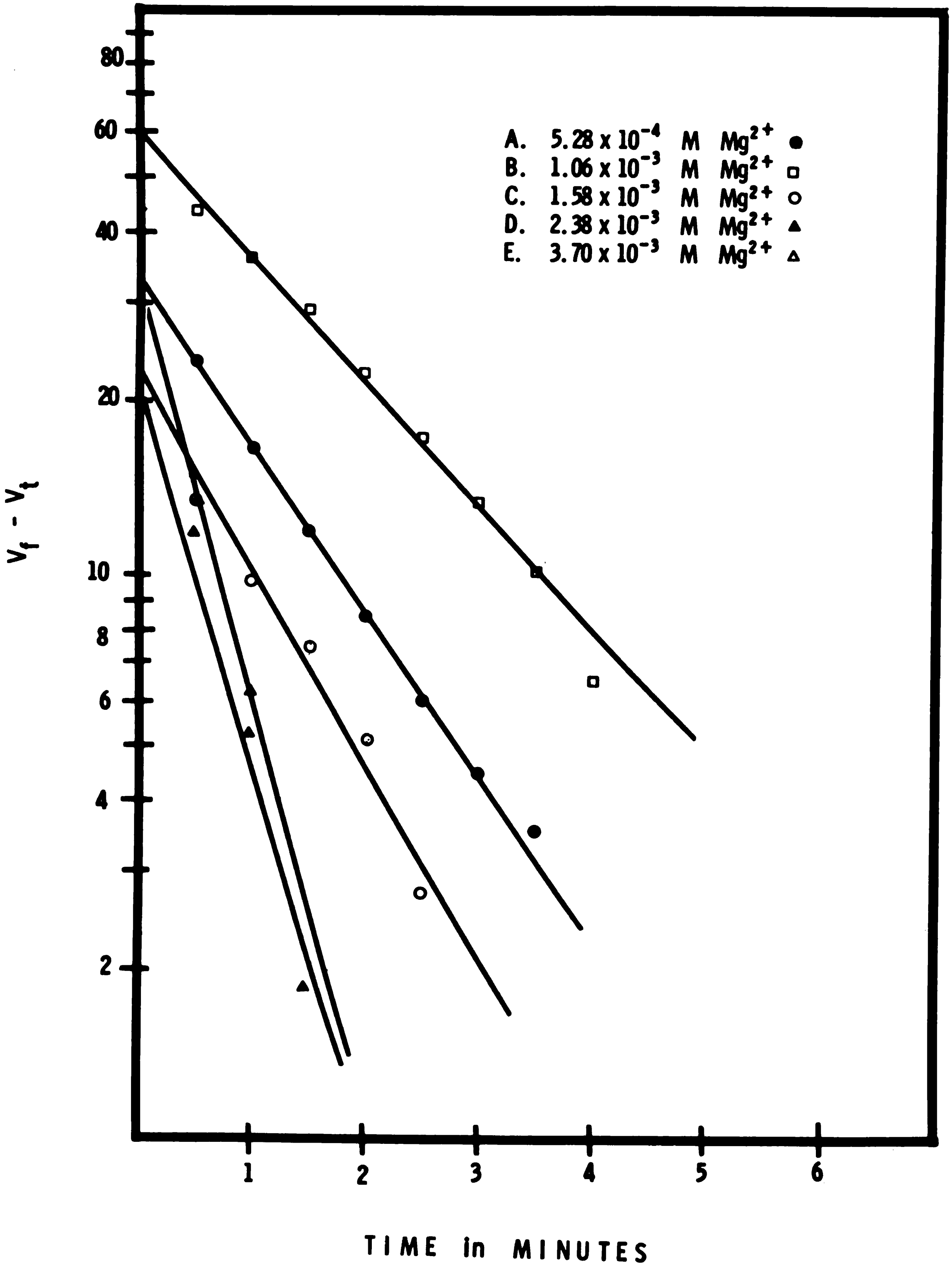

80
60
40
20
10
8
6
4
2
$V_f - V_t$
A. 5.28×10^{-4} M Mg^{2+} ●
B. 1.06×10^{-3} M Mg^{2+} □
C. 1.58×10^{-3} M Mg^{2+} ○
D. 2.38×10^{-3} M Mg^{2+} ▲
E. 3.70×10^{-3} M Mg^{2+} △
1
2
3
4
5
6
TIME in MINUTES

Figure 20

FIGURE 21: EFFECT OF DIFFERENT Mg^{2+} CONCENTRATIONS ON FIRST ORDER RATE CONSTANT.

The plot represents the relationship between the first order rate constant at different Mg^{2+} concentrations.

