RABBIT MUSCLE PYRUVATE KINASE: STRUCTURAL AND CATALYTIC STUDIES

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ABSTRACT

RABBIT MUSCLE PYRUVATE KINASE:
STRUCTURAL AND CATALYTIC STUDIES

By

George Samuel Johnson

The dissociation of tetrameric rabbit muscle pyruvate kinase (mol wt = 237,000) into unfolded subunits (mol wt = 57,000) in 6 M guanidine has been shown to be reversible. A systematic study of the factors affecting the reversal of the dissociation led to conditions where up to 70 % of the initial catalytic activity was regained. The reversal procedure consisted of two phases: (1) a 100-fold dilution of guanidine-dissociated enzyme (0°) into a reversal solvent at 0° and (2) incubation of the resulting solution at a higher temperature, usually 16°. Conditions for optimum reversal of dissociation were (1) pH 8; (2) protein concentration, 0.04 mg/ml; (3) ionic strength, 0.3; (4) reducing agent, 0.06 M β-mercaptoethanol and (5) temperature--0° dilution, followed by six hr at 16°. The half-time for activity recovery was approximately 45 min at both 0.02 mg/ml and 0.09 mg/ml enzyme concentration. Two metabolites, insulin and phosphate ion, were found to greatly influence the reversal of dissociation. Insulin decreased the activity recovery upon reversal, in contrast to what would be expected for an inducer of the enzyme. Phosphate ion yielded activity recovery at 36°; neglible activity was recovered at that temperature in its absence. The reversal of dissociation was not affected significantly by the addition of a number of

metabolites including ATP, ADP, 5'-AMP, 3'-5'-AMP, lactate, FDP and NAD^+. The reassociated enzyme had the same K_m, heat stability, and sedimentation coefficient as the native enzyme.

Rabbit muscle pyruvate kinase was 90 % inactivated by binding 2-4 moles of pyridoxal-5'-phosphate (PLP) per mole of tetrameric enzyme. Incubation with PLP in 0.2 M imidazole (pH 7.5) at 25° resulted in a time-dependent loss of enzymatic activity which reached a final value in 10-20 min at 25°. Half maximal loss of activity occurred with 0.04 mM PLP. The inactivation did not cause a gross conformational change. The inactivation was first order with respect to PLP concentration and enzyme concentration; the second order rate constant was 37 M^{-1} sec^{-1}. Increasing ionic strength decreased the rate of inactivation by PLP but low concentrations (1-10 mM) of divalent cations increased it above the level with no salt. The phosphate containing metabolites, ADP, ATP, phosphoenolpyruvate, and fructose-diphosphate also decreased the rate; the effect was more pronounced with low concentrations (0.1 mM) of divalent cations. The inactivation was relatively specific for PLP since various analogues including pyridoxamine, pyridoxamine-5-phosphate, and pyridoxal caused little or no inactivation. Reduction with $NaBH_4$ at various concentrations of PLP showed that there were 2 types of binding: (1) a specific, incativating binding, involving 2-4 moles PLP bound per mole enzyme, and (2) a nonspecific, noninactivating binding which involved at least 20 additional moles PLP bound per mole enzyme. Both types involved Schiff base formation with ϵ-NH_2 groups of lysine. Reversal of the inactivation was accomplished, only with unreduced enzyme, by dilution, by dialysis, or by addition of Tris.

George Samuel Johnson

The sedimentation coefficient for rabbit muscle pyruvate kinase in the original native state has been determined to be $s^{o}_{20,w}$ = 9.6 S - 9.8 S by sucrose density centrifugation of a crude extract from frozen rabbit muscle. A similar experiment with a crude extract from frozen rabbit liver yielded a value of $s^{o}_{20,w}$ = 7.4 S and variable amounts of a faster sedimenting protein (about 9.5 S) for rabbit liver pyruvate kinase.

The effect of high pH on rabbit muscle pyruvate kinase was studied with enzyme preparations from fresh rabbit muscle and from rabbit muscle frozen 2-3 years. The fresh muscle preparation in 0.05 M Tris or glycine buffer, 0.15 M KCl, 0.001 M EDTA showed: (1) a single peak in the pH region 7.4-10; (2) at either 5° or 24°, an initial transition at pH 8.5-9.0 from $s^{o\,0.16\,\%}_{20,w}$ = 9.6 to $s^{o\,0.16\,\%}_{20,w}$ = 9.3 S; (3a) at 5°, a second transition at pH 10.5 and above yielding a slower sedimenting peak (8.0 S) and an additional peak (4 S); (3b) at 24°, a second transition at about pH 10.1-10.2, resulting in 2 or more broad peaks in sedimentation velocity experiments. The enzyme prepared from the frozen muscle under similar conditions showed different results. (1) At 24°, the initial transition occurred at much lower pH (pH 7.9-8.4). (2) The initial transition yielded a more dissociated or unfolded enzyme, $s^{o\,0.44\,\%}_{20,w}$ = 9.4 S to 8.7 S, and the peak broadened much more rapidly during sedimentation velocity experiments. (3) At 24°, a second transition (loss of well-defined peaks) occurred at pH 9.8 and above; 0.15 M KCl was required to observe this effect at pH 9.8, but not at higher pH.

The sedimentation coefficient of rabbit muscle pyruvate kinase

prepared from fresh muscle was greatly influenced by salt concentration. It increased from a value of about 9.3 S in no added salt to a maximum value of $s^{0.16\ \%}_{20,w}$ = 9.6 S in 0.1 M KCl (pH 7.5, 5° or 24°) and 0.05 M - 0.6 M $(NH_4)_2SO_4$ (pH 8.0, 5°). It then decreased with increasing salt concentration to a value of $s^{0.16\ \%}_{20,w}$ = 7.65 S in 3.0 M KCl. Similar results were obtained with increasing KCl concentrations at pH 10.0 at 24°.

Sedimentation equilibrium analysis showed that the decrease in sedimentation coefficient was due to a dissociation into dimers or monomers. The results were also consistent with a dissociation-association rapid equilibrium between monomers (or dimers) and tetramers and/or octamers.

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STRUCTURAL AND CATALYTIC STUDIES

By

George Samuel Johnson

A THESIS

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TO MY PARENTS

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The work of Mr. Y. Y. Shum in aiding with the ultracentrifugal studies, and the help of Mrs. Karen Hollen, Miss Shirley Ann Williams, and my wife, Leslye, in the preparation of this manuscript are greatly appreciated. The predoctoral fellowship support of the National Institutes of Health is gratefully acknowledged (1-F1-GM-35,507).

VITA

George Samuel Johnson was born in Cokato, Minnesota, on August 25, 1943, and lived for the first eighteen years of his life on a small dairy farm near Annandale, Minnesota. He attended high school at Annandale where he was involved in numerous extracurricular activities including sports, editor of the school yearbook, and Senior Class president. At the end of his high school career, he was elected to the National Honor Society.

Following graduation, George attended Augsburg College in Minneapolis, Minnesota. His early interests were in engineering, but under the guidance of Dr. Courtland Agre, he became interested in and eventually majored in Chemistry, graduating Cum Laude.

George's scientific experience extended through the summer months. The summer of 1963 was spent working at the USDA Rust Laboratory at the University of Minnesota where much work was done in the growth and identification of wheat and oat rusts. The following summer George developed an interest in Biochemistry and experienced his first contact with biochemical research while participating in the NSF undergraduate research program at the Department of Biochemistry, University of Minnesota. The research during this program under the guidance of Dr. Robert Jenness centered on developing procedures combining disc electrophoresis and immunoelectrophoresis.

With a career of medical research in mind, George entered grad-

uate school in the Department of Biochemistry, Michigan State University. He was awarded an NIH Predoctoral Research Fellowship to complete his graduate studies. His thesis research, under the guidance of Dr. W. C. Deal, Jr., was aimed at further understanding the structure and control of the glycolytic enzyme, pyruvate kinase. George also was accepted for membership in the American Chemical Society.

George has been awarded an NIH postdoctoral research fellowship to study phospholipid interactions in oncogenic viral infectivity. This research is to be conducted at the National Institutes of Health in Bethesda, Maryland, under the direction of Dr. Ira Pastan.

He also has been offered a staff position at the University of Ghana in Accra, Ghana, Africa, and is considering acceptance of this position upon completion of the research at NIH.

ORGANIZATION OF THE THESIS

The three areas of research are covered individually in the three chapters of this thesis. For convenience to the reader each chapter is presented as an independent entity in the format of a scientific paper, with its own Abstract, Materials and Methods, Introduction, Results, and Discussion sections. The only deviation from this format is that the references for all three chapters are combined at the end of the thesis.

Chapter One has already been published under the title "Metabolic Control and Structure of Glycolytic Enzymes VIII. Reversal of the Dissociation of Rabbit Muscle Pyruvate Kinase into Unfolded Subunits", by George S. Johnson, Marlene Steinmetz Kayne, and William C. Deal, Jr. (1969), Biochemistry 8, 2455.

A preliminary report of the results of Chapter Two has been published, Federation Proceedings 28, 864, 1969. The detailed results have been accepted for publication in the Journal of Biological Chemistry, under the title, "Specific Inactivation of Rabbit Muscle Pyruvate Kinase by a Specific Binding of 2-4 Moles of Pyridoxal 5'-Phosphate".

Chapter Three is also being prepared for publication.

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LIST OF ABBREVIATIONS

PEP	phosphoenolpyruvate
P_i	orthophosphate
PP_i	pyrophosphate
Tricine	N-tris(hydroxymethyl)methylglycine
Bicine	N,N-bis(2-hydroxyethyl)-glycine
FDP	fructose-1,6-diphosphate
PLP	pyridoxal-5'-phosphate
EDTA	(ethylenedinitrilo)tetraacetic acid

INTRODUCTION

APPROACH TO THE RESEARCH

The aim of the research was to better understand the structural and control properties of the important glycolytic enzyme, pyruvate kinase, isolated from rabbit muscle.

A reasonable point for control of protein biosynthesis is at the level of folding of the nascent polypeptide chain. By completely unfolding a purified enzyme *in vitro* and determining the variables required for reconstitution of its original molecular structure and correlating these with *in vivo* conditions, it was expected that possible mechanisms for its control might be revealed. The metabolite NAD^+ had been found to be required for the refolding of yeast glyceraldehyde-3-phosphate dehydrogenase and it had been postulated that NAD^+ might control the synthesis of the enzyme (Deal, 1969). It seemed likely that such a control mechanism might exist for pyruvate kinase; it is a key control point in glycolysis. Chapter One thus describes the elucidation of the variables affecting the refolding and reassembly of pyruvate kinase from completely unfolded subunits.

During the study on the effect of various metabolites on the reversal process, it was discovered that the important biological cofactor, pyridoxal 5'-phosphate (PLP) inactivated the enzyme at low concentrations (10^{-5} M). This inactivation was interesting from a

a physiological point of view. PLP has long been known to be an important cofactor in enzymatic catalysis; it has an _in vivo_ concentration of about 0.465×10^{-5} M in rat muscle (Long, 1961). PLP, with its active aldehyde group, is an excellent reagent for chemical modification of proteins through formation of a Schiff base with protein side chain amino groups. Since little was known about the functional groups involved in the catalytic mechanism, and the possibility of participation of an amino group in the catalytic reaction had been postulated (Mildvan and Cohn, 1966), it seemed likely that an analysis of this inactivation would give further understanding of the catalytic mechanism of pyruvate kinase. The results of this study are presented in Chapter Two.

Several different sedimentation coefficients, ranging from $s^{o}_{20,w}$ = 10.17 S to 8.53 S, have been reported for native rabbit muscle pyruvate kinase (Warner, 1958; Kayne, 1966; Wilson _et al._, 1967). A time-dependent transition from a 10.07 S species to an 8.53 S species has been reported (Kayne, 1966) to occur during storage of the purified enzyme. The mol wt of the subunits of rabbit muscle pyruvate kinase has been determined to be 57,000 (Steinmetz and Deal, 1966). The 10 S species (mol wt 237,000; Warner, 1958) can thus be assumed to be a tetramer. However, there is no readily apparent explanation for the 8.5 S species. Additional studies were needed to determine the original _in vivo_ native state of the enzyme, and determine what significance, if any, could be attributed to the altered, non-native states. With this in mind we undertook a detailed analysis to determine the original native state of the enzyme and possible conditions which could alter it. These results are presented in Chapter Three.

LITERATURE REVIEW: PYRUVATE KINASE

INTRODUCTION. Pyruvate kinase is a key enzyme in glycolysis and gluconeogenesis. It was first discovered by Lohmann and Meyerhof (1934), and since has been purified and extensively studied. Crystalline preparations of pyruvate kinase were first obtained from rabbit muscle (Bücher et al., 1955; Teitz and Ochoa, 1958), and later from liver (Tanaka et al., 1967a). Also highly pure preparations have been obtained from yeast (Haeckel et al., 1968; Hunsley and Suelter, 1967). Its properties have been discussed in great detail in an excellent review by Boyer (1962) and more recently by Kayne (1966) and Hollenberg (1969).

This review emphasizes the more recent studies on the structural and functional properties of pyruvate kinase from rabbit muscle, and also discusses studies of pyruvate kinases from sources other than muscle.

REACTIONS CATALYZED BY PYRUVATE KINASE. In the cell, pyruvate kinase participates in glucose metabolism by catalyzing the conversion of phosphoenolpyruvate to pyruvate with the transfer of a "high energy" phosphate to ADP (Reaction (1)).

$$\text{(1)}\quad \text{PEP} + \text{ADP} + \text{H}^{+} \xrightleftharpoons[\text{Mg}^{2+}]{\text{K}^{+}} \text{pyruvate} + \text{ATP}$$

In addition, pyruvate kinase has been shown to catalyze two additional reactions: the phosphorylation of fluoride ion (Reaction (2)) (Flavin et al., 1956; Teitz and Ochoa, 1958) and of hydroxylamine (Reaction (3)) (Kupiecki and Coon, 1960; Cottam et al., 1968).

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

$$(3)\quad \text{ATP} + \text{hydroxylamine} \xrightarrow{HCO_3^-} \text{ATP} + \text{phosphoryl hydroxylamine}$$

Reactions (1), (2), and (3) require a monovalent cation, preferably potassium, for catalysis. Reactions (1) and (2) require Mg^{2+} or Mn^{2+} but not Zn^{2+}, whereas Reaction (3) is most rapid with Zn^{2+} or Co^{2+} but is not active with Mg^{2+}. Reactions (2) and (3) are essentially irreversible, and require bicarbonate for catalysis (Boyer et al., 1942; Tietz and Ochoa, 1958; Kupiecki and Coon, 1960).

Pyruvate kinase (reaction (1)) has a specific activity (μmoles substrate cleaved per minute per mg protein) at 25° of about 180 (Steinmetz and Deal, 1966). This corresponds to a turnover number of 10^3 per second. The reaction is slightly reversible (this will be discussed in a later section), and the maximum initial velocity of the forward reaction is 150 to 200 times that of the back reaction (McQuate and Utter, 1959). Pyruvate kinase has a specific activity of 38 or 58, when catalyzing reaction (2) or (3), respectively (Kupiecki and Coon, 1960).

THE PYRUVATE KINASE REACTION. Reaction Equilibrium and Energetics. The free energy of hydrolysis of the substrate PEP is unusually high; ΔF° = -12,800 cal/mole. This is sufficiently high to phosphorylate

ADP and still allow a large free energy change for the overall reaction (Mahler and Cordes, 1966).

Because of this large free energy change, the pyruvate kinase reaction was initially thought to be essentially irreversible (Meyerhof et al., 1938). The reaction was first shown to be reversible by Lardy and Ziegler (1945). The equilibrium constant for the reaction was first reported to be $K = 2 \times 10^3$ at 30°, and $K = 1.65 \times 10^3$ at 20°, both at an unspecified pH (Meyerhof and Oesper, 1949). This corresponds to a free energy change of about -4500 cal/mole for the reaction. McQuate and Utter (1959) later found that it was pH dependent, decreasing with increasing pH. The equilibrium constants they determined at 30° were: $K = 6.61 \times 10^3$ at pH 7.4, and $K = 0.37 \times 10^3$ at pH 9.0. In vivo the reaction in various tissues and organisms does not appear to be at equilibrium under either aerobic or anaerobic conditions (Lowry and Passonneau, 1964; Williamson, 1965; Hess et al., 1965).

Reaction Mechanism. The pyruvate kinase reaction appears to involve a direct transfer of the phosphoryl group from PEP to ADP; exchange studies with O^{18} of water show that the reaction occurs without exchange of the phosphate oxygens with those of substrate or water (Harrison et al., 1955), and no evidence could be detected for a phosphoryl-enzyme intermediate (Hess et al., 1961). The substrates PEP and ADP bind independently, and ATP is a competitive inhibitor to both substrates, presumably by binding at the ADP site and the γ-phosphoryl group of the ATP overlapping the PEP binding site (Reynard et al., 1961). Rose (1960) studied the enolization of

pyruvate in the reverse reaction. His results show that the enolization (detritiation of α-methyl hydrogens) is 1.8 times as fast as the phosphorylation, but that the substrate, ATP, and the activators, K^+ and Mg^{2+}, are required. This suggests that pyruvate, not enolpyruvate, is the true substrate, and that enolization is not the rate limiting step. Potassium appears to function by altering the protein conformation (Kayne and Suelter, 1965; Melchior, 1965; Sorger et al., 1965). The divalent metal activator binds to the enzyme by combining with imidazole and another ligand which may be an α-amino group or an atypical sulfhydryl group, and the ternary complex of enzyme-metal-substrate is the true reactive species (Mildvan and Cohn, 1965; 1966).

From the above results the following mechanism for the reaction can be formulated (Mildvan and Cohn, 1966): 1) The divalent metal ion binds to the protein acting as a bridge to aid the binding of the two substrates; 2) the K^+ alters the enzyme conformation to aid the binding or the proper positioning of the substrates for a direct transfer of the phosphoryl group from PEP to ADP.

PYRUVATE KINASE MOLECULAR STRUCTURE. Rabbit muscle pyruvate kinase (mol wt 237,000, (Warner, 1958)), was first reported to dissociate into subunits of mol wt 150,000 in 6 M urea (Morawiecki, 1960). However, more extensive sedimentation studies established that it is converted into a dimeric species (mol wt 115,000) in low urea concentrations (4-8 M) (Steinmetz and Deal, 1966). The molecular species in 1.5-2.0 M urea retains most of its catalytic activity, but all detectable activity is lost in 4 M urea (Steinmetz and Deal, 1966;

Cottam et al., 1969). By several types of analysis, the subunits (mol wt 57,000) appear to be highly similar if not identical in amino acid composition (Cottam et al., 1969). There is disagreement as to the exact "native" molecular state of rabbit muscle pyruvate kinase. Sedimentation coefficients ranging from 8.5 S to 10.17 S have been reported (Warner, 1958; Kimberg and Yielding, 1962; Kayne and Suelter, 1965; 1968; Kayne, 1966; Wilson et al., 1967; Cottam et al., 1969). A transition in sedimentation coefficient from 10.0 S to 8.5 S during storage of purified enzyme has been observed (Kayne, 1966; Cottam et al., 1969).

There is evidence that the molecular weights of pyruvate kinases from other sources may differ greatly from that of the muscle enzyme. The molecular weights of pyruvate kinase from rat liver (Type "L") and yeast have been determined to be 208,000 and 167,000, respectively (Tanaka et al., 1967a; Kuczenski, R. T., and Suelter, C. H., submitted for publication). (See also Table 2.)

IMPORTANCE IN GLYCOLYSIS AND GLUCOGENESIS. It is advantageous for pyruvate kinase activity to be regulated to meet the metabolic needs of the cell. When the supply of available carbohydrate is increased, the need for glycolysis is increased; hence pyruvate kinase activity should increase. However, under conditions of stress or low carbohydrate diet, the necessary glycogen store may be depleted and a tissue or organism may be required to reform necessary glucose via glucogenesis. (The term "glucogenesis" is used to designate formation of glucose from all precursors other than glycogen (White et al., 1968).

Since glucogenesis normally involves pyruvate and PEP as

intermediates, the pyruvate kinase activity must be inhibited under these conditions. Moreover, because of the thermodynamic barrier hindering PEP synthesis from pyruvate, additional reactions involving utilization of high energy compounds are required to circumvent the pyruvate kinase reaction.

This unfavorable energy barrier has been overcome by two basic mechanisms: use of the energy of ATP and GTP, or the energy of pyrophosphate.

In glucogenic tissues and organisms (those capable of conducting glucogenesis) such as liver, kidney, and yeast, pyruvate and four-carbon compounds are converted into PEP by a combination of the reactions catalyzed by pyruvate carboxylase (EC 6.4.1.1) (Reaction (4)) and PEP carboxykinase (EC 4.1.1.32) (Reaction (5)) (Utter et al., 1964).

(4) $ATP + pyruvate + CO_2 + H_2O \rightleftharpoons + P_i + oxaloactate$

(5) $GTP + oxaloacetate \rightleftharpoons GDP + PEP + CO_2$

The enzyme, phosphoenolpyruvate synthetase (Reaction (6)) has been discovered in Escherichia coli (Cooper and Kornberg, 1965); and the enzyme, pyruvate-phosphate dikinase (ATP: pyruvate, phosphate diphosphotransferase) (Reaction (7)), has been discovered in bacteria (Reeves et al., 1968; Evans and Wood, 1968), leaves of tropical grasses (Hatch and Stack, 1968), and in Entamoeba histolytic (Reeves, 1968).

(6) $pyruvate + ATP \rightleftharpoons PEP + AMP + P_i$

(7) $AMP + PP_i + PEP \rightleftharpoons ATP + P_i + pyruvate$

It is clear that if pyruvate kinase is active in conjunction

with either of these reversal systems, the result would be reformation of pyruvate from the reformed PEP, with a net loss of high energy compounds.

MECHANISM FOR CONTROL OF PYRUVATE KINASE. Control of Muscle Pyruvate Kinase Activity. Although mammalian muscle tissue has not been shown to undergo glucogenesis and glycolytic flux in muscle appears to be controlled at the level of phosphofructokinase activity (Williamson, 1965), a few inhibitors have been discovered for muscle pyruvate kinase (see Table 1). It is inhibited by the products of the reaction, ATP and pyruvate (Reynard et al., 1961), by Ca^{2+} (Boyer, 1962), AMP (Kerson et al., 1967), diethylstilbestrol (Kimberg and Yielding, 1962), and acetyl CoA (Weber et al., 1967). Computer simulation studies have been done on mammalian pyruvate kinase in an attempt to organize these effects and elucidate the control exerted by pyruvate kinase in the glycolytic pathway (Kerson et al., 1967).

A controversy has recently developed concerning the ATP inhibition of pyruvate kinase. Does ATP directly inhibit the reaction or is its effect through a chelation of the Mg^{2+} required for catalysis?

The inhibition of pyruvate kinase at ATP was first discovered by Meyerhof and Oesper (1949). It was later observed by McQuate and Utter (1959), who attributed the inhibition to a chelation of the Mg^{2+} ion. The inhibition was extensively studied by Reynard et al. (1961) who concluded, using kinetic studies, that the ATP inhibition was competitive with the substrates ADP and PEP with a K_i of 1.2×10^{-4} M. They concluded that the ATP bound at the ADP binding site with the transferable γ-phosphoryl group of the ATP overlapping the

PEP site. Mildvan and Cohn (1966) arrived at similar conclusions using NMR techniques.

Thus it seemed established that ATP was inhibiting by a direct binding; however, Wood (1968) contended that there was no inhibition by ATP of muscle pyruvate kinase at high Mg^{2+} concentrations, and that the inhibition at low Mg^{2+} concentrations could be attributed to a chelation of the Mg^{2+} by ATP. Similar conclusions were reached by Holmsen and Strom (1969), except that they did note a slight inhibition by ATP at higher concentration of Mg^{2+}. Their data did not fit the substrate-phosphate overlap mechanism proposed by Reynard et al. (1961).

The argument for a chelation mechanism for the ATP inhibition was countered by Boyer (1969). He showed that the ATP inhibition was independent of removal of free Mg^{2+} by ATP. This was done by "buffering" the free Mg^{2+} with glycerol 1-phosphate which binds Mg^{2+} moderately tightly.

The ATP inhibition has been demonstrated for pyruvate kinases from sources other than muscle (see Tables 1 and 2).

Control of Pyruvate Kinases From Sources Other Than Muscle. "In vivo" Studies. The total activity of rat liver pyruvate kinase was found to increase ten-fold with rats fed a high carbohydrate diet over those with a low carbohydrate diet (Krebs and Eggleston, 1965). Additional studies showed a twenty-fold increase in yeast pyruvate kinase grown on a 2 % glucose medium rather than a 0.6 % glucose medium (Hommes, 1966). Both enzymes increased on a high glycerol diet (Takeda et al., 1967; Gancedo et al., 1967). Liver pyruvate kinase

decreased in rats made diabetic by alloxan injection. Insulin returned the level to normal; the return was blocked by either actinomycin or ethionine (Weber et al., 1965).

Dietary regulation of rat liver pyruvate kinase was further studied by Szepesi and Freedland (1968). They observed that pyruvate kinase activity was increased by a 90 % casein diet, but only if the rats were pre-fed a 90 % carbohydrate diet for four days. This increase was inhibited by cycloheximide but not actinomycin. They concluded that carbohydrate induces the necessary m-RNA formation, but protein synthesis could be stopped by lack of necessary protein in the diet. Thus the increase in pyruvate kinase activity with a high protein diet is via protein synthesis from pre-existing m-RNA.

"In vitro" Studies. Pyruvate kinases from several sources have been shown to be under metabolic control as well (see Tables 1 and 2). Moreover, the kinetic properties of these enzymes are consistent with the allosteric mechanism of enzymatic catalysis (Monod et al., 1965).

Plots of enzymatic activity of yeast pyruvate kinase activity versus the concentration of the substrate or effector, PEP, K^+, NH_4^+, or ADP (at low concentrations of PEP) are sigmoidal, indicating a cooperativity of binding. The K_m of each effector is decreased by the addition of FDP, which transforms the sigmoid plots into Michaelis-Menten hyperbolic plots (Hess et al., 1966; Hess and Haeckel, 1967; Hunsley and Suelter, 1967).

This "feed forward" activation by FDP (Hess et al., 1966) has also been observed in pyruvate kinase from rat liver, but not muscle

(Taylor and Bailey, 1967); developing loach embryo (Milman and Yurowitzski, 1967); adipose tissue (Pogson, 1968); Escherichia coli (Maeba and Sanwal, 1968); and desert locust fat body, but not flight muscle (Bailey and Walker, 1969).

Tanaka et al. (1967b) reported the existence of two electrophoretically and immunologically distinct species of pyruvate kinase from rat liver, designated type "M" and "L" (see Table 1). The "M" type has properties similar to muscle pyruvate kinase and does not appear to be under rigid dietary or metabolic control, whereas the "L" type varies with the diet as described above, follows sigmoid kinetics, and is activated by FDP. A type "L" pyruvate kinase (determined by immunological properties) has been observed in erythrocytes which is not activated by FDP (Tanaka et al., 1967b) suggesting the possibility of two type "L" enzymes, or two forms of one enzyme. The latter possibility seems more likely since the potential for FDP activation of liver type "L" enzyme can be destroyed with incubation (Tanaka et al., 1967b) or storage (Susor and Rutter, 1968). Adipose tissue also contains two interconvertible forms of pyruvate kinase; one sensitive and the other insensitive to FDP activation (Pogson, 1968).

An abnormality in erythrocyte pyruvate kinase activity has been observed in the disease hemolytic anemia. This abnormality has been found to be of two types: 1) low total enzyme activity with a normal K_m for PEP (Valentine et al., 1961; Koler et al., 1964), 2) normal enzyme activity at saturating concentrations of PEP but a ten-fold increased K_m for PEP (Paglia et al., 1968; Sachs et al., 1968).

The occurrence of a different, very labile, pyruvate kinase from tumors has recently been reported (Criss, 1969; Weber, 1969).

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*Refers to inhibition relative to substrate in parentheses
**DES = diethylstilbestrol

TABLE 1. PROPERTIES OF PYRUVATE KINASES FROM MUSCLE, LIVER AND YEAST+

Source	Substrate Binding - K_m (M)		Inhibitor - K_i (M)		Activator - K_a (M)	
MUSCLE (mol wt - 237,000)[b]	PEP	7.8×10^{-5} [c]	ATP	1.2×10^{-4} [e]	Mg^{2+}	4.5×10^{-4} [e]
		3.2×10^{-5} [e]	Pi	3.5×10^{-5} (PEP) [d]*	Mn^{2+}	0.71×10^{-4} [g]
	ADP	2.1×10^{-4} [e]		1.8×10^{-2} (ADP) [d]		
		3.6×10^{-4} [c]	AMP	1.37×10^{-2} (PEP) [h]	K^+	1.1×10^{-2} [a]
	ATP	8.6×10^{-4} [c]		3.5×10^{-2} (ADP) [h]	NH_4^+, Rb^+	1.1×10^{-2} [a]
	Pyruvate	1.0×10^{-2} [c,e]	Ca^{2+}	1.4×10^{-4} [g]		
			DES**	7.8×10^{-5} [f]		
LIVER "L" (mol wt - 208,300)[p]	PEP	0.75×10^{-3} [j,p]	ATP	0.16×10^{-3} [j]	FDP	9.5×10^{-6} [q]
		0.08×10^{-3} [j] (in 10^{-4} M FDP)	Cu^{2+}	(inactivated by 6.6×10^{-6} M; completely reversed by 6.6×10^{-5} M FDP)[i]		
	ADP	0.1×10^{-3} [p]				
LIVER "M" (mol wt - 250,000)[p]	PEP	0.75×10^{-4} [p]	ATP	3.5×10^{-3} [j]		
	ADP	0.28×10^{-3} [p]				
YEAST (mol wt 167,000)[t]	PEP	4.5×10^{-3} [r]	NADP	6×10^{-3} [r]	FDP	1.5×10^{-4} [s]
		4.6×10^{-4} [r] (in 10^{-3} M FDP)	Ca^{2+}	1.2×10^{-2} [r]	K^+	2.9×10^{-2} [r]
			ATP	? [r]		4.5×10^{-3} [r] (in 3×10^{-3} M FDP)
	ADP	4.7×10^{-4} [r]	citrate	4×10^{-2} [r] in 3×10^{-3} M		
		1.8×10^{-4} [r] (in 10^{-3} M FDP)	$MgSO_4$	4×10^{-4} [r] in 2.5 mM $MgSO_4$	NH^{4+}	1.5×10^{-2} [r]

+Lower case letters identify references found on the adjacent page.

REFERENCES FOR TABLE 2

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Maeba, P., and Sanwal, B. D. (1968), J. Biol. Chem., 243, 448.

Adipose Tissue

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Erythrocyte and Leukocyte

Koler, R. D., Bigley, R. H., Jones, R. T., Rigas, D. A., Vanbellinghen, and Thompson, P. (1964), Cold Spring Harbor Sym. Quant. Biol., 29, 213.

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TABLE 2. PROPERTIES OF PYRUVATE KINASES FROM E. COLI, ADIPOSE TISSUE, ERYTHROCYTE AND LEUKOCYTE

Source	Sedimentation Coefficient (S)	Substrate Binding (K_m) (M)		Activators K_a (M)		Comments
E. Coli	4.52 S (not active)	ADP	0.1×10^{-3}	FDP	0.2×10^{-3}	FDP-V_{max} effector
	6.67 S (active species)	PEP	0.1×10^{-3}	AMP	0.1×10^{-3}	AMP-K_m effector
ADIPOSE TISSUE "A"	5.5 S	ADP PEP	0.33×10^{-3} 0.6×10^{-3}	FDP	0.029×10^{-3}	both forms inhibited by ATP; "A" shows alosteric properties; conversion of "A" into "B" mediated by FDP, ATP, EDTA or citrate
ADIPOSE TISSUE "B"	7.2 S	ADP PEP	0.67×10^{-3} 0.067×10^{-3}	———		
ERYTHROCYTE	7.1 S					binding of substrates are dependent upon the concentration of the other
LEUKOCYTE	7.1 S (?)	ADP PEP	2.08×10^{-4} 0.72×10^{-4}			binding of substrates are independent

LITERATURE REVIEW: PYRIDOXAL CATALYSIS

I have shown that rabbit muscle pyruvate kinase interacts specifically with pyridoxal-5'-phosphate and this interaction results in a pronounced inactivation (this will be discussed in Chapter 2). It is of interest to understand the mechanism of this interaction and to correlate this to the loss of enzymatic activity. In formulating and evaluating mechanisms for the effects I have found, it is useful to consider the known mechanisms of pyridoxal-5'-phosphate interaction with enzymes per se, and in enzyme catalyzed reactions.

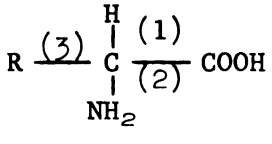
Many aspects of the interactions of pyridoxal-5'-phosphate in enzyme catalyzed reactions are described below. The interaction with enzymes per se will be discussed in Chapter 2.

Discovery of Pyridoxal Catalysis. The original experiments which led to the eventual discovery of pyridoxal catalysis showed that a variety of lactic acid bacteria required pyridoxine (pyridoxol), an analog of pyridoxal, for growth on a medium which was determined chemically to lack it (Moller, 1938; 1939). However, on addition of an animal tissue extract to the pyridoxine deficient medium, the organism grew much faster than could be attributed to the pyridoxine content of the extract. It was concluded that the pyridoxine was converted by the extract into a metabolite, called "pseudopyridoxine," which was more active for the bacterial growth than pyridoxine itself (Snell, 1942a). The higher "pseudopyridoxine" activity of the extract was found to

result from an interaction of pyridoxine and with the amino acids of the medium (Snell, 1942b), and later to the presence of the analogs, pyridoxal and pyridoxamine (Snell, 1944a, 1944b).

Pyridoxine derivatives have been shown to be involved in the decarboxylation of tyrosine in tissue extracts (Gunsalus and Bellamy, 1944). Addition of pyridoxal stimulated decarboxylation, but the addition of ATP, which led to a phosphorylated derivative of pyridoxal, markedly stimulated the decarboxylation (Gunsalus et al., 1944). A similar stimulation by a phosphorylated derivative of pyridoxal was shown for the transamination reaction (Schlenk and Snell, 1945). This cofactor, named "codecarboxylase" (Gale and Epps, 1944), was determined to be the phosphate ester of pyridoxal and was chemically synthesized (Gunsalus et al., 1945).

Reactions Catalyzed by Pyridoxal Compounds. Numerous amino acid reactions have been shown to be catalyzed nonenzymatically by pyridoxal plus metal ions or enzymatically by pyridoxal or pyridoxal-5'-phosphate with no requirement for metal ions (see reviews: Metzler, et al., 1954; Snell, 1962; Fasella, 1967). These reactions of the amino acids can be classified into three general groups of reactions involving cleavage of the three bonds to the α-carbon of the amino acid. They are shown below with examples for each.



1. Labilization of the hydrogen leading to racemization or transamination:

   alanine transaminase: L-alanine + α-ketoglutarate $\rightleftharpoons$ pyruvate + L-glutamate

   alanine racemase: L-alanine $\rightleftharpoons$ D-alanine

2. α-decarboxylation:

   histidine decarboxylase: L-histidine $\longrightarrow$ histamine + CO_2

3. Reaction of the R-Group substituent:

   a. Elimination or replacement of the entire group:

      threonine aldolase: threonine $\rightleftharpoons$ acetaldehyde + glycine

      tryptophanase: $RCH_2CHNH_2COOH + R'H \rightleftharpoons R'CH_2CHNH_2COOH + RH$

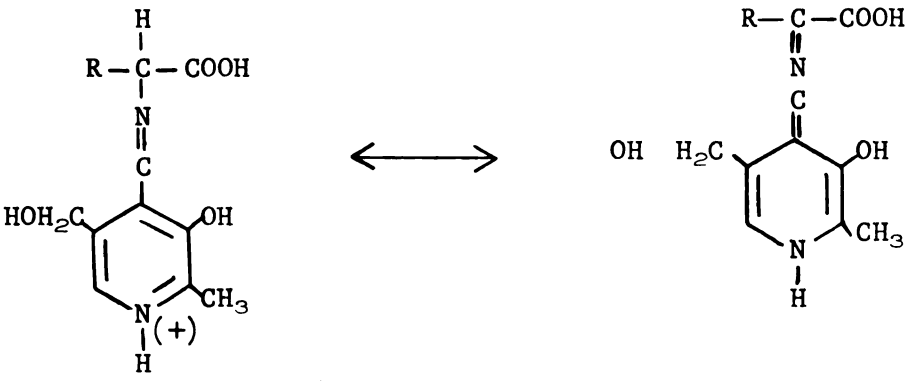
      tryptophan + $H_2O \longrightarrow$ indole + NH_3 + pyruvate

4. Modification of the R-group:

   cysteine synthetase: serine + $H_2S \longrightarrow$ cysteine + H_2O

   β-aspartate decarboxylase: aspartate $\longrightarrow$ CO_2 + alanine

General Mechanism for Pyridoxal Catalysis. The first mechanism to explain pyridoxal catalysis was proposed independently by Braunstein and Skemyakin (1953) and Metzler et al. (1954). The mechanism included the formation of a Schiff base between the amino acid reactant and the pyridoxal catalyst (shown below). The amino acid was thus assumed to be "activated" for its subsequent reactions by weakening the bonds of the α-carbon.



(a) (b)

The necessity for ketimine formation (compound b, above) as an intermediate has been postulated but not proven. Alpha-methylserine which contains no α-hydrogen atom and thus cannot form a ketimine has been decarboxylated nonenzymatically by PLP plus metal ions (Kalynakar and Snell, 1962), and degraded to formaldehyde and alanine, nonenzymatically and enzymatically (Snell, 1963). Labilization of the α-hydrogen was shown not to occur during the decarboxylation of tyrosine to tyramine by tyrosine decarboxylase (Mandeles et al., 1954); furthermore, the decarboxylation proceeded with full retention of configuration (Bellau and Burba, 1960).

