EFFECTS OF TEMPERATURE, SUBSTRATES, CATIONS, AND FRUCTOSE 1,6-DIPHOSPHATE ON THE CONFORMATIONS, SUBUNIT STRUCTURE, AND STABILITY OF YEAST PYRUVATE KINASE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
RONALD THOMAS KUCZENSKI
1970

This is to certify that the

thesis entitled

EFFECTS OF TEMPERATURE, SUBSTRATES, CATIONS, AND FRUCTOSE 1,6-DIPHOSPHATE ON THE CONFORMATIONS, SUBUNIT STRUCTURE, AND STABILITY OF YEAST PYRUVATE KINASE

presented by

Ronald Thomas Kuczenski

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date July 14, 1970

O-169

ABSTRACT

EFFECTS OF TEMPERATURE, SUBSTRATES, CATIONS, AND FRUCTOSE 1,6-DIPHOSPHATE ON THE CONFORMATIONS, SUBUNIT STRUCTURE, AND STABILITY OF YEAST PYRUVATE KINASE

by Ronald Thomas Kuczenski

This thesis describes the native and subunit molecular weights of yeast pyruvate kinase (PK), and the structural and conformational properties of the enzyme under the influence of temperature, substrates, and effectors.

Yeast PK has a molecular weight of 162,000 to 168,000 as calculated with the Svedberg equation under two sets of solvent conditions. The enzyme sediments as a single symmetrical peak with an $\underline{s}^{o}_{20,w}$ of 8.85 S and a $\underline{D}^{o}_{20,w}$ of 4.84×10^{-7} cm^2 sec^{-1} in 0.1 M tetramethylammonium cacodylate buffer, pH 6.2, containing 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, and 10^{-3} M fructose 1,6-diphosphate (FDP). In 0.1 M Tris·HCl, pH 7.5, the values obtained are 8.34 S for $\underline{s}^{o}_{20,w}$ and 4.52×10^{-7} cm^2 sec^{-1} for $\underline{D}^{o}_{20,w}$. Using the high speed equilibrium technique at low protein concentrations, a molecular weight near 165,000 was estimated for the enzyme in 0.1 M Tris·HCl, pH 7.5, containing 0.23 M KCl, 2.5×10^{-2} M $MgCl_2$, 2×10^{-3} M FDP, and 10^{-2} M phosphoenolpyruvate (PEP).

The enzyme is a tetramer, each polypeptide chain having a molecular weight of 42,000 to 45,000. Complete dissociation was obtained in 6 M guanidine hydrochloride-0.15 M 2-mercaptoethanol. Utilizing sedimentation equilibrium under

these solvent conditions, similar values for $\underline{M}_w^o$ and $\underline{M}_z^o$ were obtained, indicating the subunits have approximately equal molecular weights. Dissociation was also obtained by extensive treatment of the enzyme with maleic anhydride, resulting in a symmetrically sedimenting peak with $\underline{M}_w^o(\underline{s}/\underline{D})$ of 42,200, excluding bound maleyl groups.

The tryptophyl fluorescence of yeast PK is quenched by the addition separately or together of the activating cations K^+ and Mg^{2+}. The quenching is minimal, however, even in the presence of the substrate, PEP, when compared to the quenching observed in the presence of the activator, FDP, either in the presence or absence of the cations or PEP. Titration of the enzyme with FDP in the presence of Mg^{2+} monitored by the fluorescence change reveals a marked dependence of the FDP binding constant on the nature of the effectors present. Addition of 0.23 M K^+ increases the apparent K_D for FDP from 0.48 mM to 3.1 mM. $(CH_3)_4N^+$ has a similar though smaller effect. On the other hand, the addition of PEP markedly reduces the apparent K_D for FDP to 0.069 mM. K^+ is required to obtain the reduced K_D, whereas $(CH_3)_4N^+$ will not function. Adenosine 5'-diphosphate also promotes a decrease in the apparent K_D for FDP, but no monovalent cation requirement is observed. Changing the temperature from 30 to 0^o in the presence or absence of K^+ or PEP decreases the apparent K_D for FDP by an order of magnitude.

Yeast PK has also been shown to be susceptible to inactivation at low temperatures. Addition of micromolar

amounts of the allosteric activator, FDP, markedly enhances, by as much as 1000-fold, the rate of loss in activity both at 0° and at 23°. Addition of Mg^{2+} prevents the inactivation. At both temperatures a biphasic inactivation results, with the rate of both steps increasing as the protein concentration is decreased.

At 0° the inactivation is accompanied by a decrease in the sedimentation coefficient of the enzyme from 8.6 S to 3 S followed by a slower decrease to 1.7 S. Both the extent and the rate of the first step are dependent on protein and FDP concentrations, consistent with the establishment of an inactivation equilibrium between a tetrameric and a dimeric form of the enzyme, involving the binding of at least two moles of FDP with an apparent geometric average dissociation constant of 63 μM.

On the other hand, at 23°, only the rate of the first step of the inactivation is dependent on protein and FDP concentrations. The extent of inactivation proceeds to loss of half of the activity of native enzyme independently of protein and FDP concentrations. This step is accompanied by the formation of an apparent dimer with $\underline{s}_{20,w} = 4.2$ S, and is followed by dissociation to inactive subunits.

Both steps of the inactivation at 23° are dependent on FDP concentration, the binding of which is inhibited by high ionic strength. In the absence of added ionic strength, FDP binds to the enzyme with an apparent geometric average dissociation constant of 0.66 mM. This value is increased

to 5 mM at 0.934 μ. While the rate of the first step of the inactivation is independent of ionic strength at saturating FDP, the rate of the second step is markedly inhibited.

The overall mechanism of inactivation involves the binding of at least two moles of FDP, followed by a two-step dissociation of the native tetramer to inactive subunits. The ionic strength effects, the temperature effects, and the different time-course of inactivation at the two temperatures, however, suggest heterologous subunit interactions leading to different dimers at 0° and at 23°. These data are consistent with the inclusion of a large number of hydrophobic bonds between the subunits of the 23° dimer, and in the association of the 0° dimers in the native tetramer.

The analogous effects of temperature and ionic strength on the binding of FDP to yeast PK as observed through measurement of fluorescence changes and through the inactivation phenomenon suggest that similar structural transitions of the enzyme accompany both the fluorescence change and the inactivation and involve exposure of predominantly hydrophobic residues of the polypeptide chains. These effects are discussed with respect to the mechanism of kinetic activation of yeast pyruvate kinase by FDP.

EFFECTS OF TEMPERATURE, SUBSTRATES, CATIONS, AND FRUCTOSE 1,6-DIPHOSPHATE ON THE CONFORMATIONS, SUBUNIT STRUCTURE, AND STABILITY OF YEAST PYRUVATE KINASE

By

Ronald Thomas Kuczenski

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1970

To Sharon and Tracy

ACKNOWLEDGMENTS

I thank Dr. Clarence H. Suelter for the freedom of development which he allowed me, as well as for his encouragement and guidance throughout the four years required to complete this thesis. I am especially grateful for the amount of time he spent in both scientific and other-world discussions.

I also express my appreciation to Dr. George M. Stancel, Dr. James Hunsley, and Dr. Karl L. Smiley, Jr. for helpful discussions.

Financial assistance in the form of a predoctoral fellowship from the National Institutes of Health is acknowledged.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
CHA	cyclohexylammonium cation
$\underline{D}_{20,w}$	diffusion coefficient corrected to 20° and water
$\underline{D}^{o}_{20,w}$	diffusion coefficient corrected to 20° and water and extrapolated to zero protein concentration
DHAP	dihydroxyacetone phosphate
$\underline{E}$	extinction coefficient
FDP	fructose 1,6-diphosphate
G-3-P	glyceraldehyde 3-phosphate
GTP	guanosine 5'-triphosphate
GuHCl	guanidine hydrochloride
K_D	dissociation constant
$\langle K_D \rangle_{g.ave.}$	geometrical average dissociation constant
$\underline{M}_w$	weight-average molecular weight
$\underline{M}^{o}_w$	weight-average molecular weight extrapolated to zero protein concentration
$\underline{M}^{o}_w(\underline{s}/\underline{D})$	weight-average molecular weight determined from the Svedberg equation using sedimentation and diffusion coefficients extrapolated to zero protein concentration
$\underline{M}_z$	z-average molecular weight
$\underline{M}^{o}_z$	z-average molecular weight extrapolated to zero protein concentration

NADH	nicotinamide adenine dinucleotide, reduced form
$\underline{n}_H$	Hill slope
PEP	phosphoenolpyruvate
PK	pyruvate kinase
S	Svedberg, unit of sedimentation velocity
$\underline{s}_{20,w}$	sedimentation coefficient corrected to 20° and water
$\underline{s}^o_{20,w}$	sedimentation coefficient corrected to 20° and water and extrapolated to zero protein concentration
Tris	tris(hydroxymethyl)amino methane

INTRODUCTION

An interest in the characteristics of yeast pyruvate kinase was stimulated by the rather convincing evidence (Hommes, 1964; Pye and Eddy, 1965; Hess and Brand, 1965) that the pyruvate kinase reaction was rate limiting in both whole yeast and yeast extracts, and that the enzyme functioned as a control point in the glycolytic scheme. The successful purification of a stable homogeneous preparation of the enzyme (Haeckel *et al.*, 1968; Hunsley and Suelter, 1969a) has enabled initiation of studies of the kinetic and physical properties of this protein.

The elucidation of the kinetic properties of yeast pyruvate kinase (Hunsley and Suelter, 1969b) has revealed marked differences between the kinetics of this enzyme and the kinetics of the preparation from rabbit muscle (Reynard *et al.*, 1961). While the muscle enzyme exhibits strictly Michaelis-Menten kinetics with respect to all substrates and cofactors, almost the complete opposite is true for the yeast enzyme. The latter not only possesses homotropic cooperativity for the substrate phosphoenolpyruvate as well as the monovalent and divalent required cations, but is also markedly activated heterotropically by fructose 1,6-diphosphate in a feed-forward fashion, an observation which is consistent with the postulated role for the enzyme in

glycolytic control of yeast metabolism. Fructose 1,6-diphosphate apparently has no specific effects on muscle pyruvate kinase. The only apparent kinetic similarity between the two enzymes, in fact, is their requirement for monovalent and divalent cations as cofactors.

It was our intention to examine the physical properties and conformational changes of yeast pyruvate kinase using some of the techniques as they were applied to muscle pyruvate kinase (e.g., Suelter, 1967; Kayne and Suelter, 1968) with the hope that the similarities or differences revealed by such studies would lead to insights into not only the mechanism of enzyme action, but also the role of conformational transitions and subunit interactions in ligand-induced cooperative effects. In addition, the marked instability of the yeast enzyme, as first described by Washio and Mano (1960) and the role of glycerol in stabilizing the enzyme (Hunsley and Suelter, 1969a) set the stage for the discovery of two properties of yeast pyruvate kinase: cold lability and fructose 1,6-diphosphate-induced instability, which will be described in this communication. Considerable advantage was taken of these properties in elucidating the structural and conformational characteristics of the enzyme.

Preliminary reports of this work have been presented (Kuczenski and Suelter, 1970a,b; Kuczenski and Suelter, 1970c,d, submitted to Biochemistry for publication).

LITERATURE SURVEY

It has been a commonly accepted and well-warranted procedure in enzymology to attribute to purified enzymes a greater stability at temperatures lowered to near 0°. Likewise, stabilization has been attempted (and frequently achieved) by the addition of substrates and/or specific metabolic effectors of the enzyme. The assumption that these effects might be mediated by conformational transitions of the protein implies the existence of conformations of varying degrees of stability. However, since there are no theoretical grounds for suggesting that conformations at 0° or in the presence of specific metabolites must be the most stable, it seems only logical to assume that, for some enzymes at least, low temperatures or the presence of substrates could induce less stable conformers. The fact, though, that Grisolia, in publishing a review on substrate-induced inactivations in 1964, was prompted to acknowledge his indebtedness to editors and their antagonism "... for having thus forced (him) to further prove this point," might be favorably or unfavorably interpreted as revealing a tendency of scientists to adopt a rather zealous "show me" attitude.

Nevertheless, the observations of Grisolia concerning substrate-induced instability have been convincingly

documented in recent literature. In addition, an increasingly large number of reports have been appearing which support the contention that lowered temperatures may be detrimental to the vitality of isolated enzymes. In this review, we will be concerned firstly and primarily with those enzymes which are cold labile, and secondly those enzymes which experience cold lability induced by substrates or effectors. The decision to limit the review in the latter case, was made not only because of particular relevance to the subject of this thesis, but more importantly because a surprisingly large percentage of substrate-destabilized enzymes described in the recent literature fall into this category. The formal division of the review into two sections is also arbitrary, and considerable overlap will be apparent.

Cold Lability

One of the earliest discoveries of a cold labile enzyme[1] was reported by Hofstee (1949) in describing the properties of Jack bean urease. He attributed the loss in activity to an enzyme aggregation on the basis of indirect evidence, however he did not pursue the matter further.

It was not until the early 1960's that other reports of cold labile enzymes began to appear, with the number since

---

[1]The term "cold labile enzyme" is used throughout this thesis to refer to those enzymes which experience enhanced instability at temperatures lower than room temperature or normal physiological temperature.

then increasing steadily. In 1960, Shukuya and Schwert described the purification and properties of glutamic decarboxylase from E. coli. Dilute solutions of the enzyme were more stable at 20° than at 0°. Addition of bovine serum albumin protected against the inactivation, while pyridoxal phosphate, a required cofactor, not only protected but also reversed the inactivation. Recent electron microscopic data (Tikhonenko et al., 1968) has revealed that low temperature incubations yield a disaggregation of the polymeric enzyme from its native state. Strausbauch and Fischer (1970) have determined from sedimentation data that the enzyme is a hexamer which participates in a dissociation-association equilibrium. They suggested that the hexamer is the active species, and that conditions which lead to dissociation might yield inactivation, as seen with dilute solutions of the enzyme at 0°. Similarly, dilute solutions of D(-)β-hydroxy butyric acid dehydrogenase were highly unstable at 0° (Shuster and Doudoroff, 1962). A partial restoration of activity could be obtained by rewarming. The stability of the enzyme increased at increased protein concentrations, as did the reactivation, suggesting an equilibrium which the authors considered might involve a dissociated enzyme species.