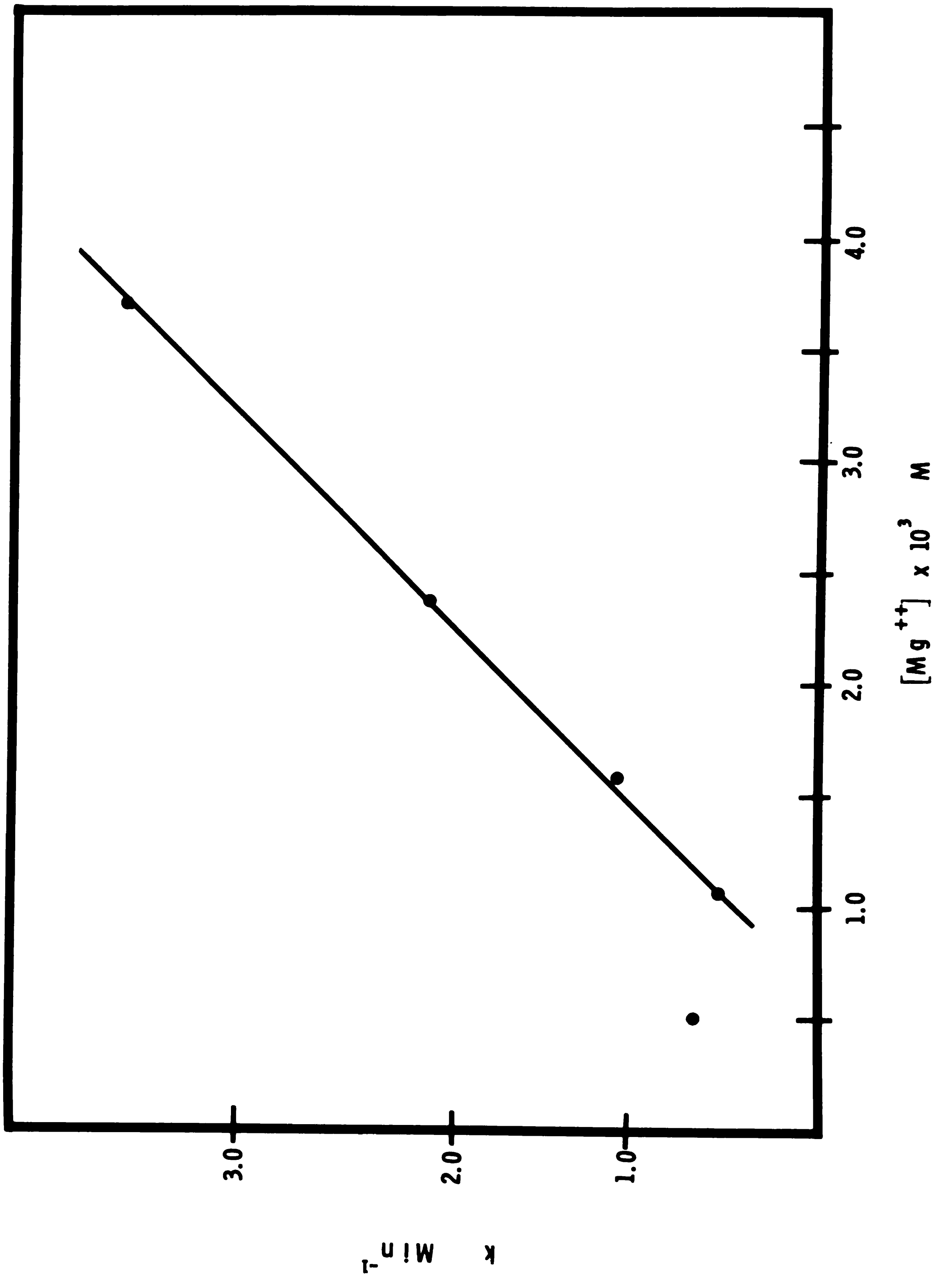


Figure 21

3.7×10^{-3} M fall on a straight line, indicating that there may be a direct relationship between Mg^{2+} concentration and rate of activation. The $t^{1/2}$ values for these different Mg^{2+} concentrations are shown in Table 14. The half time of activation decreases with increasing Mg^{2+}. The protein concentration used for these experiments was 0.5 μg/ml, which places the velocity in the linear range of v vs protein concentration (refer to Figure 17).

When final velocity determined from the above experiments was plotted vs Mg^{2+} concentration, a sigmoidal relationship is observed. This would indicate that perhaps the FDP concentration is not saturating at the lower Mg^{2+} concentrations. If the velocity and substrate concentrations are plotted in a Hill plot, the K_m is determined to be 2.0×10^{-3} M with $n_H = 1.79$ (Figure 22). This indicates that at 1 mM FDP the enzyme is not saturated for FDP or FDP cannot eliminate the sigmoidicity.

TABLE 14

First Order Rate Constants and Half Times of Activation for Hysteresis Produced at Different Mg^{2+} Concentrations

$[Mg^{2+}]M$	k min^{-1}	$t^{1/2}$ min.
5.28×10^{-4}	.65	1.06
1.06×10^{-3}	.53	1.30
1.58×10^{-3}	1.05	.66
2.38×10^{-3}	2.02	.34
3.7×10^{-3}	3.59	.19

FIGURE 22: THE RELATIONSHIP BETWEEN V_f OF MANTON-GAULIN PK AND TOTAL Mg^{2+} CONCENTRATION.

The final velocities for the hysteretic activity plots for Manton-Gaulin PK were compared at different concentrations of total Mg^{2+}.