However, there are many other systems where ketimine formation clearly occurs. The rate of appearance of the ketimine and the corresponding disappearance of the aldime (compound a, above) has been measured spectrophotometrically in methanol (Matsushima and Martel, 1967). The disappearance of a Cotton effect due to a loss of the asymmetric center upon ketimine formation has also been shown (Torchinsky and Koreneva, 1965). Spectral properties of the quinoid-like structure of the ketimine have been observed in several enzyme systems (Schirch and Jenkins, 1964).

Evidence for Schiff base formation between the 4-formyl group of PLP and the amino group of an amino acid is provided by spectral data. Pyridoxal phosphate in bicarbonate buffer at pH 7.2 displays an absorption maximum at 388 mμ. The absorption maximum is shifted to 413 mμ, with the formation of an additional bond at 280 mμ, within ten minutes after addition of an amino acid (Blakely, 1955; Metzler, 1957). The absorbance at 413 mμ is attributed to the protonated Schiff base; the absorption is shifted to 367 mμ at higher pH values. The midpoint for this transition is at pH 10.5 (Metzler, 1957).

NONENZYMATIC PYRODOXAL CATALYSIS. In nonenzymatic catalysis, the metal ions have two functions: (1) chelation with the 5-hydroxyl group of the pyridoxal and the imino group of the pyridoxal-amino acid complex, aiding electron withdrawal (Snell, 1963), (2) maintenance of the required molecular planarity (Dunthan, 1966). The heterocyclic nitrogen atom functions in electron withdrawal, while the remaining groups appear to be functionless (Metzler et al., 1954).

Enzymatic Pyridoxal Catalysis. The metal ion is replaced by a complex protein molecule in catalysis by pyridoxal in enzyme systems. Otherwise the same functional group of the pyridoxal molecule are operative in the enzymatic catalysis as in the nonenzymatic catalysis.

The formation of a Schiff base with an amino group of an enzyme was first proposed by Jenkins and Sizer (1957). At pH 4.8 they observed an absorption band at 430 mμ resulting from the interaction of pyridoxal-5'-phosphate with glutamic-aspartic trans-

aminase. This proposal was given further proof by sodium borohydride reduction of the enzyme-pyridoxal complexes with subsequent isolation of pyridoxal-lysine (Matsuo and Greenberg, 1959).

With the pyridoxal cofactor bound to the enzyme via a Schiff base, an additional step is required in enzymatic, pyridoxal catalysis, namely "transaldimination"--the transfer of pyridoxal from the enzyme to the amino acid substrate (Snell, 1962).

The enzymatic mechanism of pyridoxal catalysis is undoubtedly extremely complex encompassing several factors. The complexity is shown by spectral data indicating at least four enzyme-pyridoxal substrate complexes (Schirch and Jenkins, 1964; Jenkins and D'Ari, 1966). These complexes can be attributed to the interaction of pyridoxal with protein amino acids such as serine, cysteine, and histidine. Further analysis of these complexes will contribute to a better understanding of the catalytic mechanism.

CHAPTER ONE

REVERSAL OF THE DISSOCIATION OF RABBIT MUSCLE PYRUVATE KINASE INTO UNFOLDED SUBUNITS

ABSTRACT

The dissociation of tetrameric rabbit muscle pyruvate kinase (mol wt = 237,000) into unfolded subunits (mol wt = 57,000) in 6 M guanidine has been shown to be reversible. A systematic study of the factors affecting the reversal of the dissociation led to conditions where up to 70 % of the initial catalytic activity was regained. The reversal procedure (from: Deal, W. C. (1969), Biochemistry 8, 2795 (1969)) consisted of two phases: (1) a 100-fold dilution of guanidine-dissociated enzyme (0°) into a reversal solvent at 0° and (2) incubation of the resulting solution at a higher temperature, usually 16°. Conditions for optimum reversal of dissociation were (1) pH 8; (2) protein concentration, 0.04 mg/ml; (3) ionic strength, 0.3 (4) reducing agent, 0.06 M β-mercaptoethanol and (5) temperature--0° dilution, followed by six hr at 16°. The half-time for activity recovery was approximately 45 min at both 0.02 mg/ml and 0.09 mg/ml enzyme concentration. Two metabolites, insulin and phosphate ion, were found to greatly influence the reversal of dissociation. Insulin decreased the activity recovery upon reversal, in contrast to what would be expected for an inducer of the enzyme. Phosphate ion yielded activity recovery at 36°; neglible activity was recovered at that temperature in its absence. The reversal of dissociation was not affected significantly by the addition of a number of metabolites including ATP, ADP, 5'-AMP, 3',5'-AMP, lactate, FDP and NAD^{+}. The reassociated enzyme had the same K_m, heat stability, and sedimentation coefficient as the native enzyme.

INTRODUCTION

We have previously shown (Deal, 1967; Deal and Constantinides, 1967; Deal, 1969) that, contrary to the widely accepted view, some proteins apparently require[1] metabolites for *in vitro* reassembly from their unfolded subunits. The experimental evidence was that for *in vitro* reversal of dissociation of yeast glyceraldehyde-3-phosphate dehydrogenase, NAD^+ was required. It was suggested that the *in vitro* requirement for NAD^+ may reflect a similar *in vivo* requirement for NAD^+ for proper folding of nascent polypeptide chains as they are being synthesized, thereby providing for control of enzyme synthesis. The possibility was raised that similar effects might occur with other enzymes.

As part of an analysis of the metabolic control and structure of rabbit muscle pyruvate kinase (Steinmetz and Deal, 1966; G. S. Johnson and W. C. Deal, Jr., in preparation) it was of interest to determine whether rabbit muscle pyruvate kinase could be reassembled *in vitro* from its unfolded subunits (Steinmetz and Deal, 1966; Kayne, 1966), and if so, whether metabolites were required for, or had any effect upon, the process.

Pyruvate kinase has often been postulated to be a control point in glycolysis and the activities of pyruvate kinases from various sources are known to be affected by various compounds (Hess *et al.*, 1966; Milman and Yurowitski, 1967; Tanaka *et al.*, 1967; Taylor and Bailey, 1967; Maeba and Sanwal, 1968). Also, the synthesis of pyruvate

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[1]This means that in the absence of the metabolite, the rate of correct folding is extremely slow. The metabolite may, or may not, be absolutely essential for correctly formed enzyme.

kinase from various sources is known to be affected by various conditions. Rat liver pyruvate kinase, but not kidney cortex pyruvate kinase, decreases upon starvation or low carbohydrate diets and increases with high carbohydrate diets (Krebs and Eggleston, 1965). This effect may be explained by the observation that insulin induces pyruvate kinase synthesis in alloxan diabetic rats (Weber et al., 1965; Weber et al., 1966). This suggested that the synthesis of pyruvate kinase might be regulated as an important step in metabolic control, and that metabolites or related substances might be the agents which would accomplish such regulation.

Pyruvate kinase has been well characterized, and its properties have been discussed in an excellent review by Boyer (1962). Morawiecki (1960) reported that pyruvate kinase dissociated into subunits of mol wt 150,000 in 6 M urea and concluded that the enzyme consisted of at least two polypeptide chains. Our previous work (Steinmetz and Deal, 1966) and that of others (Cottam et al., 1969) has shown that tetrameric rabbit muscle pyruvate kinase (mol wt 237,000) is completely dissociated into unfolded subunits (mol wt 57,000) by high concentrations of urea (4 M or greater) or guanidine. Optical rotatory dispersion analysis indicated that the urea denaturation, and presumably the guanidine denaturation, resulted in essentially complete unfolding of the polypeptide chains as well (Kayne, 1966).

Although Morawiecki (1960), using a dialysis procedure, was able to obtain activity recovery of pyruvate kinase exposed to 2.5 M urea--which completely inactivates, (Morawiecki, 1960), but only partially dissociates it (Steinmetz and Deal, 1966)--he concluded that

the dissociation in 5 M urea was irreversible, since only 5 % of the activity could be regained.

This paper describes a systematic analysis of factors affecting reversal to determine the optimal conditions necessary for the renaturation of rabbit muscle pyruvate kinase. Up to 70 % of the native activity was regained upon careful dilution of the denatured enzyme into the proper renaturation solvent. Independently, Cottam et al., (1969) obtained 35-50 % activity recovery upon removal of the denaturing agent by dialysis or by gel filtration.

Two metabolites were found to influence the reversal of dissociation in a specific way. Insulin inhibited the reversal, in contrast to what would be expected for an inducer of the enzyme. The presence of phosphate ion yielded significant activity recovery at 36°; none was obtained in its absence at that temperature.

RESULTS

Preliminary experiments using the reversal[2] solvents and procedures (Deal, 1967; Deal, 1969) for glyceraldehyde-3-phosphate dehydrogenase immediately gave significant recovery of pyruvate kinase activity upon appropriate dilution of the guanidine-enzyme solution into the reversal solvent. A systematic study of variables influencing the reversal was then conducted, using a successive approximation technique. All results reported are for experiments where all variables except that under study were at their optimal values, unless indicated otherwise.

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[2]The words "reversal" and "renaturation" stand for "reversal of dissociation or unfolding or inactivation."

EFFECT OF pH AND BUFFER SPECIES. Using imidazole, tris, and glycine buffers (0.05 M), the pH of the reversal solvent was varied from pH 6.2 to pH 9.6. The range for optimum reversal was found to exist between pH 7.0 and pH 9.0 (Figure 1). Except for imidazole, all the buffers tested, including Tris, glycine, bicine and tricine (Good et al., 1966), bicarbonate, and phosphate, gave essentially identical recoveries of activity upon reversal at 16° (but see the phosphate effect at 37°). However, at a given pH, the recoveries appeared to be lower in imidazole. To eliminate the possibility that impurities in the imidazole might have been interferring with the reversal, the experiments were repeated using imidazole recrystallized from absolute ethanol; however, this did not improve the recovery.

EFFECT OF SALT SPECIES AND IONIC STRENGTH. An ionic strength optimum of 0.2 to 0.5 was found for ammonium sulfate, potassium chloride, and magnesium chloride (Figure 2). Similar results were obtained with sodium chloride. The cations of all these salts have been shown to bind pyruvate kinase (Kayne and Suelter, 1965; Suelter et al., 1966). Tetramethylammonium chloride, whose cation does not bind, gave similar effects. These results suggested that the salt requirement was solely an ionic strength effect and not salt specific.

EFFECT OF TEMPERATURE AND TIME OF INCUBATION. To determine the optimal time and temperature of incubation for reversal, reversal samples were first diluted at 0° (the first stage of reversal) and then exposed to various temperatures ranging from 0° to 37°. The samples were assayed at the indicated times (Figure 3). The results indicated that 12-16°

Figure 1. EFFECT OF pH ON THE REVERSAL OF PYRUVATE KINASE

The enzyme was dissociated in a guanidine hydrochloride solution. Then reversal was accomplished by dilution of the samples into the reversal solvent at 0°, followed by incubation at 16°. The pH of the reversal solvent was varied from pH 6.2 to pH 9.6 using imidazole, Tris, and glycine buffers. The optimal reversal conditions (see Methods) were used except that the 0.05 M buffer species was varied. All samples were assayed at pH 7.5 after an eighteen hour incubation at 16°.

Figure 1

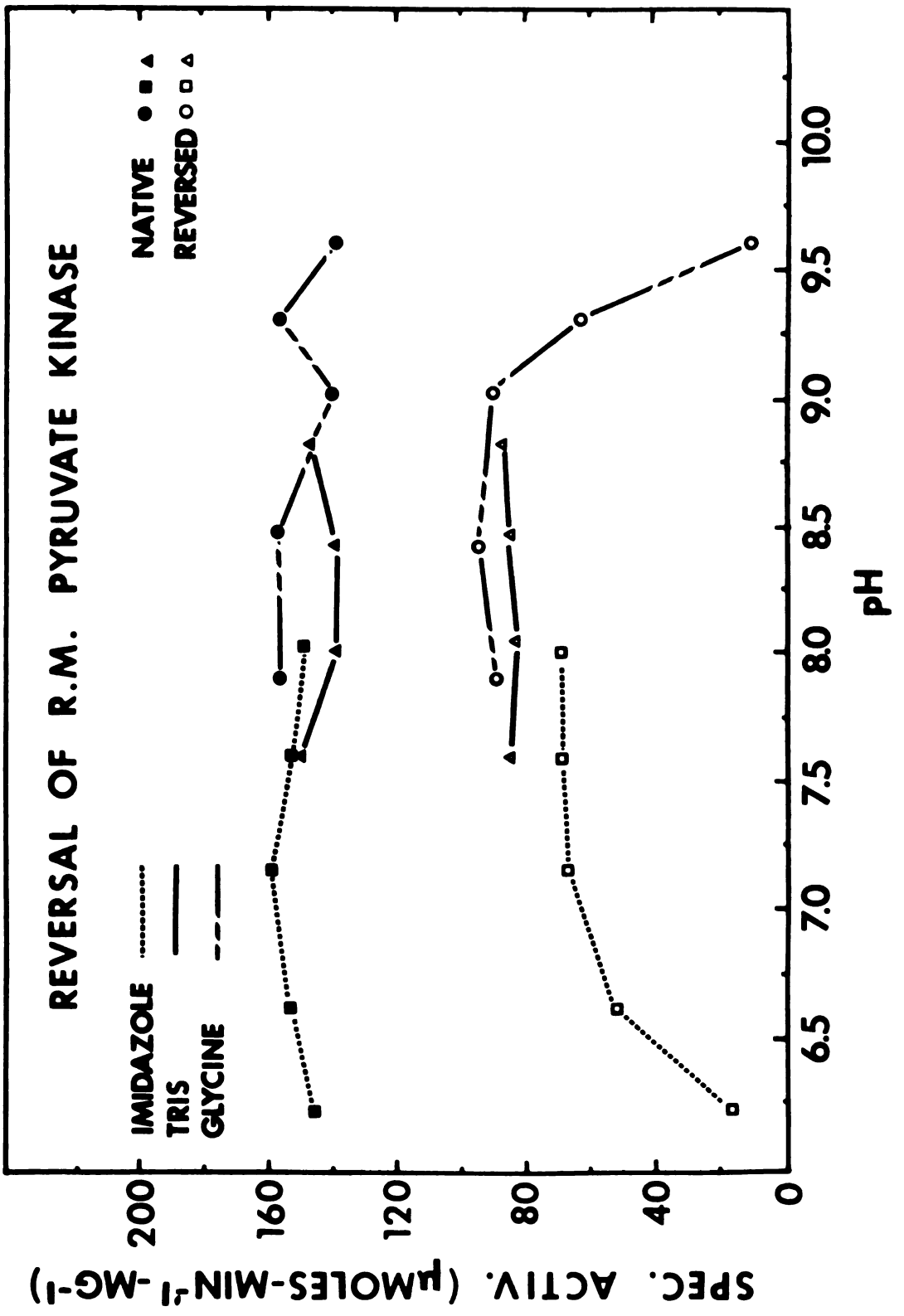

REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
REVERSED
IMIDAZOLE
TRIS
GLYCINE
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
0
40
80
120
160
200
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

Figure 2. EFFECT OF IONIC STRENGTH ON THE REVERSAL OF PYRUVATE KINASE

The optimal reversal conditions were used except for the variations in salt species and concentration. The concentration of the added salts in the reversal solvent was varied so that the ionic strength ranged from 0 to 2.15. The reversed enzyme was assayed after a six hour incubation at 16^{o}. The contribution of the buffer, approximately 0.025, is not included in the values of ionic strength. Each experimental point represents the average of two results with two test samples.

Figure 2

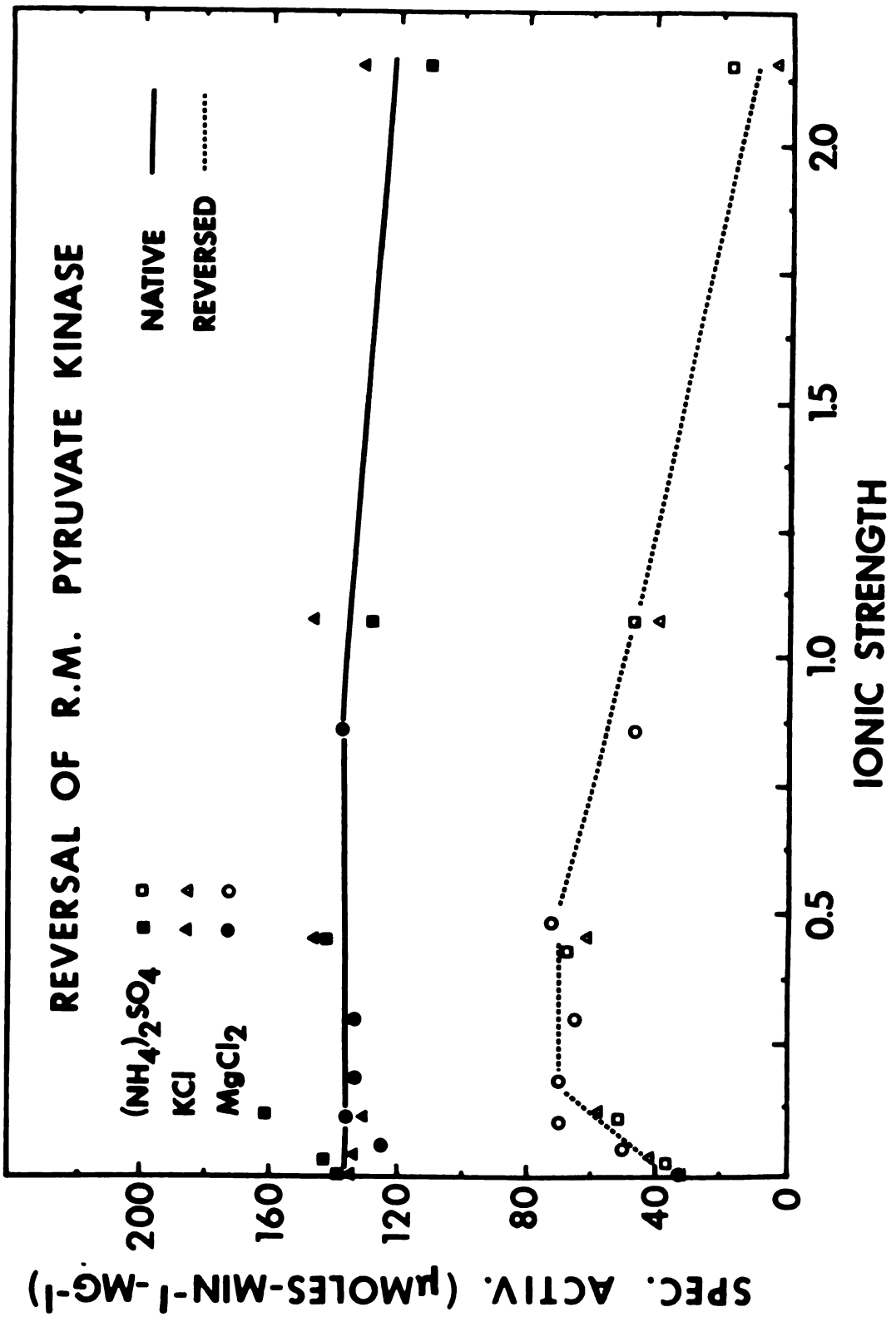
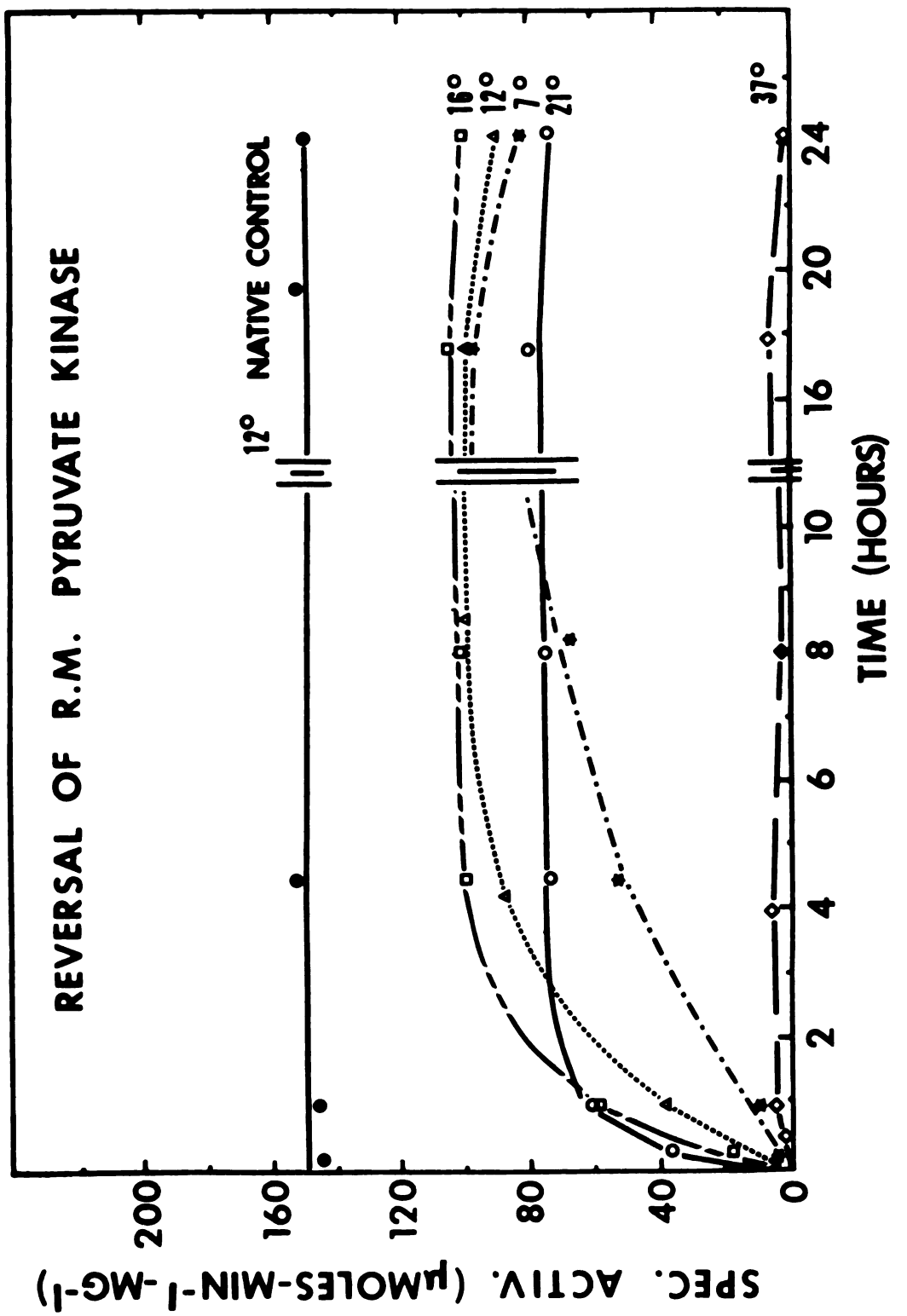

REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
REVERSED
$(NH_4)_2SO_4$
KCl
$MgCl_2$
SPEC. ACTIV. (μMOLES-MIN^{-1}-MG^{-1})
0
40
80
120
160
200
0.5
1.0
1.5
2.0
IONIC STRENGTH

Figure 3. EFFECT OF TEMPERATURE AND LENGTH OF INCUBATION OF THE REVERSAL OF PYRUVATE KINASE

The optimal reversal conditions were used. The dissociation enzyme was diluted into the reversal solution at approximately 2^{o}. The various enzyme samples were then immediately taken to the indicated temperatures and assayed at the indicated times.

Figure 3


REVERSAL OF R.M. PYRUVATE KINASE
12° NATIVE CONTROL
16°
12°
7°
21°
37°
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
TIME (HOURS)
0
40
80
120
160
200
2
4
6
8
10
16
20
24

was the temperature range for optimal reversal. They also showed that the activities of the reversed enzyme samples, and those of the controls, were constant for 24 hr (additional data not shown indicated complete stability in this system for up to 42 hr). Maximal reversal was obtained in 2-3 hr at 21°, but about 5 hr exposure was needed for the 12° and 16° samples to attain maximal recovery. Essentially no reversal occurred in the samples at 0° (not shown).

THE PHOSPHATE EFFECT. The failure to obtain significant reversal under these conditions at 37° was surprising, since this is the temperature at which the chains must fold *in vivo*. Since a visible precipitate was observed at the 0.04 mg/ml concentration at 37°, it was thought that a lower enzyme concentration might lead to less aggregation and a greater recovery of activity. However, even with concentrations of 0.02 and 0.009 mg/ml in the renaturation solution at 37°, no activity was recovered. Nor did a 30 min preincubation at 0° provide any increase in recovery.

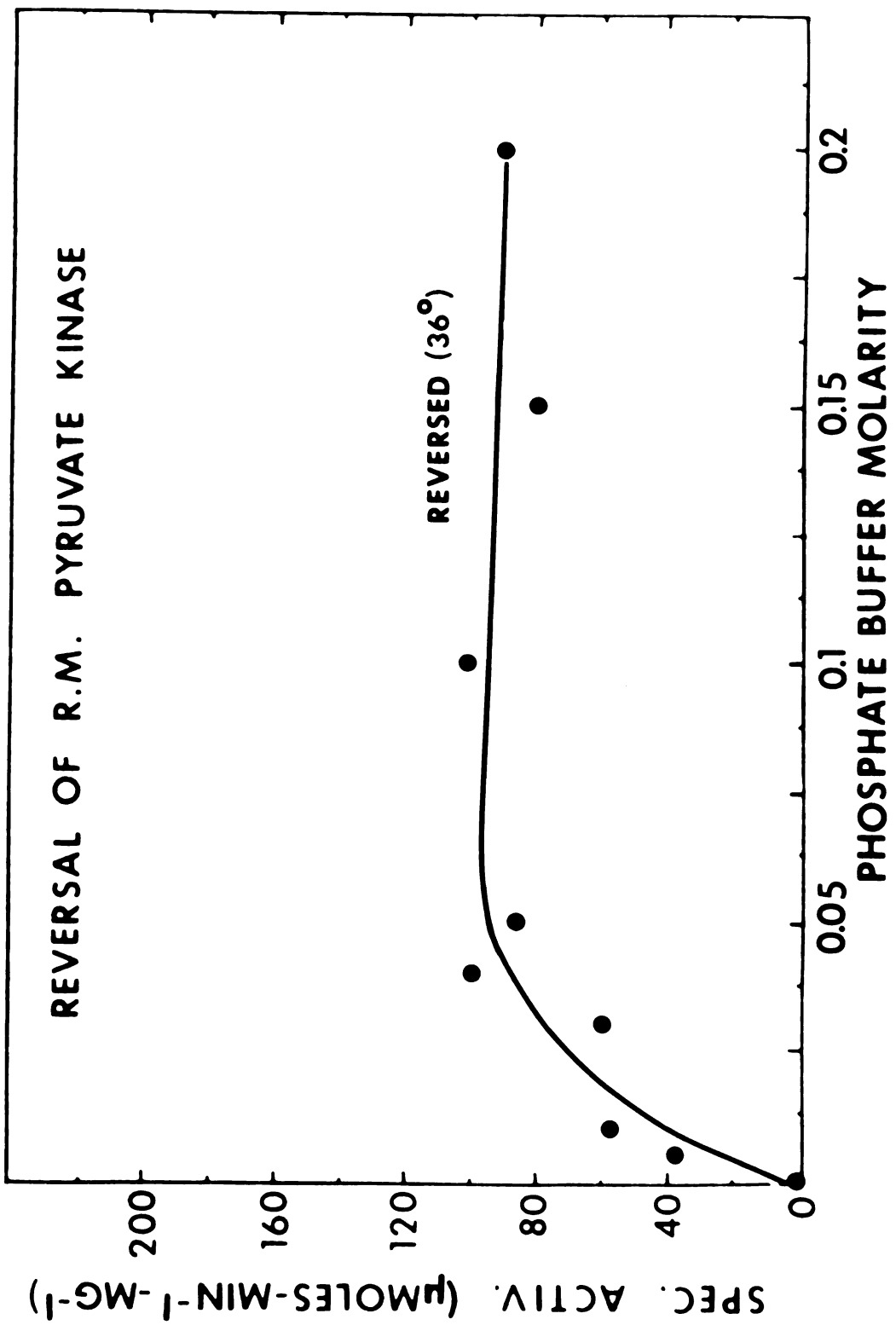
In an effort to obtain recovery at 37° in Tris buffer, four other reversal conditions were used: (1) the optimal conditions (see Methods), with the omission of the 0.4 M KCl; (2) the conditions described in the legend for Figure 5; (3) the conditions in (2) minus the EDTA, and (4) the conditions in (2) minus the ADP. However, none gave significant activity recovery.

Other buffers were used and reversal was finally obtained at 36° with phosphate, but not with bicarbonate or imidazole buffers. Maximum recovery occurred in the range of 0.04 M to 0.1 M phosphate (Figure 4). An unusual characteristic of the phosphate -36° system

Figure 4. EFFECT OF PHOSPHATE BUFFER ON THE REVERSAL AT 36°

The denatured enzyme was diluted (0.04 mg per ml) into the reversal solvent and incubated for 15 min at 0° followed by incubation for 20 min at 36° before analysis. In addition to the phosphate buffer, the reversal solution contained 0.2 M KCl, and 0.06 M β-mercaptoethanol.

Figure 4


REVERSAL OF R.M. PYRUVATE KINASE
REVERSED (36°)
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
200
160
120
80
40
0
0.05
0.1
0.15
0.2
PHOSPHATE BUFFER MOLARITY

was the dependence of activity recovery upon time of preincubation at 0° (see Methods). Preincubation times of 30 sec and 90 min at 0° yielded activity recoveries of 10 % and 30 % respectively. Denatured enzyme diluted at 36° directly, instead of at 0°, precipitated. In contrast, denatured enzyme diluted at 16° directly yielded 30 % activity recovery with no precipitation. A 5 min preincubation at 0° was adequate to yield maximal recovery at 16°.

EFFECT OF PROTEIN CONCENTRATION. Since some phase or phases of the reversal process involved the successive association of the subunits into the dimeric and then tetrameric species, it was expected that the concentration of enzyme in the reversal mixture might be important for activity recovery. To test the effect of enzyme concentration on the reversal, stock enzyme solution was diluted with the dissociation solvent, the final composition[3] of which was 3.5 M guanidine-HCl, 0.06 M β-mercaptoethanol, 0.02 M Tris-HCl buffer (pH 8.0), 0.07 M KCl, 0.001 M EDTA, plus residual 0.01 M imidazole buffer (Kayne, 1966). After a one-hr incubation in the dissociation solvent, varying aliquots of the enzyme solution were diluted appropriately with the dissociation solvent to yield various protein concentrations. Samples from these solutions were then diluted 100-fold, to yield reversal samples containing various enzyme concentrations ranging from 0.002 to 0.4 mg/ml. Native enzyme controls were

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[3]The enzyme was stored in aqueous solution (0.02 M imidazole buffer) at a concentration of 20-80 mg/ml. The 3.5 M guanidine was a compromise to allow the highest possible protein concentration in the dissociation solution with a sufficient guanidine concentration to insure complete dissociation and unfolding of the enzyme. Since guanidine usually seems to accomplish the same result as urea at roughly one-half the concentration, 3.5 M guanidine seemed adequate to insure dissociation and unfolding in this experiment (Steinmetz and Deal, 1966).

not run for this particular series because of the extensive amounts of enzyme required. The data obtained (Figure 5) indicated that enzyme concentrations in the narrow range of 0.03 to 0.08 mg/ml were optimal. One technical problem preventing analysis of a wider range was that the enzyme precipitated at protein concentrations greater than 0.2 mg/ml in the reversal solution.

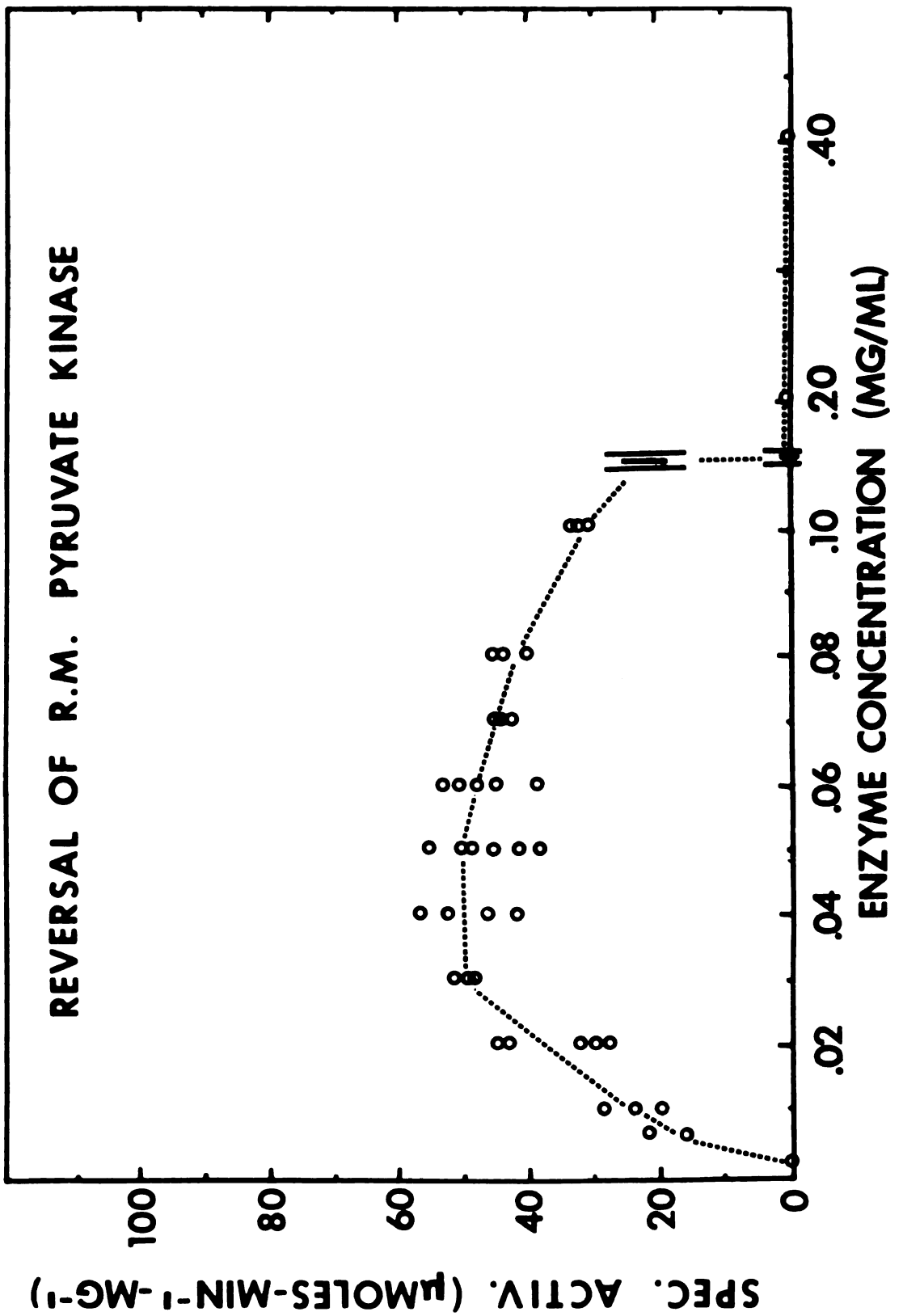
EFFECT OF SUCROSE. The presence of sucrose in the reversal solution increased the percent recovery at higher, but not at lower, enzyme concentrations. Reversal solutions with 10 % sucrose gave 45 % recovery of activity at 0.12 mg/ml enzyme and 11 % recovery at 0.2 mg/ml; under otherwise identical conditions, samples without sucrose yielded 15 % and 0 % recovery respectively. However, at a protein concentration of 0.4 mg/ml, no reversal was obtained, even in the presence of 10 % sucrose--and a precipitate formed during the incubation. In contrast to the enhancement by sucrose of reversal recovery at higher protein concentrations, there was no effect of either 10 % or 20 % sucrose on the reversal recovery using a protein concentration of 0.04 mg/ml, the optimal enzyme concentration (Figure 5).

HALF-TIMES FOR REVERSAL. Since native pyruvate kinase consists of tetramers, and also apparently exists as dimers or monomers (3.6 S species) under certain conditions (Steinmetz and Deal, 1966), it was of interest to see whether association had to occur in order for activity to be regained. The concentration dependence of the half-times of activity recovery at 16° was studied to provide information on this question. The half-time for recovery, determined experimen-

Figure 5. EFFECT OF ENZYME CONCENTRATION ON THE REVERSAL OF PYRUVATE KINASE

(See text for special dissociation and reversal procedures.) The enzyme concentration in the reversal solvent was varied from 0.002 to 0.4 mg per ml. The reversal solution also contained 0.1 mM ADP, 0.1 M Tris-HCl (pH 8.75), 0.165 M ammonium sulfate, 0.05 M β-mercaptoethanol, and 0.001 M EDTA. The data shown are the results of several experiments.

Figure 5


REVERSAL OF R.M. PYRUVATE KINASE
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
0
20
40
60
80
100
.02
.04
.06
.08
.10
.20
.40
ENZYME CONCENTRATION (MG/ML)

tally as the time at which 50 % of the maximum activity was recovered, was found to be 50 min for both the 0.09 mg/ml and 0.02 mg/ml samples; the half-time was 44 min for the 0.04 mg/ml samples (Figure 6). These differences were presumably not significant, since the two extremes of concentration had the same half-times. Although these results were not conclusive, they did suggest that at these concentrations a first-order process, a folding step, was rate-limiting for the recovery of activity.

The half-time for reversal at 16° was surprisingly long. However, the half-time for reversal at 36° in phosphate buffer and 0.04 mg/ml was determined to be only 4-5 min (Figure 7). This is closer to the rate of folding expected *in vivo*.