In 1963, Dua and Burris successfully purified and stabilized a N_2-fixing enzyme from Clostridium pasteurianum which exhibited a maximum stability at 22°. Inactivation, which was biphasic, was again inversely proportional to protein concentration, and partial reactivation could be

accomplished by rewarming. The extent of the reactivation, however, decreased as the incubation time at 0° increased, suggesting both reversible and irreversible inactivation steps. The authors attempted stabilization of the enzyme by the addition of 0.1 M glycerol, but were unsuccessful. Reference was made to an apparent stabilization by 50% (v/v) glycerol of a yeast adenosinetriphosphatase (Meyerhof and Ohlmeyer, 1952). Although the latter authors did not recognize this enzyme's cold lability (as will be seen later), their use of high concentrations of glycerol to stabilize the ATPase was similar to the use by Lovelock and Bishop (1959) of glycerol, glucose, and dimethylsulfoxide to protect red blood cells and spermatozoa against freezing damage, and to the use of glycerol to stabilize catalase against freezing damage (Shikama and Yamazaki, 1961).

Racker and coworkers (1963) pointed out that the beef heart mitochondrial ATPase was also stabilized by 20-50% glycerol, after having shown that the yeast enzyme, like the beef heart enzyme (Pullman et al., 1960) was cold labile. In an effort to explore the nature of the inactivation, Penefsky and Warner (1965) examined the effect of temperature on the sedimentation coefficient of the enzyme. At 25° the ATPase sedimented as a single peak with $s^{o}_{20,w}$ = 12.9 S, while incubation of the enzyme at 0° for 5 hr yielded a mixture of species with sedimentation coefficients of 3.5 S and 9.1 S. The amount of 3.5 S species was inversely proportional to protein concentration. Both activity and the 12.9 S

species could be recovered by warming the sample to 25°.

On the other hand, glycogen phosphorylase b apparently undergoes a much more complex inactivation mechanism (Graves et al., 1965). The sedimentation coefficient of the enzyme increased from 8.4 S to 20.2 S as the inactivation progressed. Rewarming in the presence of pyridoxal phosphate gave complete reactivation only at protein concentrations greater than 0.1 mg/ml. Not only the extent, but also the initial rate of inactivation were dependent on the first power of protein. At the same time, however, the course of the inactivation followed pseudo-first-order kinetics to a lesser extent as protein concentration was increased, and the rate of inactivation then rapidly decreased. As a result, the enzyme, as measured by its half-life, became apparently more stable as protein concentration increased, a dependence on protein opposite to the dependence on protein of the initial rate of inactivation. The authors proposed a mechanism of inactivation involving conversion of the native enzyme to an inactive species and formation of an inactive species-native enzyme complex which was no longer cold labile, to account for the protein concentration effects. Both the observation that fully inactivated phosphorylase b added to active enzyme has no effect on the rate of inactivation, as well as the requirements for reactivation described above, however, seem incompatible with the absence, in the proposed mechanism, of a dissociated enzyme species as an intermediate during the inactivation.

An equally complex low temperature inactivation phenomenon has been described for 17-β-hydroxysteroid dehydrogenase from human placenta (Jarabak et al., 1966). The enzyme undergoes a biphasic, pseudo-first-order inactivation which depends inversely on protein concentration. However, the inactivated product, which on polyacrylamide moves as a mixture of defined species more slowly than the native active species, is eluted from Sephadex G-100 with the void volume. Rewarming partially reverses the inactivation only on protein which elutes with the native enzyme or after the native enzyme. The mechanism appears to involve a reversible dissociation, followed by an irreversible aggregation. 50% glycerol completely stabilizes, and 1 M PO_4^{3-} or 10 μM substrate or inhibitor (diethylstilbesterol) partially stabilize.

Steer liver arginosuccinase (Havir et al., 1965) apparently inactivates independent of protein concentration, at 0°, although the authors only examined concentrations of 1 and 6 mg/ml. The inactivation is two-step, however, and only the first step can be reversed by rewarming. While the enzyme at low ionic strength is cold labile, the addition of Tris buffer enhances the inactivation, whereas potassium phosphate stabilizes.

Pyruvate carboxylase from chicken liver mitochondria clearly undergoes a dissociation during cold inactivation (Scrutton and Utter, 1965). The sedimentation coefficient changes from 15 S to 7 S at low temperatures, but, as the

au

pr

i.

pr

co

pa

Ir

it

si

se

a

ac

ia

it

ar

re

(H

b

co

t

de

de

Th

pr

ir

th

authors pointed out, the amount of 7 S material was not proportional to the extent of inactivation. Addition of 1.5 M sucrose [or 2.5 M methanol (Irias et al., 1969)] provided complete stabilization. Acetyl CoA, a required cofactor, also stabilized. The inactivation can be only partially reversed by rewarming in the presence of ATP. Irias et al. (1969), in further describing the cold lability of the enzyme, characterized the inactivation as consisting of two pseudo-first-order steps in which the second step was irreversible. Low concentrations of urea at 23° appear to mimic the effects of low temperatures, and acetyl CoA protects against this inactivation as well. The data are consistent with some dissociation phenomenon, and it could be argued that the 7 S species described by Scrutton and Utter arises from the irreversible inactivation step.

Several other cold labile enzymes have been described recently. These include chicken liver fatty acid synthetase (Hsu and Yun, 1970), and UDP-galactose 4-epimerase from bovine mammary gland (Tsai et al., 1970). Both enzymes are completely stabilized by glycerol at 0° (20% and 50% respectively). Interestingly, the latter enzyme at 25° is destabilized by glycerol. Lee and Muench (1970) have described an interesting cold labile enzyme from E. coli. The prolyl tRNA synthetase, which must be purified in the presence of 40% glycerol to retain activity, participates in an active dimer-inactive monomer equilibrium. At 0°, in the absence of glycerol, the inactive monomer predominates,

and on storage, this species irreversibly inactivates. Conversion of the monomer to dimer can be obtained either by incubation at 37° in the absence of glycerol, or at 0° in the presence of glycerol.

Several characteristics of cold labile enzymes can be summarized. Polyhydroxic compounds such as glycerol appear to protect against low temperature inactivation for all those enzymes which have been examined from this point of view. That Dua and Burris (1963) saw no such effect with glycerol may indicate that they did not use sufficiently high concentrations (only 0.1 M) of the polyhydroxic compound. In addition, a dissociation step in the mechanism can be implicated for most cold labile enzymes. At least as early as 1955, von Hippel and Waugh described a low temperature-induced protein dissociation involving casein, which could be reversed by rewarming the mixture to 32°. Most of the enzymes described in this review could be at least partially reversed by rewarming. Only glycogen phosphorylase b, and possibly urease appeared to undergo aggregation reactions in the functional inactivation step, but, in neither case was the data conclusive. Finally, the inactivation as measured as a function of time appears, in most cases, to follow biphasic pseudo-first-order kinetics, in which the second step is frequently irreversible. As will be described in this thesis, yeast pyruvate kinase is typical as a cold labile enzyme with respect to most of these phenomena.

Metabolite-Induced Inactivation

As mentioned previously, Grisolia reviewed the subject of substrate-mediated destabilization in 1964. A considerable impetus was given to writing such a review from experiments in his laboratory with frog liver carbamoyl phosphate synthetase. The unexpected observation was made that acetyl glutamate, a required cofactor, in the presence or absence of Mg^{2+}, or the substrate ATP in the absence of Mg^{2+} led to a marked instability of the enzyme (Caravaca and Grisolia, 1959), while ATP plus Mg^{2+} stabilized. In a later communication (Raijman and Grisolia, 1961), the authors showed this inactivation to be much more rapid at lowered temperatures.

Recently, Guthöhrlein and Knappe (1968) examined the same enzyme from rat liver in more detail. The enzyme, which is stabilized by 20% glycerol, undergoes a slow activation phenomenon in the catalytic assay at temperatures below 15°. The rate and extent of activation are proportional to the acetyl glutamate concentration and are first order. If the enzyme is stored at 0°, then assayed at 10°, the activation kinetics are biphasic, from which the authors suggested the existence of two different inactive species, I_1 and I_2. Prolonged storage at 0° resulted in a slow irreversible inactivation. Addition of acetyl glutamate at temperatures above 25° converts the enzyme to the fully active species, whereas the same ligand at 0°, in the absence

of substrates, yields complete conversion of the enzyme to the more slowly-activated inactive species (I_2). This conversion to I_2 is directly proportional to acetyl glutamate concentration. Sedimentation data indicated that the active enzyme and I_1 were similar in molecular weight, whereas I_2 was markedly reduced in size. Hence, the same ligand, depending on the temperature and the presence of substrates, could lead to either activation, or to an inactive conformer which readily dissociated at the lower temperatures.

The pyruvate carboxylase system described in the previous section (Irias et al., 1969) also involves a substrate-enhanced inactivation. Acetyl CoA alone or in conjunction with ATP, Mg^{2+}, HCO_3^- and pyruvate stabilizes the enzyme. Pyruvate alone, however, produces an enhanced low temperature inactivation.

Threonine deaminase in crude extracts of B. subtilis is cold labile in the presence of the cofactor pyridoxal phosphate (Hatfield and Umbarger, 1970). It is also destabilized by the same ligand at 37°, but the ligand completely stabilizes at 22°. Treatment of the crude extract with $CaPO_4$ gel eliminates the cold lability and produces the unusual effect of inducing a room temperature lability. The authors did not pursue this phenomenon.

Yeast glyceraldehyde 3-phosphate dehydrogenase is stable at 0°, but addition of ATP produces a rapid inactivation, accompanied by a complete dissociation of the enzyme to subunits (Stancel and Deal, 1969). The subunits have

been described by the authors as "folded," because of their relatively high sedimentation coefficient. Complete reassociation may be achieved by rewarming only in the presence of sucrose and the dissociating agent, ATP. On the basis of other data (Yang and Deal, 1969) the previous authors suggested that the rapid conformational transition which later results in the dissociation was the physiologically significant effect of ATP. The enzyme thus becomes more susceptible to proteolytic digestion.

Similarly, Rosen et al., (1967) observed that D-fructose 1,6-diphosphatase, which is inhibited by AMP, is dissociated by the ligand at pH 9.2 only at low temperatures; the substrate, FDP, protects against this dissociation. If the enzyme is desensitized chemically to AMP inhibition, it is no longer cold labile in the presence of the inhibitor. The authors describe the effect as an "... extreme manifestation of more subtle alterations ... which occur in the presence of substrate at physiological pH."

Glutamate dehydrogenase provides an interesting final example of substrate induced instability. In 1957, Fincham described a mutant of Neurospora crassa which did not grow at 20°, but did at temperatures above 25°. The mutant glutamate dehydrogenase was reversibly inactivated at 21° or below, but was stable at higher temperatures.

Eisenkraft et al., (1969) later showed that the glutamate dehydrogenase from beef liver had to be purified in the presence of 50% glycerol to maintain stability. Concentrations

of NADH less than 10 μM destabilized the enzyme, whereas concentrations above 10 μM stabilized it. The inactivation was reversible (proportional in extent to the activity remaining) by the addition of ADP. Inactivation kinetics were pseudo-first-order, and the rate was directly proportional to the concentration of a 310,000 molecular weight "monomer." Since the enzyme participates in an equilibrium with a dimeric form, the authors suggested that the dimer was a stable species under the conditions described, and that the monomer was the "active species in the inactivation." The irreversible step in the inactivation apparently involves a dissociation of the monomer to a lower molecular weight species. Henderson and Henderson (1969) further characterized the inactivation using GTP, an inhibitor of the enzyme, as the inactivating ligand. In D_2O, the enzyme is stable at low temperatures, and GTP no longer inactivates. But, kinetically, GTP acts as a stronger inhibitor in D_2O than in H_2O. D_2O also prevents the NADH-induced dissociation. The authors interpreted the results in terms of the monomer-dimer equilibrium discussed above, suggesting that if the dimer were the active species, and GTP favored the inactive monomer, then the monomer would appear to be an intermediate in the irreversible dissociation to subunits promoted by both GTP and NADH. If D_2O favored the inactive monomer over both the active dimer and the subunits, it would not only stabilize against irreversible dissociation, but also increase the inhibitory effect of GTP in the catalytic assay.

An induced inactivation promoted by either low temperatures or specific ligands, or the combination of the two, can thus provide data concerning the structural properties of enzymes. An inactivation, like a stabilization, promoted by a ligand which has been observed to affect the kinetics of an enzyme-catalyzed reaction, is direct evidence for a conformational transition resulting from the interaction with the ligand. Since internal bonds between regions of polypeptide chains or between subunits of a polymeric protein are implied to be weakened or strengthened during a destabilization or a stabilization, effects of temperature or solvents such as glycerol or D_2O on the secondary effects of ligand-protein interactions can provide insights into the mechanism by which such interactions are mediated. Although the evidence is not conclusive, physico-chemical and thermodynamic data are available for interpretation of the nature of those bonds which can be affected at low temperatures or in the solvent systems described above, and will be discussed later.

METHODS AND MATERIALS

I. Enzymes

Pyruvate kinase was isolated from fresh "Budweiser" bakers' yeast (Anheuser-Busch, Inc.), Saccharomyces cerevisiae, according to the procedure of Hunsley and Suelter, (1969a). The enzyme was stored as a concentrated suspension at 4° in 90% saturated (3.6 M) $(NH_4)_2SO_4$. The minimum specific activity of any preparation used in these studies was 210 μmoles/min per mg. Prior to use, the enzyme was chromatographed at room temperature on a column of Sephadex G-25 (coarse). Aliquots were tested with saturated $BaCl_2$ to ensure them free of ammonium sulfate.