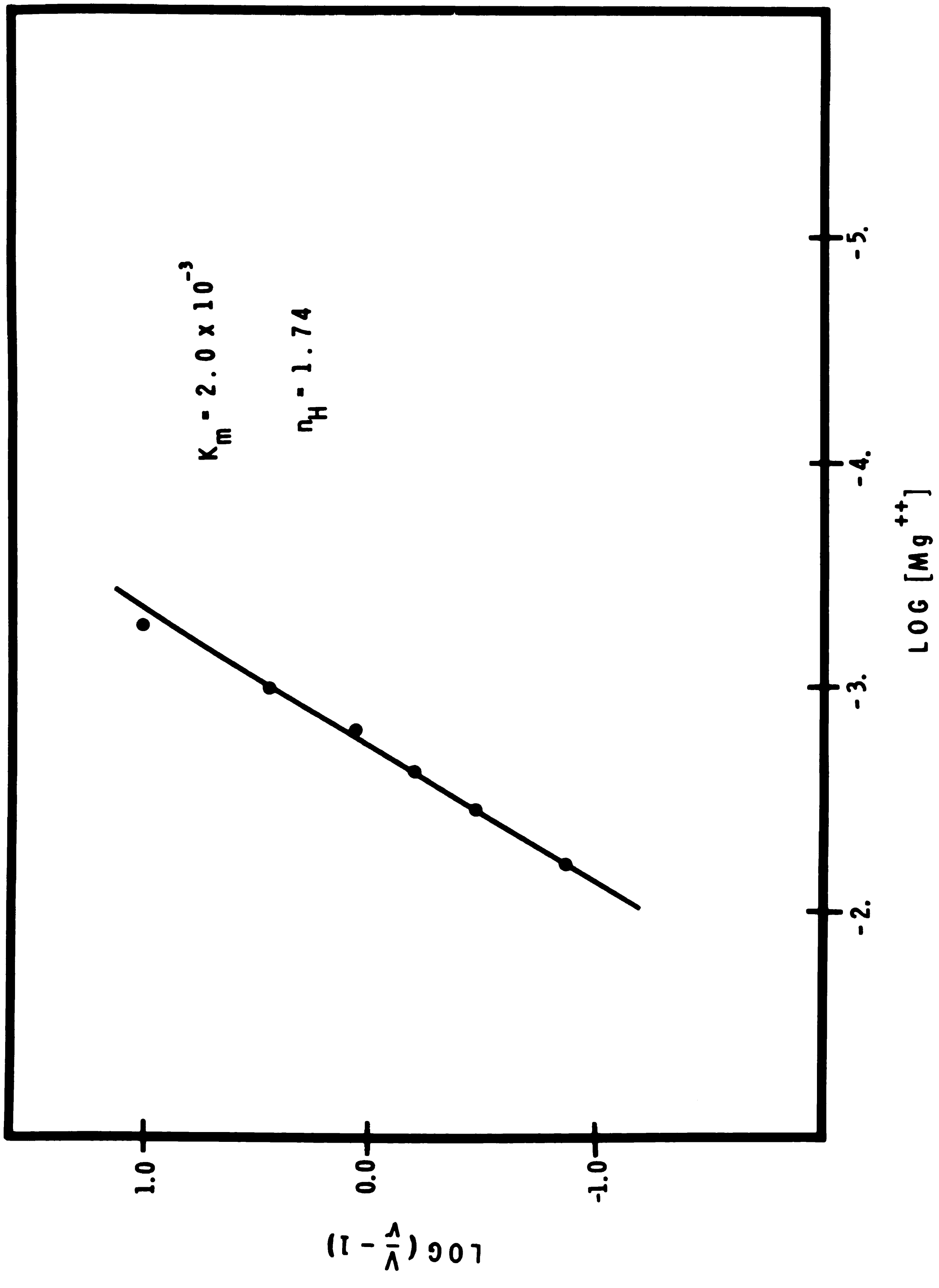

$K_m = 2.0 \times 10^{-3}$
$n_H = 1.74$
1.0
0.0
-1.0
$\mathrm{LOG}\ (\frac{V}{v} - 1)$
-2.
-3.
-4.
-5.
$\mathrm{LOG}\ [Mg^{++}]$

Figure 22

DISCUSSION

The lysis procedure used in the preparation of pyruvate kinase reported in this manuscript releases four times more pyruvate kinase and 2.6 times more total protein per pound of yeast than the toluolysis method of Hunsley and Suelter (1969a). The Manton-Gaulin lyses the yeast in 10 minutes, while toluolysis requires 15 hours. This means that separation of PK from the other proteins, including proteases, can begin much sooner, thus reducing the risk of proteolytic degradation. The fact that the entire lysing procedure can be conducted at 4° C in the presence of 1 mM DFP with controlled pH is an additional asset to prevent activation of the yeast proteases and therefore proteolysis. The yield of PK could be greatly increased if a more efficient method of separating the protease could be found. The purification summary (Table 3) indicates that nearly two-thirds of the pure pyruvate kinase is sacrificed after the Sephadex G-100 column due to proteolytic contamination.

The enzyme purified by this method, as stated previously, still shows a protease contamination before the Sephadex G-100 chromatography. The fact that the protease appears to be in an inhibited form is somewhat encouraging, but the fact that it is there raises the question of whether the purified enzyme is a degraded form. There are several physical criteria which are consistent with the conclusion that this is not a degraded form.

(1) The results of SDS gel electrophoresis show a single, sharp band which has the same mobility when different preparations are compared.

(2) Multiple N-terminals were not detected.

(3) Isoelectric focusing produces a single peak of activity.

(4) The amino acid composition from two different preparations was shown to be almost identical within experimental error.

(5) Antibody prepared to the pure enzyme produces a single precipitin line in Ouchterlony double diffusion or immunoelectrophoresis.

The purified enzyme has been compared to the enzyme in cell-free extract by several different methods, and all results suggest that the enzymes are identical. Previous work has shown that cell-free extract electrophoresed on analytical disc gels results in only one protein band containing pyruvate kinase activity (Yun *et al.*, 1975). When the mobility of this band was compared with that of the pure enzyme by the Hedrick and Smith (1966) method, they were found to be identical. Antibody prepared to the pure enzyme produces single, fused precipitin lines against enzyme in the cell-free extract and pure enzyme. Immunoprecipitation of the pure enzyme from cell-free extract with subsequent solubilization in 1% SDS and electrophoresis on SDS gels produces a single protein band which comigrates with the purified enzyme. The SDS gel technique could distinguish a difference of 1000 in molecular weight, which would be 8 amino acids. Therefore, it would appear that the purified pyruvate kinase is identical or very nearly identical to native pyruvate kinase.