EFFECT OF REDUCING AGENT SPECIES AND CONCENTRATION. Since the reversal experiments were conducted under aerobic conditions, the possibility existed that random formation of disulfide bonds might be hindering the specific refolding of the enzyme. To test this possibility, a reversal study was performed with various concentrations of the reducing agent β-mercaptoethanol ranging from 0 to 0.44 M. Maximal reversal was obtained with β-mercaptoethanol concentrations in the range of 0.05 to 0.15 M (Figure 8). Negligible recovery was obtained from the sample reversed without β-mercaptoethanol.

Other reducing agents were also tested. Reversal solutions containing 0.06 M dithiothreitol or 3-mercapto-1,2-propanediol gave results similar to those with 0.06 M β-mercaptoethanol. Glutathione, which is expected to exist in fairly high concentrations *in vivo*, also gave good recovery of activity. However, in the glutathione system both the control enzyme and the reversed enzyme were unstable during incubation,

Figure 6. EFFECT OF ENZYME CONCENTRATION ON THE HALF TIME OF REVERSAL OF PYRUVATE KINASE

The optimum reversal conditions were used except for the omission of the 0.4 M KCl. Samples of the dissociated enzyme were diluted into the reversal solution to the final enzyme concentration indicated, at approximately 2°. These diluted enzyme solutions were then placed into a 16° temperature bath and the enzyme was assayed at 25° at the appropriate times. The time at which the samples were brought to 16° was designated zero time. All the assays were linear, indicating that no change in activity occurred during the assays.

Figure 6

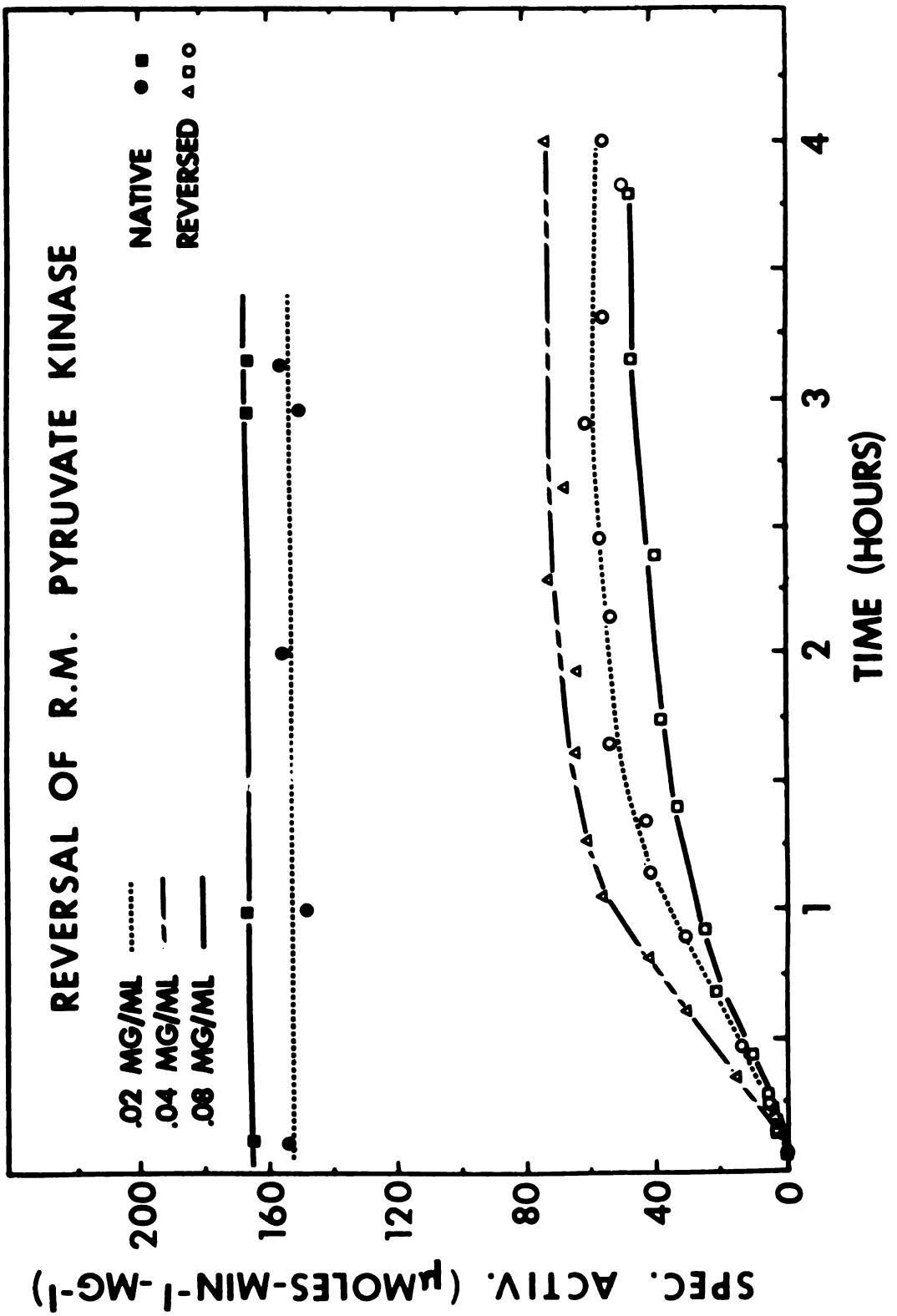

REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
REVERSED
.02 MG/ML
.04 MG/ML
.08 MG/ML
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
TIME (HOURS)
0
40
80
120
160
200
1
2
3
4

Figure 7. HALF TIMES FOR REVERSAL AT 36°

The denatured enzyme was diluted (0.04 mg per ml) into the reversal solvent and incubated for 1 min or 15 min at 0° in the reversal solution. The enzyme solutions were placed in a 36° bath and the enzyme was assayed at 25° at the appropriate times. The time at which the samples were brought to 36° was designated zero time. All assays were linear indicating that no change in activity occurred during the assays. The renaturation solutions contained 0.05 M potassium phosphate buffer, pH 8.0, 0.2 M KCl, and 0.06 M β-mercaptoethanol.

Figure 7

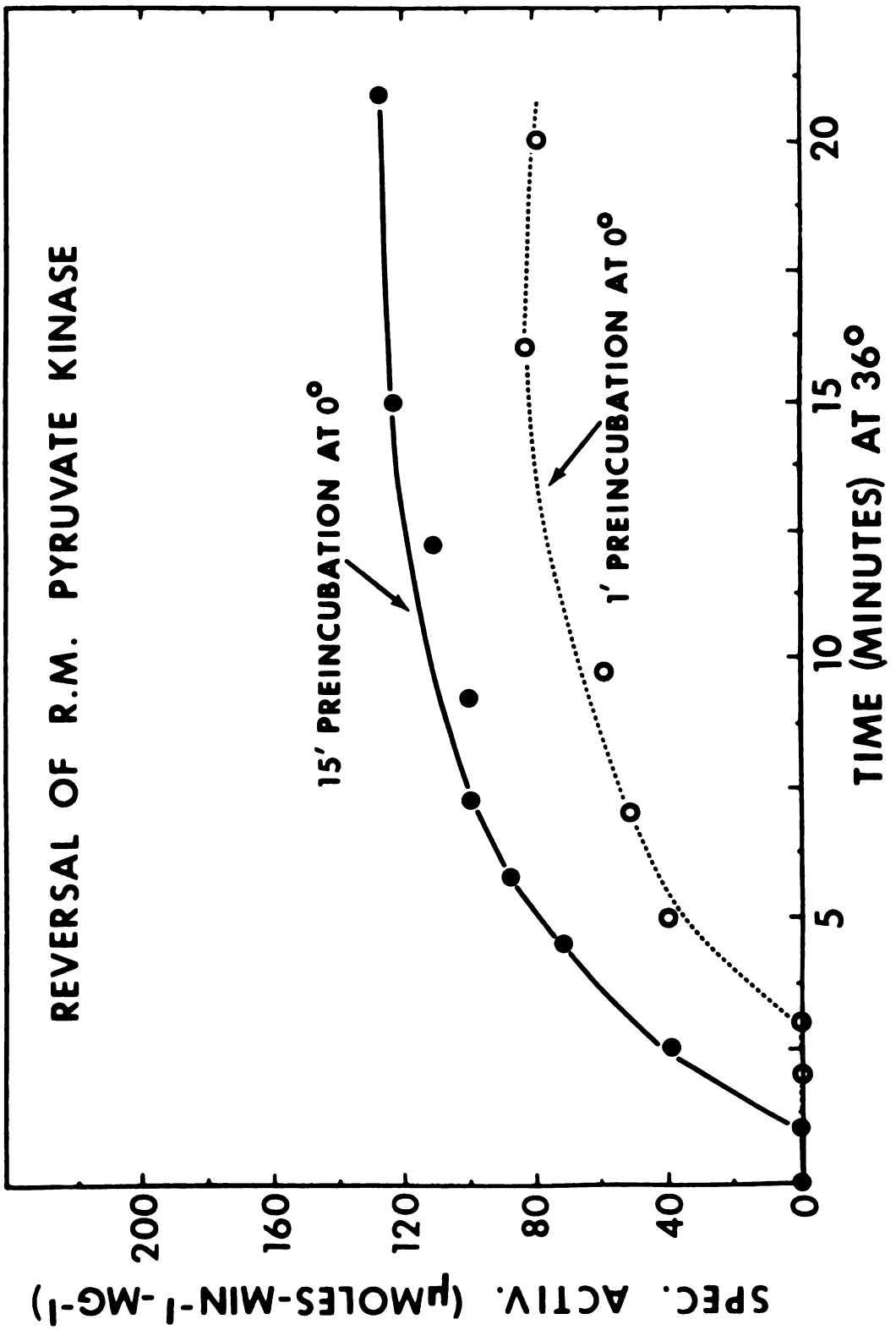
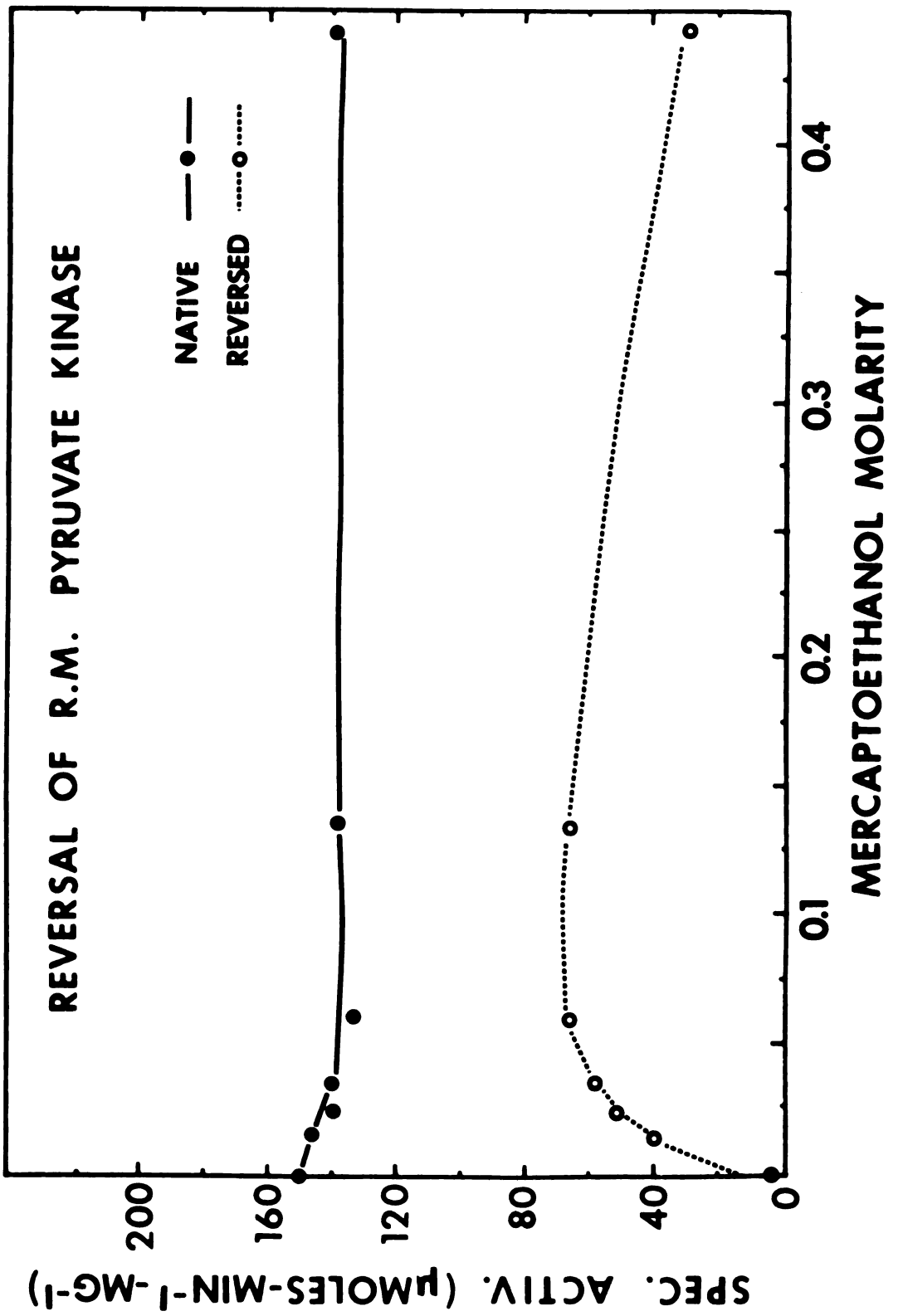

REVERSAL OF R.M. PYRUVATE KINASE
15' PREINCUBATION AT 0°
1' PREINCUBATION AT 0°
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
TIME (MINUTES) AT 36°
0
40
80
120
160
200
5
10
15
20

Figure 8. EFFECT OF β-MERCAPTOETHANOL ON THE REVERSAL OF PYRUVATE KINASE

The optimum reversal solution was used except for the omission of 0.4 M KCl. The concentration of β-mercaptoethanol in the reversal solution was varied from 0 M to 0.45 M. The renatured enzyme was assayed after a six hour incubation at 16°. Each point represents the average of results with two test samples.

Figure 8


REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
REVERSED
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
200
160
120
80
40
0
0.1
0.2
0.3
0.4
MERCAPTOETHANOL MOLARITY

losing approximately 50 % of their original activity within ten hr.

EFFECT OF ADDED METABOLITES. Previous work in this laboratory had shown that the refolding of yeast glyceraldehyde-3-phosphate dehydrogenase was aided by the metabolite NAD^+ (Deal, 1967; Deal and Constantinides, 1967; Deal, 1969). To test for a similar type of effect on the reversal of pyruvate kinase, reversal solutions 5 mM in ATP, ADP, 3',5'-AMP, PEP, or lactic acid were tested. All the metabolites were tested in the presence of 0.1 M $MgCl_2$. Also, 3',5'-AMP, ADP, and ATP were tested in 0.4 M KCl. Occasionally ADP and ATP in 0.4 M KCl appeared to give about 5-10 % enhancement of activity recovery over that in their absence, but this was not observed in 0.1 M $MgCl_2$. None of the other metabolites significantly aided the reversal under these conditions.

It seemed possible that in this system ionic strength might have an effect superimposed on metabolite effects, as it had in the reversal system of yeast glyceraldehyde-3-phosphate dehydrogenase. The requirement of that enzyme for NAD^+ was essentially absolute at low ionic strength (0.15 M KCl) and this requirement was virtually abolished when the ionic strength was raised to 0.8 (Deal, 1969). However, reduction of the KCl concentration in the reversal mixture from 0.4 M to 0.2 M still did not yield any pronounced metabolite effects at 16°, using the previously mentioned metabolites and some additional metabolites (Table 3).

Since phosphate had previously been found to uniquely aid recovery at 36°, it also seemed possible that it might aid the recovery at 16° and perhaps produce a synergistic enhancement of

TABLE 3. EFFECT OF METABOLITES ON THE REVERSAL IN 0.2 M KCl.[a]

Conditions	% of Ref. Act.[b,c] 16°	Conditions	% of Ref. Act.[b] 16°
0.05 M Tris, pH 8.0	100	0.05 M K_2HPO_4, pH 8.0	107
0.05 M Tris, pH 8.0		0.05 M K_2HOP_4, pH 8.0	
+ 2.5 mM ADP	100	+ 5 mM ADP	98
+ 2.5 mM ATP	111		
+ 5 mM 5'-AMP	86	+ 5 mM ADP	
+ 5 mM 3',5'-AMP	86	+ 5 mM AMP	106
+ 2.5 mM FDP	90		
		+ 5 mM ADP	
+ 2.5 mM FDP		+ 5 mM AMP	
+ 5 mM 3',5'-AMP	91	+ 5 mM α-D-glucose	111
+ 0.73 mM Phosphoenol-pyruvate	90	+ 5 mM ADP	
+ 2.5 mM β-NAD^+	90	+ 5 mM α-D-glucose	100
+ 5 mM α-D-glucose	100		
+ 5 mM glucose-6-phosphate	90		
+ 2.5 mM ADP	106		
+ 10 mM $MgCl_2$			

[a]The reversed enzyme was assayed after incubation for 5-7 hr at 16° in the reversal solution which, in addition to the buffer, contained 0.2 M KCl and 0.06 M β-mercaptoethanol.

[b]The reference activity is that obtained after reversal for 5-7 hr at 16° in a reversal solution which contained 0.05 M Tris (pH 8.0), 0.2 M KCl, and 0.06 M β-mercaptoethanol, and no metabolite. A reversal sample is the reference.

[c]No activity was recovered at 37° under any of these conditions.

reversal with other metabolites. However, it only slightly aided the reversal at 16° and there was no significant effect of added metabolites in the presence of phosphate (Table 3).

Insulin, an inducer of rat liver pyruvate kinase (Weber et al., 1965), showed a pronounced interference with reversal at 16° and an even more pronounced interference at 37° (Table 4). Insulin had no effect on the native enzyme. Ribonuclease and bovine serum albumin at the same concentration (0.2 mg/ml to 0.01 mg/ml) had no significant effect on the reversal recovery of pyruvate kinase, suggesting that the effect was specific for insulin and not a general protein effect.

COMPARISON OF NATIVE AND REVERSED ENZYME. The native and reversed enzymes were found to have identical characteristics as shown by measurements of sedimentation coefficients (Figure 9), K_m values for ADP (Figure 10) and K^+ (Figure 11), and heat stability profiles at 50° (Figure 12).

DISCUSSION

The systematic analysis of variables influencing the refolding and reassembly of guanidine-dissociated and unfolded rabbit muscle pyruvate kinase into its active native tetrameric structure led to conditions where substantial activity recovery (up to 70 %) was obtained. In contrast Morawiecki (1960) had earlier reported the dissociation in high concentrations of urea to be essentially irreversible, since he had obtained less than 5 % recovery of activity. Also in limited studies Cottam et al. (1969) obtained 35-50 %

TABLE 4. EFFECT OF INSULIN ON THE REVERSAL.[a]

Conditions	% of Ref. Act.[b,c] 16°	Conditions	% of Ref. Act.[b] 16°	37°
0.05 M Tris, pH 8.0	100	0.05 M K_2HPO_4, pH 8.0	107	36
0.05 M Tris, pH 8.0		0.05 M K_2HPO_4, pH 8.0		
+ 0.2 mg of bovine serum albumin	107	+ 0.2 mg/ml of insulin	34	6
+ 0.2 mg/ml of ribonuclease	90	+ 0.08 mg/ml of insulin	59	7
+ 0.2 mg/ml of insulin	32	+ 0.2 mg/ml of insulin		
+ 0.08 mg/ml of insulin	64	+ 2.5 mM FDP	27	3
+ 0.01 mg/ml of insulin	92			
		+ 0.2 mg/ml of insulin		
+ 0.2 mg/ml of insulin		+ 5 mM α-D-glucose		
+ 0.5 mM 3',5'-AMP	40	+ 5 mM ADP	30	3

[a]The reversed enzyme was assayed after incubation for 5-7 hr at 16° in the reversal solution which, in addition to the buffer, contained 0.2 M KCl and 0.06 M β-mercaptoethanol.

[b]The reference activity is that obtained after reversal for 5-7 hr at 16° in a reversal solution which contained 0.05 M Tris (pH 8.0), 0.2 M KCl, and 0.06 M β-mercaptoethanol, and no metabolite. A reversal sample is the reference.

[c]No activity was recovered at 37° under any of these conditions.

Figure 9. SUCROSE DENSITY GRADIENT CENTRIFUGATION PATTERN OF NATIVE AND REVERSED PYRUVATE KINASE

The optimum reversal conditions were used except for the omission of 0.4 M KCl and the substitution of 0.1 M bicine for 0.05 M Tris-HCl. Approximately 0.01 mg of the native or renatured enzyme was layered on the sucrose gradient, and centrifuged for eighteen hours at 40,000 rpm at 2^{o}. The sucrose solutions also contained 0.02 M imidazole-HCl (pH 7.15), 0.15 M KCl, and 0.001 M EDTA.

Figure 5

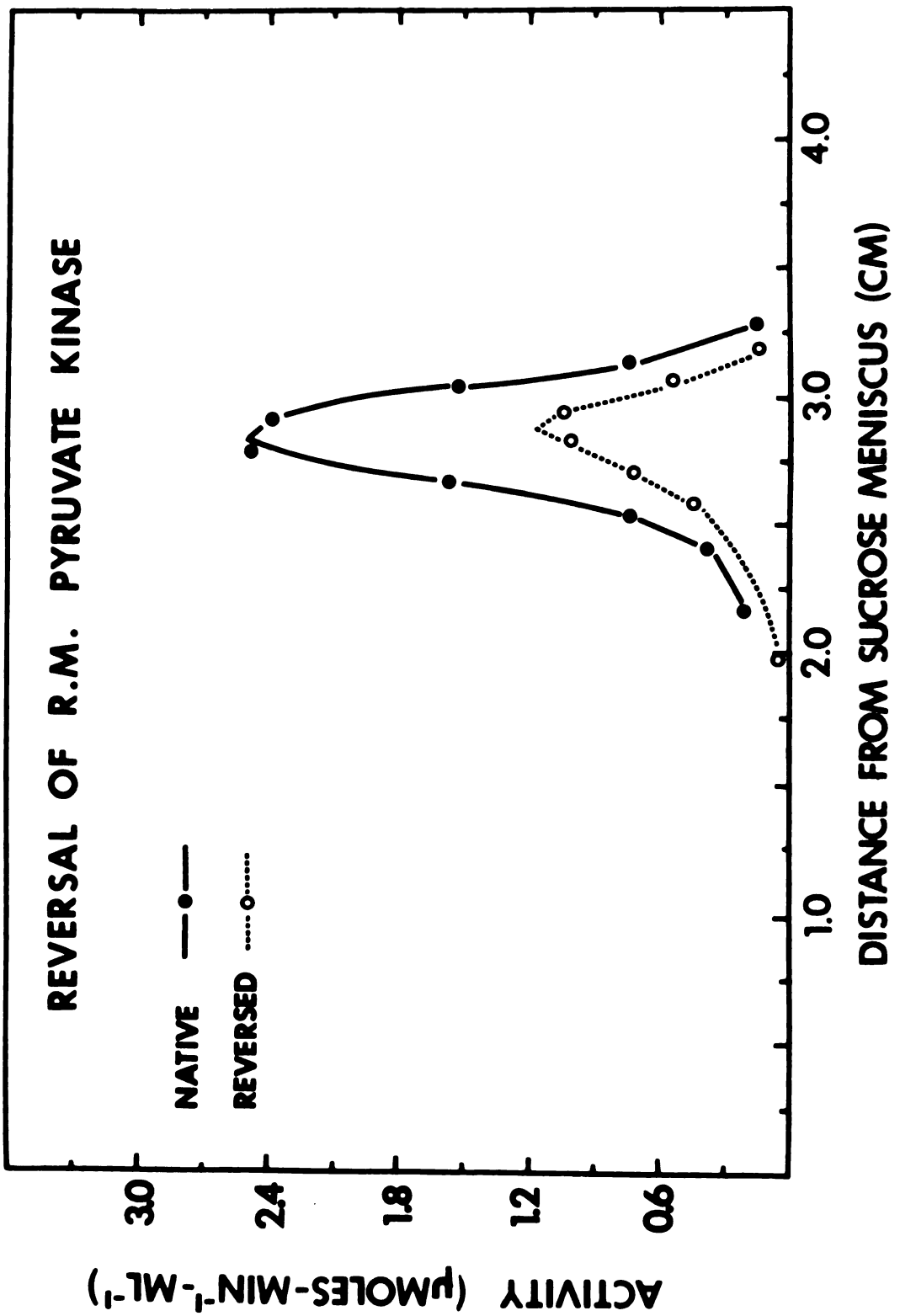

REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
REVERSED
ACTIVITY (μMOLES-MIN⁻¹-ML⁻¹)
0.6
1.2
1.8
2.4
3.0
1.0
2.0
3.0
4.0
DISTANCE FROM SUCROSE MENISCUS (CM)

Figure 10. LINEWEAVER-BURK ANALYSIS OF NATIVE AND REVERSED ENZYME WITH RESPECT TO ADP

The denatured enzyme was reversed under optimal conditions. The reversed enzyme was dialyzed for eleven hr at 4^{o} versus 100 volumes of 0.05 M Tris-HCl, pH 8.0. The final concentrations after dialysis were 0.05 M Tris-HCl, pH 8.0; 0.006 M KCl; 0.002 M $MgCl_2$. Aliquots of this enzyme solution were assayed at 25^{o}. Native enzyme was treated similarly. In addition to the ADP, the assay solution contained 0.1 M imidazole, pH 7.5; 0.1 M KCl; 2×10^{-2} M $MgCl_2$; and 3.3×10^{-4} M PEP.

Figure 10

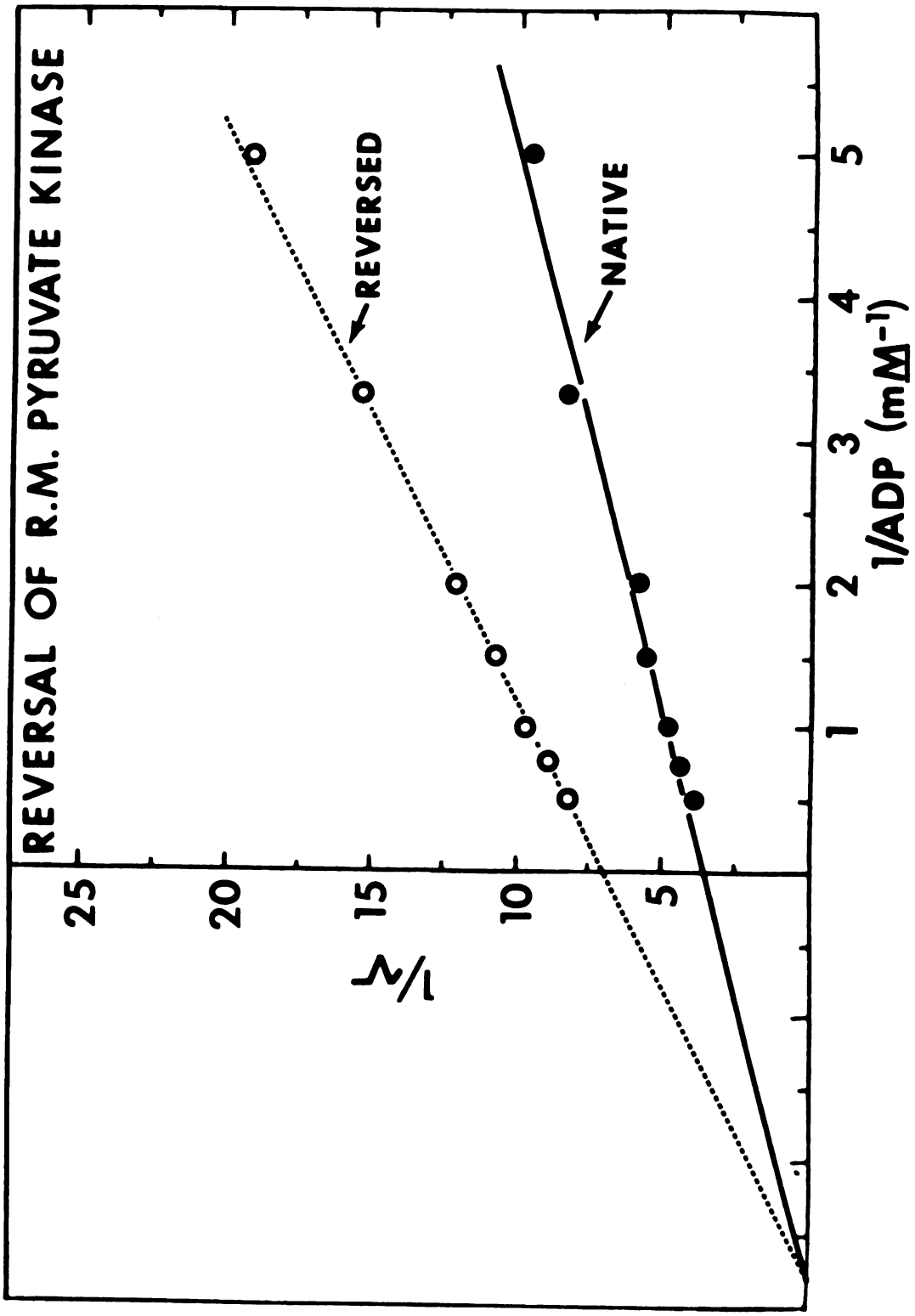

REVERSAL OF R.M. PYRUVATE KINASE
REVERSED
NATIVE
1/V
25
20
15
10
5
1
2
3
4
5
1/ADP (mM⁻¹)

Figure 11. LINEWEAVER-BURK ANALYSIS OF NATIVE AND REVERSED ENZYME WITH RESPECT TO KCl

See legend for Figure 10 for details. The assay solution also contained 2×10^{-3} M ADP.

Figure 11

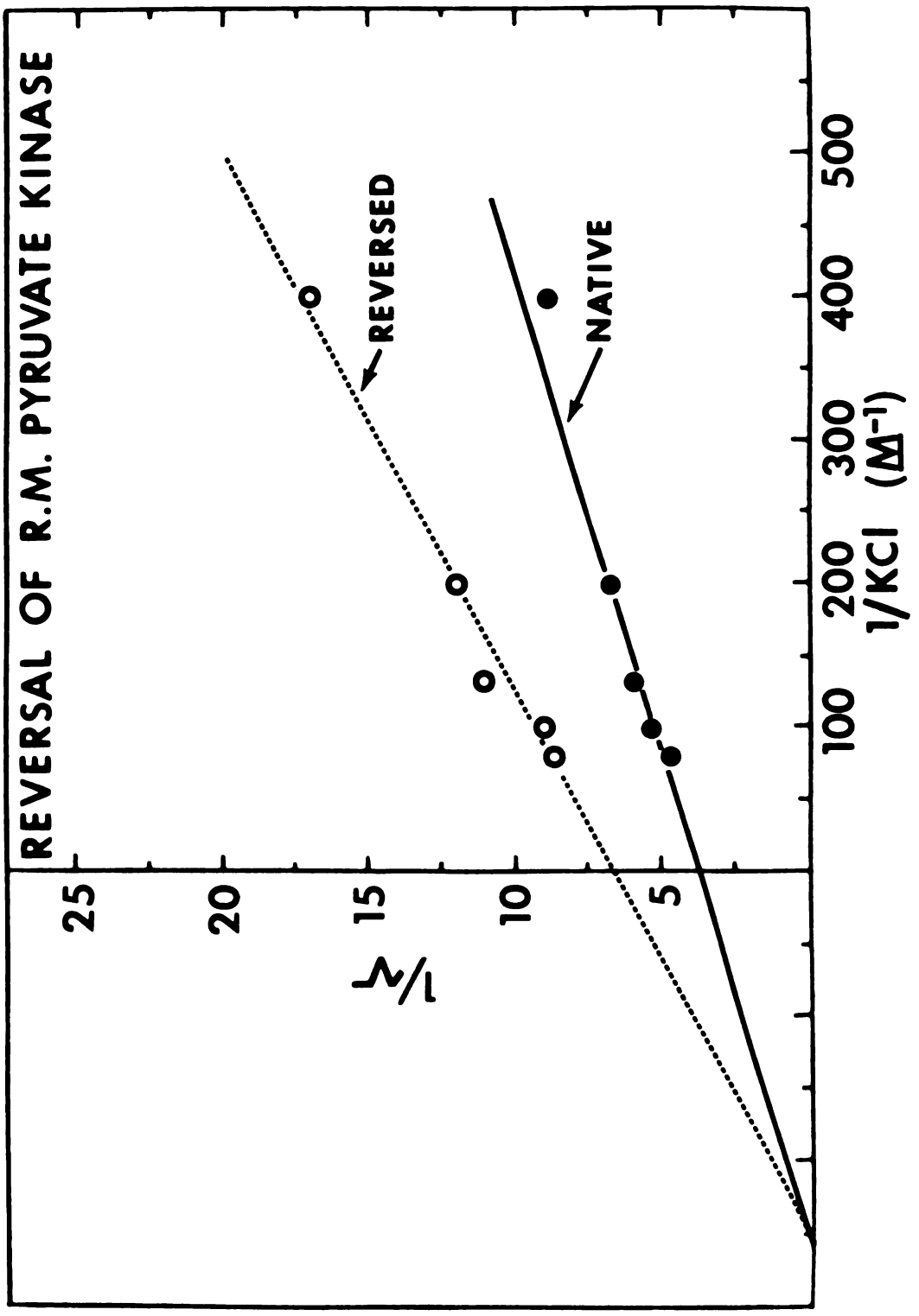
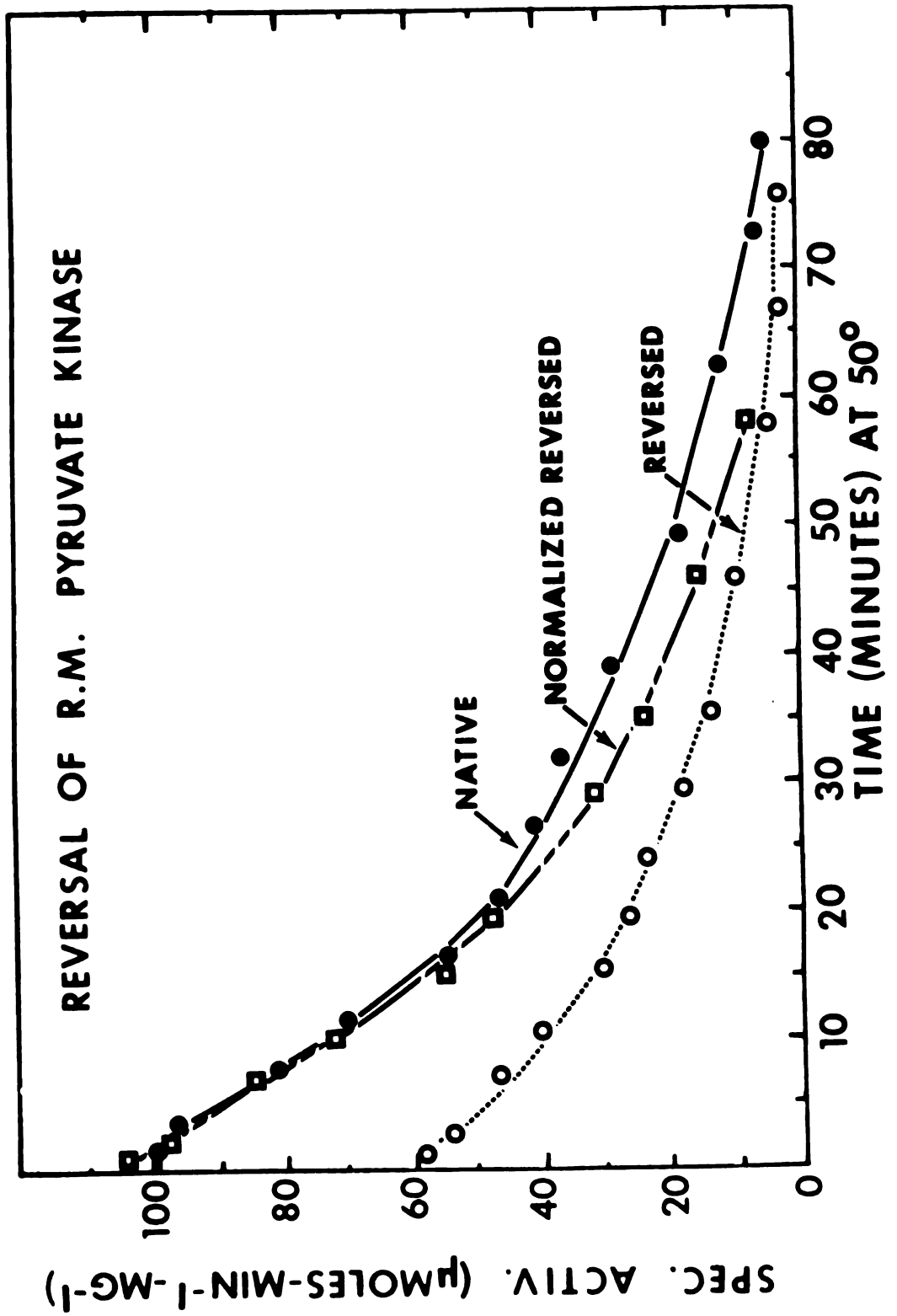

REVERSAL OF R.M. PYRUVATE KINASE
REVERSED
NATIVE
1/v
25
20
15
10
5
100
200
300
400
500
1/KCl (M⁻¹)

Figure 12. HEAT STABILITY OF NATIVE AND REVERSED ENZYMES AT 50°

The enzyme was treated as described in the legend for Figure 10. The enzyme solutions (native-0.02 mg/ml; reversed-0.036 mg/ml) were placed in a 50° temperature bath, and the enzymes were assayed at the appropriate times. The time at which the samples were brought to 16° was designated zero time. To better compare the stability of the two enzymes the reversed activity was multiplied by a factor of 1.8 (Normalized Reversed) to normalize it to that of native activity.