Lactic dehydrogenase used in the coupled assay system was the Sigma type II rabbit muscle enzyme, and was desalted free of $(NH_4)_2SO_4$ using a column of Sephadex G-25, when cation content of the assay was to be critically controlled. Ammonium sulfate suspensions of rabbit muscle aldolase were Sigma products, and rabbit muscle α-glycerophosphate dehydrogenase-triose phosphate isomerase mixed crystals were Calbiochem products. Rabbit muscle pyruvate kinase was isolated from frozen rabbit muscle (Pel-Freeze Biologicals) by a modification (Kayne and Suelter, 1965) of the procedure of Tietz and Ochoa (1958).

II. Chemicals

All water was either double glass distilled or glass distilled and deionized (Crystalab Deeminite). $(CHA)_4FDP$, $(CHA)_3PEP$, NaADP, NaNADH, Dimethyl Di-(CHA) form of DHAP, and the diethyl acetal Ba form of G-3-P were Sigma products. NaADP was converted to the Tris salt by treatment with Dowex 50W-X8 in the Tris form. $(CH_3)_4NCl$ from Aldrich was recrystallized from absolute ethanol and passed over a column of Chelex 100 in the Tris form to remove contaminating heavy metals. Maleic anhydride was also an Aldrich product. GuHCl was either Mann Ultra Pure and used directly, or was obtained from Eastman as the carbonate and converted to the hydrochloride according to the procedure of Kawahara *et al.*, (1965). Mercaptoethanol (Sigma) was redistilled before use. All other chemicals and reagents were used without further purification.

III. Experimental Methods

1. Assay of activity and determination of enzyme concentration

Yeast pyruvate kinase concentration was estimated from the absorbance at 280 nm ($E_{1\ cm}^{0.1\%} = 0.653$) (Hunsley and Suelter, 1969a). Standard assays were performed at 30° by employing the linked lactic acid dehydrogenase assay modified from Bücher and Pfleiderer (1955). The reaction mixture contained in 1 ml: 100 μmoles $(CH_3)_4N$ cacodylate, pH 6.2; 24

μmoles $MgCl_2$; 100 μmoles KCl; 10 μmoles $(CHA)_3PEP$; 10 μmoles NaADP, pH 7.0; 1.0 μmoles $(CHA)_4FDP$; 0.15 μmoles NaNADH; and 33 μg lactic dehydrogenase (Hunsley and Suelter, 1969b). Aliquots of enzyme were added to the reaction mixture at 30° in a 1 cm silica cuvette to initiate the reaction, and the change in optical density at 340 nm was recorded on a Gilford model 2000 modified Beckman DU ultraviolet spectrophotometer. The initial rate was converted to micromoles of pyruvate formed per minute using the extinction coefficient for NADH (Horecker and Kornberg, 1948).

ADP and PEP concentrations were estimated by a modification of the Bücher and Pfleiderer (1955) pyruvate kinase assay in the presence of excess rabbit muscle PK. FDP was estimated in the presence of excess aldolase as modified from the assay of Rutter et al. (1966).

2. Ultracentrifugal analyses

A Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and Rayleigh interference optics, and an RTIC unit was used for all ultracentrifugal experiments. Sedimentation velocity experiments were run at 59,780 rpm using a model AnD rotor, or at 50,760 rpm with an AnE rotor. Diffusion coefficient experiments were performed in double-sector capillary-type synthetic boundary cells at 4908 rpm and the coefficients were calculated using height-to-area analysis (Schachman, 1957).

A molecular weight for native enzyme was also determined using the meniscus depletion technique of Yphantis

(1964). Runs were performed at 20° with a rotor speed of 15,200 rpm using Rayleigh interference optics and the six-channel Kel-F centerpiece designed by Yphantis (1964). Rayleigh patterns were recorded on Kodak II-G photographic plates.

The short-column sedimentation equilibrium technique of Van Holde and Baldwin (1958) was used for molecular weight determinations in 6 M GuHCl. These experiments were run for 30 hr (results remained constant from 30 to 40 hr) near 20° with a solution column depth of 1.7 mm. Enzyme for these experiments was prepared by extensive dialysis (48 hr) against the appropriate GuHCl solution at 4°.

A molecular weight for the subunits of yeast PK was also determined with maleylated enzyme. A modification of the procedure of Freedman et al. (1968) was used to prepare maleylated yeast PK. Stock enzyme (20 mg) in 1.0 ml was dialyzed extensively against 0.05 M sodium borate buffer, pH 9.0, at 0°. Maleic anhydride in acetone (100 µl of a 0.5 g/ml solution) was added to this solution in 10-µl aliquots over a 30-min period. pH 9 was maintained with 5 N NaOH utilizing a Radiometer TTT-1-SBR2-SBU1-TTA31 automatic recording tetrator. The protein was then dialyzed for 2 days against several changes of 0.1 M KCl-0.1 M potassium borate, pH 9.0.

Densities of solutions were determined by pyncnometry. Viscosities of GuHCl solutions were interpolated from data of Kawahara and Tanford (1966). All other viscosities were

based on data from Bates and Baxter (1929), or from Svedberg and Pederson (1940).

Calculations, including statistical analyses of the data, were performed on a Control Data Corporation 3600 digital computer using fully tested programs [sedimentation velocity, diffusion coefficients, and low-speed equilibrium, (W. C. Deal, Jr., in preparation); high speed equilibrium, with a modification of a program from Small and Resnick (1965)].

3. Fluorescence analyses

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with an X-Y recorder. Fluorescence at right angle to the illumination was measured with the indicated temperature maintained using a constant temperature accessory. Several determinations were checked using front-face illumination and emission, but this technique was discarded since results were identical to those obtained using right angle illumination and emission.

Titrations of fluorescence change were routinely begun with an initial volume of 2.0 ml of sample in a fused quartz cell (capacity 4.8 ml). Small aliquots of the titrant, which was contained in a solution of identical composition as the sample (including protein concentration) were added and mixed with a magnetic stirrer.

RESULTS

I. Temperature Stability of Yeast Pyruvate Kinase

Figure 1 shows the effect of temperature on the stability of yeast pyruvate kinase at 0.5 mg/ml in 0.1 M Tris·HCl, pH 7.5. Over a period of 72 hr less than 10% of the activity was lost at 23° whereas at 0° over 95% of the activity was lost over the same time interval. Each point represents an assay of an aliquot of enzyme diluted directly into the assay mix at 30°. Assays were linear over the 1-3 min observation period.

Before each stability study, but after treatment with Sephadex, the protein was allowed to stand at 23° for 3 hr. Little or no change in specific activity could be observed over this time period. This preincubation step was introduced in this and all future experiments to eliminate the unusual results obtained without preincubation (Figure 2). Preincubation of the enzyme at 23° for 3 hr eliminates the initial activation observed at 0.05 mg/ml (lower curves), but not at 0.5 mg/ml (upper curves). Both samples were eluted from the Sephadex column at about 0.6 mg/ml. The activation observed at low protein concentration was qualitatively reproducible, and was not observed above 0.10 mg/ml. The activation was observed when dilution to 0.05 mg/ml

Figure 1. Effect of Temperature on the Stability of Yeast Pyruvate Kinase

Protein was diluted to 0.5 mg/ml after chromatography on Sephadex in 0.1 M Tris·HCl, pH 7.5. Each point represents an assay of an aliquot of enzyme removed from the incubation mixture.

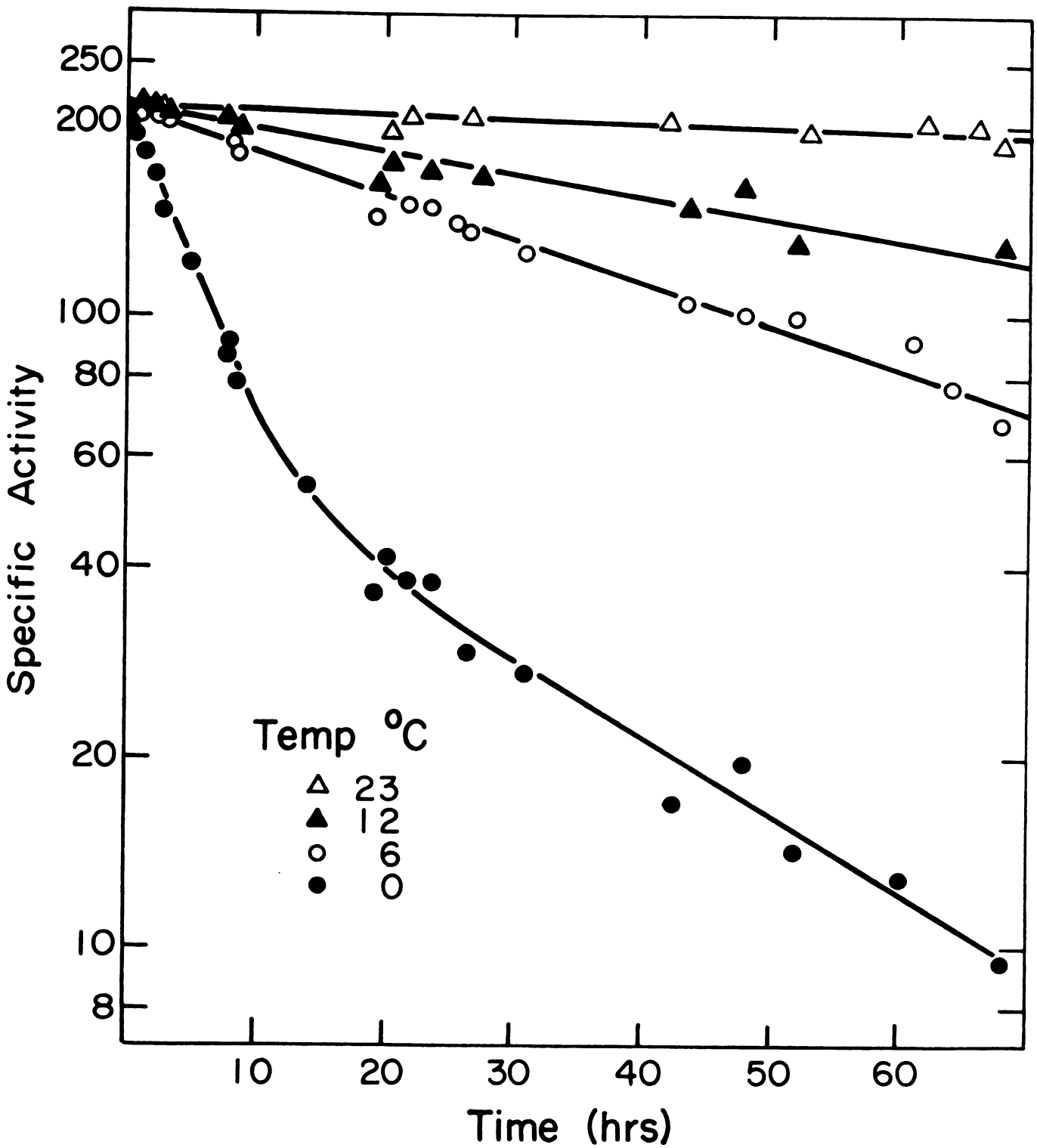
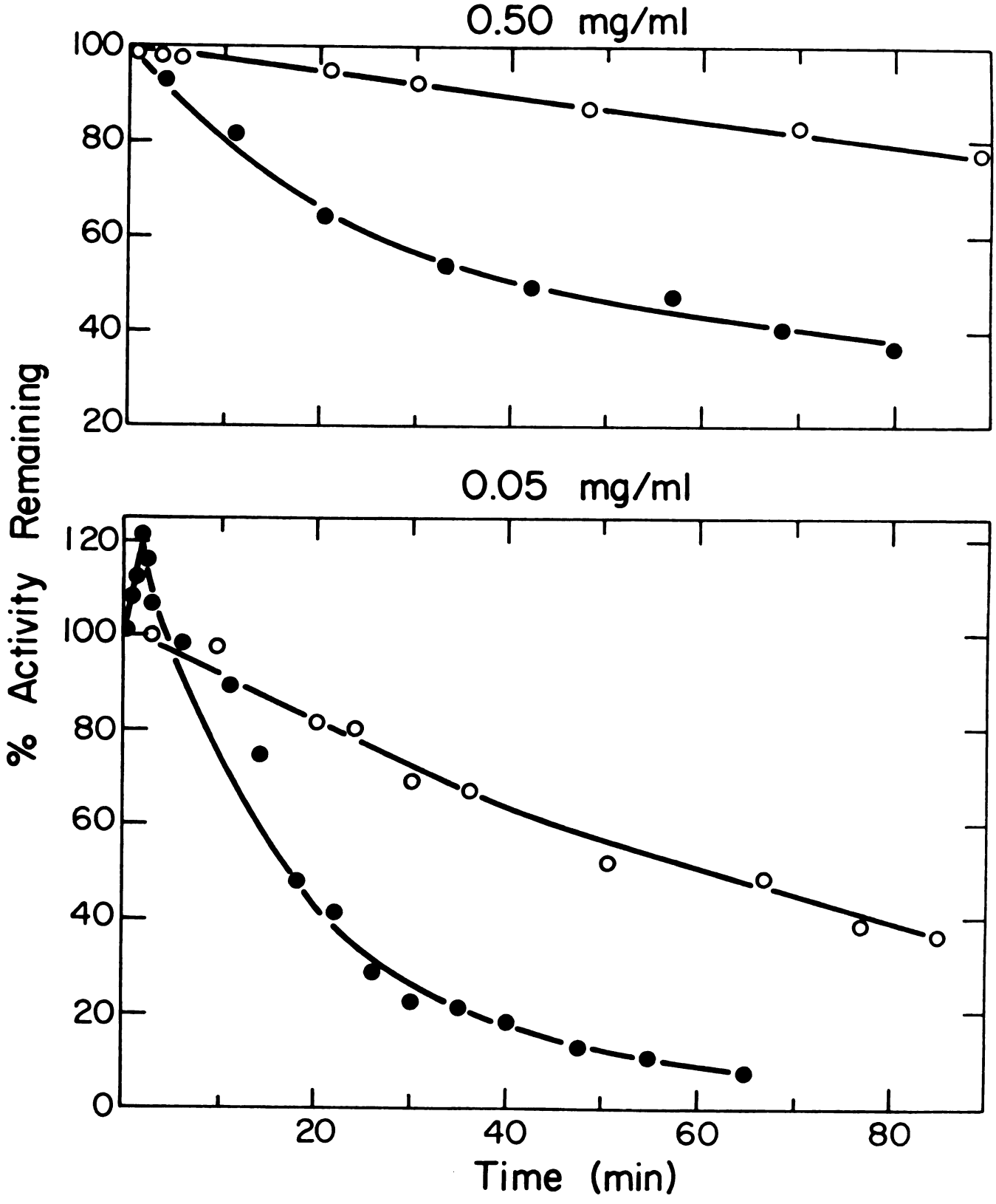

250
200
100
80
60
40
20
10
8
Specific Activity
Temp °C
23
12
6
0
10
20
30
40
50
60
Time (hrs)

Figure 2. Effect of Preincubation at 23° on the Stability of Yeast Pyruvate Kinase at 0°

Closed circles represent enzyme Sephadexed into 0.1 M Tris·HCl, pH 7.5 at 23°, then immediately diluted to the indicated protein concentration at 0°. Open circles represent enzyme treated with Sephadex and allowed to stand for 3 hr. at 23°, then diluted to the final concentration at 0°.