The native molecular weight determined by equilibrium ultracentrifugation was 209,000. Since the subunit molecular weight determined by SDS gel electrophoresis was 57,500, the enzyme is most likely a tetramer. The discrepancy between molecular weight determined on the basis of the subunit (230,000 for the tetramer) and that determined by ultracentrifugal analysis of the native enzyme is not uncommon in yeast pyruvate kinase. Fell *et al.* (1974) obtained a native molecular weight of 214,000 and a subunit molecular weight of 56,000 (224,000 for the tetramer). Bischofber *et al.* (1971) have obtained a native molecular weight of 191,000 and an SDS subunit molecular weight of 60,000 (240,000 for the tetramer) for the enzyme from *S. carlsbergensis*. For the enzyme from the latter organism, the subunits have been shown to be identical (Bornmann and Hess, 1974). Why there would be this large a difference between the SDS subunit weight and the native enzyme is not known. It could perhaps reflect a reduced ability of PK to bind SDS. There have been reports of other proteins with such characteristics (Nelson, 1971). Reduced binding of SDS would cause slower mobility which would result in a larger molecular weight.

The N-terminal analysis revealed that the amino terminal of PK must be blocked, since the dansylation method used to detect the N-terminal is reported to detect nanomole levels of amino acids (Gray and Hartley, 1963) and for bakers' yeast PK, nothing was detected. PK from brewers' yeast was reported to be acetylated. PK from other sources such as rabbit skeletal muscle (Cottam *et al.*, 1969) and human erythrocytes (Chern *et al.*, 1972) are also blocked.

The enzyme purified by the technique reported here has a maximum velocity of 340 u/mg. This is considerably higher than the 220 u/mg reported for the Hunsley and Suelter (1969a) preparation. However, the other kinetic parameters appear to be very similar, if not identical. Table 15 compares the K_m values for those parameters examined. The values for Hunsley and Suelter (1969a) were determined from published Hill plots. The fact that the K_m values are so similar is amazing since the enzymes differ by at least 40,000 in their molecular weight. This must mean that the portion removed by proteolysis had little to do with catalysis. The only kinetic property which was really different from that of

TABLE 15

Comparison of the K_m Values for Substrates and Cations for Two Preparations of PK

	Hunsley and Suelter (1969b)	This work
PEP	1.78×10^{-3}	2.69×10^{-3}
PEP + FDP	1.25×10^{-4}	1.01×10^{-4}
ADP	3.98×10^{-4}	5.0×10^{-4}
ADP + FDP	1.99×10^{-4}	3.25×10^{-4}
K^+ + FDP	5.0×10^{-2}	1.04×10^{-2}
Mg^{2+} + FDP	1.58×10^{-3}	2.00×10^{-3}

PK purified by Hunsley and Suelter (1969b) was the hysteresis observed at subsaturating Mg^{2+}. This property will be discussed later. The enzyme purified by this technique is similar to that prepared by the technique of Hunsley and Suelter (1969a) in several other ways. The enzyme is inactivated quite rapidly at 0° C. It is protected from this inactivation by the presence of cations or glycerol. The presence of FDP promotes an even more rapid inactivation at 0° C and also promotes inactivation at 23° C.

One striking difference between PK purified by the present technique and that of Hunsley and Suelter (1969a) is the hysteresis observed at subsaturating Mg^{2+}

concentrations. It can be concluded from the present investigations that hysteresis is caused by an ADP inhibition of the FDP activation at subsaturating Mg^{2+} concentrations. PPi appears to have the same inhibitory ability as ADP, presumably because of the structural similarity of the phosphate groups. Since none of the other degradative products of ADP produced inhibition, it strongly suggests that it is the diphosphate portion of the molecule which is necessary for the inhibition. Since EDTA produces no hysteretic effect, this cannot be a simple chelation of Mg^{2+} mechanism and therefore must be related to direct binding of ADP or PPi to the enzyme. The time dependent activation appears to be a first order process, as demonstrated by the linearity of the first order plots.

Frieden (1970) states that there are two mechanisms which are primarily responsible for slow enzyme responses: (a) isomerization processes and (b) displacement (under certain conditions) of a tightly bound ligand by another with a different effect on activity. A more complex process which would include either or both of the above mechanisms is polymerization or depolymerization of an enzyme. By Frieden's evaluation process it would appear that pyruvate kinase is undergoing a slow displacement of ADP by FDP. This could mean that ADP bound to the enzyme either prevents FDP binding or prevents the necessary conformational change of the enzyme for full activity after FDP has bound. Thus, ADP must be displaced before full activity will be produced. What makes this situation even more complex, however, is that Mg^{2+} concentration is also involved in some way. As the concentration of Mg^{2+} increases, the half time of activation decreases. The present experimental results are not sufficient to clearly elucidate a mechanism, but the Mg^{2+} involvement might indicate that the affinity of the enzyme for FDP may decrease with decreasing Mg^{2+} concentration allowing the affinity for ADP to be greater than that for FDP, thus creating the slow displacement described above. By this means Mg^{2+} concentration could control the rate of PK reaction and could serve as an *in vivo* regulator.