Figure 12


REVERSAL OF R.M. PYRUVATE KINASE
NATIVE
NORMALIZED REVERSED
REVERSED
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
TIME (MINUTES) AT 50°
0
20
40
60
80
100
10
20
30
40
50
60
70
80

recovery of activity upon removal of the denaturing agent by dialysis or gel filtration. Thus, the yeast glyceraldehyde-3-phosphate dehydrogenase reversal conditions and procedures (Deal, 1967; Deal, 1969) which served as the starting point for this study, have been found to give substantial recovery of activity for rabbit muscle pyruvate kinase, as they have for a number of other enzymes (see Deal, 1969).

As might be expected, the general optimum requirements for reversal of rabbit muscle pyruvate kinase are similar to those of these other enzymes. For the yeast glyceraldehyde-3-phosphate dehydrogenase reversal system and for pyruvate kinase in particular, two key factors in obtaining significant reversal were: (1) the removal of the denaturing agent by dilution of the guanidine-enzyme (0°) into a reversal solvent at 0° and then incubating the sample to higher temperatures ($12\text{-}35^{\circ}$) to produce the refolding-reassociation reactions and (2) the use of low protein concentrations, about 0.05 mg/ml, for the reversal process. Both of these factors seemed to operate to avoid nonspecific aggregations which would have caused the protein to precipitate, or irreversibly aggregate.

POSSIBLE EFFECTS OF METABOLITES. When this research was begun, the possibility seemed good that metabolites might affect the folding and synthesis of pyruvate kinase and/or its activity. Because of the possible direct relationship presumed (Deal, 1969) between folding control by metabolites *in vitro* and regulation of protein synthesis by inducers *in vivo*, it was natural to consider as prime candidates for "folding control" those compounds thought likely to be inducers (or repressors) of pyruvate kinase. In this connection the report by

Weber and co-workers (Weber et al., 1965; Weber et al., 1966) that insulin is an inducer of rat liver pyruvate kinase was of special interest. This raised the possibility that insulin, or some product of insulin action, might control the folding of pyruvate kinase. Unfortunately, the primary products of insulin action have not yet been elucidated. Therefore, within this group, the only feasible test was that of the ability of insulin to directly enhance reversal.

This study showed that under the conditions used, insulin did affect the reassembly of pyruvate kinase, albeit in a negative way rather than the positive way expected. It also showed that the binding was specific for insulin; neither bovine serum albumin nor ribonuclease had any effect on the reversal recovery. Furthermore, the insulin effect was not a nonspecific inhibition of enzyme activity, since the activity of the native enzyme was not significantly affected by insulin under identical conditions. Since insulin does interact with the unfolded polypeptide chains of pyruvate kinase, and since other variables may superimpose their effects onto those of insulin in vivo, the possibility cannot be ruled out that the insulin interaction might favor assembly in vivo, rather than impede it.

It also seemed reasonable that the metabolites expected to influence enzyme activity might be expected to be prime candidates for affecting the rates of synthesis of the enzyme. A number of compounds have been shown to affect the activity of muscle pyruvate kinase. The ion, K^+, was reported to be required for the enzyme to be in the proper conformation for activity (Melchior, 1965). The binding of substrates and activating cations was reported to have

resulted in a change in protein conformation (Kayne and Suelter, 1965). Other studies showed an interaction of the Mg^{2+}-ADP complex with the enzyme (Melchior, 1965). Weber et al. (1967) showed that acetyl CoA was an inhibitor of both liver and rat muscle pyruvate kinase.

Also, a number of compounds have been shown to affect the activity of the pyruvate kinase enzymes obtained from various sources other than muscle. Pyruvate kinase from E. coli was activated by both AMP and FDP (Maeba and Sanwal, 1968) and the enzyme from developing loach embryos was activated by 3',5'-AMP and FDP (Milman and Yurowitzki, 1967). The pyruvate kinases from yeast (Hess et al., 1966) and liver (Taylor and Bailey, 1967) were strongly activated by FDP. Although there has been no evidence that pyruvate kinase from rabbit muscle was activated by FDP, Taylor and Bailey (1967) have suggested that in vivo it might have possessed this characteristic and lost it during isolation and purification of the enzyme.

Of the metabolites and ions tested in this study, only phosphate and insulin affected the in vitro folding and assembly of pyruvate kinase from its unfolded subunits. Phosphate seemed to be absolutely required for this process at 36°, although not at 16°. Since this is near the temperature at which the in vivo folding of rabbit muscle pyruvate kinase occurs, this may be a very significant requirement.

In this regard, it is of interest that the rabbit muscle pyruvate kinase apparently can exist in two temperature-dependent states (Kayne and Suelter, 1968). Since the midpoint of the transition from one state to the other is in the range of 16°-22°, it is possible and even likely that the low temperature form, which does

not require phosphate for folding, may not occur *in vivo*. That is, possibly only the high temperature form occurs *in vivo* and it may require phosphate for folding. However, an alternative possibility is that phosphate keeps the enzyme in the low temperature form which reverses easily. Also, the possibility cannot be overlooked that *in vivo* other effects might operate in conjunction with the phosphate effect. These subjects are receiving further study.

In general, the muscle is expected to have a considerably different set of metabolic priorities and types of controls than liver or yeast because it does not have the ability to carry out gluconeogenesis, nor does it have, in significant amounts, many of the other metabolic pathways (such as the pentose phosphate cycle) which liver and yeast possess. It will thus be of much interest to compare these results for rabbit muscle pyruvate kinase with pyruvate kinases from other sources.

MATERIALS AND METHODS

ENZYME PREPARATION AND ASSAY. Pyruvate kinase (EC 2.7.1.40) was isolated from frozen rabbit muscle (Pel Freez Biologicals, Inc.) using the modifications (Steinmetz and Deal, 1966) of the method of Tietz and Ochoa (1958). The enzyme was assayed at 25° by coupling the pyruvate kinase reaction to the lactic dehydrogenase reaction and following the decrease in absorbancy at 340 mμ (Kubowitz and Ott, 1944) using a Beckman DU spectrophotometer attached to a Gilford multiple sample absorbance recorder. Enzyme concentrations were determined by measuring the optical density at 280 mμ and using the extinction coef-

ficient of ϵ_{280} mμ = 0.54 ml mg^{-1} cm^{-1} (Bücher and Pfleiderer, 1955). The specific activity of the native enzyme was found to be 140-160 μmoles of NADH consumed/min/mg protein. The enzyme concentration in the assay was 0.2 μg/ml and the reaction was monitored for 1 min.

REAGENTS. Reagent grade chemicals were obtained from the following commercial sources: Tris (Trizma base), PEP (tricyclohexylamine salt), glucose-6-phosphate (disodium slat), bovine serum albumin, bovine pancreas ribonuclease, AMP (sodium salt), bovine pancreas insulin, FDP (sodium salt), 3',5'-AMP (sodium salt), and lactic acid from Sigma (St. Louis); ATP (sodium salt), ADP (sodium salt), β-NADH (disodium salt), and β-NAD^+ (disodium salt) from P-L Biochemicals (Milwaukee); tricine and bicine (Good et al., 1966) and dithiothreitol from CalBiochem; imidazole and tetramethylammonium chloride from Eastman (Rochester, N.Y.); glycine from General Biochemicals (Chagrin Falls, Ohio); 3-mercapto-1,2-propanediol from Aldrich Chemical Co. (Milwaukee); β-mercaptoethanol from Matheson Scientific (Elk Grove Village, Illinois); α-D-glucose from Pfanstiehl Laboratories, Inc. (Waukegan, Illinois); and beef heart lactic dehydrogenase from Worthington (Freehold, New Jersey).

Urea (Baker Analytical Reagent) was recrystallized from absolute ethanol and allowed to dry at 50° to remove residual ethanol. Guanidine-HCl was prepared (Anson, 1941) from Guanidine carbonate (Eastman, Rochester, N.Y.), and recrystallized from absolute ethanol. The crystals were then dried at 50°. All pH measurements and buffer adjustments were made at 24°.

DISSOCIATION PROCEDURE. Unless otherwise indicated, the denaturation in all experiments was conducted in the following manner: stock enzyme solution (20 mg/ml, 0.02 imidazole (pH 7.0), 0.001 M EDTA) was diluted to 4 mg/ml in freshly prepared dissociation solution and allowed to remain there for one hr in an ice bath. The dissociation solution was at pH 8 and consisted of 7 M guanidine-HCl or 7 M urea, 0.12 M β-mercaptoethanol, 0.04 M Tris-HCl and 0.001 M EDTA.

Activity recoveries using urea or guanidine were identical. The buffer species or pH of dissociation were not important since dissociation at pH 6.5 (potassium phosphate), pH 7.3 (imidazole), and pH 8.0 (Tris) all gave essentially the same activity recoveries upon reversal. Also, omission of EDTA or β-mercaptoethanol from the dissociation did not affect the reversal recovery. The use of a 100-fold dilution of urea- or guanidine-enzyme, which resulted in only 0.06 M residual denaturing agent, yielded high recoveries. This level of residual denaturing agent had no effect on the activity of native control samples. Although this does not exclude the possibility that the level of residual denaturing agent might interfere with the subunit refolding process, it does seem unlikely.

REVERSAL PROCEDURE. The renaturation conditions and procedures followed were those previously described for the renaturation of glyceraldehyde-3-phosphate dehydrogenase (Deal, 1967; Deal and Constantinides, 1967; Deal, 1969). The optimal conditions for the reversal were: (1) pH 8, 0.05 M Tris-HCl, (2) protein concentration, 0.04 mg/ml, (3) salt, 0.4 M KCl plus 0.1 M $MgCl_2$, (4) reducing agent, 0.06 M β-mercaptoethanol, and (5) temperature--0° dilution, followed by six hr at 16°. These optimal

conditions were used for reversal except where designated in the appropriate legends. The dissociated enzyme was diluted with careful swirling into the reversal solution which had been previously cooled in an ice bath to approximately 0°. The samples were then taken to 16° for incubation. Although in some cases the conditions used varied slightly, they remained in the optimal range plateau in all cases. The reducing agent was added to the stock reversal solvent just prior to the protein dilution to prevent undesirable air oxidation of the sulfhydryl groups. In each experiment, control samples were subjected to identical treatment except for the presence of urea or guanidine. Duplicate samples were usually run.

COMPARISON STUDIES. The sucrose density centrifugation experiment followed the procedure of Martin and Ames (1961). The Spinco Model L ultracentrifuge was used and the SW-39 rotor was run at 40,000 rpm at 2° for 18 hr.

For the heat stability and Lineweaver-Burk analyses, the reversed and native enzymes (0.04 mg/ml) in 0.05 M Tris (pH 8.0), 0.3 M KCl, 0.1 M $MgCl_2$, and 0.06 M β-mercaptoethanol were dialyzed 12 hr against 50 volumes of 0.05 M Tris, pH 8.0.

The half-time for the inactivation at 50° under these conditions was about 18 min. This temperature appears to be near the transition point, since at 42° there was only 25 % activity loss in one hr.

CHAPTER TWO

INACTIVATION OF TETRAMERIC RABBIT MUSCLE PYRUVATE KINASE BY SPECIFIC BINDING OF 2-4 MOLES OF PYRIDOXAL-5'-PHOSPHATE

ABSTRACT

Rabbit muscle pyruvate kinase is 90 % inactivated by binding 2-4 moles of pyridoxal-5'-phosphate (PLP) per mole of tetrameric enzyme. Incubation with PLP in 0.2 M imidazole (pH 7.5) at 25° results in a time-dependent loss of enzymatic activity which reaches a final value in 10-20 min at 25°. Half maximal loss of activity occurs with 0.04 mM PLP. The inactivation does not cause a gross conformational change. The inactivation is first order with respect to PLP concentration and enzyme concentration; the second order rate constant is 37 M^{-1} sec^{-1}. Increasing ionic strength decreases the rate of inactivation by PLP but low concentrations (1-10 mM) of divalent cations increase it above the level with no salt. The phosphate containing metabolites, ADP, ATP, phosphoenolpyruvate, and fructose-diphosphate also decrease the rate; the effect is more pronounced with low concentrations (0.1 mM) of divalent cations. The inactivation is relatively specific for PLP since various analogues including pyridoxamine, pyridoxamine-5-phosphate, and pyridoxal cause little or no inactivation. Reduction with $NaBH_4$ at various concentrations of PLP shows that there are 2 types of binding: (1) a specific, inactivating binding, involving 2-4 moles PLP bound per mole enzyme, and (2) a nonspecific, noninactivating binding which can involve up to about 20 additional moles PLP bound per mole enzyme. Both types involve Schiff base formation with ϵ-NH_2 groups of lysine. Reversal of the inactivation is accomplished, only with unreduced enzyme, by dilution, by dialysis, or by addition of Tris.

INTRODUCTION

Pyridoxal-5'-phosphate (PLP) is required for many enzymatically catalyzed reactions, such as transamination and decarboxylation, in which it participates through formation of a Schiff base. (For a review, see (Fasella, 1967)). In addition, PLP has a different function in several enzyme systems. It is required for the proper quaternary structure of tryptophanase (Morino and Snell, 1967) and L-aspartate β-decarboxylase (Tate and Meister, 1969). Also PLP is required for glycogen phosphorylase activity but reduction of the PLP-enzyme complex with sodium borohydride does not alter the enzymatic activity (Fisher et al., 1958).

Aside from its normal functions in enzyme catalysis, PLP, with its active aldehyde group, is an ideal reagent for chemical modification of enzymes as a means of identifying and studying enzyme functional groups involved in catalysis. For example, through formation of a Schiff base with ε-amino groups of lysine residues, it inactivates the following enzymes: glutamic dehydrogenase (Anderson et al., 1966), hexokinase (Grillo, 1968), 6-phosphogluconate dehydrogenase (Rippa et al., 1967), and fructose-1,6-diphosphate aldolase (Shapiro et al., 1968). It also inactivates adenylic acid deaminase (Kaldor and Weinbach, 1966) but the mechanism for inactivation has not been determined. With aldolase and 6-phosphogluconate dehydrogenase, PLP is thought to bind specifically to the same site as the phosphate of the substrate. It also binds to bovine plasma albumin (Dempsey and Christensen, 1962), through at least three types of binding sites, all involving the ε-amino groups of lysines. Treatment of fructose-1,6-diphosphatase with PLP

followed by reduction with sodium borohydride yields an active pyridoxamine-phosphate derivative of the enzyme which is no longer sensitive to allosteric AMP inhibition (Marcus and Hubert, 1968; Krulwich et al., 1969). PLP lowers the oxygen affinity of hemoglobin (Benesch et al., 1969).

In the course of a study of the effects of metabolites upon the reassembly of rabbit muscle pyruvate kinase (E.C. 2.7.1.40) from unfolded subunits (Johnson et al., 1969), we discovered that pyruvate kinase was inactivated in the presence of PLP. Since little was known about the functional groups involved in the catalytic mechanism of pyruvate kinase, we undertook a detailed study of this effect to learn more about the mechanism of catalysis as well as to evaluate this effect as a possible mechanism for in vivo control of the enzyme.

We present here a detailed analysis of the inactivation of pyruvate kinase by PLP. Evidence is presented for a PLP binding to 2-4 specific lysine groups. The results also provide further information on the roles of substrates and divalent cations in the reaction mechanism. A preliminary report of this research has been published (Johnson and Deal, 1969).

RESULTS

Incubation of rabbit muscle pyruvate kinase with various concentrations of PLP in 0.2 M imidazole (pH 7.5) at 25° resulted in a time-dependent loss of enzymatic activity which reached a final value within 10-20 min (Figure 13). No further change occurred with continued incubation up to 7 hr, so it was assumed that these represented equilibrium values. The midpoint or 50 % point for the inactivation

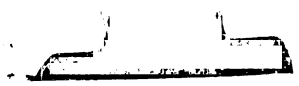
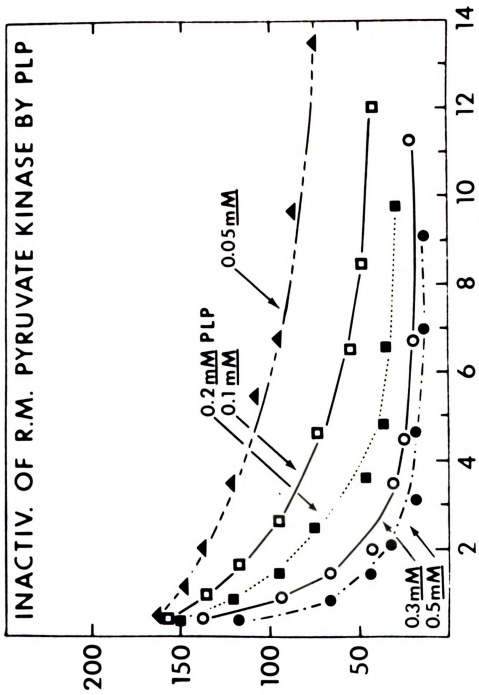


Figure 13. EFFECT OF PYRIDOXAL-5'-PHOSPHATE (PLP) CONCENTRATION ON THE RATE OF INACTIVATION OF RABBIT MUSCLE PYRUVATE KINASE

Samples of pyruvate kinase (0.042 mg per ml) were incubated with various concentrations of PLP in 0.2 M imidazole, pH 7.5, at 25°. Aliquots were assayed at the indicated times.

Figure 13


INACTIV. OF R.M. PYRUVATE KINASE BY PLP
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
TIME – MINUTES
0.2 mM PLP
0.1 mM
0.05mM
0.3mM
0.5mM
200
150
100
50
2
4
6
8
10
12
14

transition was determined to be 0.04 mM from a graph of equilibrium activity values as a function of PLP concentrations (Figure 14). Inhibition was most pronounced at pH 7.5 (Figure 15) so this pH was chosen for the detailed analysis.

The inhibition was relatively specific for PLP. Incubation for 30 min at 25° with 1 mM pyridoxal caused only a 20 % loss of activity (see Table 5 for additional data). Similar treatments with 5 mM pyridoxamine, 5 mM pyridoxamine-5'-phosphate, 0.1 mM acetaldehyde, or 0.1 mM benzaldehyde had no effect on the activity.

Additional experiments were performed to check directly for instantaneous inhibition of pyruvate kinase activity by pyridoxal compounds during the assay. In direct spectrophotometric assays, which couple the reactions of pyruvate kinase and lactate dehydrogenase, an apparent inhibition was observed with PLP, pyridoxamine, or pyridoxamine-5'-phosphate, but not with pyridoxal. The apparent K_i, about 1 mM, was not due to inhibition of pyruvate kinase, since this effect was not observed in the direct measurement of pyruvate kinase activity using the pH stat. Direct assays of lactate dehydrogenase in the presence of these compounds confirmed that the interference was with this reaction.

SPECTRAL ANALYSIS. The spectrum of the mixture of PLP and pyruvate kinase was consistent with that expected for the formation of a Schiff base between PLP and the ϵ-NH_2 group of lysine residues (Shaprio et al., 1968) showing absorption bands at 432 mμ and 342 mμ (Figure 16). The 432 mμ absorbance has been attributed to the presence of a protonated Schiff base and the 342 mμ absorbance to an unsubstituted aldimine

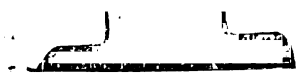


Figure 14. EFFECT OF PLP CONCENTRATION ON THE PYRUVATE KINASE ACTIVITY OBTAINED AT EQUILIBRIUM

Data from the experiment described in Figure 1. The data shown are the final limiting values obtained after 15-20 minutes incubation.

Figure 14

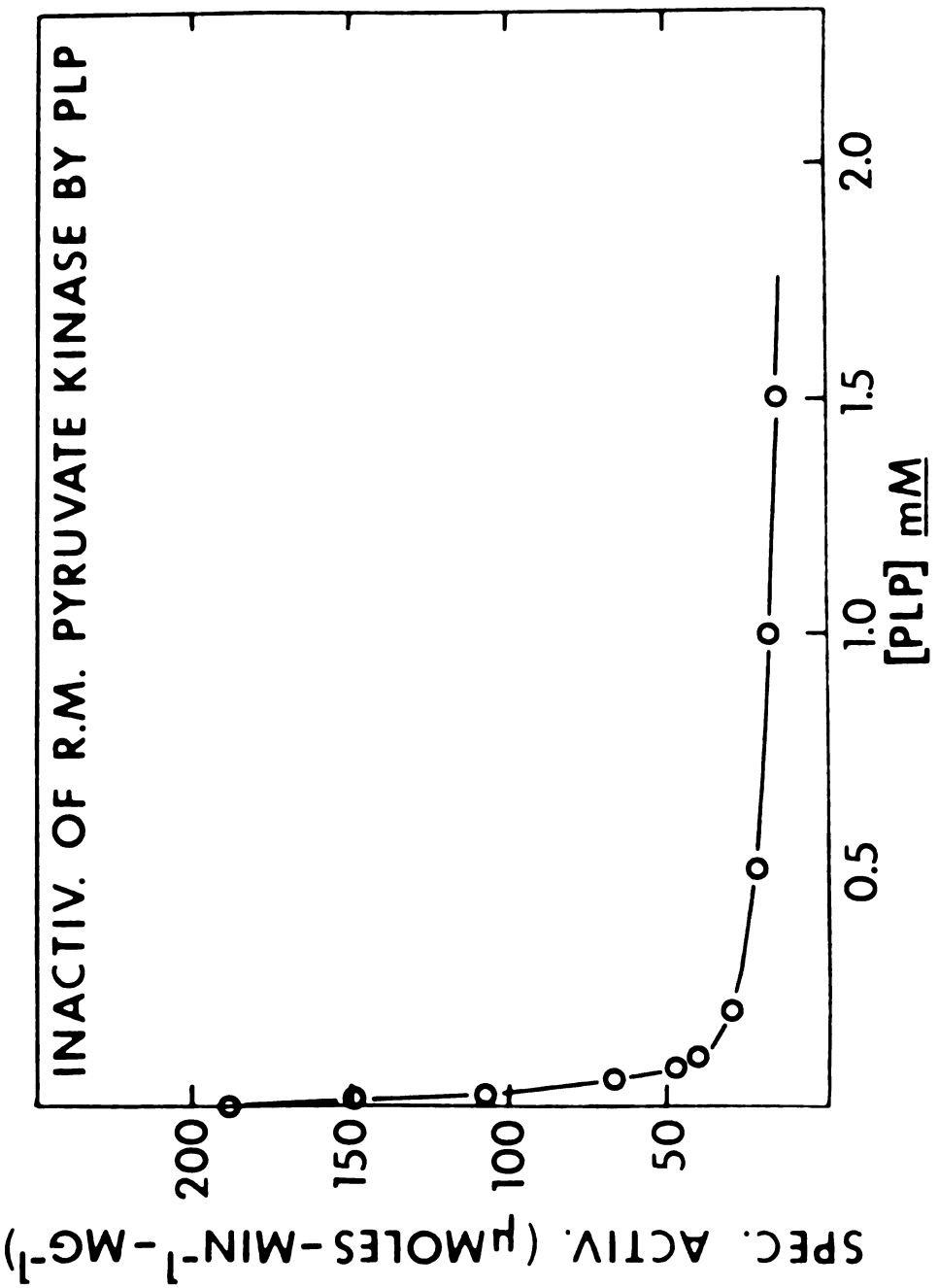

INACTIV. OF R.M. PYRUVATE KINASE BY PLP
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
200
150
100
50
0.5
1.0
1.5
2.0
[PLP] mM

Figure 14. EFFECT OF PLP CONCENTRATION ON THE PYRUVATE KINASE ACTIVITY OBTAINED AT EQUILIBRIUM

Data from the experiment described in Figure 1. The data shown are the final limiting values obtained after 15-20 minutes incubation.

Figure 14

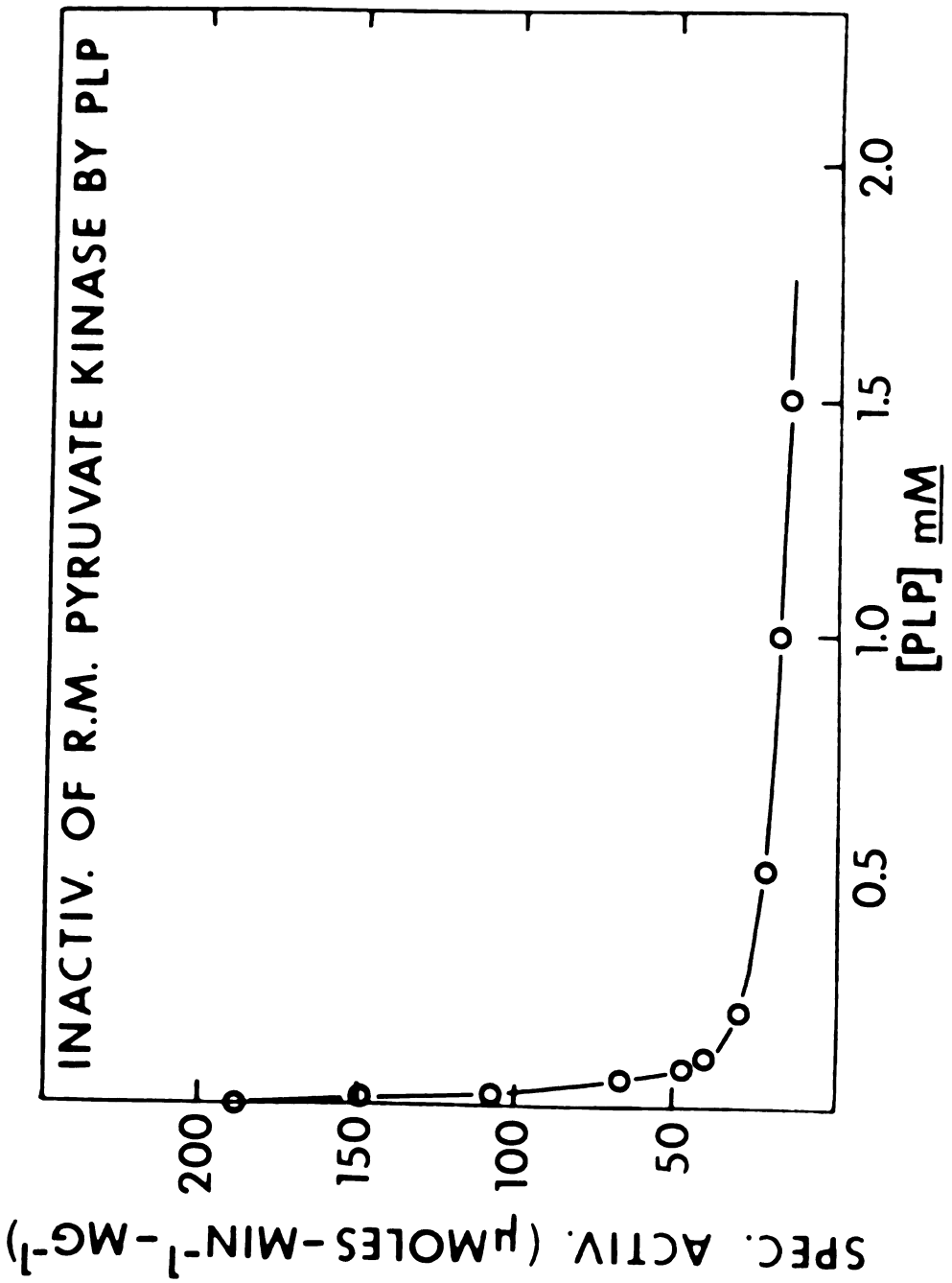

INACTIV. OF R.M. PYRUVATE KINASE BY PLP
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
200
150
100
50
0.5
1.0
1.5
2.0
[PLP] mM

Figure 15. EFFECT OF pH ON THE INACTIVATION

The stock enzyme solution (see Methods) was diluted to 3.2 mg per ml in 0.2 M imidazole, pH 7.5. Enzyme samples at various pH values were prepared by diluting this enzyme with various 0.2 M buffers and an appropriate quantity of PLP to give a final protein concentration of 0.047 mg per ml and a final PLP concentration of 0.25 mM. These solutions were incubated for 90 minutes at 25°. Aliquots were assayed in the various buffers at the indicated values of pH. Controls were treated similarly except PLP was omitted.

Figure 15

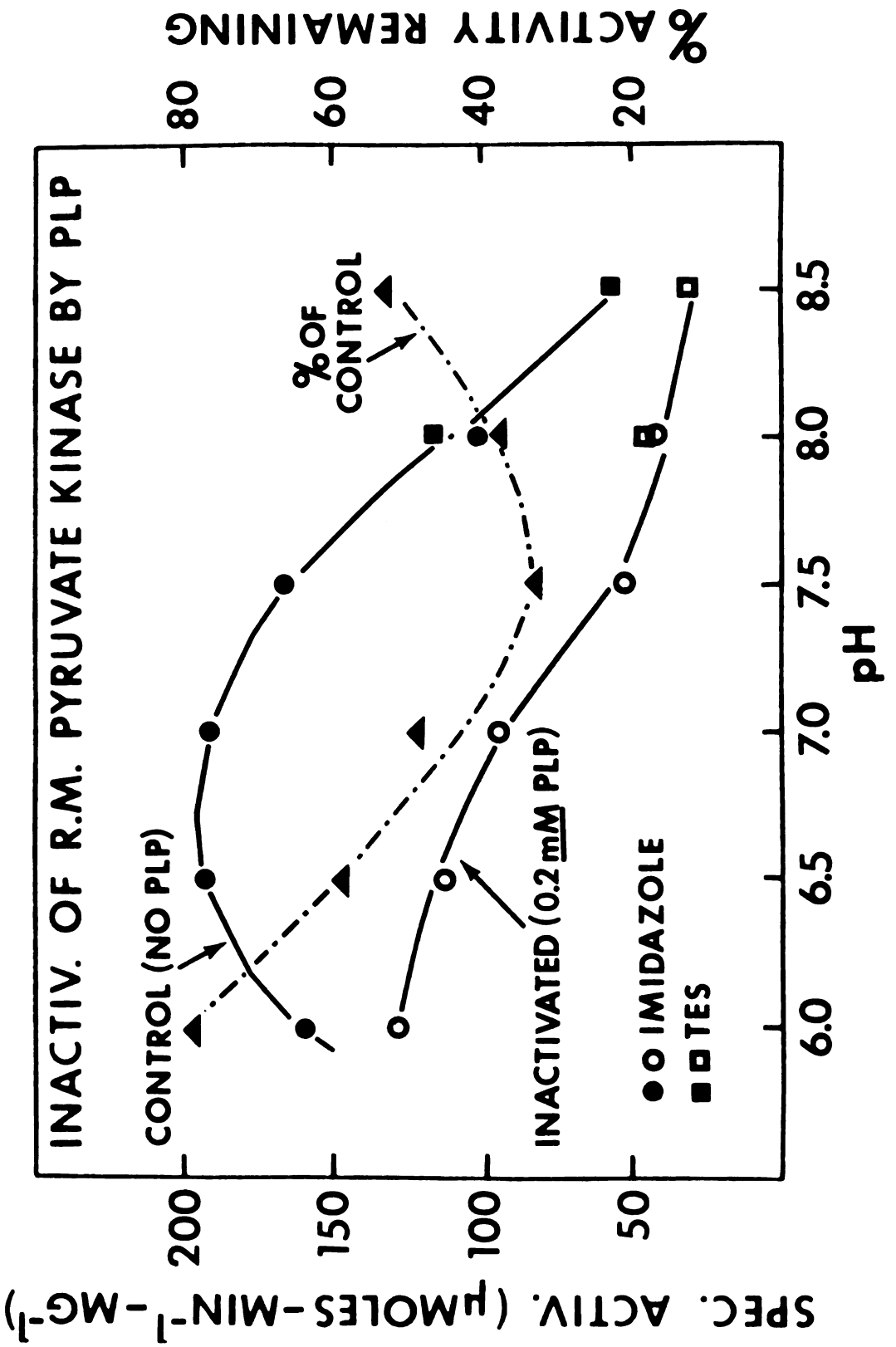
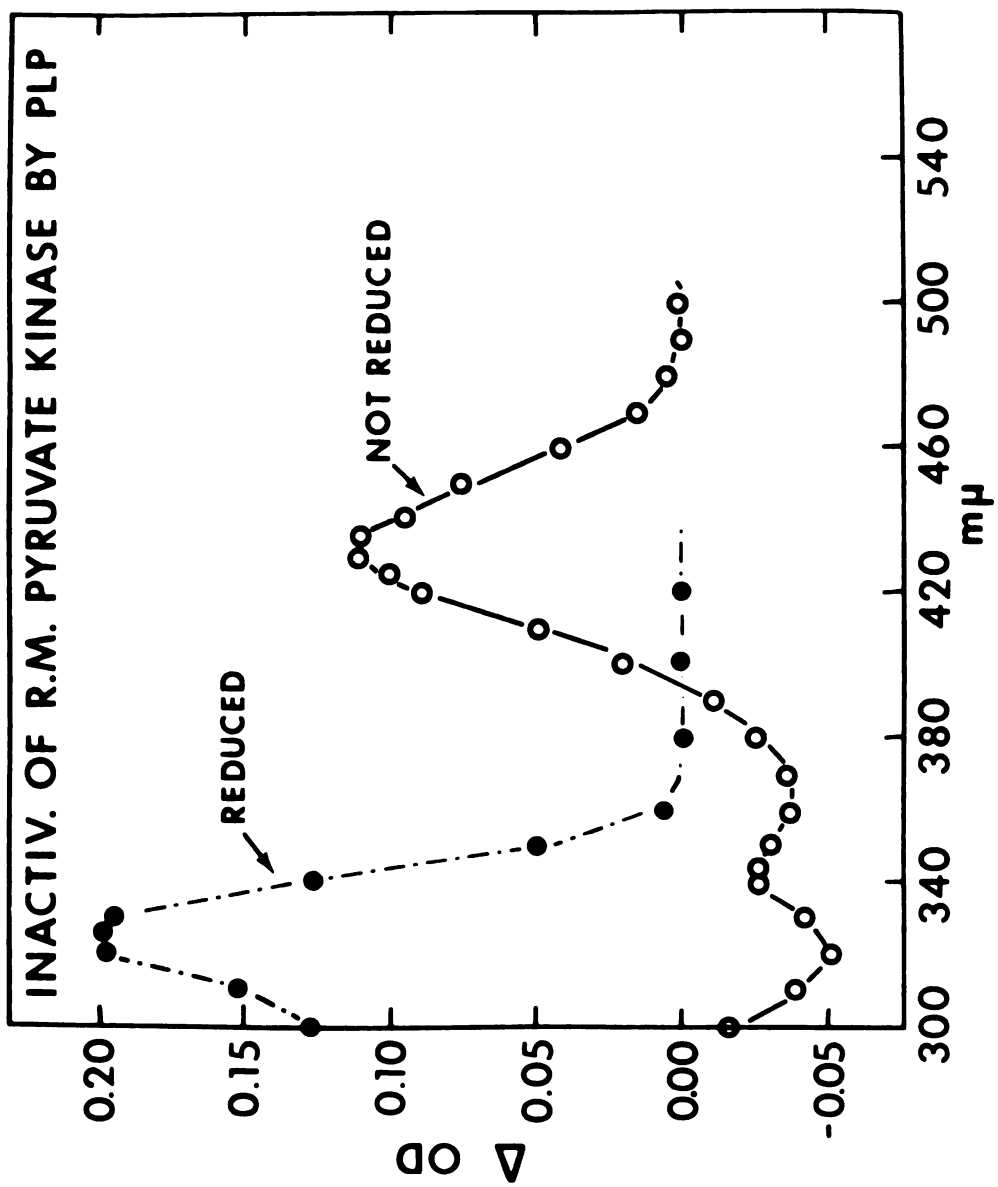

INACTIV. OF R.M. PYRUVATE KINASE BY PLP
% ACTIVITY REMAINING
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
pH
CONTROL (NO PLP)
INACTIVATED (0.2 mM PLP)
% OF CONTROL
● ○ IMIDAZOLE
■ □ TES

Figure 16. ABSORPTION SPECTRA OF THE REDUCED AND NONREDUCED PYRUVATE KINASE-PLP COMPLEXES

Nonreduced: Pyruvate kinase (2.7 mg per ml) was incubated with 0.08 m$\underline{M}$ PLP for 30 minutes at 25°. The double-tandem reference cuvettes contained 0.08 m$\underline{M}$ PLP in one half and 2.7 mg per ml pyruvate kinase in the other half. Spectra obtained using a Cary 15 Spectrophotometer. Reduced: Pyruvate kinase (0.4 mg per ml) was treated and reduced with sodium borohydride as described in Methods. The reference was a sample treated identically except PLP was omitted. Spectra obtained using a Beckman spectrophotometer equipped with a Gilford absorbance indicator attachment.

Figure 16


INACTIV. OF R.M. PYRUVATE KINASE BY PLP
REDUCED
NOT REDUCED
Δ OD
0.20
0.15
0.10
0.05
0.00
-0.05
300
340
380
420
460
500
540
mμ

(Metzler, 1957; Snell, 1958). The PLP-pyruvate kinase complex reduced with $NaBH_4$ had an absorption maximum (Figure 16) at 325 mμ as expected (Fischer et al., 1959; Matsuo and Greenberg, 1959).

REDUCTION WITH SODIUM BOROHYDRIDE. Reducation of the pyruvate kinase-PLP complexes at various concentrations of PLP yielded covalent complexes with different ratios of moles PLP bound per mole enzyme (Figure 17). The biphasic character of the curve suggested two different types of binding sites: (a) a strongly binding, inactivating type and (b) a weakly binding; noninactivating type. It appeared that there were about 4 strongly binding sites and about 20 or more weakly binding sites. The data show that up to 25 moles of PLP were bound per mole of protein. However, since amino acid analysis (Kayne, 1966; Cottam et al., 1969) indicates the rabbit muscle pyruvate kinase contains about 150-160 lysine residues, it is likely that considerably more PLP would be bound at higher PLP concentrations.