0.50 mg/ml
100
80
60
40
20
0.05 mg/ml
120
100
80
60
40
20
0
% Activity Remaining
20
40
60
80
Time (min)

was made at 23 or 0° either from Sephadex-chromatographed stock solution or from ammonium sulfate precipitate, and either into a glass or polypropylene reaction vessel. The 23° dilution, however, resulted in a higher activation over a much shorter period of time.

To determine whether any changes in the sedimentation pattern of the enzyme could be observed as a result of the preincubation period, aliquots of the ammonium sulfate precipitate were treated in two ways. One was chromatographed over Sephadex into Tris buffer, diluted to 6.0 mg/ml, and allowed to stand at room temperature for 3 hr. The second was centrifuged in the preparatory centrifuge for 10 min at 0°, the precipitate was collected, and at the end of the 3-hr preincubation period for the former sample, was dissolved in 0.1 M Tris buffer. Both samples were immediately centrifuged at 20°. The sample preincubated after treatment with Sephadex sedimented as a single symmetrical peak (8.0 S), while the other contained three peaks with approximate $\underline{s}$ values of 7.7, 11.5, and 13.6 S, in a ratio of approximately 4:3:3.

II. Molecular Weight of Yeast Pyruvate Kinase

1. Native enzyme

i. $\underline{s}/\underline{D}$ molecular weights

Initial attempts to determine the moleculae weight of the native enzyme were performed in 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, 10^{-3} M $(CHA)_4FDP$, and 0.1 M $(CH_3)_4N$ cacodylate buffer,

pH 6.2, the conditions for optimal catalytic activity. The sedimentation constant in this solvent, found by extrapolation to zero protein concentration ($\underline{s}^{o}_{20,w}$) was 8.85 S (Figure 3). In a similar manner, the diffusion coefficients extrapolated to zero protein gave a value of $\underline{D}^{o}_{20,w} = 4.84 \times 10^{-7}$ cm^2/sec (Figure 4). These values, together with a partial specific volume of 0.734 cc/g as calculated from the amino acid content (Hunsley and Suelter, 1969a) using the procedure of McMeekin and Marshall (1952), yielded a weight average molecular weight ($\underline{M}^{o}_{w}(\underline{s}/\underline{D})$) of 166,500.

During the above experiments, a slow precipitation of protein, particularly at higher protein concentrations, was observed. This problem was eliminated in 0.1 M Tris, pH 7.5, and thus the studies were repeated in this solvent. Values of $\underline{s}^{o}_{20,w} = 8.34$ S (Figure 5), and $\underline{D}^{o}_{20,w} = 4.52 \times 10^{-7}$ cm^2/sec (Figure 6) yielded a molecular weight of $\underline{M}^{o}_{w}(\underline{s}/\underline{D}) = 168{,}100$. The diffusion coefficient was determined both after a 3 hr preincubation, and after an 18 hr preincubation. Varying the time of preincubation after 3 hr had no effect on the sedimentation pattern of the enzyme.

ii. High speed equilibrium technique

The molecular weights of the native enzyme as determined by the meniscus depletion technique are plotted in Figure 7 as number-average molecular weight *vs.* concentration in fringes for enzyme in 0.1 M Tris·HCl, pH 7.5, containing 0.23 M KCl, 2.5×10^{-2} M $MgCl_2$, 2×10^{-3} M FDP, and 10^{-2} M PEP. The enzyme retained 92% of its initial activity

Figure 3. Sedimentation Coefficient of Yeast Pyruvate Kinase as a Function of Protein Concentration in 0.1 M $(CH_3)_4N$ Cacodylate Buffer, pH 6.2, Containing 0.1 M KCl, 2.6 x 10^{-4} M MgCl, and 10^{-3} M $(CHA)_4FDP$, at 20°

The line is a least squares plot.

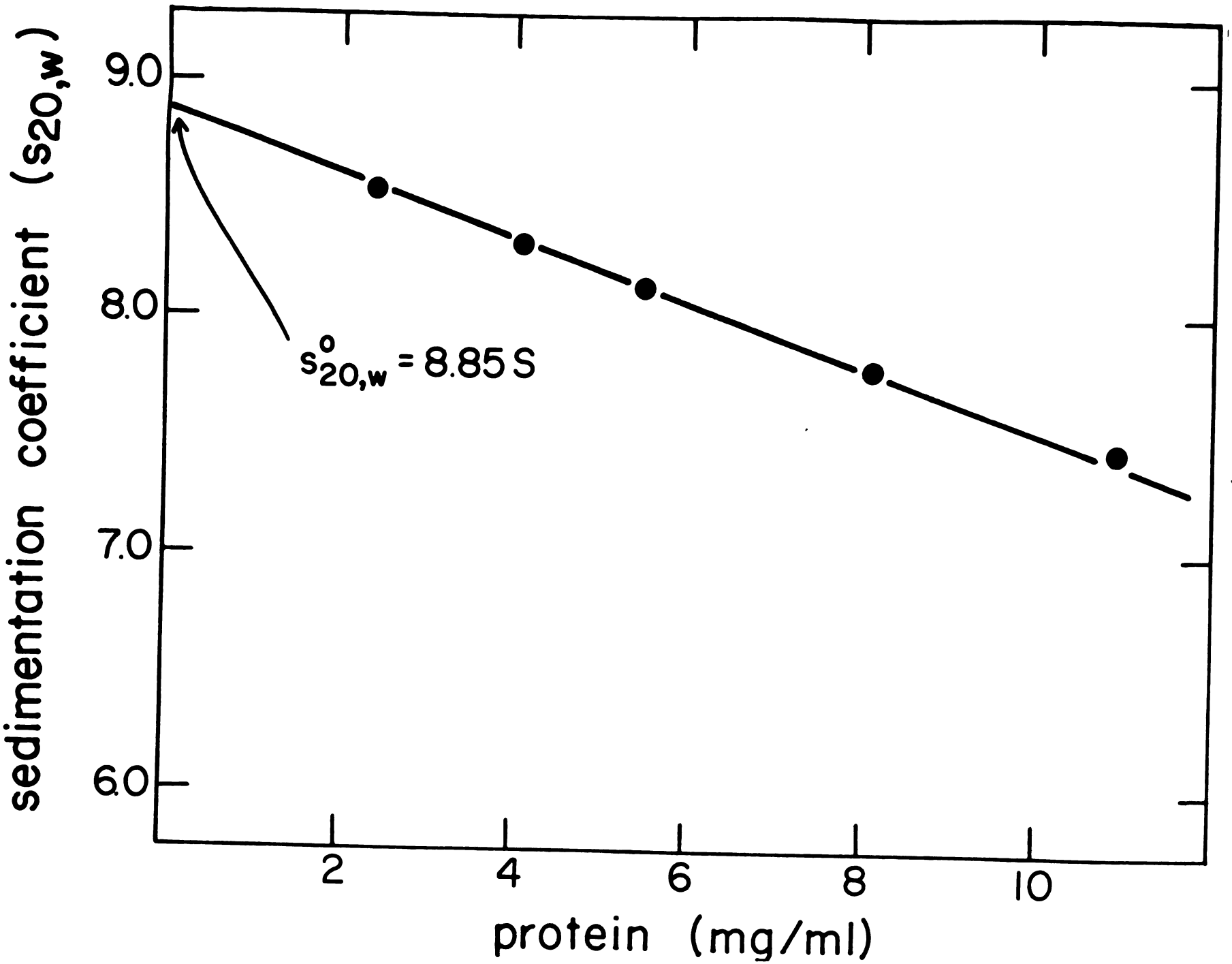

sedimentation coefficient ($s_{20,w}$)
9.0
8.0
7.0
6.0
$s^o_{20,w}$ = 8.85 S
2
4
6
8
10
protein (mg/ml)

Figure 4. Diffusion Coefficient of Yeast Pyruvate Kinase as a Function of Protein Concentration

Conditions were as described in Figure 3. The line is a least squares plot.

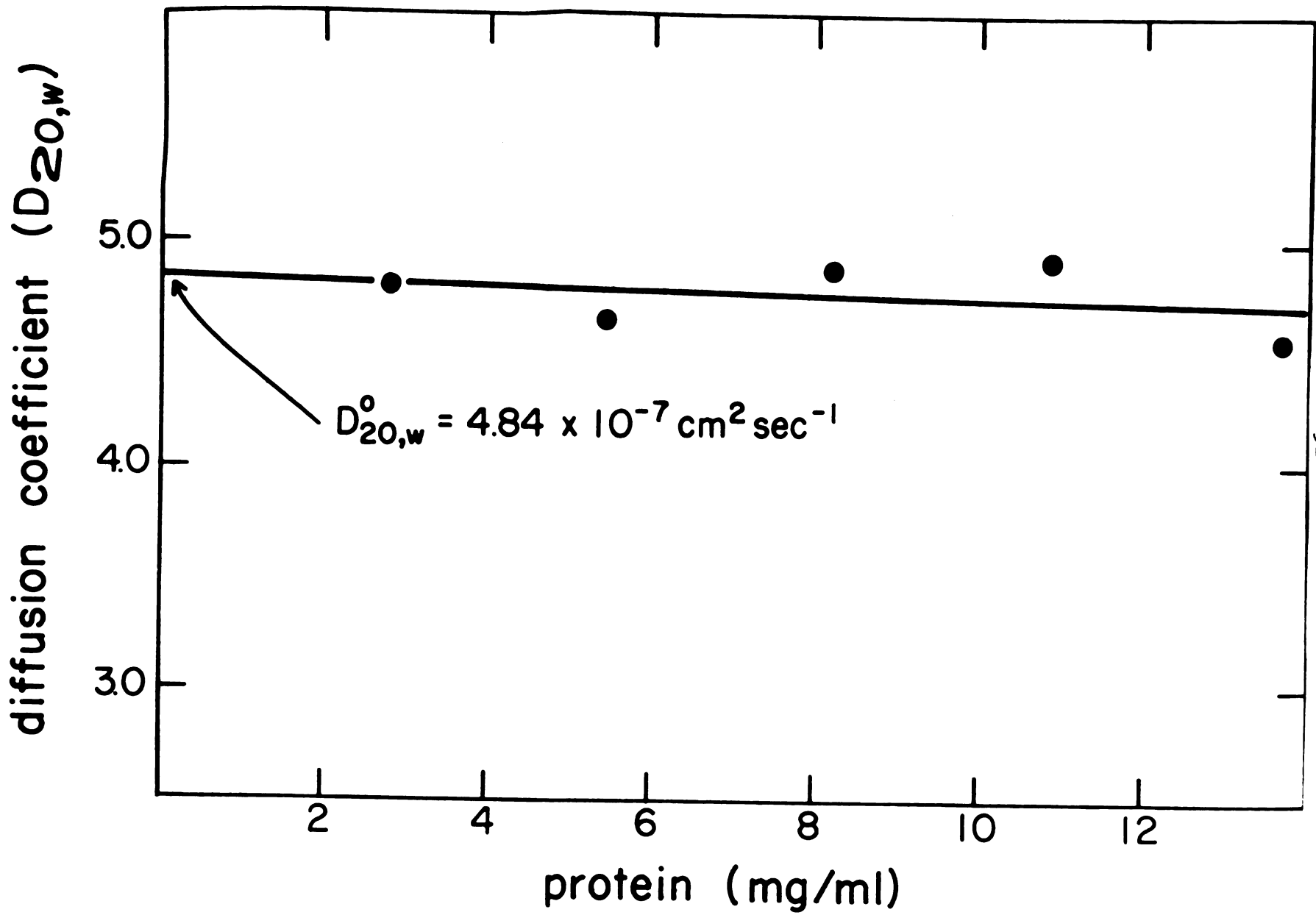

diffusion coefficient ($D_{20,w}$)
5.0
4.0
3.0
$D^{o}_{20,w} = 4.84 \times 10^{-7}\ cm^2\ sec^{-1}$
2
4
6
8
10
12
protein (mg/ml)

Figure 5. Sedimentation Coefficient of Yeast Pyruvate Kinase as a Function of Protein Concentration in 0.1 M Tris·HCl, pH 7.5

The extrapolation to zero protein concentration is a least squares plot.