An inhibition of rat liver PK by ATP and alanine also results in a hysteretic activation by FDP (Spivey *et al.*, 1974). These experiments were conducted in the presence of saturating Mg^{2+}, however. Interestingly, the rat liver enzyme at a concentration of 17.6 to 51.2 μg/ml showed smaller specific activities than compared to samples preincubated with FDP. At lower enzyme levels, 0.18 μg/ml the maximum velocity is the same whether or not the enzyme is preincubated with FDP. Unfortunately, there was no plot of specific activity at various protein concentrations, with and without preincubation. Consequently, there is really no way of telling whether these investigators were observing the same phenomenon as was communicated here (Figure 18). Their explanation for the phenomenon was that at sufficiently high concentration of enzyme only a fraction of the total needs to be transformed to obtain a catalytic rate that is large relative to further rate of change in the fraction activated. From there on an apparently constant catalytic rate might be observed until beyond the initial velocity range.

The unusual results obtained by plotting specific activity vs μg PK with FDP preincubation (Figure 18) might indicate that during the 15 second FDP preincubation the PK at lower concentrations was being dissociated into dimers or monomers which produced a lower activity. If this is true, this might indicate that the reduced final activity produced in the absence of an FDP preincubation could also be due to the fact that PK is dissociating in the assay. However, the results of the rate of activation vs μg of PK (Figure 19) indicate that the rate of activation is independent of protein concentration below 5 μg. Elucidation of the role protein concentration plays in this activation phenomenon awaits further experimentation.

Since there are now two other preparations of pyruvate kinase from yeast which are reported to be free of protease and proteolytic degradation, it would be of interest to compare the properties of the purified enzyme with those from this preparation. Table 16 shows such a comparison. The molecular weights for both native and subunit are within a reasonable range. However, the

TABLE 16

Comparison of the Properties of PK from Three Different Preparations

MEASUREMENT TECHNIQUE	*S. carlsbergensis* prepared by the method of Haekel *et al.* (1968) or of Roschlau & Hess (1972)[1]	*S. cerevisiae* the present communication	*S. cerevisiae* prepared by the method of Fell *et al.* (1974)
MW - sedimentation equilibrium	191,000	209,000	213,000
Subunit molecular weight	55,000-60,000	57,500	56,000
$E_{280}^{0.1\%}$	0.76	0.51	0.66
Cold lability	no	yes	yes
Protease contamination	yes	yes	no
Lysis procedure	toluolysis	Manton-Gaulin	freeze-thawing
N-terminal	acetylated	blocked	blocked
C-terminal	valine	---	---
Isoelectric point	6.08	6.7	---

[1] Additional reference: Bischofberger *et al.* (1971).

extinction coefficients indicate that the other two preparations have more aromatic residues. The isoelectric point of the enzyme from brewers' yeast is considerably lower, indicating less basic amino acids or more acid ones. It would be difficult to say the preparations are identical based on these comparisons. Perhaps further investigation will resolve the differences.

Table 17 compares the amino acid composition of the brewers' yeast enzyme with that of this preparation. The total number of amino acids and hydrophobic content are nearly identical. This preparation has more basic amino acids than the brewers' yeast enzyme. The acidic amino acids are nearly equal. This might explain the difference in isoelectric point. Other large differences occur in threonine, valine, methionine, and isoleucine. All others are very similar. On the basis of amino acid composition, the enzymes from brewers' and bakers' yeast appear to be very similar but not identical.

The question arises, what was it about the Hunsley and Suelter (1969a) preparation that produced a degraded form of the enzyme? The Yun *et al.* (1975) modification of the Hunsley and Suelter (1969a) method of purifying pyruvate kinase involved (1) slightly modifying the toluolysis procedure so that less toluene was used in the presence of βME; (2) inclusion of 5 mM EDTA, 5 mM βME, and 25% glycerol (instead of 50%) in the purification buffers; and (3) completion of entire preparation at

TABLE 17

Comparison of the Amino Acid Content of PK from *S. carlsbergensis* and *S. cerevisiae*

Amino acid	*S. carlsbergensis* (Bischofberger *et al.*, 1970[1])	*S. cerevisiae* (present work)
Lysine	111	132
Arginine	83	89
Histidine	28	24
Aspartic acid	212	212
Threonine	157	133
Serine	101	99
Glutamic acid	147	140
Proline	83	87
Glycine	120	122
Alanine	157	161
Half cysteine	24	20
Valine	166	178
Methionine	28	42
Isoleucine	111	127
Leucine	120	128
Tyrosine	55	53
Phenylalanine	52	54
Tryptophan	9	---
TOTAL AMINO ACIDS	1,760	1,801 (less tryptophan)
HYDROPHOBIC CONTENT	30%	32%
$E_{280}^{0.1\%}$	0.76	0.51

[1] Calculated from published values.

room temperature. This procedure produced an enzyme of increased molecular weight, 209,000, and specific activity of 340 /mg (protein based on $E_{280}^{0.1\%} = 0.51$). The Fell *et al.* (1974) modification of the Ashton (1971) preparation of PK, a preparation nearly identical to that of Hunsley and Suelter (1969a), involved changing the lysis procedure to a low temperature toluolysis, freeze-thawing technique. This preparation produced an enzyme with increased molecular weight, 213,000, and increased specific activity to 340 v/mg (protein based on $E_{280}^{0.1\%} = 0.66$). The purification procedure described in this communication reports a modification of the Yun *et al.* (1975) procedure where the lysis was changed to a mechanical method (Manton-Gaulin) with only two other modifications to remove extra protein. The resulting enzyme had an identical molecular weight to that of Yun *et al.* (1975) and identical specific activity.