The data (Figure 17) also show that PLP binding at the strongly binding sites accounted for almost all of the inactivation of the enzyme and that PLP binding at the weakly binding sites did not seem to inactivate the enzyme; it retained 10 % residual activity, even with 25 moles PLP bound per mole enzyme. This suggested that the strongly-binding, inactivating sites may be specific and that the weakly binding, noninactivating sites may be non-specific, since they include as many as 25 of the approximately 150-160 remaining potential binding sites (Kayne, 1966; Cottam et al., 1969).

However, the data in Table 5 showing that 1.4 moles PLP per mole enzyme could be bound with only 5 % inactivation, not the pre-

Figure 17. ACTIVITY OF PYRUVATE KINASE AS A FUNCTION OF MOLES OF PLP COVALENTLY REDUCED ONTO IT

The PLP covalent complexes with pyruvate kinase were prepared by sodium borohydride reduction of mixtures of pyruvate kinase (0.4 mg per ml) and PLP (incubation concentrations in parentheses) as described in Methods. The results also show the correlation between moles PLP bound per mole enzyme and PLP incubation concentration.

Figure 17

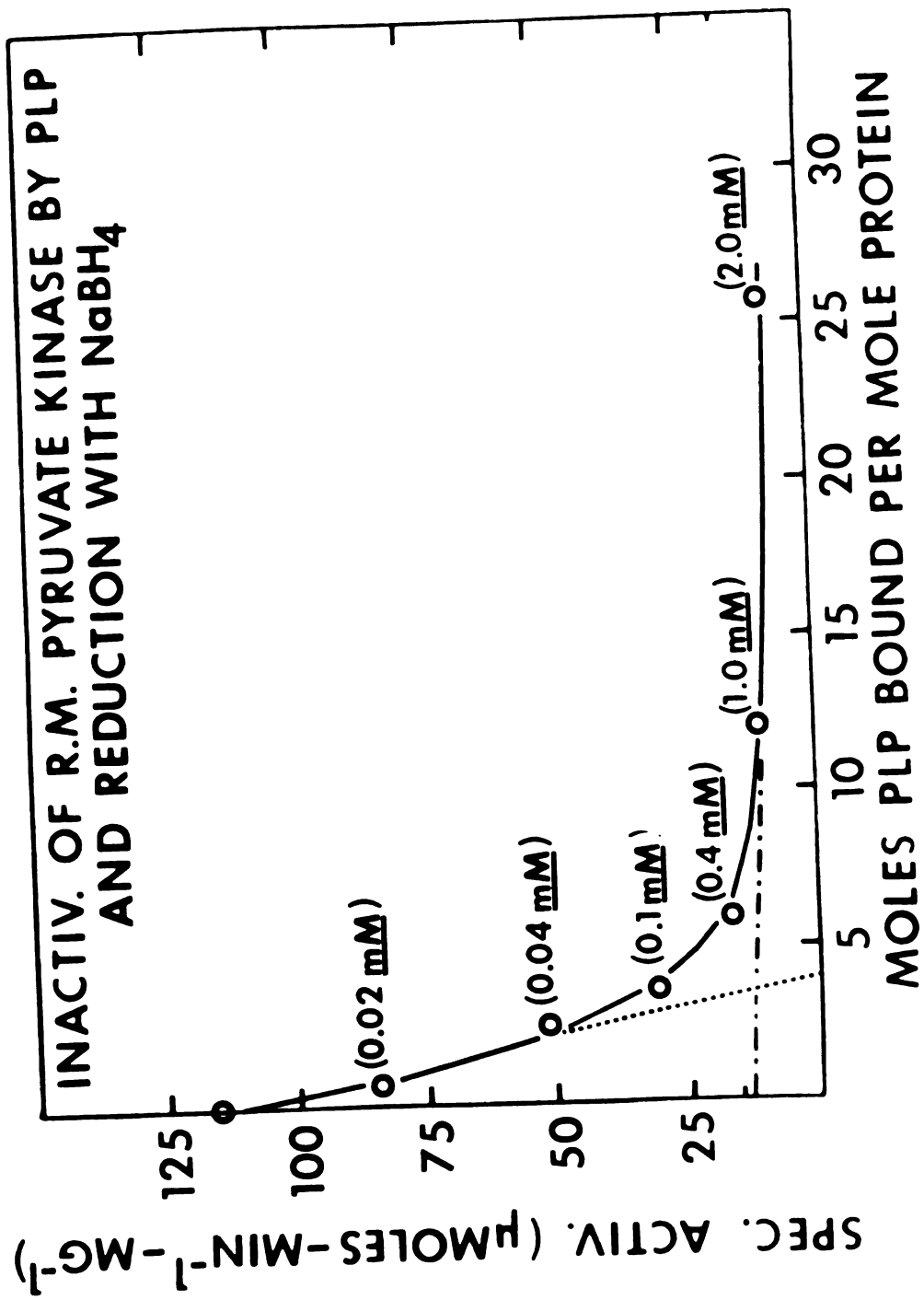

INACTIV. OF R.M. PYRUVATE KINASE BY PLP AND REDUCTION WITH $NaBH_4$
SPEC. ACTIV. (μMOLES-MIN^{-1}-MG^{-1})
MOLES PLP BOUND PER MOLE PROTEIN
125
100
75
50
25
5
10
15
20
25
30
(0.02 mM)
(0.04 mM)
(0.1 mM)
(0.4 mM)
(1.0 mM)
(2.0 mM)

TABLE 5. SODIUM BOROHYDRIDE REDUCTION OF COMPLEXES OF PYRUVATE KINASE AND PYRIDOXAL COMPOUNDS

The solutions containing pyruvate kinase (0.39 mg per ml) in 0.2 M imidazole, pH 7.5, were incubated for 30 min at 25° with the indicated concentration of inactivation compound. The complexes of pyruvate kinase and pyridoxal compound were reduced with sodium borohydride and dialyzed for 18-24 hr at 4°.

Compound Present	Moles Comp. red./Mole prot.	% Act. Remaining
None	---	100
0.2 mM pyridoxal-5'-Phosphate	4.7	17
0.8 mM pyridoxal-5'-Phosphate[a]	1.4	95
0.8 mM pyridoxal	1.6	96
4.0 mM pyridoxal	3.3	70
2.0 mM pyridoxal then, 0.2 mM pyridoxal-5'-Phosphate[b]	---	17

[a] No 30 min incubation prior to the reduction.

[b] 30 min incubation with pyridoxal, then 30 min incubation with PLP.

dicted 30 %, indicated that the distinction between types of binding sites was not absolute. Thus, as many as 2 of the 4 strongly binding sites may be noninactivating.

The specific (strongly binding) and nonspecific (weakly binding) sites were not distinguishable by their spectral properties, since complexes with 3.6 or 12 moles of PLP bound per mole of enzyme (Figure 17) had the same absorption maximum (325 mμ) and had no apparent differences in the shapes of their curves except for the increased extinction due to increased numbers of PLP bound.

Enzyme complexes with pyridoxal were also reduced with sodium borohydride (Table 5). These results show that pyridoxal did bind and inactivate but much less effectively than PLP. Sodium borohydride had no effect on the activity of the native enzyme.

RATE OF INACTIVATION. Since the rate of pyruvate kinase inactivation was dependent upon the concentration of PLP employed (Figure 13), experiments were conducted to determine the order of the reaction. Logarithmic plots of residual activity versus time (Figure 18) were linear, indicating that the reaction was first-order with respect to enzyme concentration. In addition, the rate of inactivation was directly proportional to PLP with the four concentrations tested (Figure 19), showing that the inactivation was first-order with respect to PLP concentration.

However, increasing the concentration to 0.5 mM did not increase the rate of inactivation proportionally (Figure 19). But this point is subject to large experimental error, as can be seen from the very steep curve at this concentration (Figure 13).

Figure 18. LOGARITHMIC PLOTS OF PYRUVATE KINASE ACTIVITY WITH TIME AT VARYING PLP CONCENTRATIONS

The data from Figure 13 is plotted to allow calculation of the pseudo first-order rate constants for the inactivation. (See Methods and legend for Figure 13.)

Figure 18

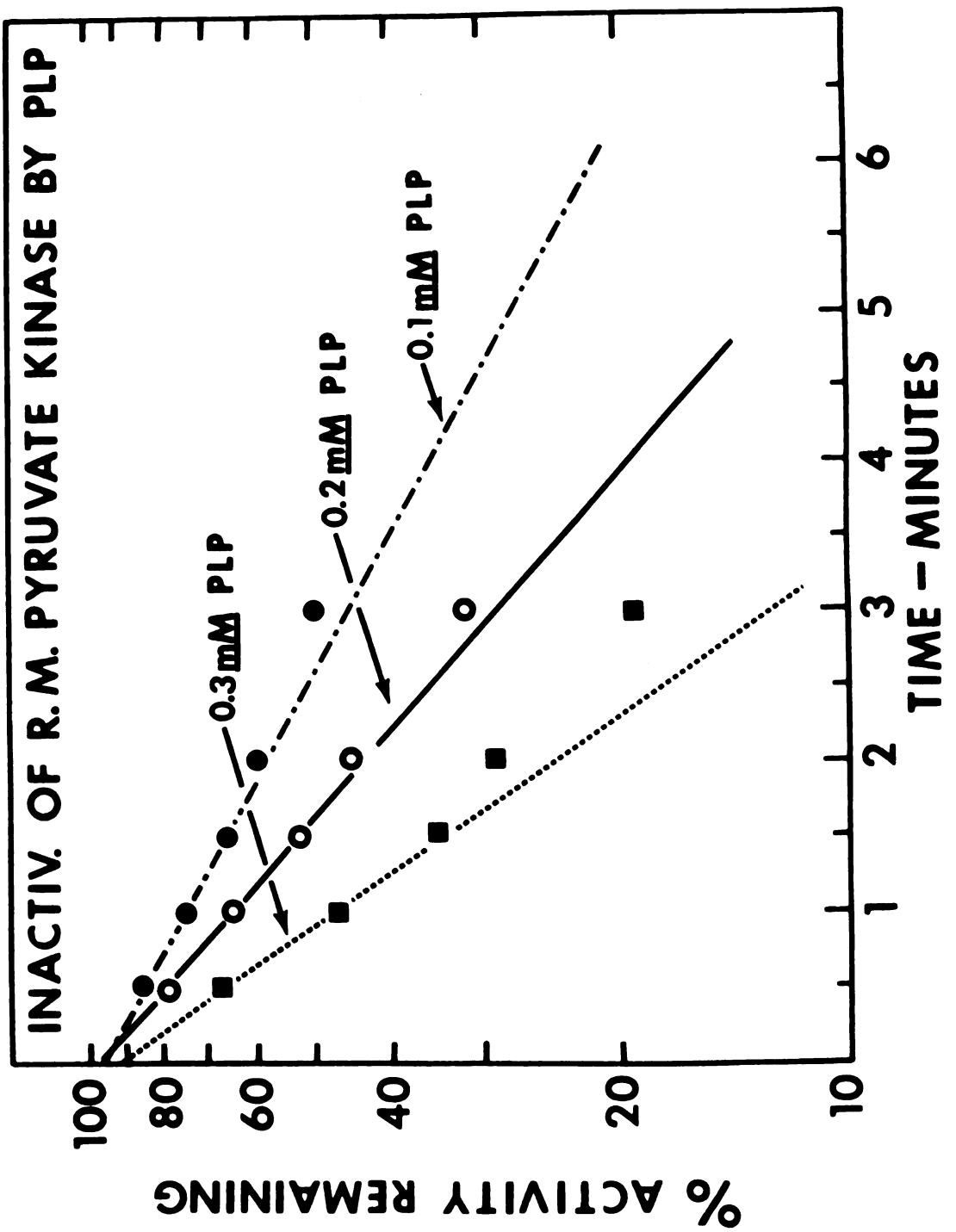
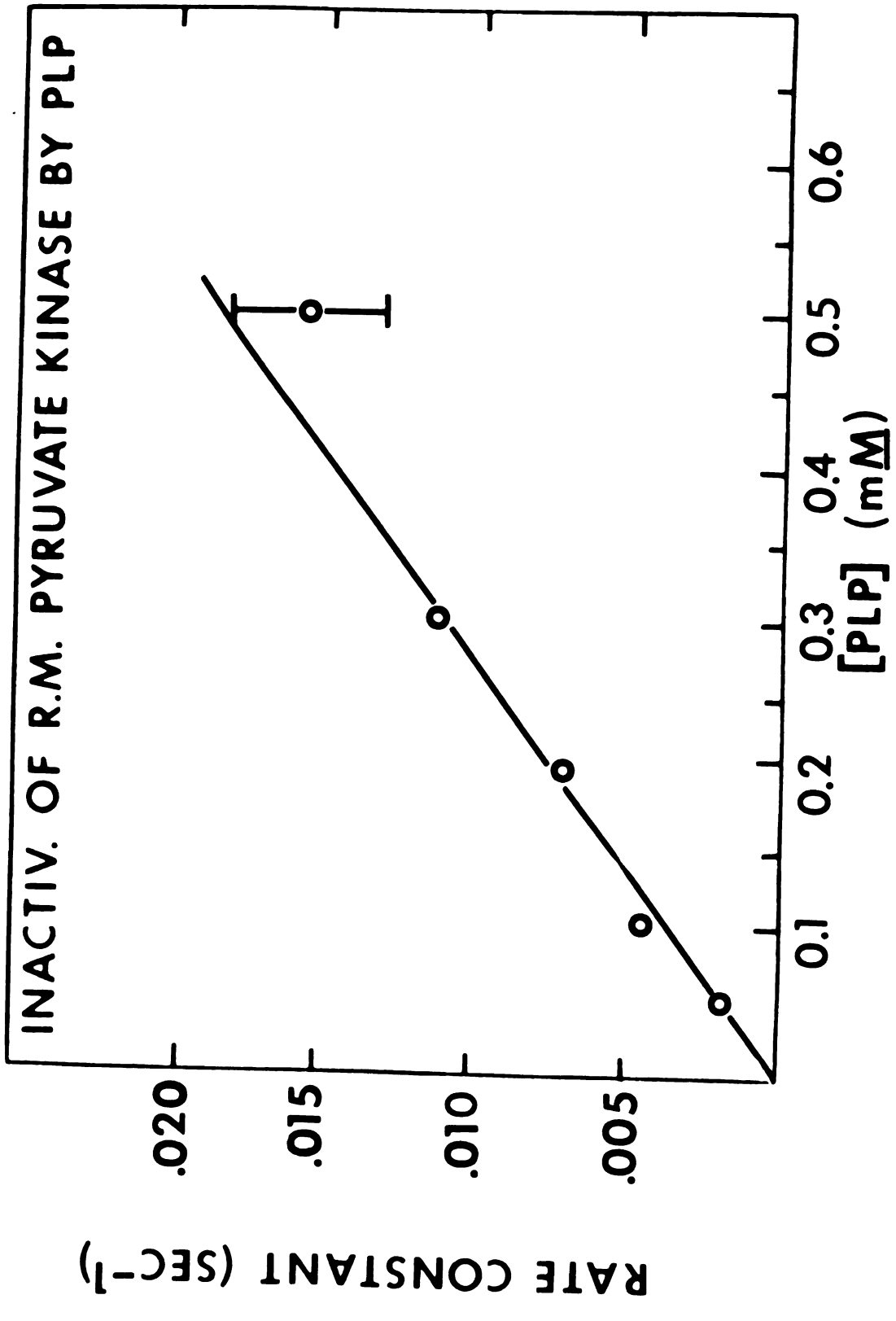

INACTIV. OF R.M. PYRUVATE KINASE BY PLP
0.3mM PLP
0.2mM PLP
0.1mM PLP
% ACTIVITY REMAINING
100
80
60
40
20
10
1
2
3
4
5
6
TIME – MINUTES

Figure 19. DETERMINATION OF THE SECOND-ORDER RATE CONSTANT FROM THE GRAPH OF PSEUDO FIRST-ORDER RATE CONSTANTS VS. PLP CONCENTRATION

The data are from the experiments described in Figure 13. The slope of the line is equal to the second-order rate constant (see Eqn. 3).

Figure 19


INACTIV. OF R.M. PYRUVATE KINASE BY PLP
RATE CONSTANT (SEC⁻¹)
.020
.015
.010
.005
0.1
0.2
0.3
0.4
0.5
0.6
[PLP] (mM)

Since the rate of inactivation can be expressed as

$$\text{Eqn (1)} \quad -\frac{dA}{dt} = k_2 \, (PLP) \, (A)$$

where A = activity = enzyme concentration, and k_2 is the second-order rate constant, then

$$\text{Eqn (2)} \quad -\frac{dA}{dt} = k_2 \, (PLP) \, (A) = k_1{}^P \, (A)$$

where

$$\text{Eqn (3)} \quad k_1{}^P = k_2 \, (PLP)$$

and $k_1{}^P$ is a pseudo first-order rate constant. Values of $k_1{}^P$ as a function of PLP concentration were calculated from the data in Figure 18. From Eqn (3), the plot of the pseudo first-order rate constant, $k_1{}^P$, vs PLP concentration should have a slope equal to k_2, the second-order rate constant. From such a plot (Figure 19) the second-order rate constant was found to be $k_2 = 37 \ \underline{M}^{-1} \ sec^{-1}$.

Since most of the activity of pyruvate kinase was lost upon binding only 4 moles PLP per mole enzyme, it seemed possible that PLP was binding at or near the active site. Thus it appeared that a substrate or a required cation, or another metabolite might affect the inactivation. Accordingly, several such compounds were tested. The species of salt used and certain metabolites were found to affect the rate of inactivation (Figure 20, Figure 21, and Table 6), but not the equilibrium (final) activity values. All the equilibrium values were within 10 % of that with no added salts or metabolites.

Figure 20. EFFECT OF MONOVALENT CATIONS ON THE PSEUDO FIRST-ORDER RATE CONSTANTS FOR INACTIVATION

Pyruvate kinase (0.042 mg per ml) was incubated in a solution containing 0.2 m$\underline{M}$ PLP, 0.2 $\underline{M}$ imidazole, pH 7.5, and the indicated concentration of monovalent cation. Aliquots were assayed at the indicated times, and rate constants were determined from the slopes (see Methods).

Figure 20

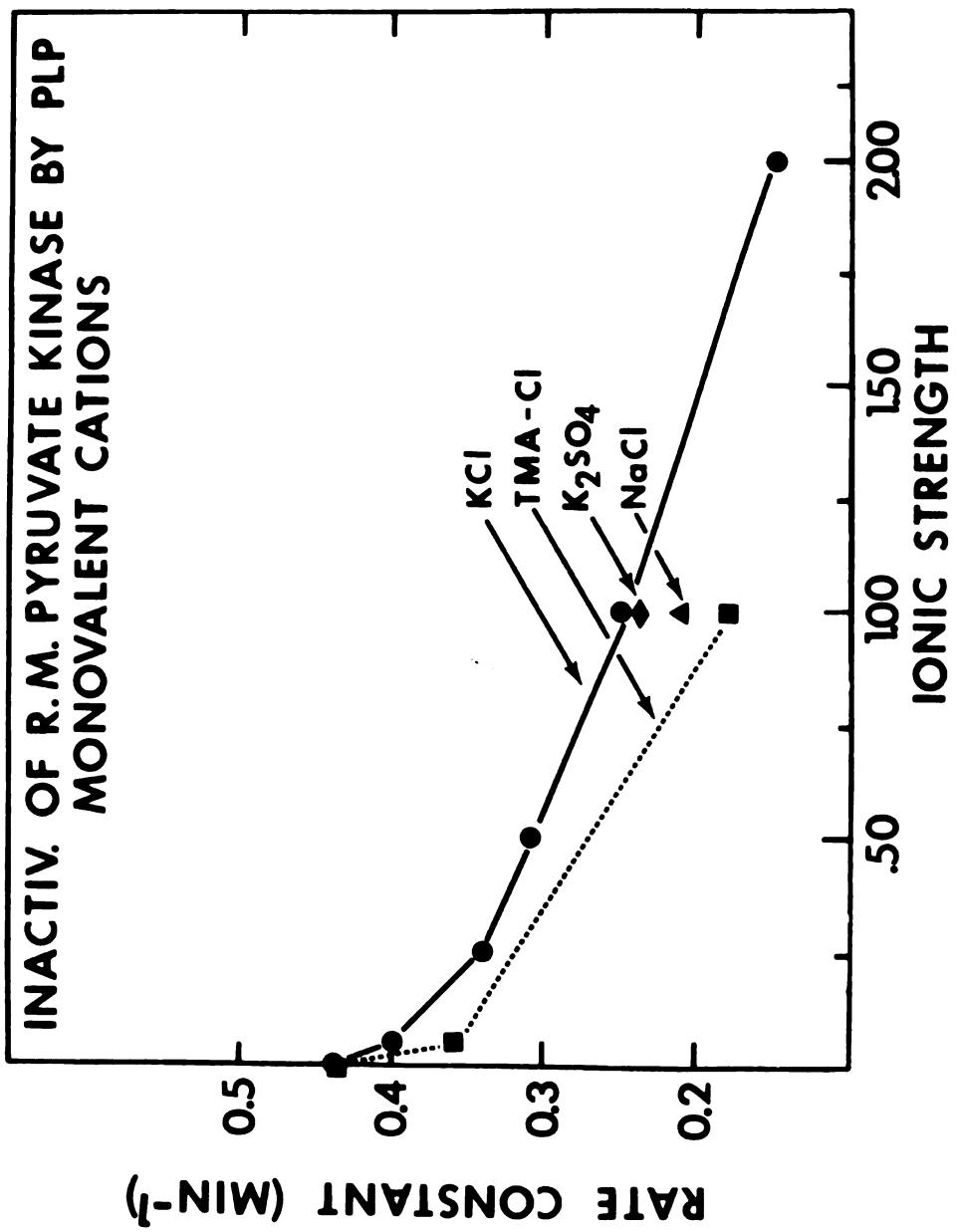

INACTIV. OF R.M. PYRUVATE KINASE BY PLP
MONOVALENT CATIONS
KCl
TMA-Cl
K_2SO_4
NaCl
RATE CONSTANT (MIN-1)
0.5
0.4
0.3
0.2
50
100
150
200
IONIC STRENGTH

Figure 21. EFFECT OF DIVALENT CATIONS ON THE PSEUDO FIRST-ORDER RATE CONSTANTS FOR INACTIVATION

The experiments were performed as in Figure 20.

Figure 21

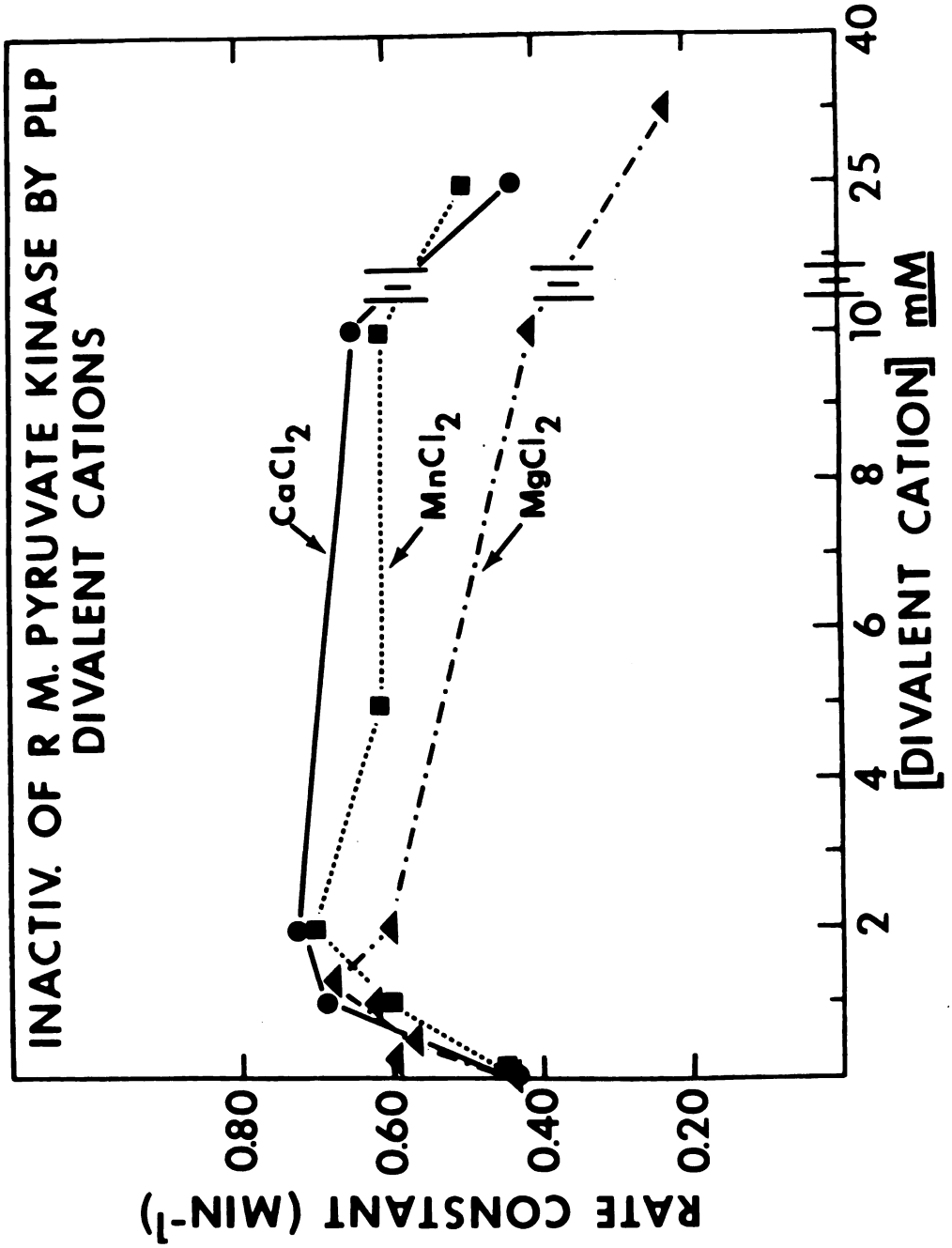

INACTIV. OF R M. PYRUVATE KINASE BY PLP
DIVALENT CATIONS
$CaCl_2$
$MnCl_2$
$MgCl_2$
RATE CONSTANT (MIN^{-1})
0.80
0.60
0.40
0.20
2
4
6
8
10
25
40
[DIVALENT CATION] mM

TABLE 6. EFFECT OF METABOLITES ON THE RATE CONSTANTS FOR INACTIVATION OF PYRUVATE KINASE BY PLP

Conditions as in Figure 8.

Contents	Rate Constant (sec^{-1})
Metabolites Plus No Added Cations	
0.2 M imidazole	0.0073
+ 1 mM ADP	0.0061
+ 1 mM ATP	0.0061
+ 1 mM P-Enolpyruvate	0.0055
+ 1 mM pyruvate	0.0073
+ 1 mM Fructose-1,6-diphosphate	0.0068
Metabolites Plus Added Monovalent Cations	
0.2 M imidazole + 50 mM KCl	0.0053
+ 1 mM ADP	0.0050
+ 1 mM P-Enolpyruvate	0.0050
+ 1 mM Fructose-1,6-diphosphate	0.0056
Metabolites Plus Added Divalent Cations	
0.2 M imidazole + 1 mM P-Enolpyruvate	0.0055
+ 0.08 mM $MgCl_2$	0.0048
+ 0.25 mM $MgCl_2$	0.0043
+ 0.5 mM $MgCl_2$	0.0045
+ 1.0 mM $MnCl_2$	0.0040
+ 5.0 mM $MnCl_2$	0.0043
+10.0 mM $MnCl_2$	0.0058
+ 1.0 mM $CaCl_2$	0.0043
0.2 M imidazole + 1 mM ADP	0.0061
+ 0.12 mM $MnCl_2$	0.0060
+ 0.25 mM $MnCl_2$	0.0060
+ 5.0 mM $MnCl_2$	0.0100
Metabolites Plus Added Monovalent and Divalent Cations	
0.2 M imidazole + 50 mM KCl + 5 mM $MnCl_2$	0.0073
+ 1 mM ADP	0.0056
+ 1 mM ATP	0.0056
+ 1 mM P-Enolpyruvate	0.0060
+ 1 mM pyruvate	0.0073
+ 1 mM Fructose-1,6-diphosphate	0.0066

The control samples without PLP were relatively stable, losing only about 5 % activity per hr. The sample containing 1 mM fructose-1, 6-diphosphate was an exception to this; it lost 20 % activity per hr.

At a constant PLP concentration (0.2 mM) the rate of inactivation (determined from Figure 22 and similar plots) was dependent upon salt species and salt concentration. The pseudo first-order rate constant decreased monotonically with increasing concentrations of monovalent cations (Figure 20) but with increasing concentrations of divalent cations it reached a maximum and then decreased (Figure 21).

The decrease in rate constant with increasing monovalent cation concentrations appears to be an ionic strength effect since the activating salts, potassium chloride and potassium sulfate (Boyer, 1962) show equivalent effects at equal ionic strengths (Figure 20). The less effective activating salt, sodium chloride, and the nonactivating salt, tetramethylammonium chloride, show an even greater protection than potassium chloride.

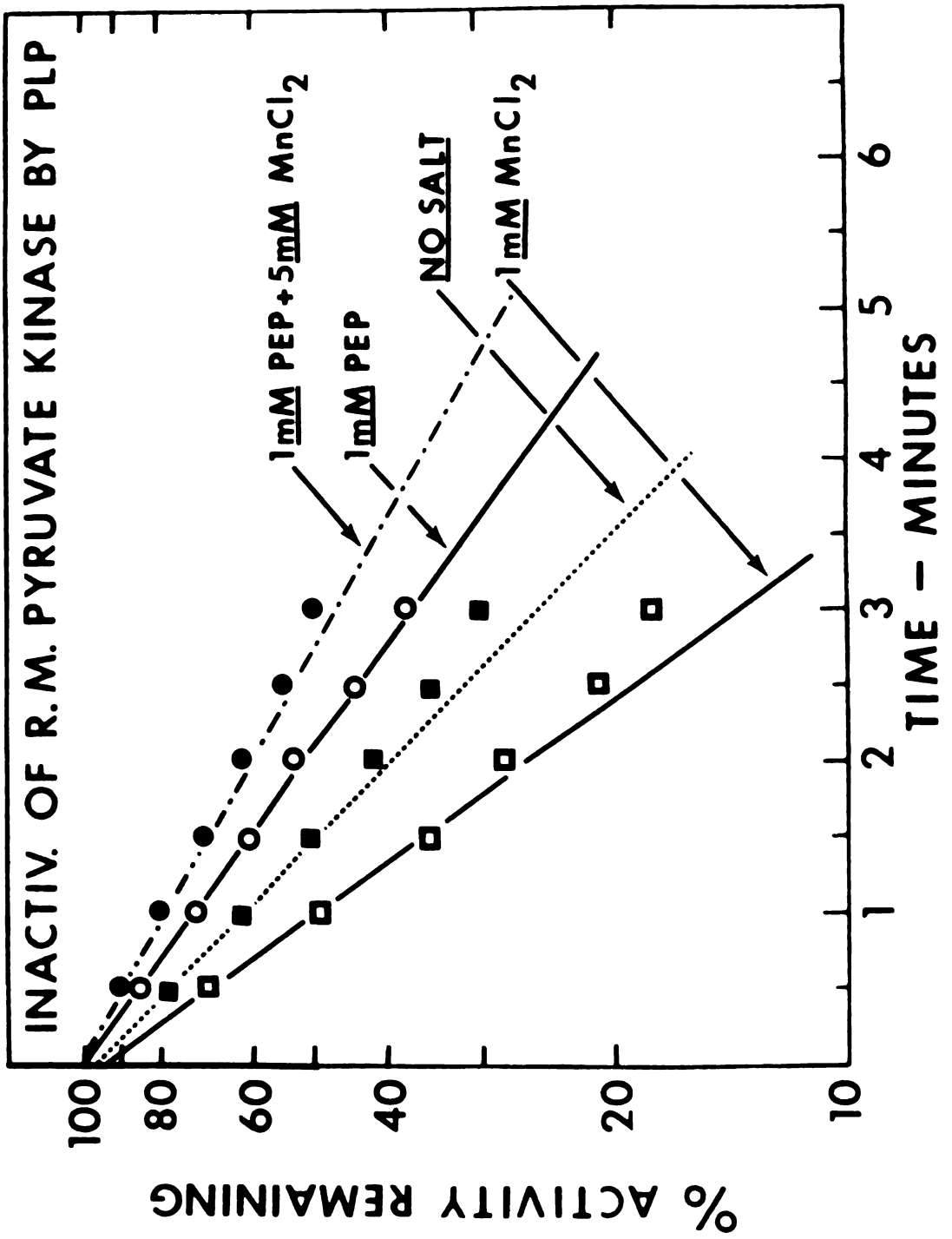
The divalent cation effect (Figure 21) was observed with activating cations (Mg^{+2} and Mn^{+2}), as well as with the inhibitory cation, Ca^{+2} (Boyer, 1962).

The metabolites ADP, ATP, phosphoenolpyruvate, and, to a slight extent, fructose-1,6-diphosphate protected the enzyme against inactivation by PLP (Table 6). In contrast, 1 mM pyruvic acid, AMP, 3',5'-cyclic AMP, fructose-6-phosphate, NAD^{+}, or inorganic phosphate, or insulin (0.2 mg per ml) did not protect. There was a large decrease in the rate of inactivation with phosphoenolpyruvate and ADP in the presence of $MnCl_2$ or $MgCl_2$ (Figure 22, Table 6). This effect was seen

Figure 22. EFFECT OF SALTS AND SUBSTRATES ON THE RATE OF INACTIVATION

The experiments were performed as in Figure 20.

Figure 22


INACTIV. OF R.M. PYRUVATE KINASE BY PLP
1mM PEP+5mM MnCl2
1mM PEP
NO SALT
1mM MnCl2
% ACTIVITY REMAINING
100
80
60
40
20
10
1
2
3
4
5
6
TIME – MINUTES

with ADP only at lower concentrations of divalent cations. This is in contrast to the increased rate of inactivation caused by divalent cations alone.

A solution of partially inactivated (in 0.2 mM PLP) pyruvate kinase with approximately 3 moles of PLP covalently bound per mole of protein was further inactivated in 0.2 mM PLP. It was found to be inactivated at the same rate and to reach the same final % inactivation as unreduced enzyme.

STRUCTURAL ANALYSIS. The enzyme inactivated by PLP was centrifuged in a sucrose density gradient containing PLP. The otherwise inactive enzyme was located by reversing the inactivation by addition of excess Tris to each fraction. No gross conformational change accompanied the inactivation since native enzyme and inactivated enzyme showed identical sedimentation properties (Figure 23).

REVERSAL OF THE INACTIVATION. Complete recovery of activity was obtained by three methods: (a) by a 40-fold dilution of the PLP inactivated enzyme with buffer; (b) by a 16 hr dialysis at 4°; or (c) by addition of 0.05 M Tris buffer, which completed with the protein amino groups to form a Schiff base with PLP.

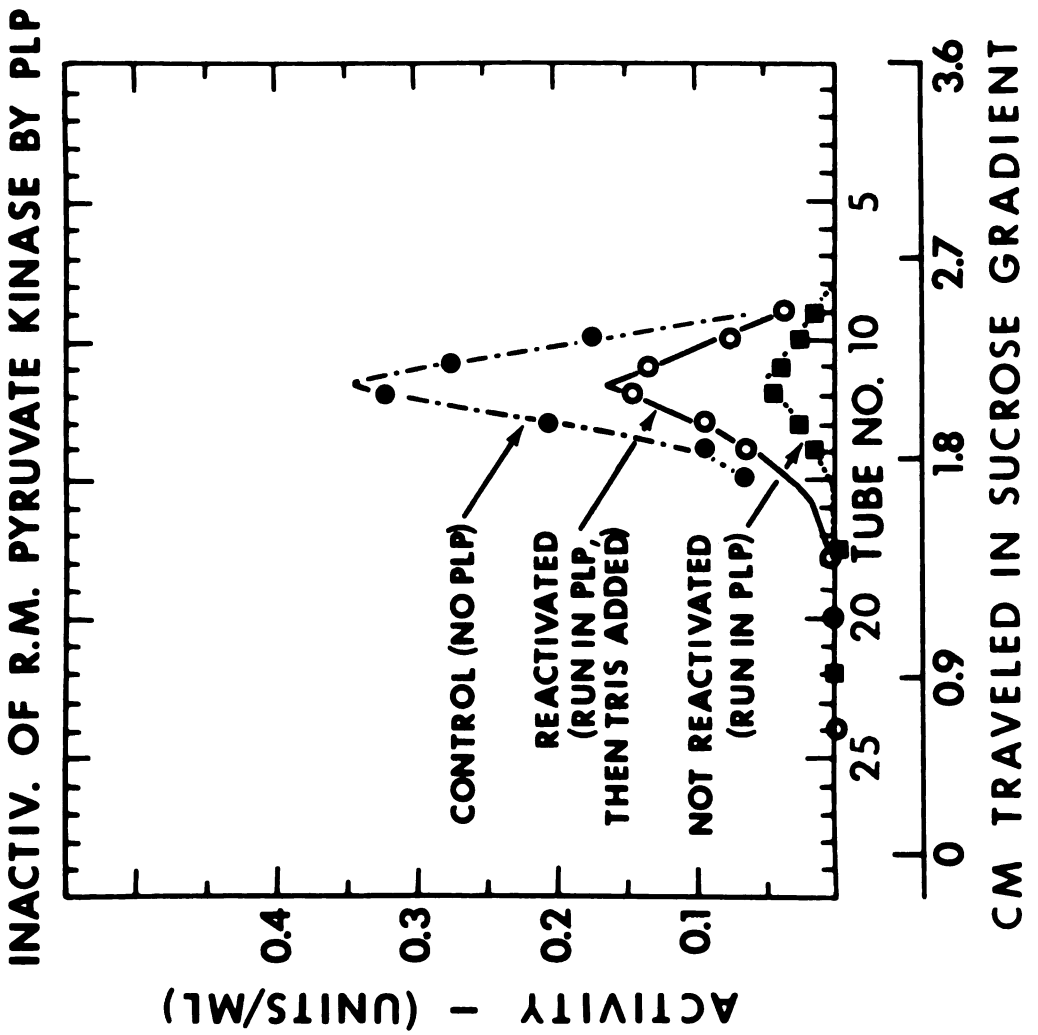
DISCUSSION

POSSIBLE BIOLOGICAL SIGNIFICANCE. There are only a few known inhibitors of muscle pyruvate kinase; moreover, none of these has been suggested to be a potential agent for metabolic control *in vivo*. This study shows that under the conditions used in this study, rabbit muscle pyruvate kinase is 50 % inactivated by 4×10^{-5} M PLP. The

Figure 23. SEDIMENTATION CHARACTERISTICS OF NATIVE AND INACTIVATED PYRUVATE KINASE

Dialyzed pyruvate kinase (0.042 mg per ml) was incubated for 15 minutes with 0.5 m$\underline{M}$ PLP at 24° in 0.2 $\underline{M}$ imidazole, pH 7.5. Aliquots (4.2 μg in 0.1 ml) were layered onto two sucrose density gradients with the same composition as the incubation mixture. After collection of fractions, one tube (NOT REACTIVATED) was assayed. For the second tube (REACTIVATED), Tris buffer was added to a final concentration of 0.05 $\underline{M}$. The fractions were incubated at 24° for 1 hour and then assayed. A third tube (CONTROL) was treated as the sample for tube 1, except PLP was omitted in the pre-incubation mixture and from the sucrose density gradients. All samples were assayed in the absence of PLP.