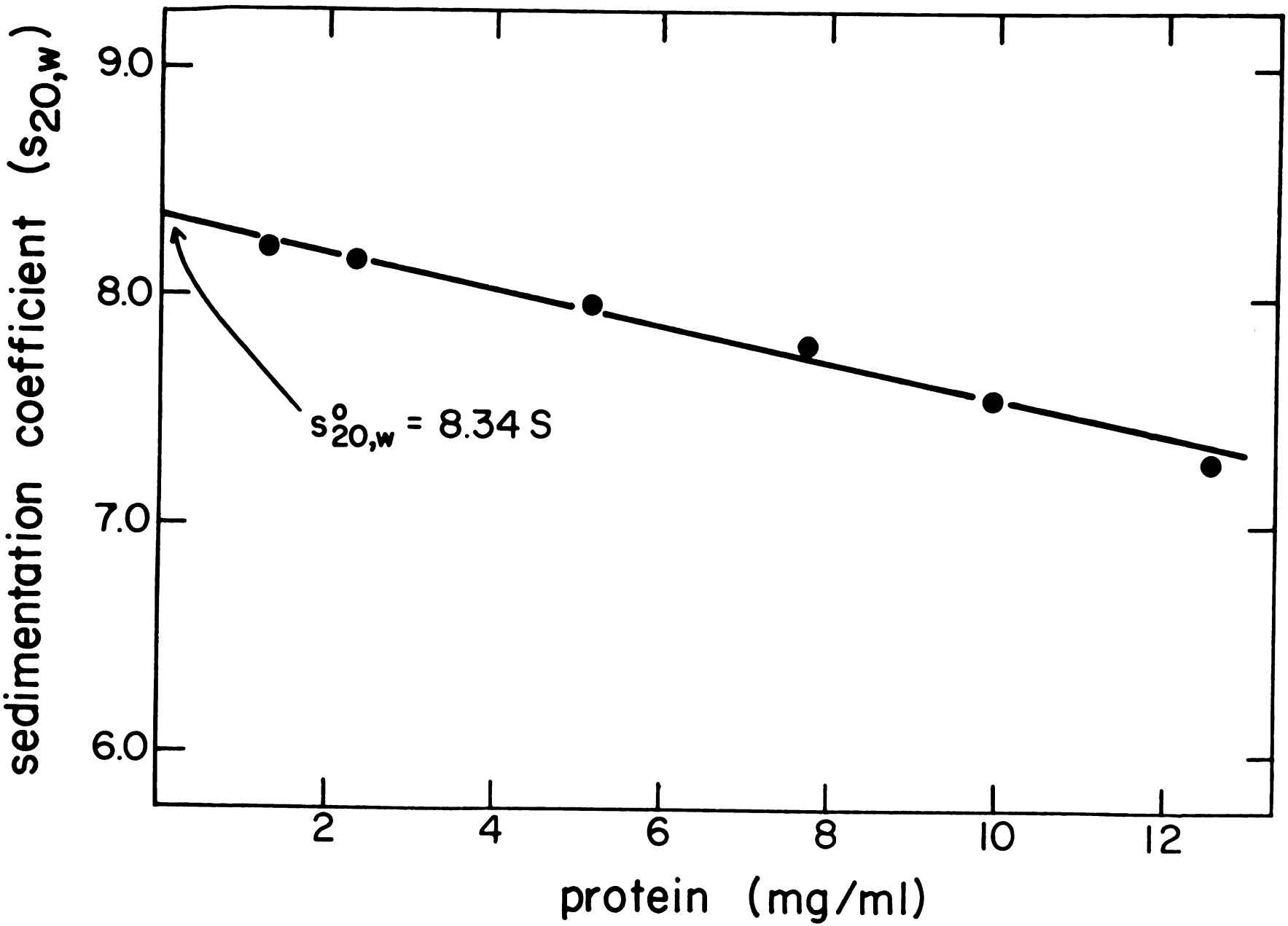

sedimentation coefficient ($s_{20,w}$)
9.0
8.0
7.0
6.0
$s^{o}_{20,w}$ = 8.34 S
2
4
6
8
10
12
protein (mg/ml)

Figure 6. Diffusion Coefficient of Yeast Pyruvate Kinase as a Function of Protein Concentration in 0.1 M Tris·HCl, pH 7.5

The closed circles represent enzyme preincubated at 23° for 18 hr before centrifugation. Open circles represent enzyme preincubated for 3 hr at 23° before centrifugation. The line is a least squares plot of the data represented by the closed circles.

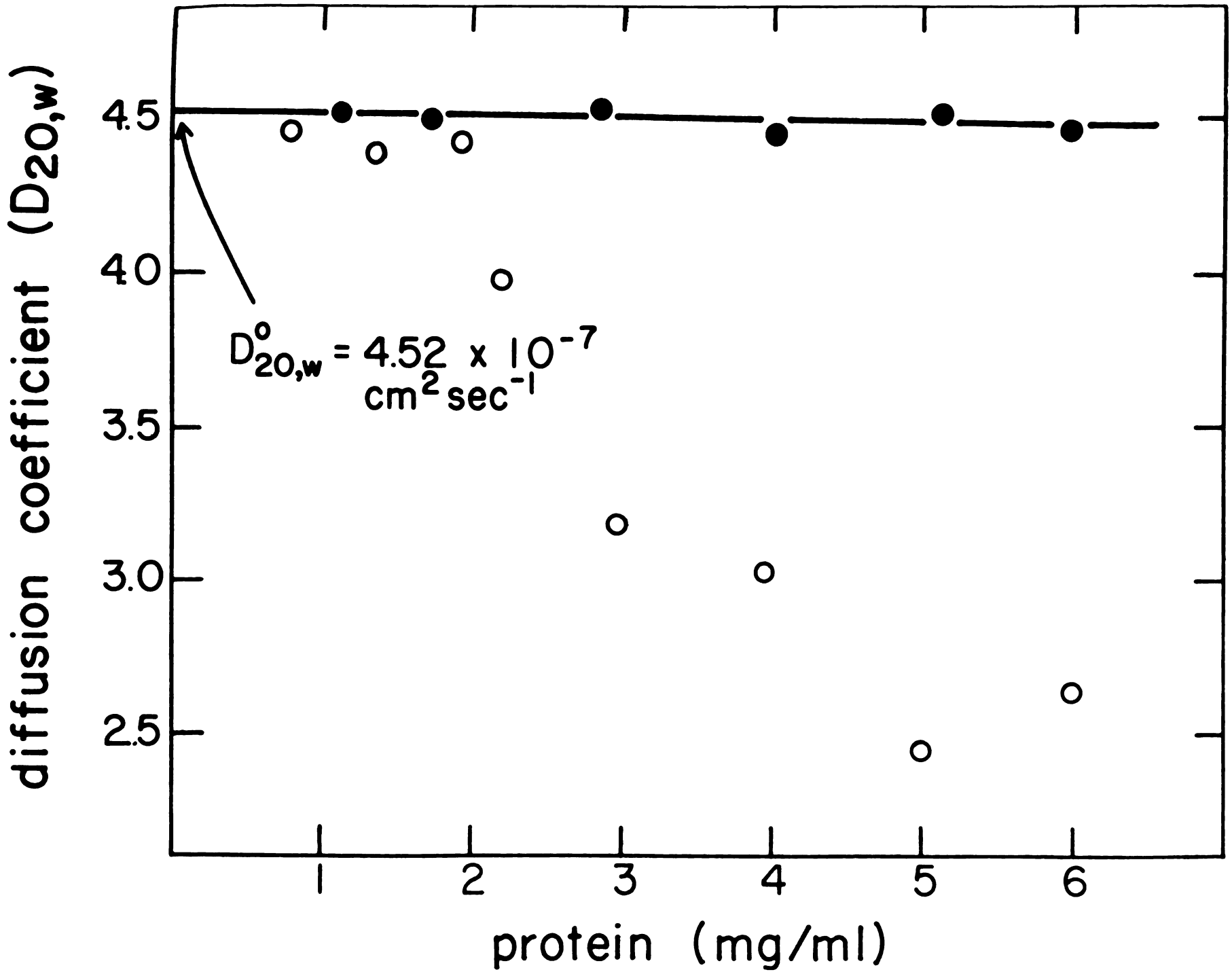
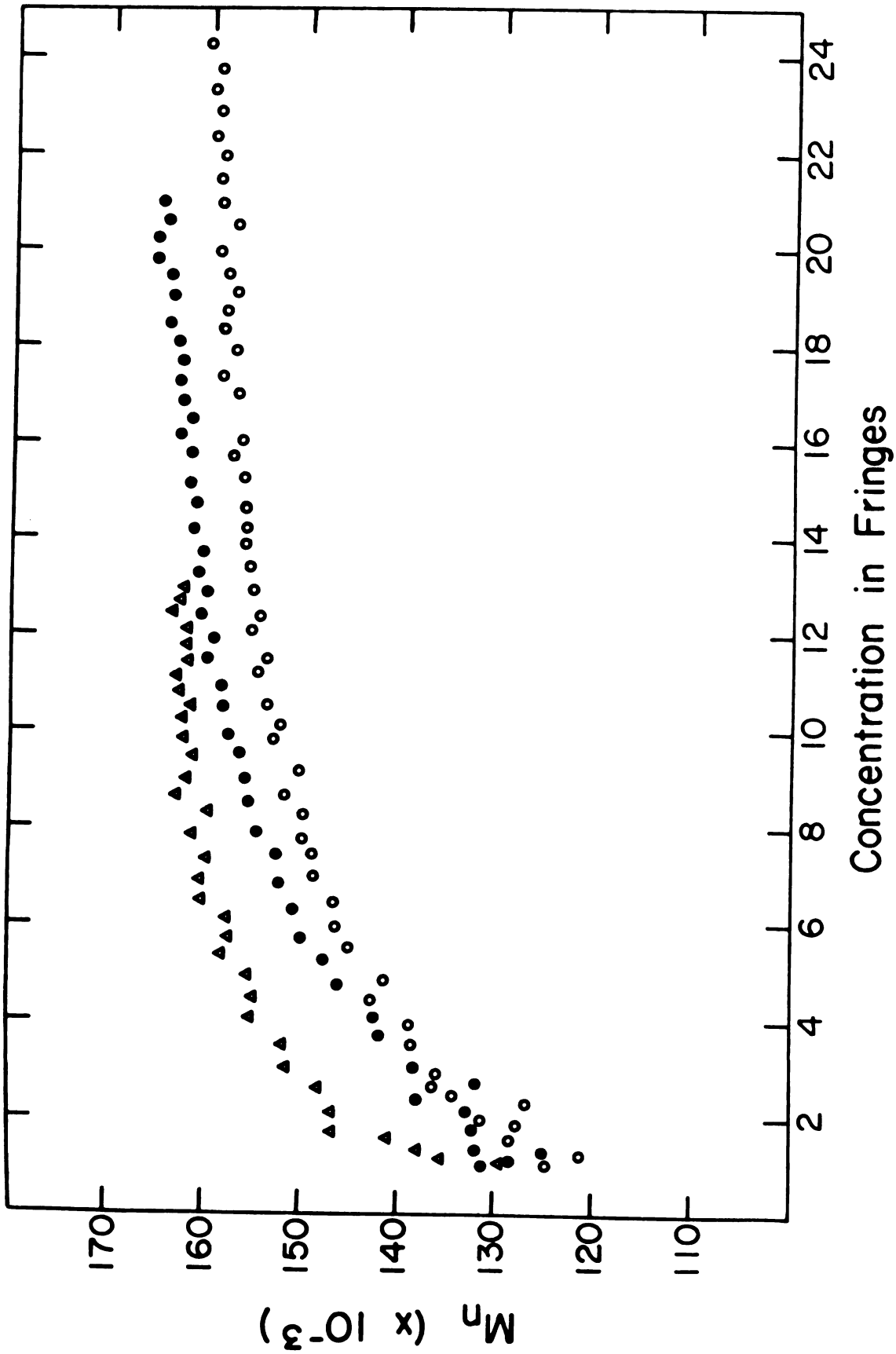

diffusion coefficient ($D_{20,w}$)
4.5
4.0
3.5
3.0
2.5
$D^{o}_{20,w} = 4.52 \times 10^{-7}$
$cm^2 sec^{-1}$
1
2
3
4
5
6
protein (mg/ml)

Figure 7. Number-Average Molecular Weight as a Function of Fringe Concentration for Native Yeast Pyruvate Kinase at 20° in 0.1 M Tris·HCl, pH 7.5 containing 0.23 M KCl, 0.025 M $MgCl_2$, 2×10^{-3} M FDP, and 10^{-2} M PEP

The open triangles represent an initial concentration of 0.20 mg/ml; closed circles, 0.40 mg/ml; and open circles, 0.60 mg/ml.


M_n (x 10^{-3})
170
160
150
140
130
120
110
2
4
6
8
10
12
14
16
18
20
22
24
Concentration in Fringes

when allowed to stand for 24 hr at 20° at 0.2 mg/ml in this solvent. The enzyme was not sufficiently stable for a 24-hr period at 0.2 mg/ml to determine the molecular weight in the absence of FDP.

2. Subunits

i. Enzyme in guanidine hydrochloride

Preliminary sedimentation equilibrium experiments with yeast PK in 6 M GuHCl indicated the need for a high concentration of reducing agent to eliminate what appeared to be a random aggregation of the protein. Figure 8 presents data, plotted according to Van Holde and Baldwin (1958), from a typical sedimentation equilibrium experiment with yeast PK in 6 M GuHCl containing 0.15 M 2-mercaptoethanol. Data for $\underline{M}_w$ and $\underline{M}_z$ of yeast PK at several concentrations are plotted and extrapolated to zero protein concentration in Figure 9. Values for $\underline{M}_w^o$ and $\underline{M}_z^o$ were calculated to be 41,400 and 45,900, respectively. Under the same solvent conditions, the enzyme sedimented as a single symmetrical peak ($\underline{s}_{20,w}^{0.8\%}$ = 1.09 S).

ii. Maleylated enzyme

To further characterize the subunits, efforts were made to dissociate the enzyme through the introduction of negative charges by maleylation. Under the conditions described in Methods, 230 moles of maleate/168,000 g of protein (11.9% of the resultant molecular weight) were incorporated as determined by the spectrophotometric assay of Freedman et al. (1968). Extensive dialysis of the maleylated enzyme did not alter the absorption coefficient

Figure 9. Determination of the Homogeneity of Yeast PK at 1.75 mg/ml in a 6 M GuHCl Containing 0.15 M 2-Mercaptoethanol, According to Van Holde and Baldwin (1958)

The rotor speed was 21,708 rpm, and the results were obtained after 30 hr at 20°. $\partial n/\partial r$ is the refractive index gradient in arbitrary units and r is the radial distance (cm). The abscissa represents the integrated area under the refractive index gradient curve, beginning at the inner meniscus. The line is a least-squares plot.