In one case the lysis procedure was changed with no modification of subsequent steps. In another, there were no real changes in the lysis but there were modifications in the subsequent steps which resulted in a more rapid purification. The only conclusion that can be drawn from all this is that the Fell *et al.* (1974) procedure probably released little if any protease activity so that subsequent purification steps made little difference. The Yun *et al.* (1975) procedure released proteases, but the remaining purification steps were conducted rapidly so that degradation did not have a chance to proceed. The present technique hopefully takes the best of both preparations with the added benefit of more efficient release of pyruvate kinase for better yields of pure enzyme.

The fact that protease activity copurifies with yeast PK indicates that the protease has a selective affinity for this enzyme. It is interesting to note that most of the enzymes discussed in the Literature Review that had been degraded or copurified with protease activity have been described as regulatory enzymes. In some cases it has been shown rather convincingly that the levels of these enzymes can be regulated by proteolytic enzymes. Although no clear-cut evidence has been presented that shows conclusively that this is a regulatory mechanism for PK, the fact that it is selectively "adsorbed" to PK throughout the purification might indicate a high affinity for PK which could lead to *in vivo* post-transcriptional regulation.

SUMMARY

The experimental work described in this dissertation began by developing a purification procedure for bakers' yeast pyruvate kinase that utilized a mechanical method for lysing the yeast in the presence of DFP at 4° C. The enzyme purified by this means had a proteolytic contamination that was removed by Sephadex G-100 chromatography. The protease could only be inhibited by DFP in the presence of SDS, which suggests that it exists as a protease-inhibitor complex. This would explain why the protease did not degrade the PK throughout the preparation. Physical characterization of the purified enzyme by a variety of techniques would indicate that the enzyme shows no microheterogeneity. When purified pyruvate kinase subunit molecular weight was compared with that of the enzyme in the cell-free extract by immunoprecipitation and subsequent SDS gel electrophoresis, they were shown to be identical. This was consistent with the purified enzyme being the "native" pyruvate kinase.

The enzyme purified by the means described here had a larger molecular weight and greater V_m than that reported for the enzyme purified by an earlier purification procedure (Hunsley and Suelter, 1969a). The kinetic parameters determined for the Manton-Gaulin enzyme appeared to be nearly identical to those determined by Hunsley and Suelter (1969b). FDP was found to be an allosteric activator which transformed the sigmoid kinetics of PEP to hyperbolic. PK was found to have an absolute requirement for the presence of both Mg^{2+} and K^+ for activity. At subsaturating concentrations of Mg^{2+}, the FDP activation was shown to be inhibited by the substrate ADP or by PPi. Thus, the FDP activation was observed as a first order, hysteretic process. The first order rate constant was found to increase linearly with Mg^{2+} concentration but was independent of protein concentration. Preincubation of PK with FDP before assaying produced final velocities that were greater than those without preincubation for PK concentrations greater than 0.5 μg/ml. The reason for these results was not elucidated by the experiments.

REFERENCES

REFERENCES

Ashton, I. and Peacock, A. (1971), *FEBS Lett.* 16, 25.

Atkinson, D. (1966), *Ann. Rev. Biochem.* 7, 85.

Atwell, J. L., Cone, R. E., and Marchalonis, J. J. (1973), *Nature New Biology* 241, 251.

Aust, A., Yun, S.-L., and Suelter, C. (1975), in *Methods in Enzymology*, Vol. 42, Part C, W. Wood, Ed., New York, New York, Academic Press, p. 176.

Bailey, K., and Webb, E. (1948), *Biochem. J.* 42, 60.

Barnard, E. (1975), in *Methods in Enzymology*, Vol. 42, Part C, W. Wood, Ed., New York, New York, Academic Press, p. 6.

Barwell, C., and Hess, B. (1971), *FEBS Lett.* 19, 1.

Baur, S., Vittetta, E. S., Sherr, C. J., Shenkein, I., and Uhr, J. W. (1971), *J. Immun.* 106, 1133.

Behnke, J., Dagher, S., Massey, T., and Deal, W., Jr., *Anal. Biochem.* (1975), in press.

Berger, L., Slein, M., Colowick, S., and Cori, C. (1946), *J. Gen. Physiol.* 29, 379.

Bischofberger, H., Hess, B., and Roschlau, P. (1971), *Hoppe-Seyler's Z. Physiol. Chem.* 352, 1139.

Bornmann, L. and Hess, B. (1974), *Eur. J. Biochem.* 47, 1.

Bücher, T., and Pfleiderer, G. (1955), in *Methods in Enzymology*, Vól. 1, S. Colowick and N. Kaplan, Eds., New York, New York, Academic Press, p. 435.

Cabib, E., and Bowers, B. (1971), *J. Biol. Chem.* 246, 152.

Cabib, E., and Farkas, V. (1971), *Proc. Nat. Acad. Sci.* 68, 2052.

Cabib, E., and Ulane, R. (1973), *J. Biol. Chem.* 248, 1451.

Cabib, E., and Ulane, R. (1973), *Biochem. Biophys. Res. Commun.* 50, 186.

Chern, C., Rittenberg, M., and Black, I. (1972), *J. Biol. Chem.* 247, 7173.

Chou, A., and Wilson, J. (1972), *Arch. Biochem. Biophys.* 151, 48.

Cohn, R., and Edsall, J. (1943), in *Proteins, Amino Acids, and Peptides*, Reinhold, p. 370.