Figure 23


INACTIV. OF R.M. PYRUVATE KINASE BY PLP
ACTIVITY - (UNITS/ML)
0.4
0.3
0.2
0.1
CONTROL (NO PLP)
REACTIVATED (RUN IN PLP, THEN TRIS ADDED)
NOT REACTIVATED (RUN IN PLP)
25
20
TUBE NO. 10
5
0
0.9
1.8
2.7
3.6
CM TRAVELED IN SUCROSE GRADIENT

concentration of PLP in muscle (rat) is 4.65×10^{-6} M (Wachstein and Moore, 1958; Long, 1961), and the concentration of free PLP may be even lower due to specific binding of PLP to other enzymes.

NUMBER OF BINDING SITES. Pyruvate kinase (mol wt 237,000 has been shown to be composed of four subunits of mol wt 57,000 (Steinmetz and Deal, 1966) which by several tests appear to be highly similar (Cottam et al., 1969). This raises the possibility that there might be four active sites per tetramer; however, only two have been found in previous studies (Reynard et al., 1961; Mildvan and Cohn, 1965). The results reported in this study do not relate directly to the number of catalytic sites in the enzyme. The data do suggest that there are 2 to 4 unusually reactive lysines. Furthermore it is possible that these are near active sites, since the characteristics of PLP binding to these unique lysine groups are so strongly and specifically influenced by the cations and substrates of the pyruvate kinase reaction. However, they do not appear to be part of the active site (see later section on Mechanism of Inactivation).

There are at least two possible explanations for the fact that the enzyme is not completely inactivated: (a) the binding by PLP renders the active sites only 10 % active or (b) an equilibrium exists between enzyme with PLP bound and enzyme without PLP bound.

In the first explanation, the first four PLP molecules bound convert the tetrameric enzyme to a species which is only 10 % active. The remaining PLP molecules bind at noninactivating sites and cause only negligible further loss of activity. This explanation agrees best with the reduction data.

The second explanation, which may be called the equilibrium mixture explanation, assumes that when PLP is bound to a specific binding site, it completely inactivates that site. Hence, an equilibrium is formed between free enzyme sites, inactivating sites with PLP bound, and noninactivating sites with PLP bound. The residual activity is presumed to come from the free enzyme sites or enzyme with only noninactivating sites bound.

This second explanation would appear to be ruled out by the data. Increasing the PLP concentration should drive the reaction completely to products, yielding complete inactivation. This was not observed; the inactivation seemed to reach a plateau at the high PLP concentrations used. However, there is one possibility which would be consistent with both the second explanation and the data. This involves postulating that the initial binding of 2-4 PLP molecules would lead to some unfolding of the enzyme, thereby exposing previously inaccessible, noninactivating sites. These newly available, noninactivating sites could then compete with inactivating sites for PLP and greatly increase the amount of PLP required for inactivation over that expected from the results during the initial stages of binding. In this case the overall results would be the sum of two or more equilibria, not one. The highest PLP concentrations used in these studies led to binding of only about 25 % of the total 150-160 lysines.

MECHANISM OF THE INACTIVATION. One question is whether PLP inactivates rabbit muscle pyruvate kinase by binding at or near the active site, or whether it binds elsewhere and inactivates the enzyme by a conformational change. There is no evidence for instantaneous inhibition of

the enzyme by PLP; all the curves of activation as a function of time extrapolate back to maximal activity at 0 time.

Since there is no instantaneous inhibition by PLP, it does not seem likely that PLP binds at the phosphate binding site of the substrates. This is further supported by the lack of pronounced protection by substrates against inactivation under equilibrium conditions. It is interesting that the phosphate-containing substrates, ADP, ATP, and P-enolpyruvate (but not pyruvate) decrease the rate of inactivation of the enzyme by PLP. It appears that they do this not by binding at the active site, but by stabilizing the native conformation, thereby hindering the conformational changes required for inactivation. The inactivation of rabbit muscle pyruvate kinase by binding of PLP to specific sites that are not catalytic sites differs from that found with certain other enzymes. PLP inhibits aldolase (Shapiro et al., 1968) and 6-phosphogluconate dehydrogenase (Rippa et al., 1967) by binding at the same site as the phosphate of the substrate.

The PLP data indicate that divalent and monovalent cations have completely different effects on the inactivation. The rate of inactivation decreases monotonically with increasing monovalent cation concentration (Figure 20) but it goes through a maximum with increasing concentration of divalent cation (Figure 21). In contrast to this, there is a decrease in the rate of inactivation with low concentrations of divalent cation in the presence of the substrates ADP or phosphoenolpyruvate. The monovalent cations and higher concentrations of divalent cations apparently retard the inactivation by stabilizing the native enzyme conformation via a relatively nonspecific ionic strength effect.

There are three general possible roles which metal ions may play in the PLP inactivation in the presence and absence of substrates: (a) they may cause a conformational change in the enzyme, (b) they may complex with PLP, (c) they may complex with the substrates. These possibilities have known counterparts in the mechanism of action of PLP and the mechanism of pyruvate kinase catalysis: (a) The monovalent cations are thought to provide the enzyme conformation necessary for catalysis (Melchior, 1965). Also, divalent cations bind to the enzyme (Mildvan and Cohn, 1965) and cause conformational changes in it (Suelter and Melander, 1963). Such conformational changes have been postulated to explain the protection by Mn^{+2} against inactivation of pyruvate kinase by p-mercuribenzoate (Mildvan and Leigh, 1964). (b) A chelation of divalent cations by PLP has been postulated in the nonenzymatic catalysis by PLP (Metzler and Snell, 1952). (c) Both phosphoenolpyruvate and ADP are known to bind divalent cations (O'Sullivan and Perrin, 1964; Wold and Ballou 1951; Mildvan and Cohn, 1966) and the divalent cation substrate species is thought to be the reactive substrate species in enzymatic catalysis (Melchior, 1965). It should be noted that with the concentration of substrates and divalent cations used (Table 6), the cation-substrate complexes would be present in sufficient concentrations (about 0.1-1 m$\underline{M}$) to be considered as a possible factor in decreasing the rate of inactivation.

It is interesting that fructose-1,6-diphosphate protects and somewhat destabilizes the control enzyme. It has previously been shown (see Introduction) to interact with pyruvate kinase from sources other than muscle; these results show that it binds to rabbit muscle pyruvate kinase as well.

METHODS AND MATERIALS

Rabbit muscle pyruvate kinase was purified and assayed as previously described (Johnson et al., 1969; Steinmetz and Deal, 1966; Teitz and Ochoa, 1958). Pyridoxal, pyridoxamine, pyridoxamine-5'-phosphate and PLP were purchased from Sigma (St. Louis). All other compounds were reagent grade products from commercial sources as previously described (Johnson et al., 1969).

INACTIVATION. Unless otherwise stated, the following procedure was used: the stock enzyme solution (60 mg per ml) was diluted to 3.4 mg per ml with 0.2 M imidazole, pH 7.5, and dialyzed 10-14 hr at 4° versus 150 volumes of the buffer. Following dialysis, the protein was further diluted to 0.042 mg per ml with solutions containing buffer plus the proper constituents (see Results). Aliquots (0.1 μg) of the resulting solutions were then assayed. There was no initial reactivation of the inactive enzyme by dilution into the assay solution since the assay was linear for up to 1 min; however, reactivation was observed in an extended assay as shown by an increase in the rate of the reaction. Accordingly, the initial rates were measured.

REDUCTION. The enzyme-PLP complex was reduced by first incubating 1 ml of enzyme solution (0.4 mg per ml) with the appropriate PLP concentration for 15-30 min at 25°, then adding 2 mg of finely divided sodium borohydride and incubating for 15 min more. The samples were dialyzed for 12-15 hr versus 100 volumes of the buffer at 4°. The ratios of moles of inactivator bound per mole of enzyme were calculated using molar extinction coefficients of ϵ_{325} = 10,000 for the ϵ-aminolysine-

PLP complex, ϵ_{325} = 9,710 for the ϵ-amino-lysine-pyridoxal complex (Fischer et al., 1963), and ϵ_{280} = 0.54 ml mg^{-1} cm^{-1} for the enzyme concentration (Bücher and Pfleiderer, 1955).

CENTRIFUGATION. The procedure used for sucrose density gradient centrifugation followed that of Martin and Ames (1961). The dialyzed protein was incubated for 15 min with 0.5 mM PLP at 24° in 0.2 M imidazole, pH 7.5; aliquots (4.2 μg protein in 0.1 ml) were layered on the sucrose density gradients (5-20 %) with the same composition. Samples were centrifuged for 16 hr at 0° and at 40,000 rpm using a Spinco Model L Ultracentrifuge with a SW 39 rotor. Fractions of ten drops each were collected from the tubes and assayed for pyruvate kinase activity.

For the reactivation analyses, Tris buffer (pH 7.5, final concentration, 0.05 M) was added to each fraction before assaying. The solutions were then incubated for 1 hr at 24° and assayed. Control samples were treated identically, except PLP was omitted.

UV SPECTRA. The UV difference spectra were recorded with a Cary Model 15 spectrophotometer using double-tandem cells as described by Kayne and Suelter (1965), and with a Beckmann spectrophotometer equipped with a Gilford absorbance indicator attachment.

CHAPTER THREE

STRUCTURAL CHANGES OF RABBIT MUSCLE PYRUVATE KINASE

ABSTRACT

The sedimentation coefficient for rabbit muscle pyruvate kinase in the original native state has been determined to be $s^{o}_{20,w}$ = 9.6 S - 9.8 S by sucrose density centrifugation of a crude extract from frozen rabbit muscle. A similar experiment with a crude extract from frozen rabbit liver yielded a value of $s^{o}_{20,w}$ = 7.4 S and variable amounts of a faster sedimenting protein (about 9.5 S) for rabbit liver pyruvate kinase.

The effect of high pH on rabbit muscle pyruvate kinase was studied with enzyme preparations from fresh rabbit muscle and from rabbit muscle frozen 2-3 years. The fresh muscle preparation in 0.05 M Tris or glycine buffer, 0.15 M KCl, 0.001 M EDTA showed: (1) a single peak in the pH region 7.4-10; (2) at either 5° or 24°, an initial transition at pH 8.5-9.0 from $s^{o\,0.16\,\%}_{20,w}$ = 9.6 to $s^{o\,0.16\,\%}_{20,w}$ = 9.3 S; (3a) at 5°, a second transition at pH 10.5 and above yielding a slower sedimenting peak (8.0 S) and an additional peak (4 S); (3b) at 24°, a second transition at about pH 10.1-10.2, resulting in 2 or more broad peaks in sedimentation velocity experiments. The enzyme prepared from the frozen muscle under similar conditions showed different results. (1) At 24°, the initial transition occurred at much lower pH (pH 7.9-8.4). (2) The initial transition yielded a more dissociated or unfolded enzyme, $s^{o\,0.44\,\%}_{20,w}$ = 9.4 S to 8.7 S, and the peak broadened much more rapidly during sedimentation velocity experiments. (3) At 24°, a second transition (loss of well-defined peaks) occurred at pH 9.8 and above; 0.15 M KCl was required to observe this effect at pH 9.8, but not at higher pH.

The sedimentation coefficient of rabbit muscle pyruvate kinase prepared from fresh muscle was greatly influenced by salt concentration. It increased from a value of about 9.3 S in no added salt to a maximum value of $s^{0.16\ \%}_{20,w}$ = 9.6 S in 0.1 M-0.5 M KCl (pH 7.5, 5° or 24°) and 0.05 M-0.6 M $(NH_4)_2SO_4$ (pH 8.0, 5°). It then decreased with increasing salt concentration to a value of $s^{0.16\ \%}_{20,w}$ = 7.65 S in 3.0 M KCl. Similar results were obtained with increasing KCl concentrations at pH 10.0 at 24°.

Sedimentation equilibrium analysis showed that the decrease in sedimentation coefficient was due to a dissociation into dimers or monomers. The results were also consistent with a dissociation-association rapid equilibrium between monomers (or dimers) and tetramers and/or octamers.

INTRODUCTION

Several different sedimentation coefficients have been reported for rabbit muscle pyruvate kinase. Warner (1958) first reported a value of $s^{o}_{20,w}$ = 10.04 S. This value has been confirmed by a number of investigators but values as low as 8.5 S have also been reported. Kimberg and Yielding (1962) reported sedimentation coefficient values of $s^{o}_{20,w}$ = 10.0-11.3 S, determined from sucrose density centrifugation experiments and $s^{0.25\ \%}_{20,w}$ = 8.85 S from analytical ultracentrifuge experiments. Kayne and Suelter (1965) observed a sedimentation coefficient of $s^{o}_{20,w}$ = 9.33 S in 0.1 M KCl and 0.001 M $MnCl_2$ and 9.22 S in 0.103 M tetramethylammonium chloride. Kayne (1966) reported an irreversible transition from $s^{o}_{20,w}$ = 10.07 to $s^{o}_{20,w}$ = 8.53 S upon extended dialysis and also upon extended storage of the enzyme in

aqueous buffered solutions at 80 mg/ml at 4°. This transition did not alter the catalytic activity. A similar time-dependent transition has also been observed with prolonged storage as the ammonium sulfate suspension of the enzyme (Cottam et al., 1969). Kayne and Suelter (1968) later reported values of $s^{o}_{20,w}$ = 8.5-8.9 S depending upon pH and salt species present. Wilson et al. (1967) published sedimentation values of $s^{o}_{20,w}$ = 9.99 S - 10-17 S in 0.05 M solutions of potassium phosphate, Tris phosphate, lithium phosphate, or potassium chloride, and 9.55 S in Tris chloride.

The mol wt of the subunits of rabbit muscle pyruvate kinase has been determined to be 57,000 (Steinmetz and Deal, 1966). The 10 S species (mol wt 237,000; Warner, 1958) can thus be assumed to be a tetramer (Steinmetz and Deal, 1966). However, the discrepancy between the often observed values of about 8.5 S and 10 S, the value generally assumed to represent native enzyme, is far outside the limits of experimental error. Furthermore, the explanation cannot be due to concentration dependence in those cases not extrapolated to zero protein concentration. The usual concentration dependence for globular proteins is -0.05 S/mg and almost never greater than -0.1 S/mg.

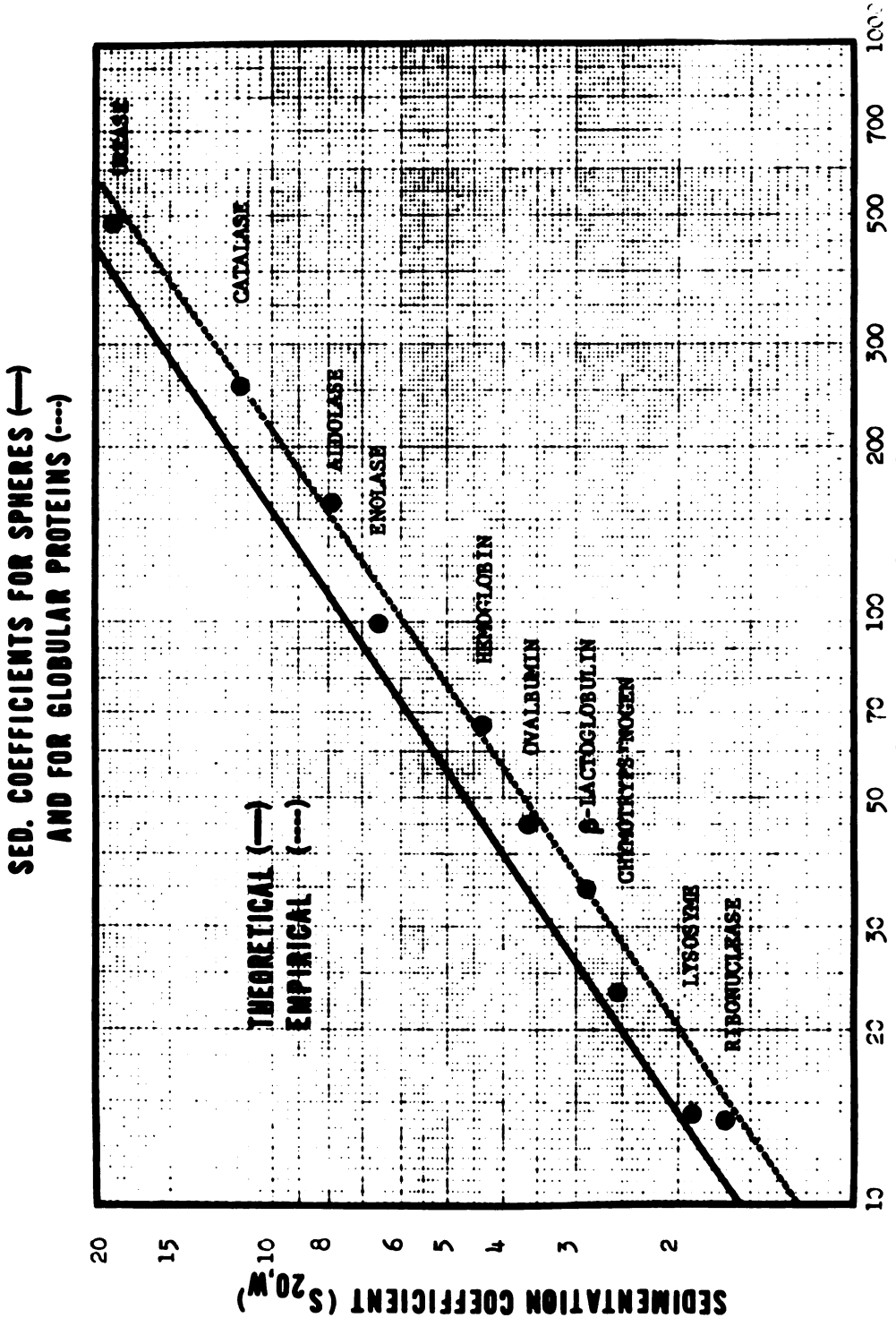
Two possible explanations for the discrepancy are (1) dissociation or (2) unfolding. However, neither of these alone is completely consistent with the reported characteristics of the 8.5 S species.

Consider the possibility of dissociation. From the graph of sedimentation coefficient versus mol wt for globular proteins (Figure 24), 8.5 S should correspond to a mol wt of about 170,000.

Figure 24. SEDIMENTATION COEFFICIENTS VS MOLECULAR WEIGHTS FOR PERFECT SPHERES AND GLOBULAR PROTEINS

The solid line represents the theoretically calculated values, and the dashed line represents the empirical results calculated from experimental data published in the literature.

Figure 24


SED. COEFFICIENTS FOR SPHERES (—)
AND FOR GLOBULAR PROTEINS (---)
THEORETICAL (—)
EMPIRICAL (---)
CATALASE
ALDOLASE
ENOLASE
HEMOGLOBIN
β-LACTOGLOBULIN
CHYMOTRYPSINOGEN
LYSOZYME
RIBONUCLEASE
SEDIMENTATION COEFFICIENT ($S_{20,w}$)
MOLECULAR WEIGHT X 10 - 3

Since the "native tetrameric" enzyme has a mol wt of about 230-240,000, this would require an asymmetric dissociation of the tetramer into a monomer and a trimer; but only one peak has been observed in the sedimentation velocity experiments (Kayne, 1966). However, this could be consistent if the dissociation involved dimers and tetramers, or monomers, dimers and tetramers in a rapid equilibrium (Gilbert, 1955). However, preliminary experiments (Kayne, 1966) show a lack of dependence of the sedimentation coefficient on protein concentration. This argues against a rapid equilibrium dissociation-association mechanism.

Consider the possibility of unfolding. The 15 % difference in sedimentation coefficients is near the maximum that could be expected for an unfolding; moreover, if the difference were due solely to unfolding, the 8.5 S species would show a greatly decreased sedimentation coefficient with increased protein concentration. However, there appeared to be no significant concentration dependence of the sedimentation coefficient (Kayne, 1966).

Thus, additional studies were necessary to determine the original <u>in vivo</u> native state of rabbit muscle pyruvate kinase and to determine the nature, origin and significance, if any, of the altered, "non-native" states. With this in mind we undertook a detailed analysis to determine the original native state of the enzyme and study conditions which could alter it, in an attempt to determine conditions whereby we could form the 8.5 S from the native state of the enzyme. The results of this study show that the original native structure has a sedimentation coefficient, $s^{0.16\ \%}_{20,w} = 9.6$ S and that it is reversibly altered by pH, temperature, and salts.

RESULTS

Analysis of the Sedimentation Properties of Pyruvate Kinase From Crude Extracts of Rabbit Skeletal Muscle and Rabbit Liver. Before attempting to analyze the sedimentation properties of purified pyruvate kinase, the sedimentation coefficient of pyruvate kinase from the crude extract of frozen rabbit muscle was determined to provide a reference to check for possible structural alterations during purification. Sucrose density centrifugation was used for this determination since the pyruvate kinase could be located by assay measurements, independent of the contaminating proteins of the crude extract.

From the data shown in Figure 25, the sedimentation coefficient of pyruvate kinase from the crude extract of rabbit muscle was determined to be $s^{o}_{20,w}$ = 9.6-9.8 S using the rabbit muscle aldolase ($s^{o}_{20,w}$ = 7.8 S; Deal et al., 1963) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase ($s^{o}_{20,w}$ = 7.5 S; Constantinides and Deal, 1969) of the crude extract, as markers.

The sedimentation properties of pyruvate kinase from a crude extract of rabbit liver were also determined (Figure 26). We assumed the marker enzymes in liver had sedimentation coefficients identical to their counterparts in rabbit muscle, and determined the sedimentation coefficient of liver pyruvate kinase to be approximately $s^{o}_{20,w}$ = 7.4 S. This suggests that the rabbit liver pyruvate kinase has a lower mol wt than rabbit muscle pyruvate kinase; it could be a dimer instead of a tetramer. A small amount of a faster sedimenting species (about 9.5 S) was also present as indicated by the slight shoulder at tubes 7-10 (Figure 26). In other experiments not shown, larger rela-

Figure 25. SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE IN A CRUDE EXTRACT FROM AGED, FROZEN RABBIT MUSCLE

The crude extract from frozen rabbit muscle was prepared as described in Methods. An aliquot of the crude extract (0.1 ml) was layered on the 5-20 % sucrose gradient. The proteins sedimented for 14 hr at 0° and 40,000 rpm. The sucrose solutions also contained 0.02 M imidazole (pH 7.0), 0.15 M KCl, 0.001 M EDTA and 0.05 M β-mercaptoethanol.

Figure 25

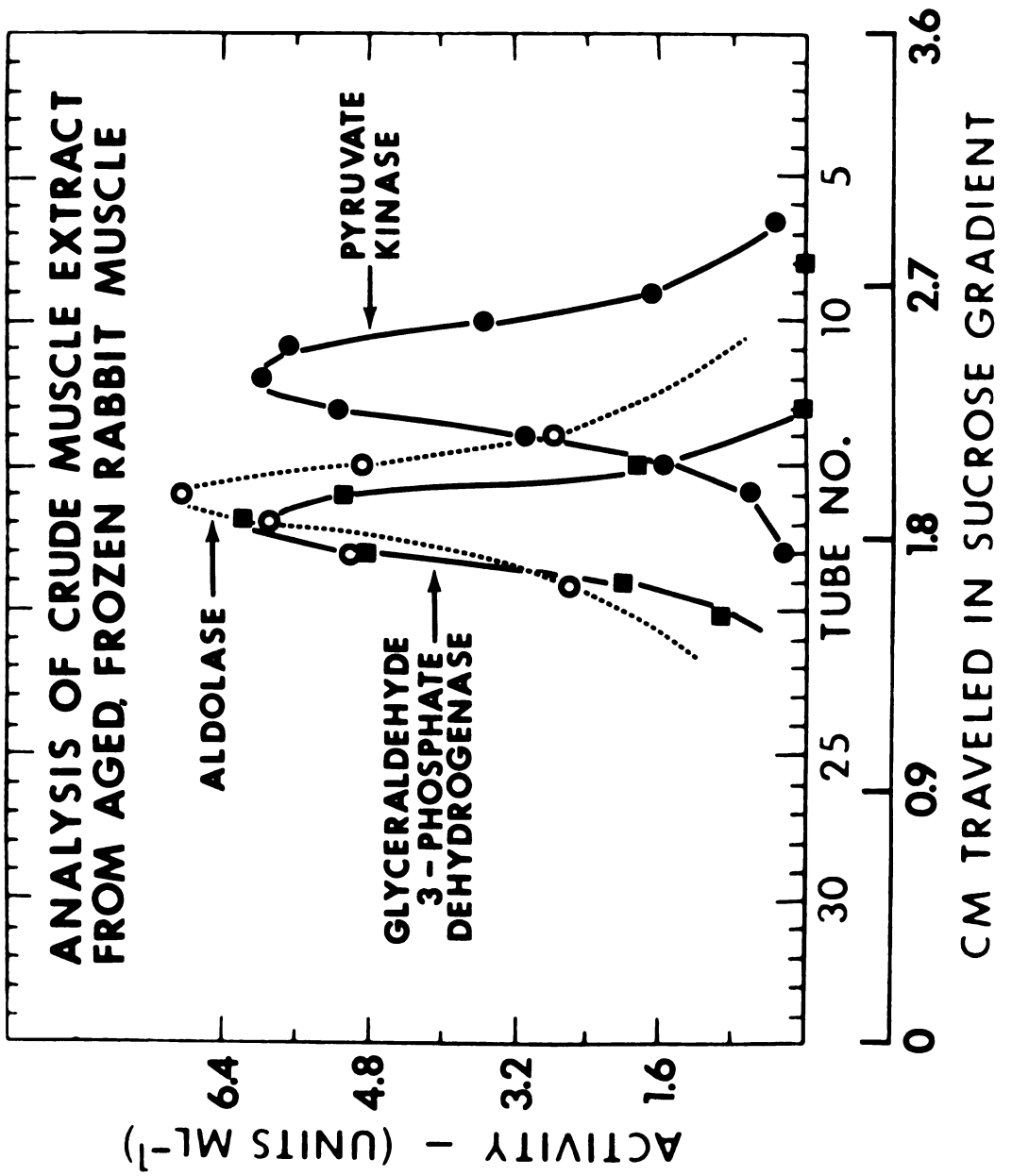
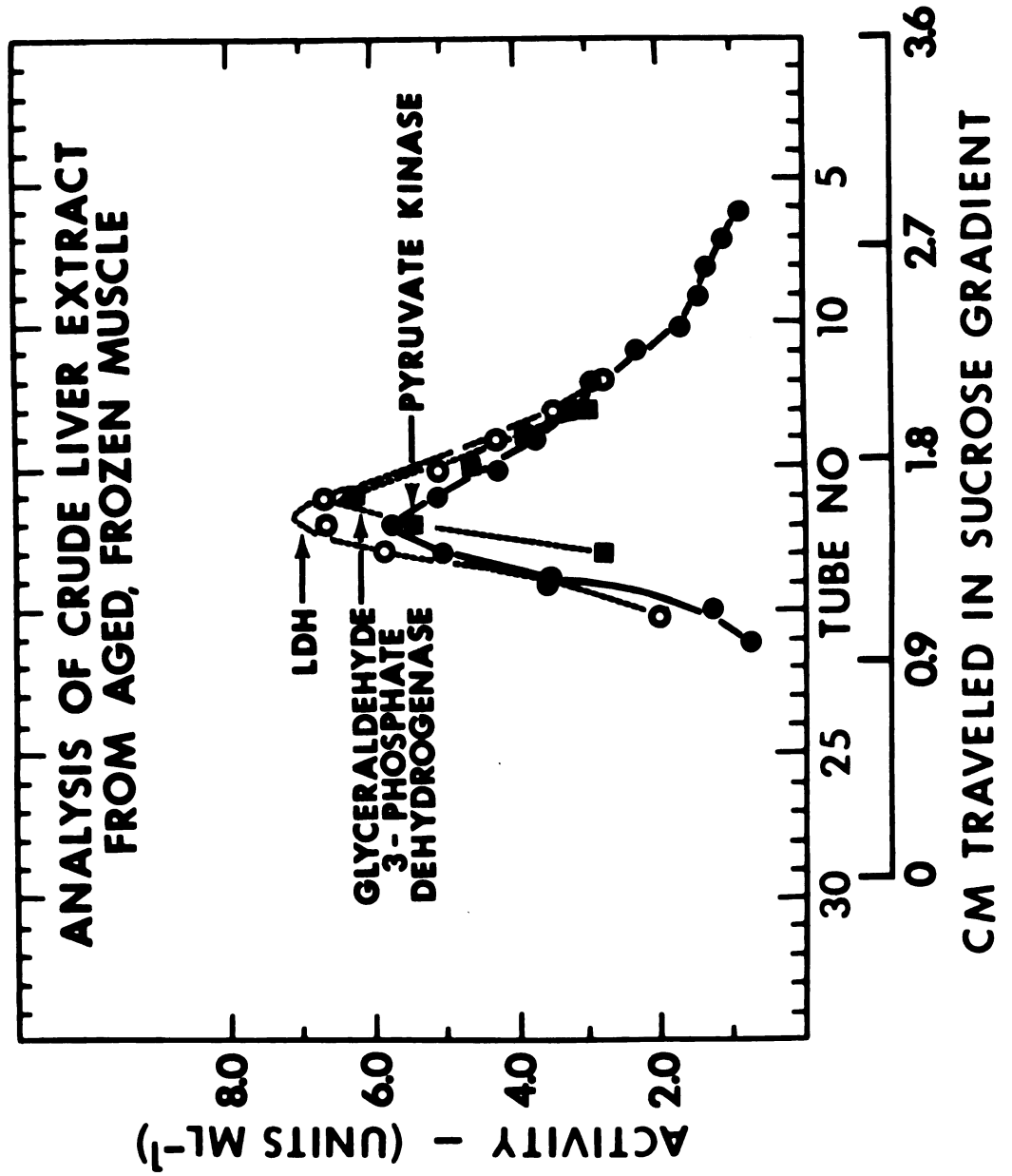

ANALYSIS OF CRUDE MUSCLE EXTRACT
FROM AGED, FROZEN RABBIT MUSCLE
ALDOLASE
GLYCERALDEHYDE
3-PHOSPHATE
DEHYDROGENASE
PYRUVATE
KINASE
ACTIVITY - (UNITS ML-1)
6.4
4.8
3.2
1.6
30
25
TUBE NO.
10
5
0
0.9
1.8
2.7
3.6
CM TRAVELED IN SUCROSE GRADIENT

Figure 26. SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE IN A CRUDE EXTRACT FROM AGED, FROZEN RABBIT LIVER

The crude extract from frozen rabbit liver was prepared as described in Methods and centrifuged as described in the legend for Figure 25.

Figure 26


ANALYSIS OF CRUDE LIVER EXTRACT
FROM AGED, FROZEN MUSCLE
LDH
GLYCERALDEHYDE
3-PHOSPHATE
DEHYDROGENASE
PYRUVATE KINASE
ACTIVITY - (UNITS ML-1)
8.0
6.0
4.0
2.0
30
25
0.9
0
TUBE NO
1.8
10
2.7
5
3.6
CM TRAVELED IN SUCROSE GRADIENT

tive amounts of the faster moving peak were observed.

Rat liver has been shown to contain two pyruvate kinases of mol wts 208,000 and 250,000 (Tanaka et al., 1967) (presumably both are tetramers). Thus, the rabbit liver enzyme may dissociate into a slower sedimenting species at the low protein concentrations in the extract. Alternatively, the rat liver pyruvate kinase may differ from the rabbit liver enzyme.

Effect of pH of the Sedimentation Coefficient of Rabbit Muscle Pyruvate Kinase. The series of experiments evaluating the effect of pH utilized two different enzyme preparations: one prepared from fresh rabbit muscle and used within one month after preparation, and the other prepared from rabbit muscle which had been frozen for 2-3 years. The use of the two preparations seemed advisable since the sedimentation properties of rabbit muscle pyruvate kinase have been shown to undergo a time dependent transition (Kayne, 1966). This would eliminate any alteration due to age or storage of the preparation, and a comparison of the results with the two preparations would show any differential sensitivity to pH of the fresh and the old enzyme.

Preliminary experiments with the old enzyme preparation showed that at low pH (pH 3.4, 0.1 M citrate), 0.05 M NaCl and 0.001 M EDTA, no well-defined peaks could be detected in the schlieren patterns. However, at high pH (0.2 M glycine) two well-defined peaks with sedimentation coefficients of about 3 S and 8 S were observed. These values are near those expected for an unfolded monomer (mol wt 57,000) and a trimer (mol wt 170,000), respectively (see Figure 24). Since this effect was observed near the pH of 8.5 used in previous studies

(Kayne, 1966), and since it raised the possibility of an asymmetric dissociation of the tetramer into a trimer and monomer, it seemed worthy of further analysis. In particular, it was of interest to see whether it occurred at pH values near neutrality.

As a function of pH, the sedimentation coefficient curves of pyruvate kinase isolated from fresh muscle (Figure 27) showed three basic characteristics: (1) At 5^{o} and 24^{o} a transition from 9.6 S to 9.3 S at about pH 8.5-9.0 was observed. Only one peak could be detected in the schlieren pattern (Figure 29, a, b). (2) At 4^{o} there was a marked decrease at pH 10.5 in the sedimentation coefficient, accompanied by the formation of an additional peak which sedimented at about 4 S (Figure 29, b). (3) At 24^{o} there was a drastic change in the sedimentation profile from pH 10.1 to 10.2; there was one well-defined, sharp peak at pH 10.1 whereas at pH 10.2 there was extensive dissociation and the pattern was such that no single, well-separated peak could be detected (Figure 29, a).

The dissociation shown at pH 10.2 (Figure 27; Figure 29, a) was reversible (see Methods). A well-defined peak (s = 9.5 S) was observed in the schlieren pattern, although a large amount of protein precipitated during the dialysis procedure.

The effect of pH on the sedimentation coefficient of pyruvate kinase isolated from the aged frozen muscle was also tested. At 4.4 mg/ml and 5^{o} (Figure 28) this enzyme showed a transition from $s^{0.44\ \%}_{20,w}$ = 9.4 S to 8.7 S at pH 9.5-9.8. This decrease in sedimentation coefficient was accompanied by a much more rapid broadening of the protein peak than was observed with the enzyme isolated from fresh rabbit muscle (Figure

Figure 27. EFFECT OF pH ON THE SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE

The enzyme samples (1.6 mg per ml) was dialyzed against 120 volumes of the appropriate buffer at the indicated pH for 12 hr at the indicated temperature. They were then centrifuged using a Spinco Model E Analytical Ultracentrifuge as described in Methods. The enzyme solutions contained 0.05 M buffer, 0.15 M KCl and 0.001 M EDTA.