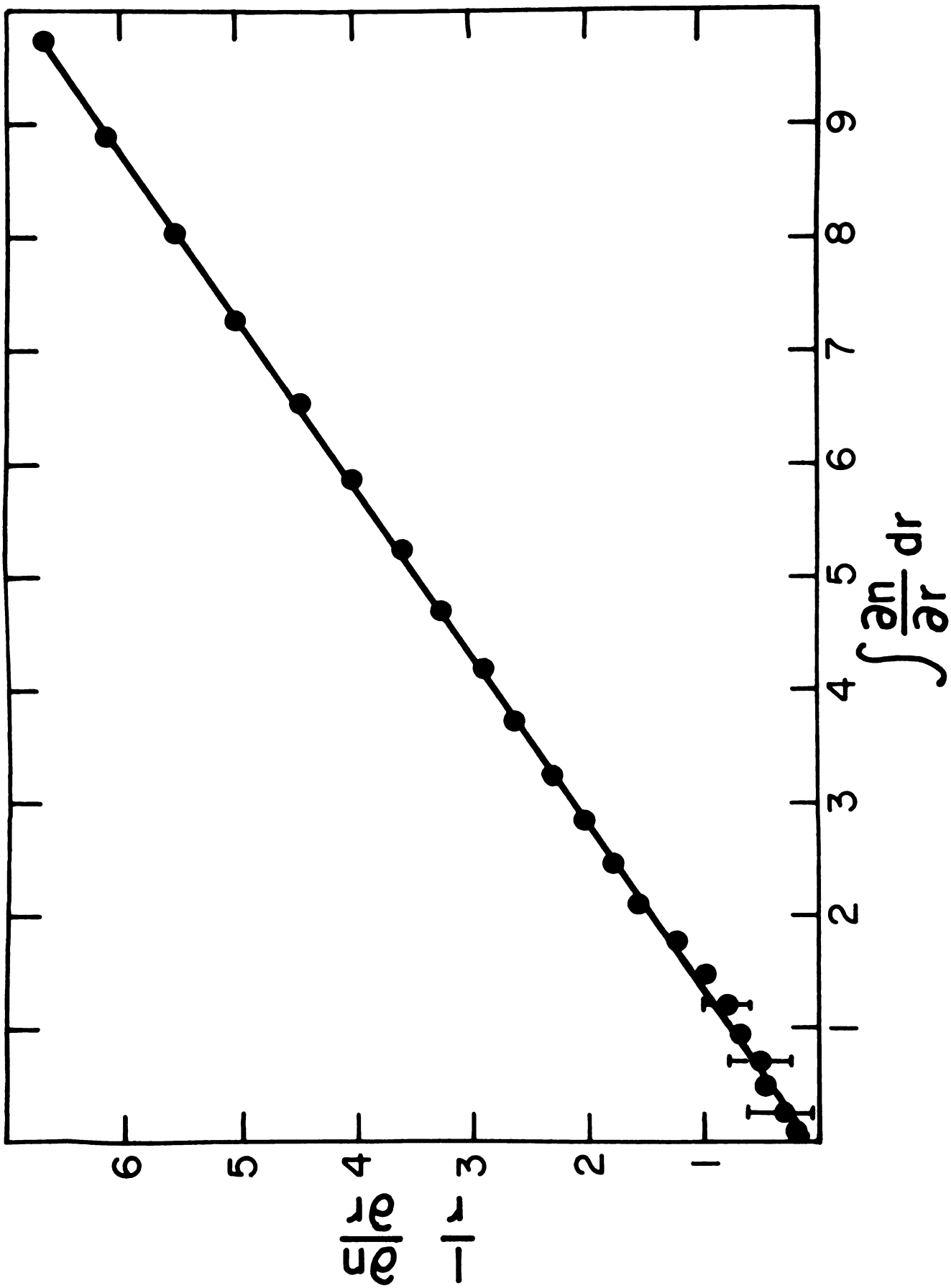
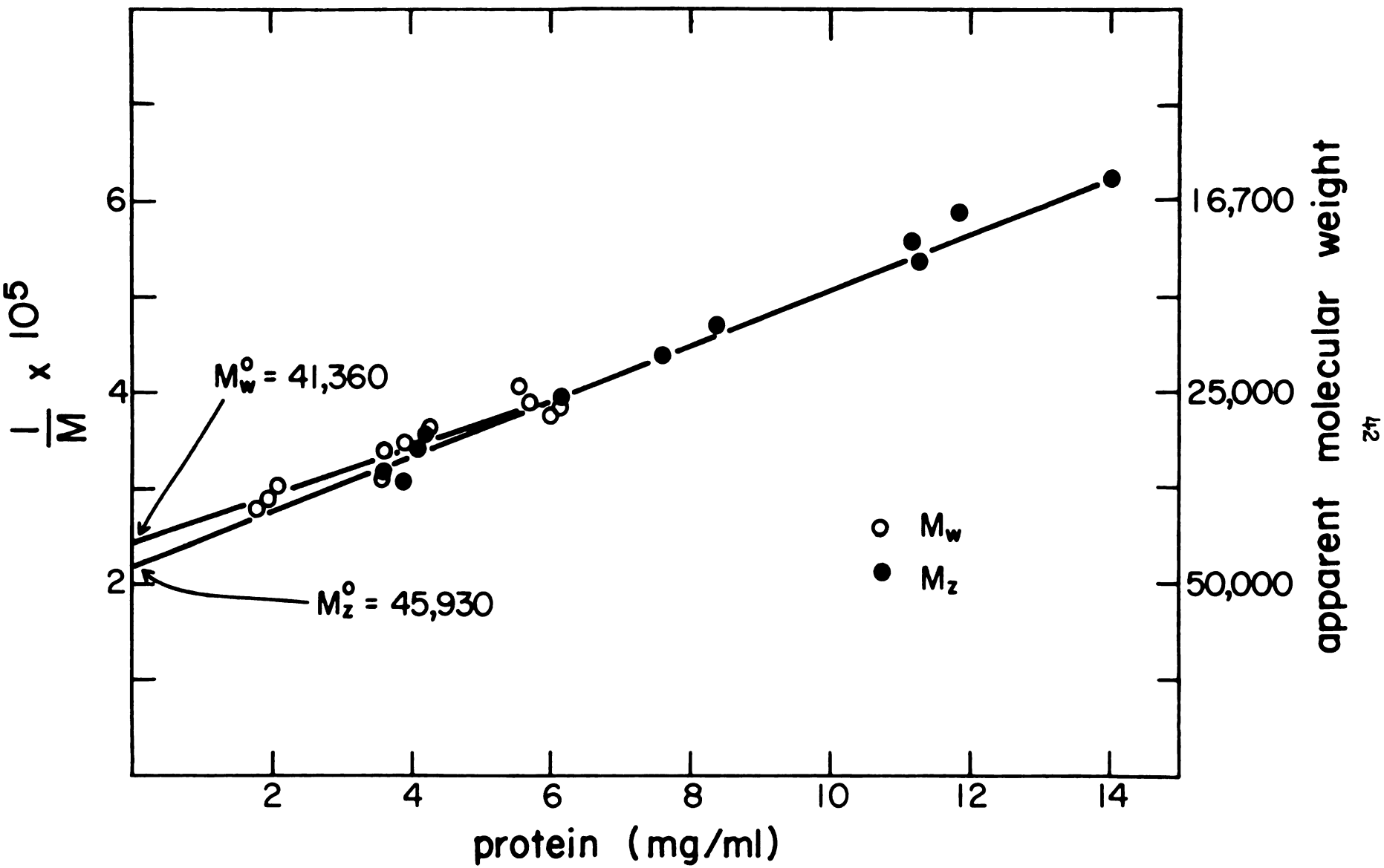

$\frac{1}{r}\frac{\partial n}{\partial r}$
$\int \frac{\partial n}{\partial r} dr$
1
2
3
4
5
6
7
8
9

Figure 9. Extrapolation of the Apparent Weight-Average ($\underline{M}_w^{app}$) and Apparent z-Average ($\underline{M}_z^{app}$) Molecular Weights of Yeast PK to Zero Protein Concentration

The solvent system contained 6 M GuHCl and 0.15 M 2-mercaptoethanol. The temperature was 20°. Centrifugation was carried out at 21,708 rpm for 30 hr. Concentrations were evaluated as $(c_m + c_b)/2$ for $\underline{M}_w^{app}$ and $(c_m + c_b)$ for $\underline{M}_z^{app}$, where c_m and c_b are the concentrations at the meniscus and bottom of the cell respectively. The lines are least-squares plots.


$\frac{1}{M} \times 10^5$
6
4
2
M_w^o = 41,360
M_z^o = 45,930
o M_w
● M_z
2
4
6
8
10
12
14
protein (mg/ml)
16,700
25,000
50,000
apparent molecular weight

of the enzyme at 250 nm, suggesting complete removal of unbound maleate.

Results for the sedimentation coefficient and the diffusion coefficient of the maleylated enzyme determined as a function of protein concentration are shown in Figures 10 and 11 respectively. The values of $\underline{s}^{o}_{20,w}$ = 1.97 S and $\underline{D}^{o}_{20,w}$ = 3.75 x 10^{-7} cm^2/sec yielded a molecular weight of $\underline{M}^{o}_{w}(\underline{s}/\underline{D})$ = 47,900. Subtracting 5700 for the contribution of the bound maleyl groups gave a net molecular weight of 42,200 for the subunits. Addition of 0.1 M 2-mercaptoethanol to the maleylated enzyme affected neither the sedimentation coefficient nor the diffusion coefficient. The sedimentation and diffusion coefficients of yeast PK under all of the conditions described above are summarized in Table I.

3. Effects of cations, substrates, and FDP on the sedimentation coefficient of yeast pyruvate kinase

Although the results presented in Figure 7 suggest that no association-dissociation equilibrium involving interaction of the enzyme with ligands is apparent, the effect of cations and other ligands, separately and together, on the sedimentation coefficient of yeast PK was measured to further rule out that possibility. The results are presented in Table II. The only changes in $\underline{s}^{o}_{20,w}$ occur in the presence of FDP with no K^+ (Expt. 4), and in the presence of FDP, K^+, and PEP (Expt. 5), both with Mg^{2+} present. In both cases, slight increases in $\underline{s}^{o}_{20,w}$ are obtained. As will be seen

Figure 10. Sedimentation Coefficient of Yeast PK after Maleylation

Centrifugations were performed at 20° in 0.1 M $K_2B_2O_7$ buffer, pH 9.0, containing 0.1 M KCl. The line is a least-squares plot.

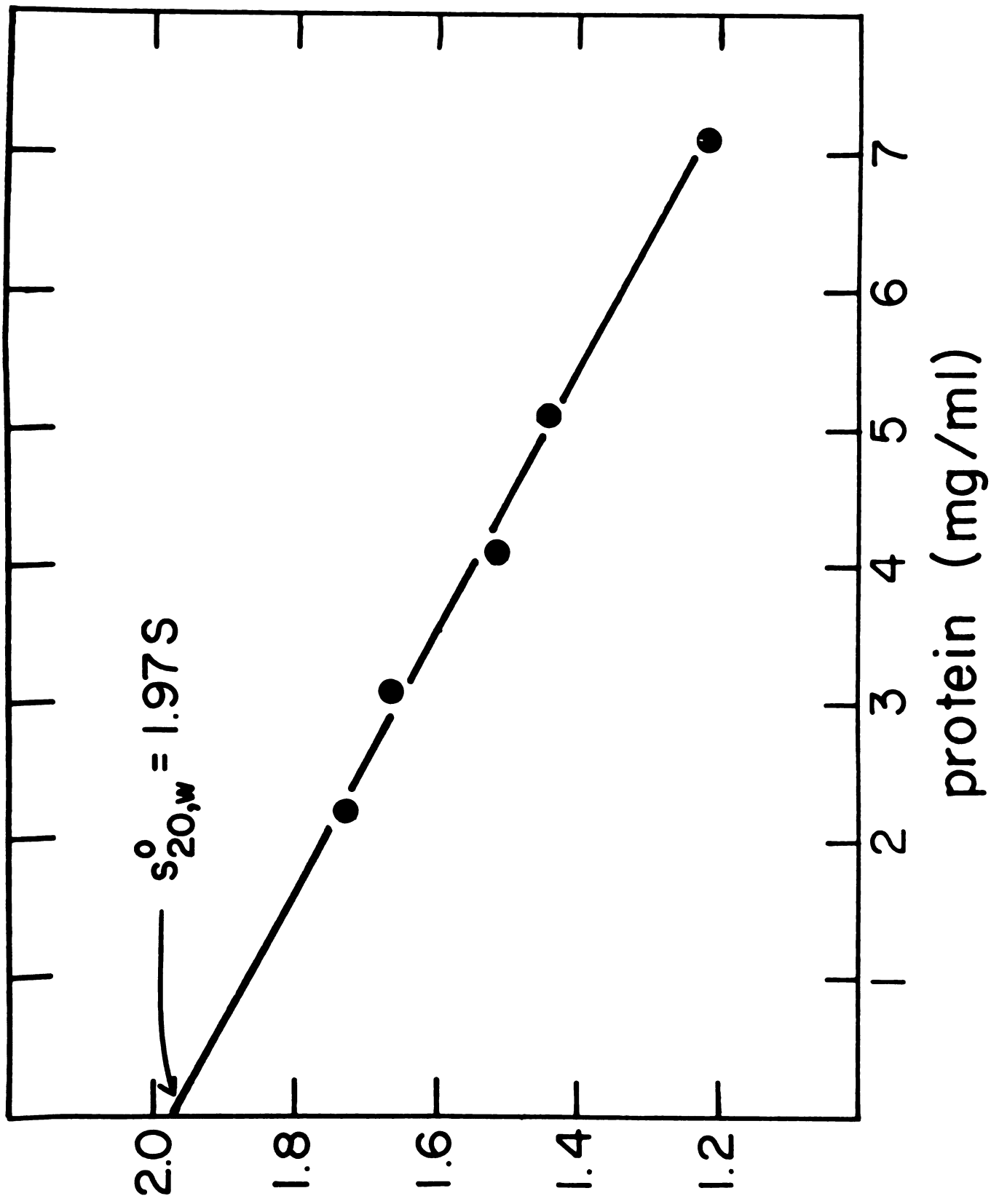

sedimentation coefficient ($s_{20,w}$)
2.0
1.8
1.6
1.4
1.2
$s^o_{20,w}$ = 1.97S
1
2
3
4
5
6
7
protein (mg/ml)

Figure 11. Diffusion Coefficient of Yeast PK after Maleylation

Centrifugations were performed at 20° in 0.1 M $K_2K_2O_7$ buffer, pH 9.0, containing 0.1 M KCl. The line is a least-squares plot.