Cottam, G., Hollenberg, P., and Coon, M. (1969), *J. Biol. Chem.* 244, 1481.

Cullen, S. E., Schwartz, B. D., Nathenson, S. G., and Cherry, M. (1972), *Proc. Nat. Acad. Sci. U.S.A.* 69, 1394.

Darrow, R., and Colowick, S. (1962), in *Methods in Enzymology*, Vol. 5, S. Colowick and N. Kaplan, Eds., New York, New York, Academic Press, p. 226.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci.* 12, 404.

Diezel, W., Bohme, H.-J., Nissler, K., Freyer, R., Heilmann, W., Kopperschlager, G., and Hoffman, E. (1973), *Eur. J. Biochem.* 38, 479.

Diezel, W., Nissler, K., Heilmann, W., Kopperschlager, G., and Hofmann, E. (1972), *FEBS Lett.* 27, 195.

Doi, E., Hayashi, R., and Hata, T. (1967), *Agr. Biol. Chem.* 31, 357.

Evans, H., and Sorger, G. (1966), *Ann. Rev. Plant Physiol.* 17, 47.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry* 10, 2606.

Fell, D., Liddle, P., and Peacock, A. R. (1974), *Biochem. J.* 139, 665.

Fernandez, M., Medrano, L., Ruiz-Amil, and Losada, M. (1967), *Eur. J. Biochem.* 3, 11.

Frieden, C. (1970), *J. Biol. Chem.* 245, 5788.

Gancedo, C. (1971), *J. Bacteriol.* 107, 401.

Gancedo, C., Salas, M., Giner, A., and Sols, A. (1965), *Biochem. Biophys. Res. Commun.* 20, 15.

Gould, N., and Leiner, I. E. (1965), *Biochemistry* 4, 90.

Gray, W. (1972), in *Methods in Enzymology*, Vol. 25, C. H. W. Hirs and S. N. Tamasheff, Eds., New York, New York, Academic Press, p. 121.

Gray, W., and Hartley, B. (1963), *Biochem. J.* 89, 59P.

Haekel, R., Hess, B., Lauterborn, W., and Wuster, K.-H. (1968), *Hoppe-Seyler's Z. Physiol. Chem.* 349, 699.

Harris, W., and Ferguson, J. (1967), *Fed. Proc.* 26, 678 .

Hata, T., Hayashi, R., and Doi, E. (1967a), *Agr. Biol. Chem.* 31, 150.

Hata, T., Hayashi, R., and Doi, E. (1967b), *Agr. Biol. Chem.* 31, 357.

Hayashi, R., Aibara, S., and Hata, L. (1970), *Biochem. Biophys. Acta* 212, 359.

Hayashi, R., and Hata, T. (1972), *Agr. Biol. Chem.* 36, 630.

Hayashi, R., Minami, Y., and Hata, T. (1972), *Agr. Biol. Chem.* 36, 621.

Hayashi, R., Moore, S., and Stein, W. (1973), *J. Biol. Chem.* 248, 2296.

Hayashi, R., Oka, Y., Doi, E., and Hata, L. (1968), *Agr. Biol. Chem.* 32, 367.

Hayashi, R., Oka, Y., and Hata, L. (1969), *Agr. Biol. Chem.* 33, 196.

Hedrick, J., and Smith, A. (1968), *Arch. Biochem. Biophys.* 126, 155.

Hess, B., and Brand, K. (1965a), in *Control of Energy Metabolism*, B. Chance, R. Estabrook, and J. Williamson, Eds., New York, New York, Academic Press, p. 111.

Hess, B., and Brand, K. (1965b), *Abstracts of the 150th National Meeting of the American Chemical Society*, Atlantic City, New Jersey, p. 27C.

Hess, B., and Haekel, R. (1967), *Nature* 214, 848.

Hommes, F. (1964), *Arch. Biochem. Biophys.* 108, 36.

Hunsley, J., and Suelter, C. (1969a), *J. Biol. Chem.* 244, 4815.

Hunsley, J., and Suelter, C. (1969b), *J. Biol. Chem.* 244, 4819.

Jacobson, K., and Murphy, J. (1972), *FEBS Lett.* 22, 80.

Jacq, C., and Lederer, F. (1970), *Eur. J. Biochem.* 12, 154.

Jacq, C., and Lederer, F. (1972), *Eur. J. Biochem.* 25, 41.

Johnson, J. C., DeBaker, M., and Boezi, J. A. (1971), *J. Biol. Chem.* 246, 1222.

Juni, E., and Heym, G. (1968a), *Arch. Biochem. Biophys.* 127, 79.

Juni, E., and Heym, G. (1968b), *Arch. Biochem. Biophys.* 127, 89.

Katsunuma, I., Schött, E., Elsässer, S., and Holzer, H. (1972), *Eur. J. Biochem.* 27, 520.

Kayne, F., and Reuben, J. (1970), *J. Am. Chem. Soc.* 92, 220.

Keiditsch, E., and Strauch, L. (1970), in *Chemistry and Molecular Biology of the Intercellular Matrix*, E. Balazs, Ed., Vol. 3, New York, New York, Academic Press, p. 1671.

Keller, F., and Cabib, E. (1971), *J. Biol. Chem.* 246, 160.

Kornberg, H. L. (1973), in *Rate Control of Biological Processes*, D. D. Davis, Ed., Cambridge Univ. Press, p. 175.

Kuczenski, R. (1970), Ph.D. Thesis, Michigan State University, East Lansing, Michigan.

Kuczenski, R., and Suelter, C. (1970), *Biochemistry* 9, 939.