Figure 27

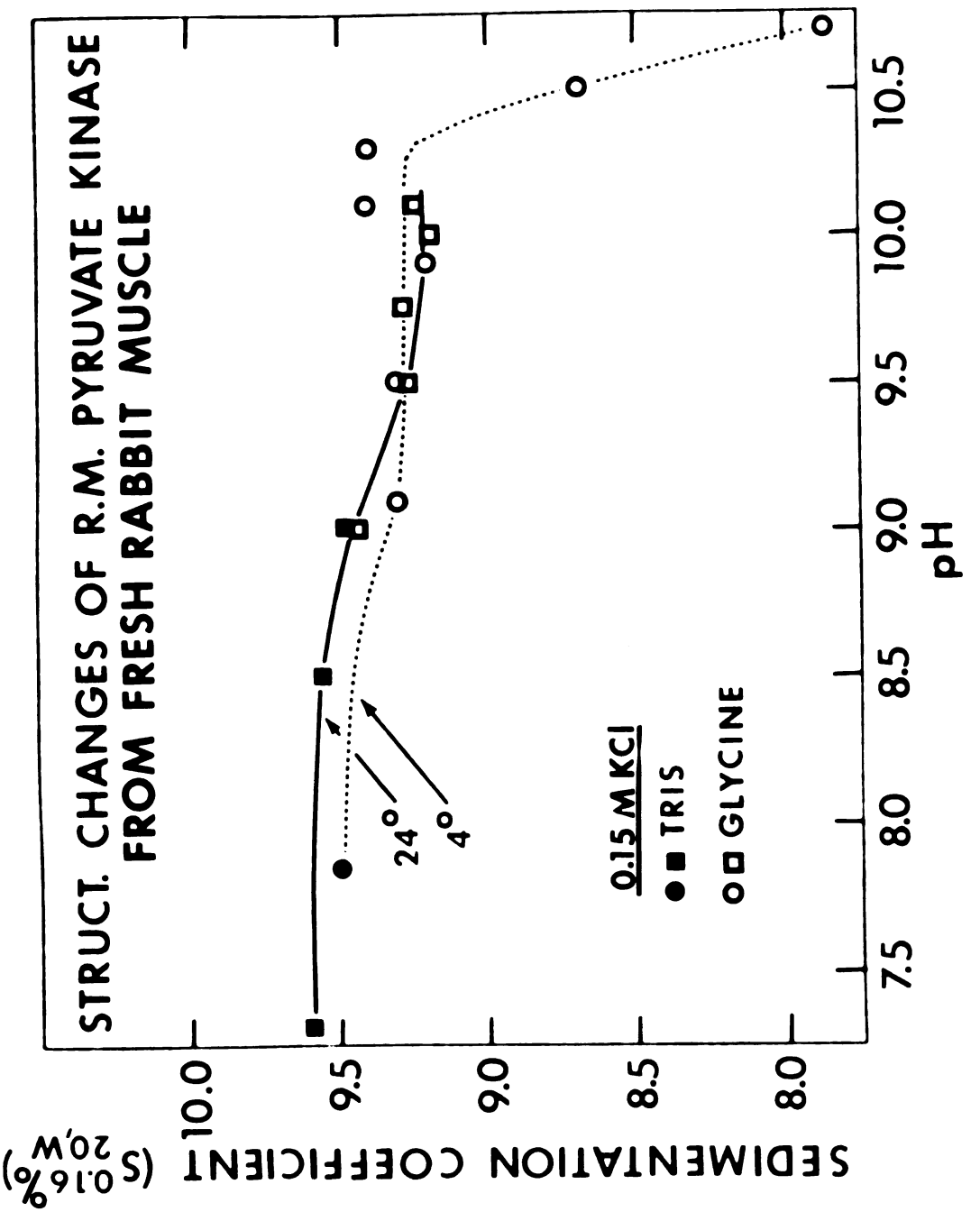

STRUCT. CHANGES OF R.M. PYRUVATE KINASE
FROM FRESH RABBIT MUSCLE
SEDIMENTATION COEFFICIENT ($S^{0.16\%}_{20,w}$)
10.0
9.5
9.0
8.5
8.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
pH
0.15 M KCl
● ■ TRIS
○ □ GLYCINE
24°
4°

Figure 27. EFFECT OF pH ON THE SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE

The enzyme samples (1.6 mg per ml) was dialyzed against 120 volumes of the appropriate buffer at the indicated pH for 12 hr at the indicated temperature. They were then centrifuged using a Spinco Model E Analytical Ultracentrifuge as described in Methods. The enzyme solutions contained 0.05 M buffer, 0.15 M KCl and 0.001 M EDTA.

Figure 27

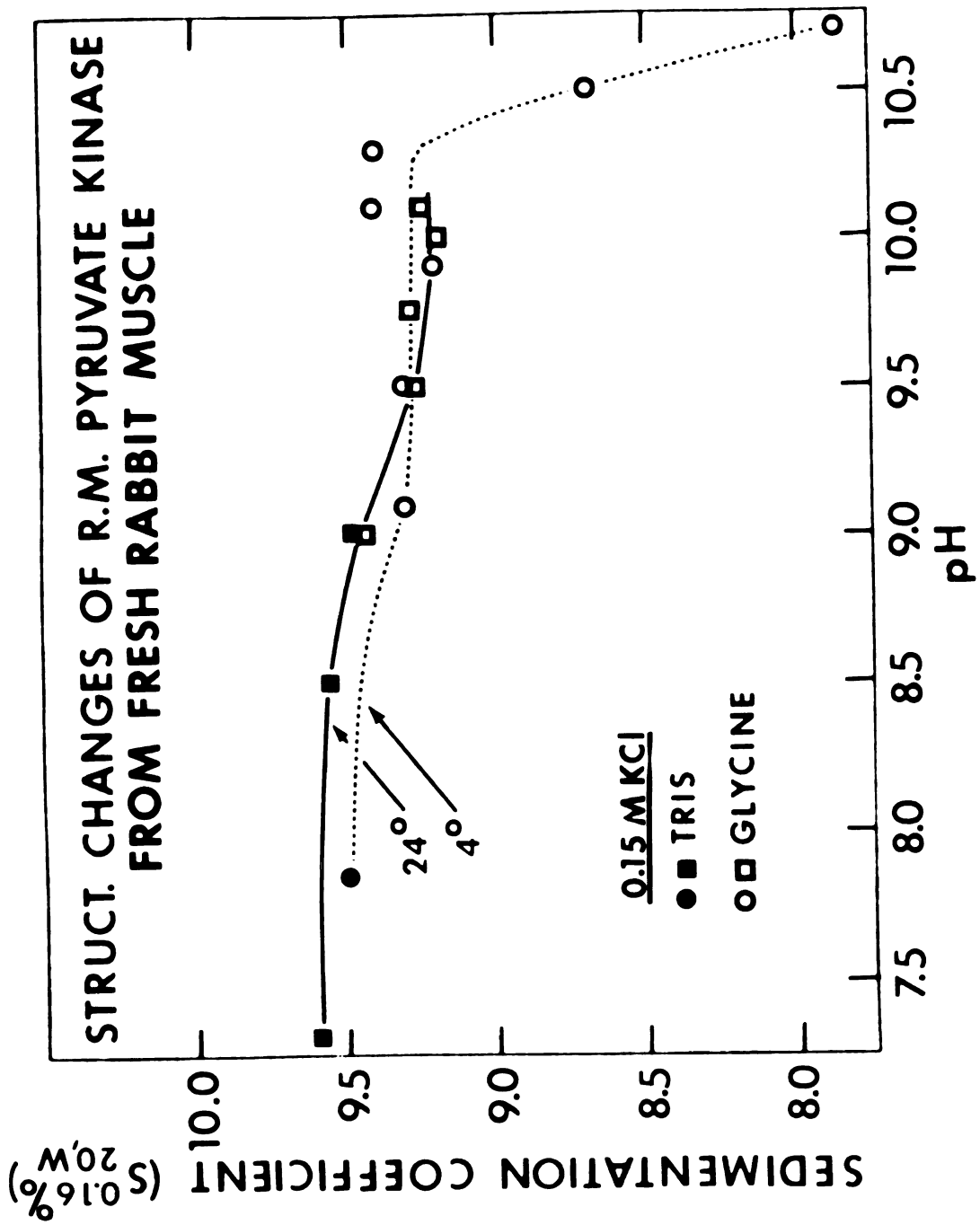

STRUCT. CHANGES OF R.M. PYRUVATE KINASE
FROM FRESH RABBIT MUSCLE
SEDIMENTATION COEFFICIENT ($S^{0.16\%}_{20,w}$)
10.0
9.5
9.0
8.5
8.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
pH
0.15 M KCl
● ■ TRIS
○ □ GLYCINE
24°
4°

Figure 28. EFFECT OF pH ON THE SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE ISOLATED FROM AGED, FROZEN RABBIT MUSCLE

The enzyme (4.4 mg per ml) was treated as described in the legend for Figure 27. The rabbit muscle used had been frozen for 2-3 years.

Figure 28

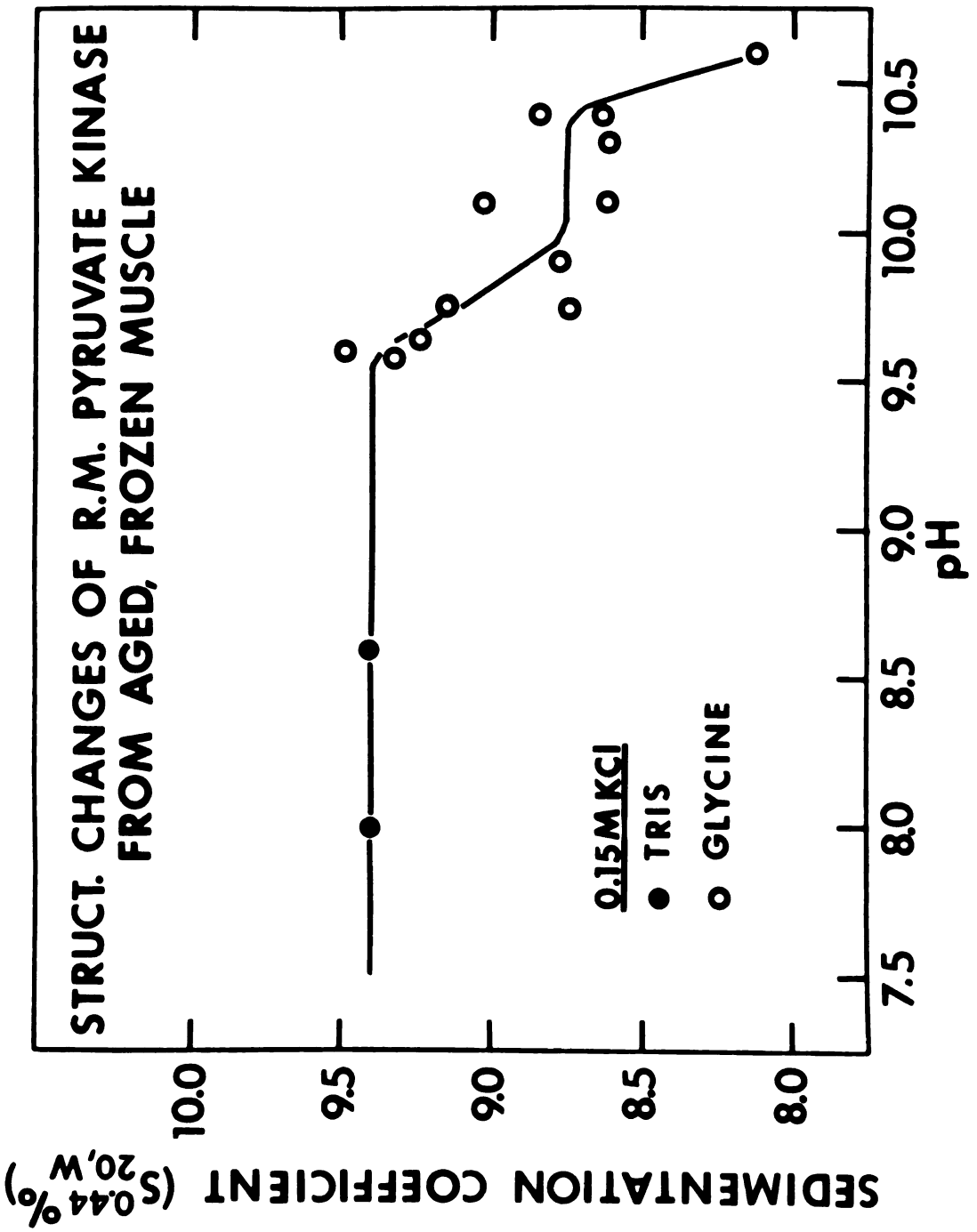

STRUCT. CHANGES OF R.M. PYRUVATE KINASE
FROM AGED, FROZEN MUSCLE
0.15M KCl
● TRIS
o GLYCINE
SEDIMENTATION COEFFICIENT ($S^{0.44\%}_{20,w}$)
10.0
9.5
9.0
8.5
8.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
pH

Figure 29. SEDIMENTATION VELOCITY PATTERNS OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE

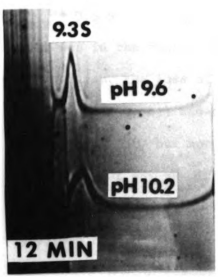
The enzyme solutions were prepared and centrifuged as described in Methods. Sedimentation is from left to right. The sedimentation coefficient indicated above the peaks are the values at the given protein concentration, corrected to 20° and water. All samples contained 0.05 M buffer, 0.001 M EDTA, and, except where indicated, 0.15 M KCl. They were run at 56,100 rpm.

(A) and (B) Upper: 1.65 mg/ml, 24°, glycine, pH 9.6; pictures taken 12 min (A) and 24 min (B) after attaining speed.
Lower: 1.65 mg/ml, 24°, glycine, pH 10.2; pictures taken 12 min (A) and 24 min (B) after attaining speed.

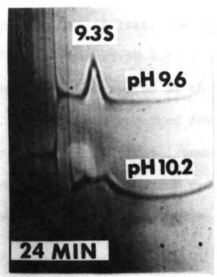
(C) Upper: 1.65 mg/ml, 5°, Tris, pH 7.9; pictures taken 24 min after attaining speed.
Lower: 1.65 mg/ml, 5°, glycine, pH 10.5; pictures taken 24 min after attaining speed.

(D) Upper: 1.65 mg/ml, 5°, Tris, pH 7.5, 1.5 M KCl; pictures taken 12 min after attaining speed.
Lower: Same as upper except the buffer contained 2.5 M KCl instead of 1.5 M.

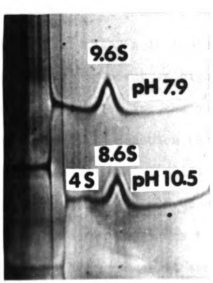
Figure 2



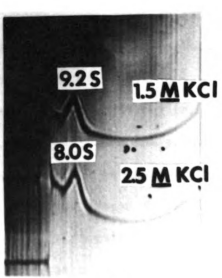
(A)



(B)



(C)



(D)

30, a). An additional 4 S peak was observed at pH 10.6.

The effect of pH on the pyruvate kinase from frozen muscle was also tested at 1.5 mg/ml and 24°. A transition similar to that shown in Figure 27 was observed; the sedimentation coefficient decreased from $s^{o}_{20,w}$ = 9.6 S to $s^{o}_{20,w}$ = 9.3 S. However, in contrast, the transition point was in the region of pH 7.9-8.4, instead of pH 8.5-9.0. A second transition, the loss of well-defined peaks, occurred at pH 9.8 and above (Figure 30, b); added salt (0.15 M KCl) was required for this effect at pH 9.8, but not at higher pH. This salt requirement was not observed at any pH with the enzyme prepared from fresh muscle.

The "aging transition" reported by Kayne (1966) and Cottam et al. (1969) was not observed.

Effect of Salt Concentration on the Sedimentation Coefficient of Pyruvate Kinase. The ionic environment of the protein has been shown to be an important determinant in protein structure (see Jencks, 1969, for a review). It seemed likely that pyruvate kinase could be sensitive to small changes in ionic strength and that perhaps gross changes in structure may occur at higher salt concentration. Potassium chloride was chosen for the analysis because it is a required cofactor for the catalytic reaction (Boyer et al., 1942) and ammonium sulfate was chosen because of its use at high concentrations in the purification procedure (Teitz and Ochoa, 1958). A further reason for the choice of the two salts was that rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is specifically dissociated into dimers by ammonium sulfate (Constantinides and Deal, 1967) and into monomers by KCl (Constantinides and Deal, 1968).

Figure 30. SEDIMENTATION VELOCITY PATTERNS OF PYRUVATE KINASE ISOLATED FROM AGED FROZEN RABBIT MUSCLE

The enzyme solutions were prepared and centrifuged as described in Methods. Sedimentation is from left to right. The sedimentation coefficients indicated above the peaks are the values at the given protein concentration, corrected to 20° and water. All samples contained 0.05 M buffer, 0.001 M EDTA and, except where indicated, 0.15 M KCl. They were run at 56,100 rpm.

(A) Upper: 4.4 mg/ml, 5°, glycine, pH 9.7; picture taken 40 min after attaining speed.

Lower: 4.4 mg/ml, 5°, glycine, pH 9.0; pictures taken 40 min after attaining speed.

(B) Upper: 1.6 mg/ml, 24°, glycine, pH 9.8; pictures taken 8 min after attaining speed.

Lower: 1.6 mg/ml, 24°, glycine, pH 9.8, no KCl; pictures taken 8 min after attaining speed.

Figure 30

Figure 31 shows the effect of increasing salt concentration on the sedimentation coefficient of pyruvate kinase isolated from fresh rabbit muscle. The sedimentation coefficient increased from a value of about 9.3 in no added salt to a maximum of $s^{0.1\,6\ \%}_{20,w} = 9.6$ S at 0.1 M-0.5 M KCl, pH 7.5, 24°, and 0.05 M-0.16 M $(NH_4)_2SO_4$, pH 8.0, 5°, and decreased with increasing salt concentration to a value as low as $s^{0.1\,6\ \%}_{20,w} = 7.65$ S at 3.0 M KCl, very close to values expected for a dimer (mol wt 115,000). The transition at pH 9.5-pH 10.0 (Figure 27) was observed in 0.15 M KCl, but not in 0.5 M KCl. At pH 10.0 sedimentation coefficient decreased with increasing KCl concentration to a value of $s^{0.1\,6\ \%}_{20,w} = 7.5$ S in 2.5 M KCl.

The decrease in sedimentation coefficient with increasing concentrations of KCl and $(NH_4)_2SO_4$ appeared to be a function of ionic strength and not of molarity of the solutions (Figure 31). This suggested that the decrease in sedimentation coefficient could be correlated to the activity of water in the solutions. The activity of water in the KCl solutions was calculated from the osmotic coefficient, Ø, of the solutions (given in Harned and Owens, 1958) by the expression:

$$\ln a_{H_2O} = - \phi \frac{M}{1000} m$$

where: a is the activity of water; M is the molecular weight of water; Ø is the osmotic coefficient, and m is the molal concentration of KCl.

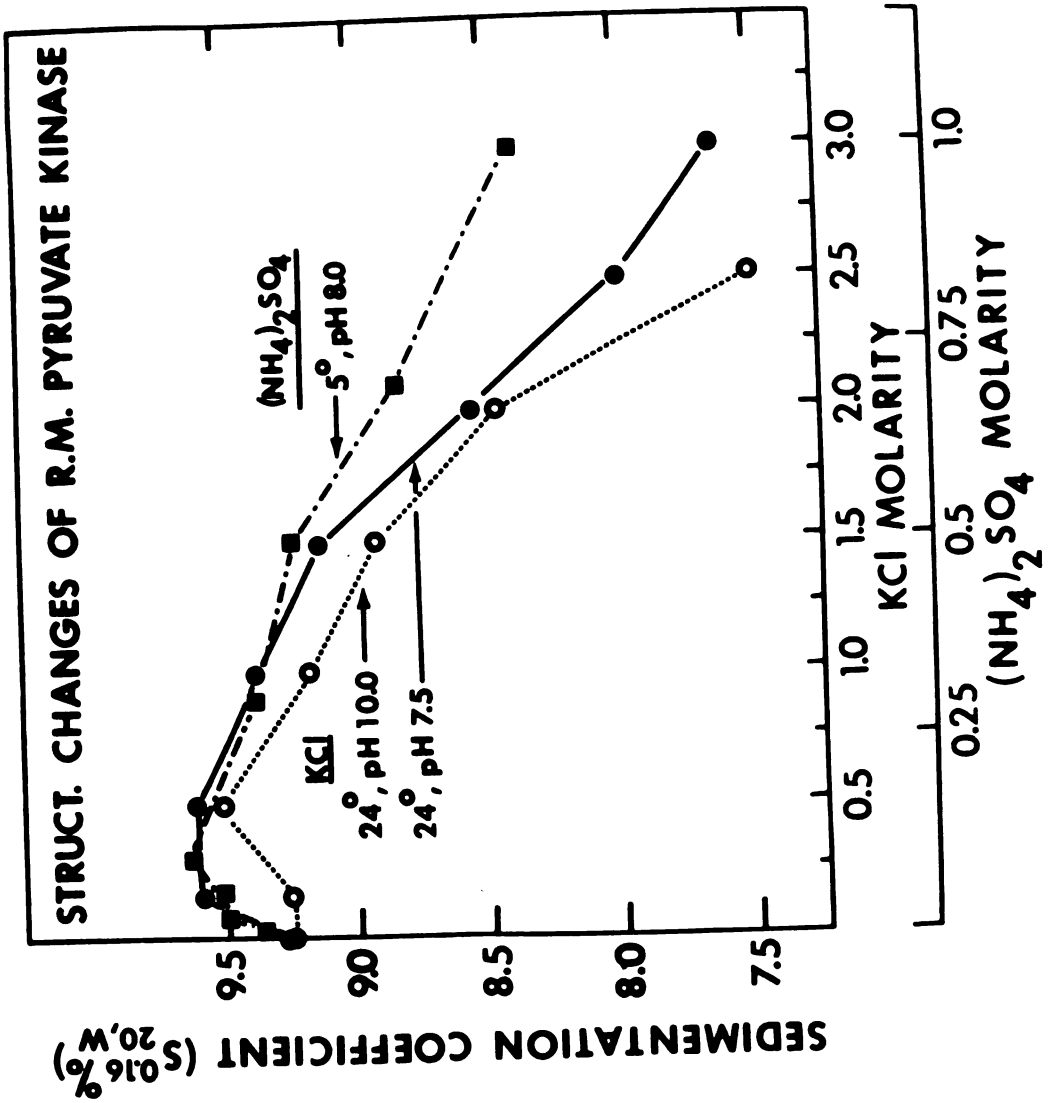
However similar data for the $(NH_4)_2SO_4$ solutions could not be located. Thus the correlation could not be made.

The results were qualitatively similar with increasing concentrations of both ammonium sulfate and potassium chloride under the condi-

Figure 31. EFFECT OF SALT CONCENTRATION ON THE SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE

The enzyme (1.65 mg per ml) was dialyzed for 12 hr at the indicated temperature versus 120 volumes of the respective buffer and salt concentration, and centrifuged using a Spinco Model E Analytical Ultracentrifuge as described in Methods. In addition to the salt, the enzyme solution contained 0.05 M buffer, and 0.001 M EDTA.

Figure 31


STRUCT. CHANGES OF R.M. PYRUVATE KINASE
$(NH_4)_2SO_4$
5°, pH 8.0
KCl
24°, pH 10.0
24°, pH 7.5
SEDIMENTATION COEFFICIENT ($S^{0.16\%}_{20,w}$)
9.5
9.0
8.5
8.0
7.5
0.5
1.0
1.5
2.0
2.5
3.0
KCl MOLARITY
0.25
0.5
0.75
1.0
$(NH_4)_2SO_4$ MOLARITY

tions shown, and also with KCl at 4°, not shown, suggesting that the results were general effects of ionic strength and not specific for any one set of conditions.

Analysis of Rabbit Muscle Pyruvate Kinase in 3.0 M KCl. The most likely explanations for the decreased sedimentation coefficient in 3.0 M KCl are a rapid equilibrium dissociation-association system or an unfolding. With either of these possibilities the sedimentation coefficient should show a pronounced protein concentration dependence. In the former case it should increase, while in the latter it should decrease, with decreasing protein concentration.

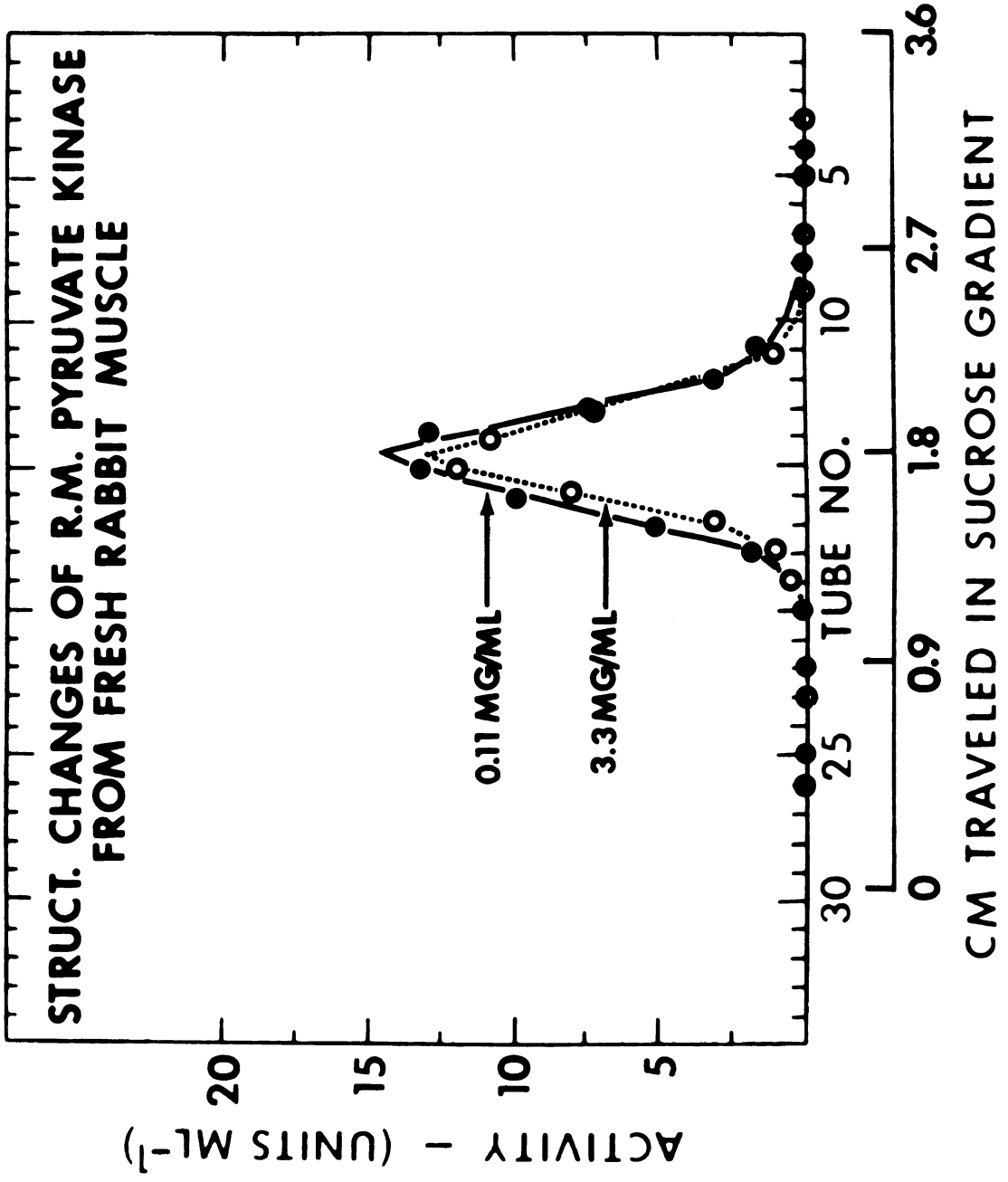
To test for a protein concentration dependence, sucrose density centrifugation was used in the protein concentration range of 3.3 to 0.055 mg/ml with the 5-20 % sucrose gradients also containing 3.0 M KCl. Contrary to expectation, there was no change in the relative sedimentation coefficients over the protein concentration range tested. The results of two extremes of concentrations are shown in Figure 32. The actual sedimentation coefficient could not be determined because of the lack of stable markers in 3.0 M KCl.

Since the sucrose may have stabilized the enzyme against dissociation or unfolding, two other methods were used to analyze for these changes. Unequivocal molecular weights can be determined from (1) sedimentation equilibrium experiments, or (2) from the sedimentation and diffusion coefficients using the Svedberg equation (see Methods). Accordingly, in simultaneous experiments, the molecular weight of pyruvate kinase in 3.0 M KCl was determined by sedimentation equilibrium experiments and by a determination of the diffusion coefficient

Figure 32. EFFECT OF PROTEIN CONCENTRATION ON THE SEDIMENTATION COEFFICIENT OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE IN 3.0 M KCl USING SUCROSE DENSITY CENTRIFUGATION

The enzyme was dialyzed at the indicated enzyme concentration for 12 hr at 24° versus 150 volumes of the buffer solutions containing 0.05 M Tris (pH 7.5, 0.001 M EDTA, and 3.0 M KCl. Aliquots (0.1 ml) of each solution were layered on the 5-20 % sucrose gradients of the same buffer composition. The protein sedimented at 40,000 rpm and 24° for 10 hr.

Figure 32


STRUCT. CHANGES OF R.M. PYRUVATE KINASE
FROM FRESH RABBIT MUSCLE
0.11 MG/ML
3.3 MG/ML
ACTIVITY - (UNITS ML-1)
20
15
10
5
TUBE NO.
30
25
20
15
10
5
0
0.9
1.8
2.7
3.6
CM TRAVELED IN SUCROSE GRADIENT

and sedimentation coefficient.

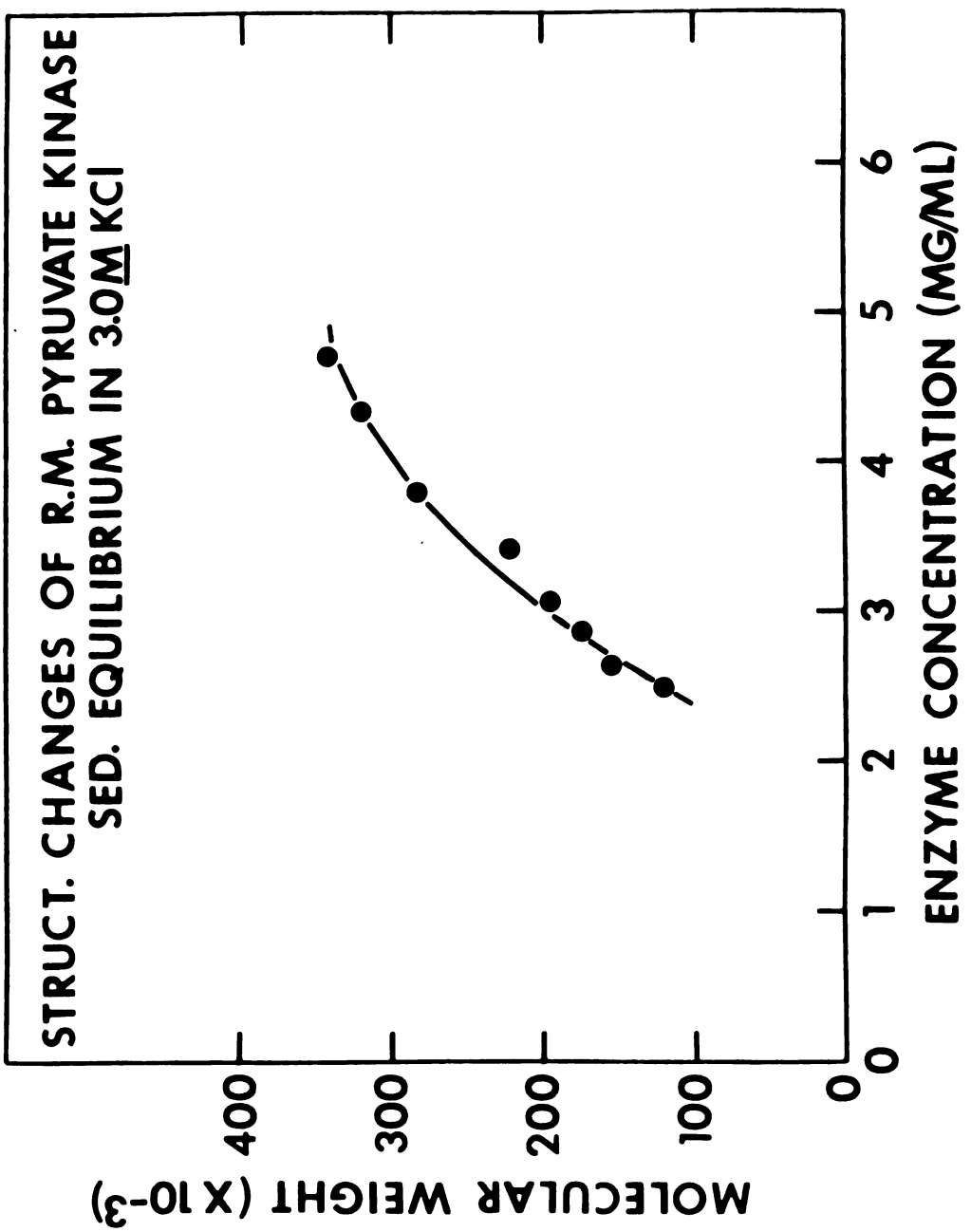
The equilibrium experiments showed (1) rabbit muscle pyruvate kinase dissociated in 3.0 M KCl, (2) a dissociation-association system existed which was dependent upon the concentration of protein in the cell, (3) possibly there was also random aggregation. Using computer programs (see Methods), it was observed (Figure 33) that at 2.5 mg/ml (top of the cell) the molecular weight was 120,000, whereas at 4.7 mg/ml (bottom of the cell) the molecular weight was 340,000. The weight average molecular weight, over the entire cell, was 232,700, and the Z-average molecular weight was 600,800.

These results suggested the possibility of a rapid equilibrium between a dimer (mol wt 115,000) or a monomer (mol wt 57,000) and a tetramer (mol wt 237,000) or an octamer (mol wt 470,000). A monomer-octamer equilibrium is the best explanation for the data: (1) The sedimentation coefficient determined in 3.0 M KCl was $s^{0.16\ \%}_{20,w} = 7.65$ S. A sedimentation coefficient of 7.65 S should correspond to a molecular weight of 120,000 (see Figure 24). However, since the rapid equilibrium dissociation-association system yielded a sedimentation coefficient which was intermediate between the two interacting components, this necessitated the involvement of the monomer and not the dimer in the equilibrium. (2) The trend of the molecular weight curve shown in Figure 33 predicts that at concentrations of about 1 mg/ml or lower, the average mol wt would be far below 120,000 (the dimer mol wt) indicating that the equilibrium must involve a monomer. (3) Molecular weights (340,000) larger than that of the tetramer were observed, indicating the presence of the octamer. (4) The equilibrium constant cal-

Figure 33. SEDIMENTATION EQUILIBRIUM ANALYSIS OF RABBIT MUSCLE PYRUVATE KINASE IN 3.0 M KCl

The enzyme was prepared and centrifuged as described in Methods. Equilibrium was reached in 24 hr at 6994 rpm and 24°. The solution contained 3.3 mg/ml enzyme, 0.05 M Tris, pH 7.5, 3.0 M KCl, and 0.001 M EDTA. Computer programs were used to calculate protein concentration and "point" weight-average molecular weights at various radial positions in the cell.

Figure 33


STRUCT. CHANGES OF R.M. PYRUVATE KINASE
SED. EQUILIBRIUM IN 3.0M KCl
MOLECULAR WEIGHT (X 10-3)
100
200
300
400
0
1
2
3
4
5
6
ENZYME CONCENTRATION (MG/ML)

culated at the two extremes of concentration was most consistent with a monomer-octamer equilibrium. However, the possibility of an equilibrium between monomers and tetramers cannot be excluded, due to the presence of aggregation.

The diffusion coefficient was determined to be $D^{0.33\ \%}_{20,w} = 3.30 \times 10^{-7}$ cm^2/sec. Combining this value with $s^{0.16\ \%}_{20,w} = 7.65$ S, the molecular weight of rabbit muscle pyruvate kinase in 3.0 M KCl was calculated to be 220,000 using the Svedberg equation (see Methods). This value was not consistent with the lower molecular weight which should be observed at low protein concentrations (1.6 mg/ml) (Figure 33), but the effect of rapid equilibrium on the diffusion coefficient has not been determined. The high molecular weight component may have had a disproportional effect on the diffusion coefficient, thus leading to an increased apparent molecular weight.

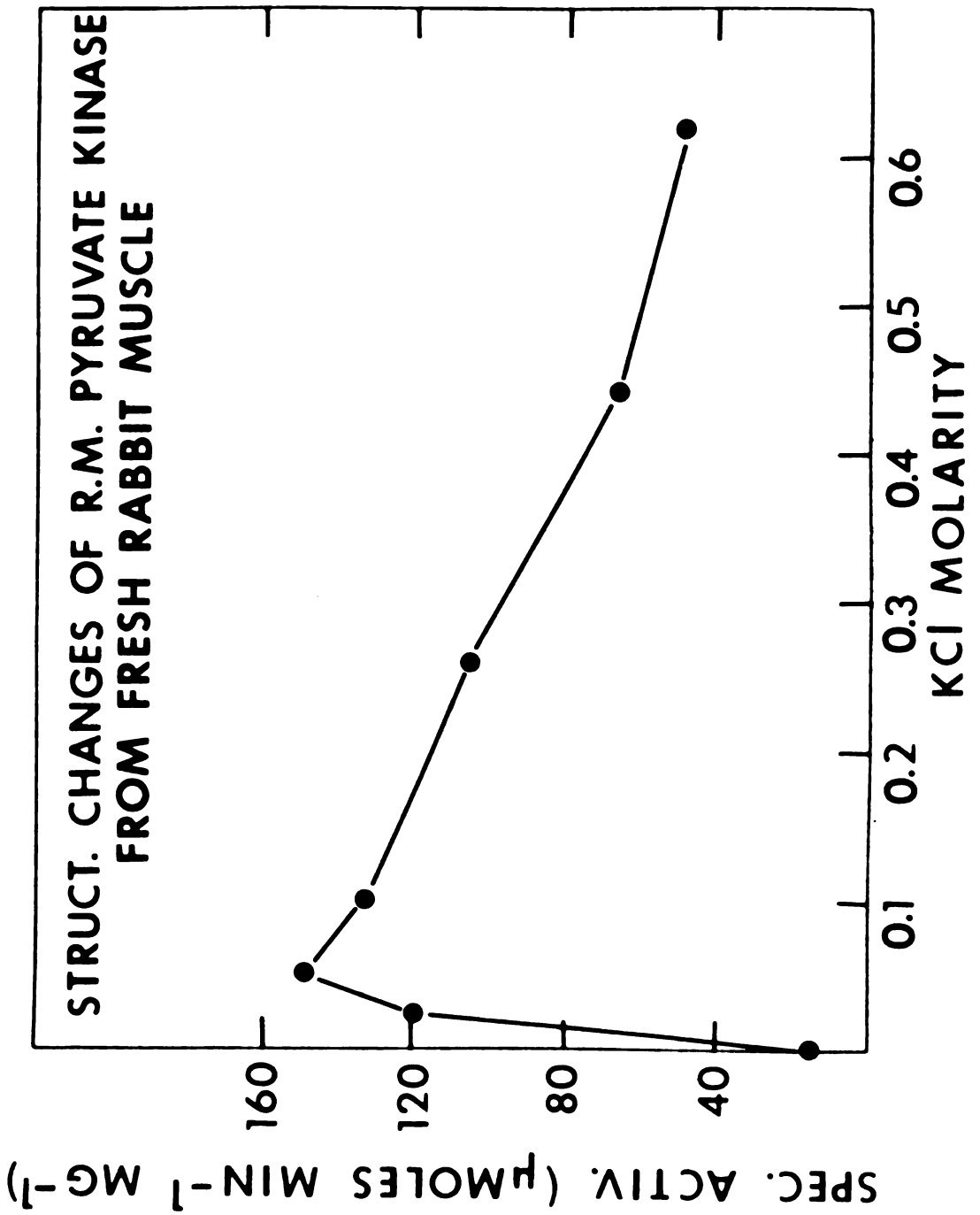
Correlation of the Change in Sedimentation Coefficient with KCl Concentration to Enzymatic Activity. The specific activity of rabbit muscle pyruvate kinase increased from about 15 with no added KCl in the assay to a maximum value of 155 in 0.06 M-0.1 M KCl. It then decreased to about 30 % of the maximum activity in 0.6 M KCl (Figure 34). Similar activity results have been previously reported (Melchior, 1965).