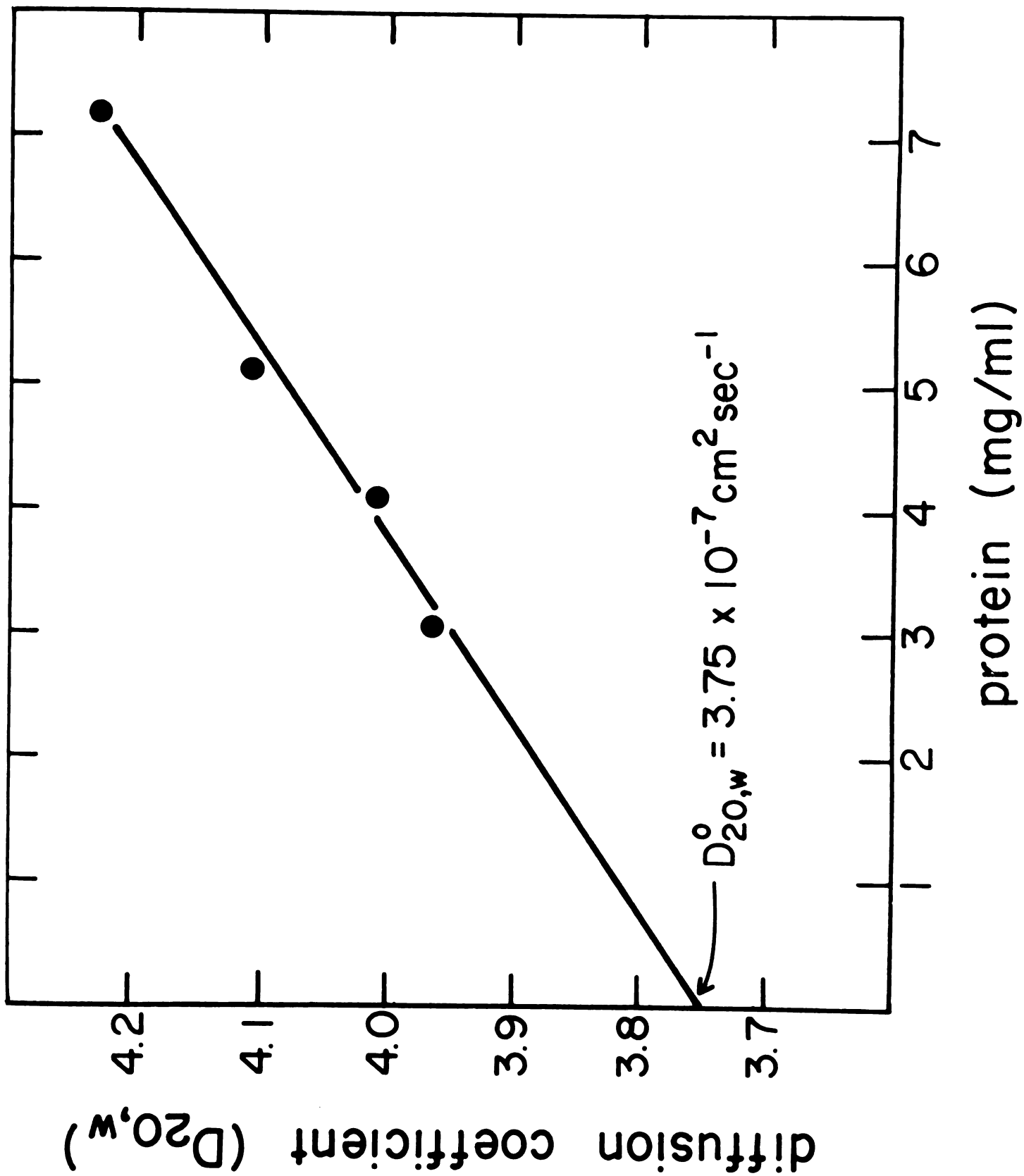

diffusion coefficient ($D_{20,w}$)
4.2
4.1
4.0
3.9
3.8
3.7
$D^o_{20,w} = 3.75 \times 10^{-7} cm^2 sec^{-1}$
1
2
3
4
5
6
7
protein (mg/ml)

TABLE I

Sedimentation and Diffusion Coefficients of
Yeast Pyruvate Kinase as a Function of Protein Concentration

All experiments were performed at 20° in (a) 0.1 M tetramethylammonium cacodylate buffer, pH 6.2 containing 0.1 M KCl, 2.6 x 10^{-2} M $MgCl_2$ and 10^{-3} M fructose-1,6-diphosphate, (b) 0.1 M Tris·HCl, pH 7.5, or (c) 0.1 M $K_2B_2O_7$ buffer, pH 9.0, containing 0.1 M KCl. Extrapolations to zero protein concentration were least squares plots, ± standard deviations.

Conditions	$\underline{s}^{o}_{20,w}$	$[\Delta \underline{s}_{20,w}/mg]^*$	$\underline{D}^{o}_{20,w}$ (x 10^{-7} cm^2 sec^{-1})	$[\Delta \underline{D}_{20,w}/mg]^*$ (x 10^{-7} cm^2 sec^{-1})	Concentration Range (mg/ml)
			Native Enzyme		
(a)	8.85S ± 0.02	-0.13S			2-11
			4.84 ± 0.19	-0.010	2-13.5
(b)	8.35S ± 0.04	-0.081S			1.3-12.5
			4.52 ± 0.03	-0.009	1.3-6.0
			Maleylated Subunits		
(c)	1.97S ± 0.02	-0.105S			2.1-7.1
			3.75 ± 0.06	+0.067	3.0-7.1

*The Δ values represent the slope of the least squares line derived from a plot of the indicated parameter vs. protein concentration.

TABLE II

Sedimentation Coefficients of Yeast Pyruvate Kinase as a Function of Cations, Substrates, and FDP

Expt. No.	Additions*	$s^0_{20,w}$	$[\Delta s_{20,w}/mg]^+$	Concentration Range (mg/ml)
1	None	8.35 S ± 0.04	-0.081 S	1.3-12.5
2	0.20 M KCl 0.25 M $MgCl_2$	8.35 S ± 0.04	-0.086 S	2.0-8.0
3	0.20 M KCl 0.025 M $MgCl_2$ 0.001 M $(CHA)_4FDP$	8.32 S ± 0.06	-0.073 S	2.0-7.6
4	0.025 M $MgCl_2$ 0.001 M $(CHA)_4FDP$	8.45 S ± 0.05	-0.061 S	2.0-7.5
5	0.20 M KCl 0.025 M $MgCl_2$ 0.001 M $(CHA)_4FDP$ 0.01 M $(CHA)_3PEP$	8.53 S ± 0.04	-0.065 S	2.0-10.1

*All runs were performed at 20° in 0.1 M Tris·HCl, pH 7.5. Extrapolations to zero protein concentration were least squares plots ± standard deviations.

+The Δ values represent the slopes of the least squares plots of sedimentation coefficient vs. protein concentration.

later, the concentration of FDP used in Expt. 3 in the presence of K^+ was not saturating so that the results obtained for that experiment are not conclusive.

III. Effects of Substrates, Cations and FDP on the Fluorescence of Yeast Pyruvate Kinase

1. Qualitative effects

The fluorescence of yeast PK is quenched by the addition of the cations K^+ and/or Mg^{2+}, but not $(CH_3)_4N^+$. However, as can be seen from Table III, the extent of the quenching produced by these cations individually or together is minimal when compared to the effect on the fluorescence of yeast PK brought about by the addition of FDP. The extent of the quenching produced by FDP was independent of cations and/or PEP, although, as will be seen, the concentration of FDP required to bring about the total fluorescence change depends on the effectors present. Because of the instability of yeast PK in the presence of FDP alone, a quantitative determination of the interaction of FDP in the absence of cations with the enzyme could not be made, although it can be stated that the fluorescence quenching by this ligand under these conditions is significantly greater than the quenching obtained in the presence of only the cations. The upper curve in Figure 12 represents the fluorescence emission spectrum of yeast PK at 0.30 mg/ml in 0.1 M Tris·HCl, pH 7.5, excited at 280 nm. Addition of 5×10^{-3} M FDP and 2.5×10^{-2} M $MgCl_2$ reduced the intensity at 344 nm by 12%,

TABLE III

Relative Fluorescence of Yeast Pyruvate Kinase

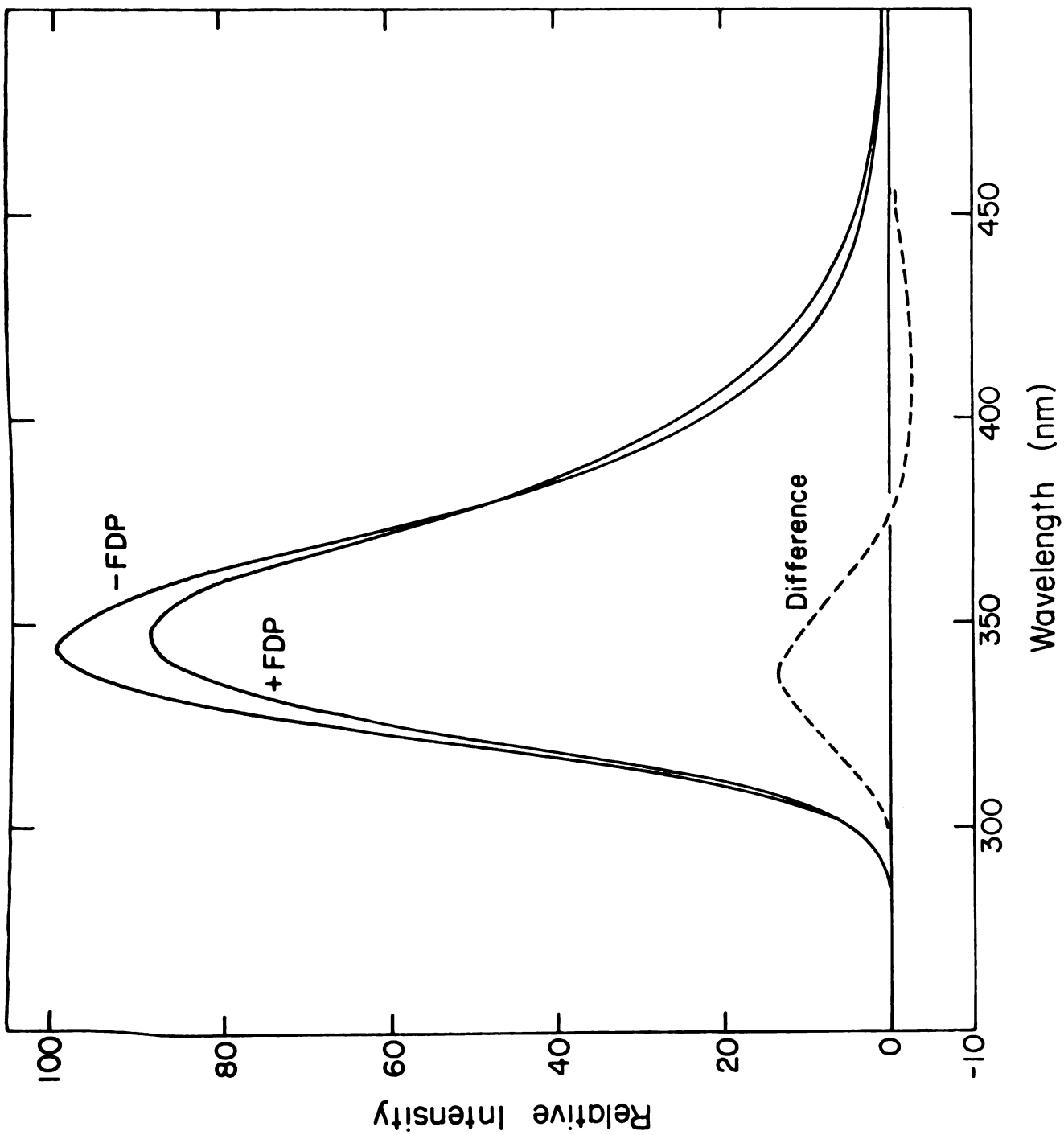
Expt. No.	Conditions+				Relative Fluorescence at 344 nm
	0.23 M KCl	0.025 M $MgCl_2$	0.01 M PEP	FDP*	
1					100
2	+				97-98
3		+			97-98
4	+	+			95-96
5			+		95-96
6	+		+		95-96
7		+	+		95-96
8	+	+	+		95-96
9		+		+	86-88
10	+	+		+	86-88
11	+	+	+	+	86-88

+All measurements were made in 0.1 M Tris·HCl, pH 7.5 at 23° with the protein concentration maintained at 0.30 mg/ml. The excitation wavelength was 280 nm.

*See Table IV for saturating FDP concentrations.

Figure 12. Fluorescence Emission Spectrum of Yeast PK in 0.1 M Tris·HCl, pH 7.5 and 0.025 M $MgCl_2$ at 23°

Protein concentration was 0.30 mg/ml for both curves, and the FDP concentration was 5×10^{-3} M. Excitation wavelength was 280 nm.