Kunitz, M., and McDonald, M. R. (1946), *J. Gen. Physiol.* 29, 393.

Layne, E. (1957), in *Methods in Enzymology*, Vol. 3, S. Colowick and N. Kaplan, Eds., New York, New York, Academic Press, p. 450.

Lazarus, N., Ramil, A., Rustum, Y., and Barnard, E. (1966), *Biochemistry* 5, 4003.

Lederer, F., and Jacq, C. (1971), *Eur. J. Biochem.* 20, 475.

Lederer, F., and Simon, A.-M. (1971), *Eur. J. Biochem.* 20, 469.

Lenney, J. (1956), *J. Biol. Chem.* 221, 919.

Lenney, J., and Dalbec, J. (1967), *Arch. Biochem. Biophys.* 120, 42.

MacFarlane, N., and Ainsworth, S. (1972), *Biochem. J.* 129, 1035.

Manney, I. (1968), *J. Bacteriol.* 96, 403.

Masters, B., Baron, J., Taylor, W., Isaacson, E., and La Spalluto, J. (1971), *J. Biol. Chem.* 246, 4143.

Matile, P., and Wiemken, A. (1967), *Arch. Mikrobiol.* 56 148.

Mildvan, A., Hunsley, J., and Suelter, C. (1971), *Johnson Foundation Colloq.: Probes of Structure and Function of Membranes and Macromolecules*, B. Chance, Ed., New York, New York, Academic Press, p. 131.

Moe, O., and Butler, L. (1972), *J. Biol. Chem.* 247, 7308.

Molano, J., and Gancedo, C. (1974), *Eur. J. Biochem.* 44, 213.

Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol.* 12, 88.

Moore, S., and Stein, W. H. (1963), in *Methods in Enzymology*, Vol. 6, S. Colowick and N. Kaplan, Eds., New York, New York, Academic Press, p. 819.

Murachi, T., and Yasui, M. (1965), *Biochemistry* 4, 2275.

Nelson, C. (1971), *J. Biol. Chem.* 246, 3895.

Nicholls, R., Atkinson, M., Burgoyne, T., and Symons, R. (1966), *Biochem. Biophys. Acta* 122, 14.

Ouchterlony, O. (1950), *Arkiv. Kemi* 1, 43.

Pon, N., and Bondar, R. (1967), *Anal. Biochem.* 19, 272.

Pringle, J. (1970), *Biochem. Biophys. Res. Commun.* 39, 46.

Pye, E., and Eddy, A. (1965), *Fed. Proc.* 24, 537.

Ramel, A., Rustum, Y., Jones, J., and Barnard, E. (1971), *Biochemistry* 10, 3499.

Roschlau, P., and Hess, B. (1972), *Hoppe-Seyler's Z. Physiol. Chem.* 353, 435.

Rose, A., and Harrison, J. (1969), in *The Yeasts*, New York, New York, Academic Press, p. 250.

Rustum, Y., Ramel, A., and Barnard, E. (1971), *Prep. Biochem.* 1, 309.

Rutter, W., Hunsley, J., Groves, W., Calder, J., Rajkumar, T., and Woodfin, B. (1966), in *Methods in Enzymology*, Vol. 9, W. Wood, Ed., New York, New York, Academic Press, p. 479.

Saheki, T., Matsuda, Y., and Holzer, H. (1974), *Eur. J. Biochem.* 47, 325.

Schlesinger, M., and Coon, M. (1960), *Biochim. Biophys. Acta* 41, 30.

Schött, H., and Holzer, H. (1974), *Eur. J. Biochem.* 42, 61.

Schulze, I., and Colowick, S. (1969), *J. Biol. Chem.* 244, 2306.

Schulze, I., Gazith, J., and Gooding, R. (1967), in *Methods in Enzymology*, Vol. 11, C. Hirs, Ed., New York, New York, Academic Press, p. 376.

Schwartz, B. D., and Nathenson, S. G. (1971), *J. Immun.* 107, 1363.

Spiviy, H., Flory, W., Peczon, B., Chandler, J., and Koippe, R. (1974), *Biochem. J.* 141, 119.

Suelter, C. (1970), *Science* 168, 789.

Suelter, C., Singleton, R., Jr., Kayne, F., Arrington, G., Glass, J., and Mildvan, A. S. (1966), *Biochemistry* 5, 131.

Vitols, E. V., North, R. J., and Linnane, A. W. (1961), *J. Biophys. Biochem. Cytol.* 9, 689.

Washio, S., and Mano, Y. (1960), *J. Biochem.* (Tokyo) 48, 874.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem.* 244, 4406.

Welton, A. F., Pederson, T. C., Buege, J. A., and Aust, S. D. (1973a), *Biochem. Biophys. Res. Commun.* 54, 161.

Welton, A. F., Pederson, T. C., Buege, J. A., and Aust, S. D. (1973b), *The Pharmacologist* 15, 170.

Wieker, H.-J., and Hess, B. (1971), *Biochemistry* 10, 1243.

Wiemken, A., Matile, P., and Moor, H. (1970), *Arch. Mikrobiol.* 70, 89.

Wilkinson, G. (1961), *Biochem. J.* 80, 324.

Wilson, R., Evans, H., and Becker, R. (1967), *J. Biol. Chem.* 242, 3825.

Wood, W. (1975), Personal communication.

Yphantis, D. (1964), *Biochemistry* 3, 297.

Yun, S.-L., Aust, A., and Suelter, C. (1975), Manuscript in preparation.