These results did not parallel the change in sedimentation coefficient as a function of KCl concentration (Figure 31). Although the time of exposure to the salt differed in the two experiments, some correlations can be made. The activity and sedimentation coefficient reached a maximum at about the same concentration of KCl (0.1 M) and

Figure 34. EFFECT OF POTASSIUM CHLORIDE OF THE ENZYMATIC ACTIVITY OF PYRUVATE KINASE ISOLATED FROM FRESH RABBIT MUSCLE

Stock enzyme solution (90 mg per ml) was diluted to 0.09 mg per ml into water at 0^{o}. Aliquots (0.9 μg) were assayed at 25^{o} in assay solutions containing the indicated concentration of KCl. In addition to the KCl the assay solution contained 0.1 M imidazole (pH 7.5), 2×10^{-2} M $MgCl_2$, 3.3×10^{-4} M PEP, 2.0×10^{-3} M ADP.

Figure 34


STRUCT. CHANGES OF R.M. PYRUVATE KINASE
FROM FRESH RABBIT MUSCLE
SPEC. ACTIV. (μMOLES MIN-1 MG-1)
160
120
80
40
0.1
0.2
0.3
0.4
0.5
0.6
KCl MOLARITY

then decreased with increasing salt concentration; the decrease in activity did not correspond to the decrease in sedimentation coefficient.

Reversal of the Transition. The inactivation and decrease in sedimentation coefficient in high concentrations of KCl was shown to be reversible. The product of a 12 hour dialysis in 0.15 M KCl, 0.05 M Tris, pH 7.5 at 24° had a sedimentation coefficient of $s^{o}_{20,w}$ = 9.6 S. Also, the enzyme which should have had little, if any, activity in 2.0 or 3.0 M KCl (Figure 34) was fully activated by dilution into the assay mix.

DISCUSSION

This study shows that in its original native state, rabbit muscle pyruvate kinase has a sedimentation coefficient of $s^{o}_{20,w}$ = 9.6 S; this value is also found by a sucrose density analysis of pyruvate kinase from a crude extract from rabbit muscle. It has also shown that the native enzyme is reversibly altered by high pH or high ionic strength. High salt 3.0 M KCl (pH 7.5, 24°) causes a decrease in sedimentation coefficient to a value of $s^{o}_{20,w}$ = 7.6 S. Moreover, a molecular weight analysis has shown that this decrease is due to a dissociation, and the results are most consistent with a rapid equilibrium between monomers and tetramers and/or octamers. The range of sedimentation coefficients observed at about pH 10.0-10.5 may also be explained by a rapid equilibrium but these systems have not yet been analyzed.

We did not observe the irreversible conversion to an 8.5 S

species previously reported (Kayne, 1966). However, we have shown that rabbit muscle pyruvate kinase appears to exist as a rapid equilibrium mixture under certain conditions. It is possible that this equilibrium may exist under normal conditions of temperature, pH, and ionic strength and strongly favor the native tetrameric enzyme under the protein concentrations previously studied.

The shift for the transition to a lower pH with the aged, frozen enzyme is particularly interesting. It is possible that conditions such as prolonged storage in high salt concentrations or at an unusual or unexpected high pH may irreversibly alter the equilibrium position. This could result from a modification of the enzyme (i.e., oxidation of sulfhydryl groups) to a form which does not readily associate.

It is possible that the high salt concentrations used in purification may cause dissociation. However, the ammonium sulfate precipitation is done at a higher protein concentration than that of this study (20 mg/ml) and the effect of this factor on the dissociation is not known.

Mechanism of Alteration of Structure by High pH and High Salt. Rabbit muscle pyruvate kinase contains a large number of the hydrophobic amino acids: alanine, glycine, valine, leucine, and isoleucine (Kayne, 1966; Cottam et al., 1969). Hence pyruvate kinase could be expected to be unstable in a hydrophobic environment. It has been dissociated into completely unfolded subunits in the highly hydrophobic environment of 4-8 M urea (Steinmetz and Deal, 1966). But the enzyme is relatively stable in the lesser hydrophobic environment of ethanol

(30 %), and the reagent is used in the purification procedure (Teitz and Ochoa, 1958). Recently, Cottam et al. (1969) reported that lower concentrations of guanidine-HCl (greater than 1 M) than urea (4 M) are required to dissociate the enzyme into completely unfolded subunits, presumably due to the dual, ionic and hydrophobic, character of the guanidine-HCl solution. This suggests that hydrophobic bonding is not the sole determinate for protein structure, but ionic and hydrogen bonding may also be important.

It is possible that the dissociation by high concentrations of salt is due to a disruption of key ionic linkages. However, an alternate explanation is that the high salt concentrations dissociate the enzyme indirectly by changing the characteristics of the solvent. Because urea is able to dissociate rabbit muscle pyruvate kinase completely into ultimate subunits, it is highly unlikely that ionic bonds could be a major determinate in subunit bonding. Such has been found to be the case for hemoglobin (Kirshner and Tanford, 1964; Kawahara et al., 1965). Two types of binding sites between subunits were postulated, one ionic and the other hydrophobic. It is likely that the dissociation by high concentrations of salt is due to a combination of the two alternate explanations.

The dissociation at high pH suggests that a charged group with a pK of about 10 is important for proper subunit binding. However, the possibility of base catalyzed cleavage of peptide linkages cannot be excluded.

The Partial Specific Volume Factor. An exact knowledge of the partial specific volume ($\bar{v}$) of pyruvate kinase is essential for valid sedimen-

tation and molecular weight determinations. It is assumed in the calculations that the partial specific is constant for all the buffer conditions tested. However, the possibility of preferential interaction of pyruvate kinase with one member of a multicomponent system, in the case of the salt species, exists, such that the specific volume of the enzyme solute complex may be quite different from that of the pure enzyme.

Previous studies have shown that high salt concentrations do not affect the partial specific volume of other enzymes. The partial specific volume of hemoglobin is unchanged in solutions up to 2.65 M NaCl (Kirshner and Tanford, 1964), and that of human transferin or bovine plasma albumin does not change in solutions up to 0.2 M KCl (Hunter, 1967).

Although a slight change in the partial specific volume at high salt concentrations is possible, it is unlikely that such a change would be great enough to significantly affect the sedimentation values. It should be noted that a change in partial specific volume from 0.741 cc/g to 0.795 cc/g would be required to account for the change in sedimentation coefficient from 0.15 M KCl to 3.0 M KCl.

MATERIAL AND METHODS

Pyruvate kinase was prepared and assayed as previously described (Teitz and Ochoa, 1958; Steinmetz and Deal, 1966; Johnson et al., 1969). The source of the enzyme was skeletal muscle from live rabbits obtained locally or from frozen muscle (Pel Freez Biologicals, Inc.) which had been frozen for 2-3 years (see Test for further details).

Analysis of the Crude Extracts From Rabbit Muscle and Rabbit Liver. Frozen rabbit muscle or frozen rabbit liver (5-10 g) was added to an equal weight of water and homogenized for 15 minutes at 0°. The solution was then centrifuged at 0° for 15 minutes at 27,000 g. The supernatant was analyzed by sucrose density gradient centrifugation (Martin and Ames, 1961). The Spinco Model L ultracentrifuge was used and the SW 39 rotor was run at 0° for 14 hours at 40,000 rpm. Aldolase and lactate dehydrogenase, which served as markers, were assayed according to the procedures of Richards and Rutter (1961) and Chasin (1967), respectively.

Preparation of the Enzyme for Ultracentrifugal Analysis. The stock enzyme solutions (70-90 mg/ml) were diluted to 1.65 mg/ml or 4.4 mg/ml with a solvent containing 0.05 M Tris (HCl) or glycine (K) buffer, 0.001 M EDTA and the indicated salt concentration. They were then dialyzed for 11-13 hours at 5° or 24° against 125 volumes of the buffer. The ammonium sulfate solutions were adjusted to proper pH with H_2SO_4, and the other solutions were adjusted with HCl or KOH. Except for the ammonium sulfate solutions, the pH of the samples was adjusted to the proper pH at 24°, and the actual pH at the lower temperatures was calculated using the correction factor, -0.03 pH units per degree (Long, 1961). This correction factor was also verified experimentally. Thus, for all except the ammonium sulfate samples, the pH values listed are the actual values at the temperatures at which the experiments were performed. Due to the presence of varying concentrations of ammonium ions in the ammonium sulfate solutions, the dependence of pH with temperature was not determined and the pH

reported is that measured at 24°.

Ultracentrifugal Analysis. All experiments were performed in a Spinco Model E ultracentrifuge equipped with phase-plate schlieren optics. The exact temperatures were obtained with a calibrated rotor and temperature indicator and control (RTIC) unit. Photographic plates were measured with a Bausch and Lomb microcomparator. Calculations were done manually or on a Control Data Corporation 3600 digital computer, using computer programs developed in this laboratory.

Sedimentation Velocity Analysis. Sedimentation velocity experiments were done with 12 mm signle sector cells, at 56,100 rpm in an An-D rotor. The sample volumes were approximately 0.55 ml. Sedimentation coefficients were calculated from the expression:

$$S = \frac{1}{(t-t_o)w^2} \quad \ln \frac{r_p\,(t)}{r_p\,(t_o)}$$

where S is the sedimentation coefficient in Svedberg units (10^{-13} seconds = S), r_p is the peak maximum in the refractive index gradient curve of the picture taken at time t_o (first picture taken), or at time t (later pictures), and w is the rotor velocity in radians/sec.

Sedimentation Equilibrium Analysis. Standard double sector cells were used for the sedimentation equilibrium experiments. Equilibrium was reached after centrifuging for 24 hours at 6994 rpm and 24° using an An-D rotor. Computer programs developed in our laboratory were used to calculate weight-average and Z-average molecular weights, averaged over the whole cell, and also the protein concentration and "point"

weight-average molecular weights at various radial positions in the cell. The "point" mol wt (M) was calculated using the expression:

$$M = \frac{RT}{(1-\bar{v}\rho)w^2} \cdot \frac{(dc/dr)}{rc}$$

where T is the absolute temperature, R is the gas constant of 8.314×10^7 ergs/mole/degree, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solution, w is the angular velocity of the rotor in radians/sec, and c and dc/dr are the concentration and concentration gradient at the point, r, in the cell.

Diffusion Coefficient Analysis. The diffusion coefficient, D, was determined with a 12 mm double sector synthetic boundary cell at 4,059 rpm using an An-D rotor. Values were calculated manually from an earlier equation (Schachman, 1957), modified to allow use of measured data without correction for the magnification factor, F:

$$D = \frac{m}{4\pi\ 60\ F^2}$$

where m is the slope of a line from a graph which plots area^2/height^2 versus time (minutes).

Molecular Weight Determination Using the Svedberg Equation. The molecular weight is calculated from the sedimentation and diffusion coefficients using the following equation (Svedberg and Pedersen, 1940):

$$M = \frac{s\ RT}{D\ (1-\bar{v}\rho)}$$

where the terms are the same as those defined above.

Buffer Correction Factors. (See Appendix II for a detailed analysis.) The viscosities of the solutions were determined in constant temperature water baths by means of a Cannon-Ubbeholde capillary dilution viscometer. Densities of the solvents were determined by use of a pyncnometer. The value of 0.741 cc/g at 20° (Kayne, 1966) was used for the partial specific volume; a change of 0.003 units per degree (Hunter, 1966) was used to correct for temperatures other than 20°. The change in viscosity due to temperature was determined from published tables (Handbook of Chemistry and Physics, 1963).

SUMMARY AND CONCLUSIONS

(1) The dissociation of tetrameric rabbit muscle pyruvate kinase into unfolded subunits has been shown to be reversible. This study confirms and greatly extends the preliminary studies reported by Kayne (1966). The reversal is accomplished by a 100-fold dilution of the dissociated enzyme solution into a renaturation mixture. Optimal conditions for the reversal (up to 70 % recovery of activity) are (a) pH 8.0; (b) protein concentration, 0.04 mg/ml; (c) ionic strength, 0.3; (d) reducing agent, 0.06 M β-mercaptoethanol; (e) temperature-0° dilution, followed by 6 hr at 16°. Insulin decreases the extent of the reversal, and phosphate is required for reversal at 36°. None of the other metabolites tested influenced the reversal.

The reasons for the lack of higher than 70 % recovery of activity are not known. Perhaps insulin may aid the folding under sets of conditions or combinations of metabolites different from those tested.

(2) Rabbit muscle pyruvate kinase is reversibly inactivated by a time dependent (half-time of 5 min) formation of a Schiff base with the biological cofactor, PLP. Reduction of the inactivated complexes shows that there are two types of binding, (1) a specific inactivating binding, involving 2-4 moles of PLP, (2) a non-specific noninactivating binding.

(3) Glutathione and FDP were found to interact with the enzyme but these phenomena were not studied in detail.

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(4) The sedimentation coefficient of rabbit muscle pyruvate kinase in its original (and presumably in vivo) state has been determined to be $s^{0.16\ \%}_{20,w}$ = 9.6 S since this is the value found under a variety of conditions with a number of different preparations and samples. Results from sucrose density centrifugation analysis of crude rabbit liver extract showed that rabbit liver pyruvate kinase has an $s^{o}_{20,w}$ = 7.4 S; this is lower than the value expected for pyruvate kinase from rat liver (Tanaka et al., 1967).

(5) The effect of high pH on the sedimentation coefficient of pyruvate kinase has been evaluated from enzyme isolated from fresh rabbit muscle and from muscle which has been frozen for 2-3 years. Both samples showed a change in sedimentation value from 9.6 S to 9.3 S at about pH 8.5-9.0 with an additional transition to about 8.5 S at pH 10.5 and at 5°. With the aged frozen enzyme the transition to 9.3 S could be observed as low as pH 7.9 at 24°. The aged frozen enzyme was unstable at pH 9.8 and above at 24°; the loss of peaks was observed in the presence of salt but not if there was no salt present.

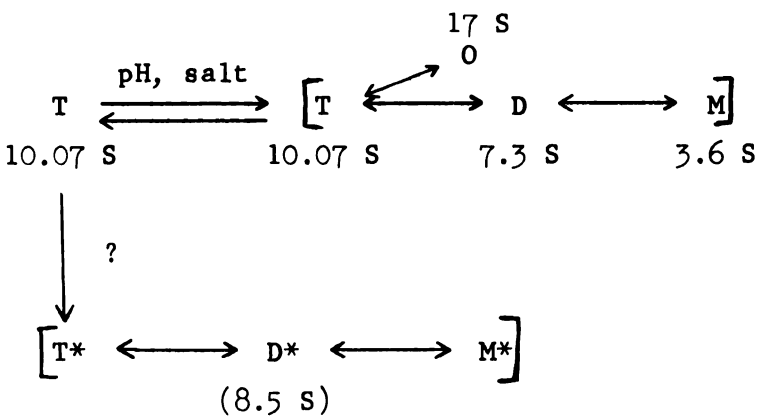
(6) The sedimentation coefficient increased from 9.3 S with no added salt to a maximum of $s^{0.16\ \%}_{20,w}$ = 9.6 S at 0.1-0.5 M KCl. It then decreased, without gradations, at higher salt concentrations, reaching a value of $s^{0.16\ \%}_{20,w}$ = 7.65 S in 3.0 M KCl. This transition was reversible by dilution or by dialysis.

(7) Sedimentation equilibrium analysis shows that the decrease in sedimentation coefficient is due to a dissociation into dimers or monomers. The results of this study are also fairly consistent with a

dissociation-association rapid equilibrium between monomers (or dimers) and tetramers and/or octamers.

(8) The conditions necessary for the observed production of a more slowly sedimenting species (8.5 S) (Kayne, 1966) at neutral pH in aqueous solutions has not been defined. A possible explanation for this irreversible transition is an alteration, such as oxidation of a critical sulfhydryl group, during prolonged storage of the enzyme at high salt concentrations or high pH.

(9) The results of the studies on the native structure of rabbit muscle pyruvate kinase are consistent with the following model:



where: T represents the native tetrameric native enzyme (mol wt 237,000, 10.07 S); D represents the dimer (mol wt 115,000, 7.3 S); M represents the monomer (mol wt 57,000, 3.6 S); O represents the octamer (mol wt 470,000, 17 S); and T* represents the irreversibly altered native enzyme which enters into the equilibrium leading to the 8.5 S sedimentation coefficient.

APPENDIX I

Derivation of Rate Equations Used to Study the Rate of Inactivation of Pyruvate Kinase by Pyridoxal 5'-Phosphate

The rate of inactivation of pyruvate kinase by pyridoxal 5'-phosphate can be expressed by the general equation:

$$(1) \quad -\frac{dA}{dt} = k_{(n+x)} (PLP)^n (A)^x$$

where: A is the active enzyme concentration; PLP is the concentration of pyridoxal 5'-phosphate; n is the order of the reaction with respect to PLP concentration; x is the order of the reaction with respect to active enzyme concentration; and k is the (n + x)th order rate constant for the inactivation.

If we assume x = 1; and (PLP) is constant,* expression (1) reduces to:

$$(2) \quad -\frac{dA}{dt} = k' (A)$$

this can be rearranged to:

$$(3) \quad -\frac{dA}{A} = k'dt$$

where k', the "pseudo first-order rate constant," is equal to:

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*Since the concentration of enzyme is 1.75×10^{-7} moles/liter and the concentration of PLP is 10^{-4} moles/liter, this is a valid assumption.

Upon int

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$$(4) \quad k' = k_{(n+1)} (PLP)^n$$

Upon integration, expression (3) yields:

$$(5) \quad \ln {}^{A}/_{A_o} = -k't$$

Now a plot of $\ln {}^{A}/_{A_o}$* versus time yields a straight line; the slope of this line is equal to -k'.

Since $k' = k_{(n+1)} (PLP)^n$

$$(6) \quad \log k' = \log k_{(n+1)} + n\log (PLP)$$

The rate of the reaction with respect to PLP,n, is given by the slope of a plot of the log k', determined at various concentrations of PLP, versus the respective concentration of PLP. The rate constant for the reaction $k_{(n+1)}$, is the intercept of the k' coordinate.

If n = 1, the plot of k' versus the respective PLP concentration should have a slope equal to the second-order rate constant for the reaction.

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*This is equivalent to the ln of per cent activity remaining.

APPENDIX II

Determination of the Correction Factors Necessary for Correcting the Observed Sedimentation Coefficients to Standard Conditions

The sedimentation coefficients observed under various conditions of solvent composition and temperature were corrected to the standard conditions of 20° and water. This appendix explains in detail the determinations and calculations used for these corrections. One result was surprising and of special interest. In the viscosity determinations the flow times for the KCl solutions were shorter than those for the reference solvent, water, which is most unusual. This confirms the results from the International Critical Tables (1929), which show this effect indirectly by listing relative viscosities of less than unity. Another important result was that the viscosities of the KCl solvents relative to water were not independent of temperature.

The Correction Expression. The complete expression for the correction of the sedimentation coefficient to 20° and water is given by the following equation:

$$\text{Eqn (1)} \quad s_{20,w} = s_{t,b} \left(\frac{\eta_{t,b}}{\eta_{20,w}}\right) \left(\frac{1-\bar{v}_{20,w}\rho_{20,w}}{1-\bar{v}_{t,b}\rho_{t,b}}\right)$$

where: $s_{20,w}$ is the sedimentation coefficient corrected to the standard conditions; $s_{t,b}$ is the observed sedimentation coefficient mea-

sured at the temperature, t, and in the solvent buffer, of the experiment; $(\eta_{t,b}/\eta_{20,w})$ is the viscosity of the buffer at temperature, t, relative to that of water at 20°, $\rho_{20,w}$ is the density of water at 20°; $\rho_{t,b}$ is the density of solvent at temperature, t; $\bar{v}_{20,w}$ is the partial specific volume of the protein in water at 20°; and $\bar{v}_{t,b}$ is the partial specific volume of the protein in the buffer at temperature, t.

The term, $\eta_{t,b}/\eta_{t,w}$ is composed of two terms:

$$\text{Eqn (2)} \quad \frac{\eta_{t,w}}{\eta_{20,w}} \times \frac{\eta_{t,b}}{\eta_{t,w}}$$

The first term is the temperature contribution to the viscosity of water. The values are found in the Handbook of Chemistry and Physics (1963). The second term is the viscosity of the buffer relative to that of water. It is generally assumed to be independent of temperature, but the results of this study have shown that this is not true for high concentrations of KCl. This term is determined by the following expression showing the temperature dependence.

$$\text{Eqn (3)} \quad \eta_{t,b}/\eta_{t,w} = (\rho_b/\rho_w)\,(T_{t,b}/T_{t,w})$$

where (ρ_b/ρ_w) is the density of the buffer solution relative to that of water and $(T_{t,b}/T_{t,w})$ is the flow time of the buffer solution through the viscometer relative to water at temperature, t.

The density, $\rho_{t,b}$ (Eqn (1)) of the solvent at temperature, t, is approximately equal to the relative density, ρ_t/ρ_w, since the value of ρ_w is very close to unity, ranging from 0.99997 g/cc at 6° to 0.99823 g/cc at 20° (Handbook of Chemistry and Physics, 1963). Thus

a determination of the relative density factor ρ_b/ρ_w is sufficient to determine $\rho_{t,b}$.

The correction factors ($\eta_{t,w}/\eta_{20,w}$) and $\rho_{20,w}$ are found in the Handbook of Chemistry and Physics (1963). The partial specific volume has been discussed in Chapter 3 and will not be further mentioned. The remainder of this Appendix will discuss an evaluation of the $\eta_{t,b}/\eta_{t,w}$ and ρ_b/ρ_w factors.

Necessity for Additional Determinations. The relative viscosity and density terms for several ammonium sulfate and potassium chloride solutions are given in the International Critical Tables (1929). However, the tables are not complete for the salt concentrations and temperatures used in the centrifuge experiments of Chapter 3. Moreover they do not include the contributions of the remaining buffer components (0.05 M buffer and 0.001 M EDTA) to the density and viscosity.

For these reasons we undertook a detailed analysis to determine the relative viscosities and relative densities of the buffer solutions used in Chapter 3.

Results. The viscosities of the solutions were determined in constant temperature water baths by means of a Cannon-Ubbelodhe capillary dilution viscometer. Densities of the solvents were determined by use of a pyncnometer.

The relative densities of the solutions are shown in Figure 35, and the relative flow times through the viscometer in Figure 36. From these values, the relative viscosities were calculated (Figure 37). The relative viscosities of a few representative solutions with

Figure 35. RELATIVE DENSITIES OF THE SALT SOLUTIONS

Densities were determined at the indicated temperatures with a pyncnometer. In addition to the salts, the buffer solutions contained 0.001 M EDTA and 0.05 M Tris HCl (pH 7.5 in the KCl solutions; pH 8.0 in the $(NH_4)_2SO_4$ solutions).

Figure 35

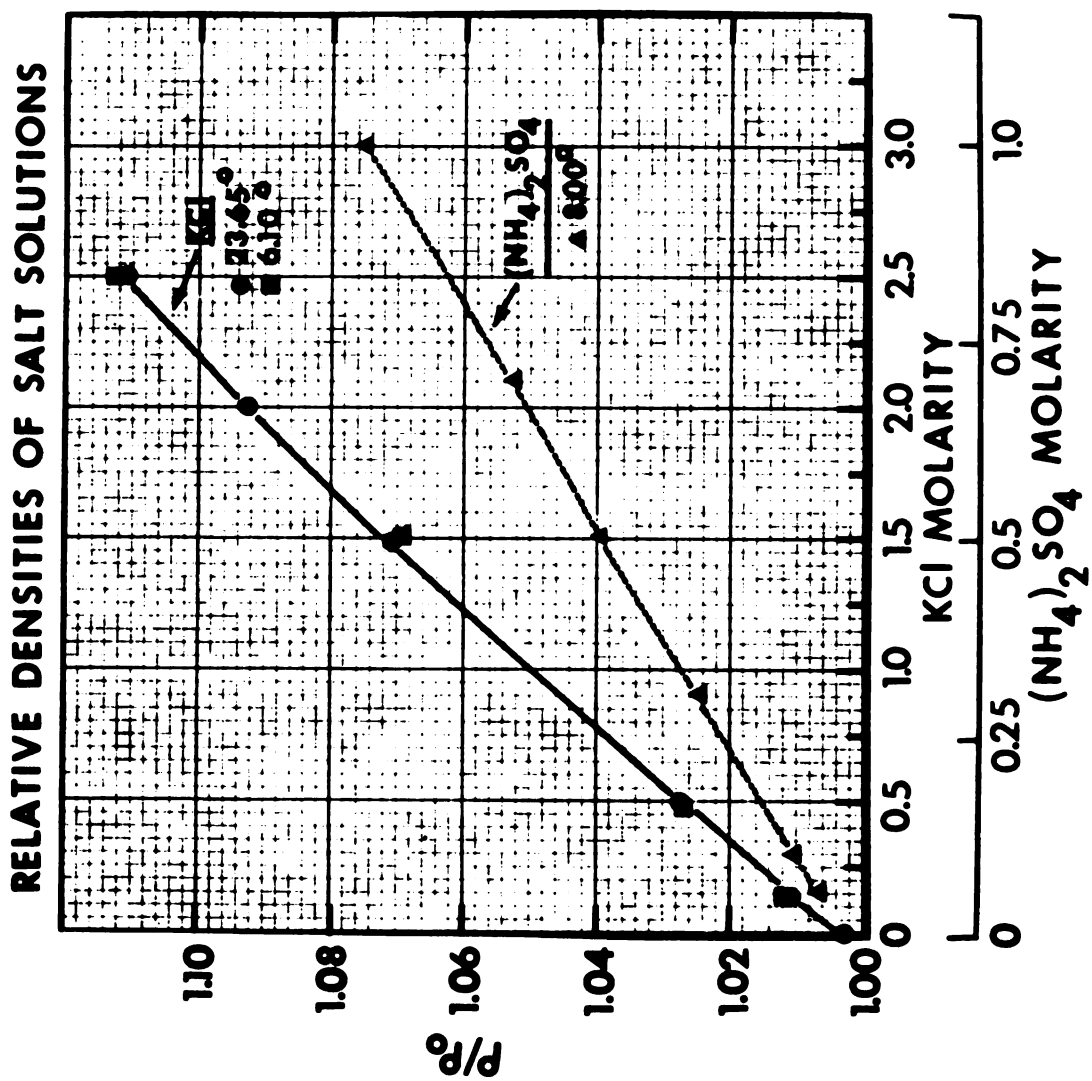

RELATIVE DENSITIES OF SALT SOLUTIONS
P/P₀
1.10
1.08
1.06
1.04
1.02
1.00
0
0.5
1.0
1.5
2.0
2.5
3.0
KCl MOLARITY
0
0.25
0.5
0.75
1.0
(NH4)2SO4 MOLARITY
KCl
23.45°
6.10°
(NH4)2SO4
8.00°

Figure 36. RELATIVE FLOW TIME OF THE SALT SOLUTIONS THROUGH THE VISCOMETER

The flow times were determined in a constant temperature water bath using a Cannon-Ubbelohde capillary dilution viscometer. The buffers described in the legend for Figure 35 were used.

Figure 36

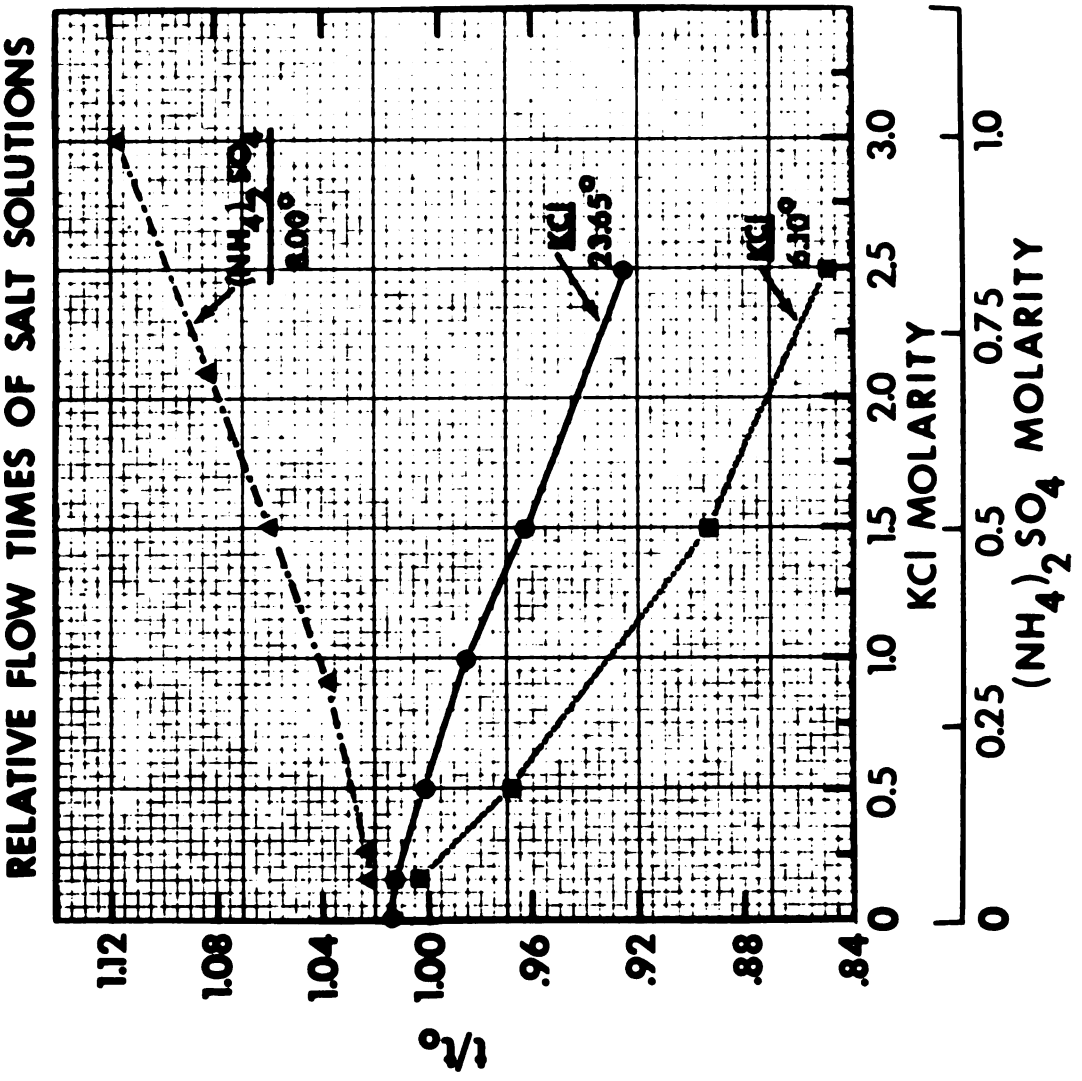
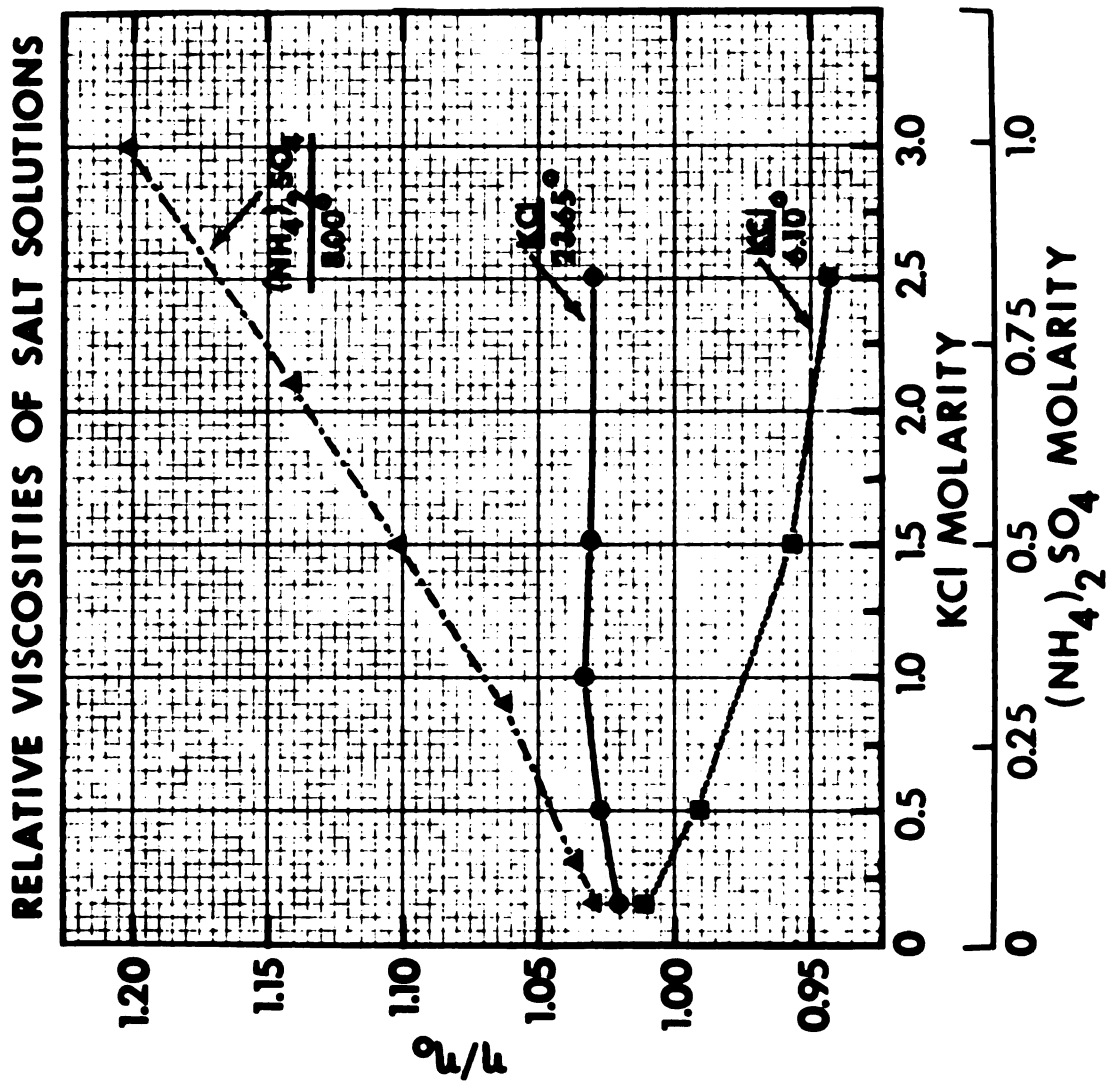

RELATIVE FLOW TIMES OF SALT SOLUTIONS
t/t₀
1.12
1.08
1.04
1.00
.96
.92
.88
.84
(NH4)2SO4 3.00°
KCl 23.5°
KCl 6.30°
0
0.5
1.0
1.5
2.0
2.5
3.0
KCl MOLARITY
0
0.25
0.5
0.75
1.0
(NH4)2SO4 MOLARITY

Figure 37. RELATIVE VISCOSITIES OF SALT SOLUTIONS

The relative viscosities were calculated from the data shown in Figures 35 and 36 using the expression:

where: = viscosity, = density, and t = time and the subscripts o refer to values for solvent alone. See legends for Figures 35 and 36 for additional details.

Figure 37


RELATIVE VISCOSITIES OF SALT SOLUTIONS
$(NH_4)_2SO_4$ 1.00°
KCl 23.45°
KCl 4.10°
η/η_0
1.20
1.15
1.10
1.05
1.00
0.95
0
0.5
1.0
1.5
2.0
2.5
3.0
KCl MOLARITY
0
0.25
0.5
0.75
1.0
$(NH_4)_2SO_4$ MOLARITY

glycine buffer replacing the Tris were determined and were found not to significantly differ from these values. The relative densities increased with increasing salt concentration of both KCl and $(NH_4)_2SO_4$. However the flow time through the viscometer showed different results. The solutions with increasing concentrations of KCl actually flowed through the viscometer faster than water, and the effect was even greater at 6.10^{o} than 23.65^{o}. This confirms the results reported in the International Critical Tables (1929), but it is interesting that this is contrary to what has been reported for NaCl which flowed through the viscometer slower than water (Svedberg and Pedersen, 1940). The results also show that the relative viscosity of the KCl solutions are not independent of temperature; the relative viscosity of the KCl solutions at 6.10^{o} are significantly lower than those at 23.65^{o}. This is contrary to what is normally assumed for viscosity corrections.

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