-FDP
+FDP
Difference
Relative Intensity
Wavelength (nm)
100
80
60
40
20
0
-10
300
350
400
450

and shifted the peak position slightly to 347 nm.

2. Titration of yeast pyruvate kinase with FDP by measurement of fluorescence change

Since the changes in the fluorescence of PK after addition of FDP were sufficiently large to allow for a determination of dissociation constants from titrations of the fluorescence change, we proceeded to examine the extent of interaction of the enzyme with cations and substrates as reflected in the binding of FDP. Except as noted, the excitation wavelength was 280 nm and the emission was monitored at 344 nm. The data were treated as shown in Figure 13 as a Hill plot (Atkinson, 1966) of the fluorescence of PK as a function of the concentration of FDP at various KCl concentrations. As can be seen from Figure 13 and Table IV, the addition of 0.10 M KCl (the optimal KCl concentration for catalytic activity of PK in the presence of FDP) increased the apparent K_D for FDP from 0.49 mM to 1.3 mM with little effect on the Hill slope (n_H). Increasing the KCl to 0.23 M (optimal for catalytic activity in the absence of FDP) increased the apparent K_D for FDP even further to 3.1 mM. On the other hand, addition of 0.01 M PEP at the latter KCl concentration markedly increased the affinity of the enzyme for FDP yielding an apparent K_D of 0.069 mM. It should be noted that identical K_D's and n_H's were obtained when fluorescence differences were determined at 340 to 355 nm, and when the excitation wavelength was 280 to 295 nm.

Figure 13. Plots of the Binding of FDP to Yeast Pyruvate Kinase as Monitored by Fluorescence Changes

Protein was maintained at 0.30 mg/ml and the temperature was 23°. The excitation wavelength was 280 nm. The number in parentheses is the Hill slope (n_H).

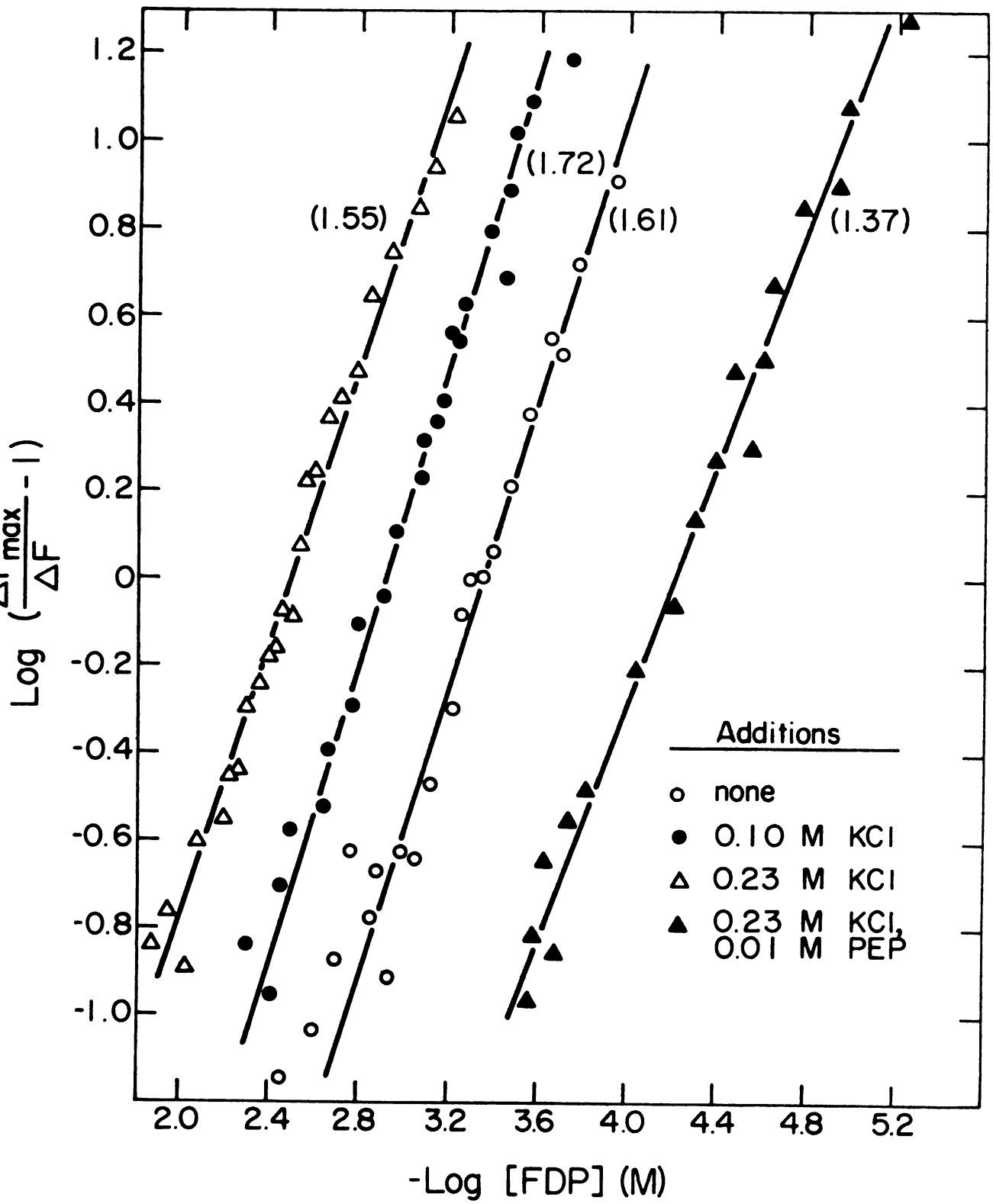

1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
Log ($\frac{\Delta F_{max}}{\Delta F}$ - 1)
(1.55)
(1.72)
(1.61)
(1.37)
Additions
none
0.10 M KCl
0.23 M KCl
0.23 M KCl, 0.01 M PEP
2.0
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
-Log [FDP] (M)

TABLE IV

Apparent Dissociation Constants of FDP and Yeast Pyruvate Kinase Obtained by Titration of Fluorescence Changes

Additions*	Temperature (°C)	K_D(mM)+	n_H+
None	0	0.12 ± 0.009	1.71 ± 0.07
	23	0.49 ± 0.029	1.58 ± 0.03
	30	0.91	1.38
0.10 M KCl	23	1.3 ± 0.08	1.68 ± 0.04
0.10 M $(CH_3)_4NCl$	23	1.0	1.86
0.23 M KCl	0	0.36	1.13
	23	3.1 ± 0.15	1.63 ± 0.08
	30	3.3	2.07
0.23 M $(CH_3)_4NCl$	23	1.2 ± 0.25	2.03 ± 0.07
5 mM Tris ADP, pH 7.5	23	0.20	1.16
0.23 M KCl 5 mM Tris ADP, pH 7.5	23	0.19 ± 0.007	1.05 ± 0.07
0.23 M $(CH_3)_4NCl$ 5 mM Tris ADP, pH 7.5	23	0.17 ± 0.017	1.02 ± 0.01

0.01 M $(CHA)_3PEP$	23	0.48	1.20
0.23 M KCl	0	0.03	
0.01 M $(CHA)_3PEP$	23	0.069 ± 0.0031	1.37 ± 0.06
	30	0.45	1.57
0.23 M $(CH_3)_4NCl$	23	0.48 ± 0.005	1.15 ± 0.05
0.01 M $(CHA)_3PEP$			

*All titrations were done in 0.10 M Tris·HCl, pH 7.5 containing 0.025 M $MgCl_2$, and the indicated additions. Protein concentration in all cases was maintained at 0.30 mg/ml.

+Values for which a range is given are averages of two determinations.

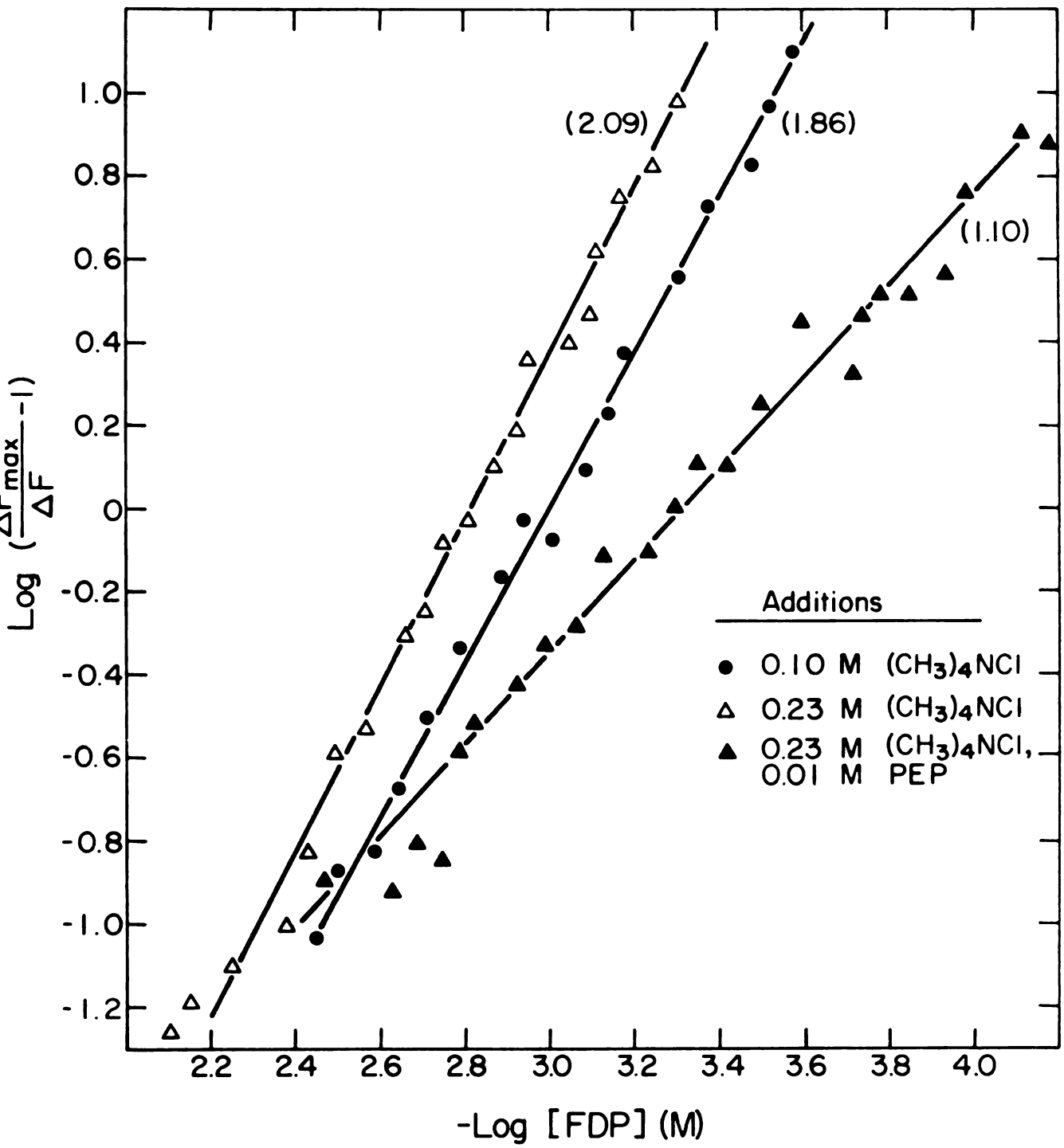
In an effort to separate specific monovalent cation effects from general ionic strength effects, dissociation constants for FDP and PK were determined in the presence of the same concentrations of $(CH_3)_4N^+$, a catalytically non-activating cation (Figure 14). The effect of $(CH_3)_4N^+$ on the apparent dissociation constant for FDP is analogous to the effect of K^+, although the extent of the increase in apparent K_D is somewhat lessened. On the other hand, a considerable increase in the Hill slope was obtained with $(CH_3)_4N^+$ which was not observed with K^+. In addition, the combined presence of PEP and $(CH_3)_4N^+$ does not yield the marked decrease in apparent K_D for FDP as was seen with 0.01 M PEP plus K^+. In fact, the apparent K_D for FDP in the presence of 0.01 M PEP with or without $(CH_3)_4N^+$ is identical to the apparent K_D for FDP obtained in the absence of PEP, that is, in the presence of Mg^{2+} alone, although the Hill slope is considerably reduced.

The effect of the second substrate of the pyruvate kinase reaction, ADP, is also included in Table IV. For these titrations, an excitation wavelength of 295 nm was used to minimize absorption due to the ADP. In the presence of ADP and either K^+ or $(CH_3)_4N^+$ the apparent K_D for FDP is reduced by an order of magnitude from the value obtained in the absence of ADP; the Hill slope in both cases is reduced from values near 2 to unity.

The influence of temperature on the quenching of PK fluorescence by FDP is also included in Table IV. Under

Figure 14. Plots of the Binding of FDP to Yeast Pyruvate Kinase as Monitored by Fluorescence Changes

Protein was maintained at 0.30 mg/ml and the temperature was 23°. The excitation wavelength was 280 nm. The numbers in parentheses are Hill slopes (n_H).


1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
Log $(\frac{\Delta F_{max}}{\Delta F} - 1)$
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
-Log [FDP] (M)
(2.09)
(1.86)
(1.10)
Additions
0.10 M $(CH_3)_4NCl$
0.23 M $(CH_3)_4NCl$
0.23 M $(CH_3)_4NCl$, 0.01 M PEP

each of the three conditions in which effects of temperature were examined, (no K^+, 0.23 M K^+, and 0.23 M K^+ plus 0.01 M PEP) the apparent K_D for FDP increased by an order of magnitude as the temperature was raised from 0 to 30°. However, no consistent trend for n_H could be seen, with values decreasing in the absence of K^+ as the temperature was raised from 0 to 30°, and values increasing in the presence of K^+ or K^+ plus PEP as the temperature was increased from 0 to 30°.

IV. Fructose 1,6-Diphosphate-Induced Inactivation of Yeast Pyruvate Kinase

1. Inactivation at 0°

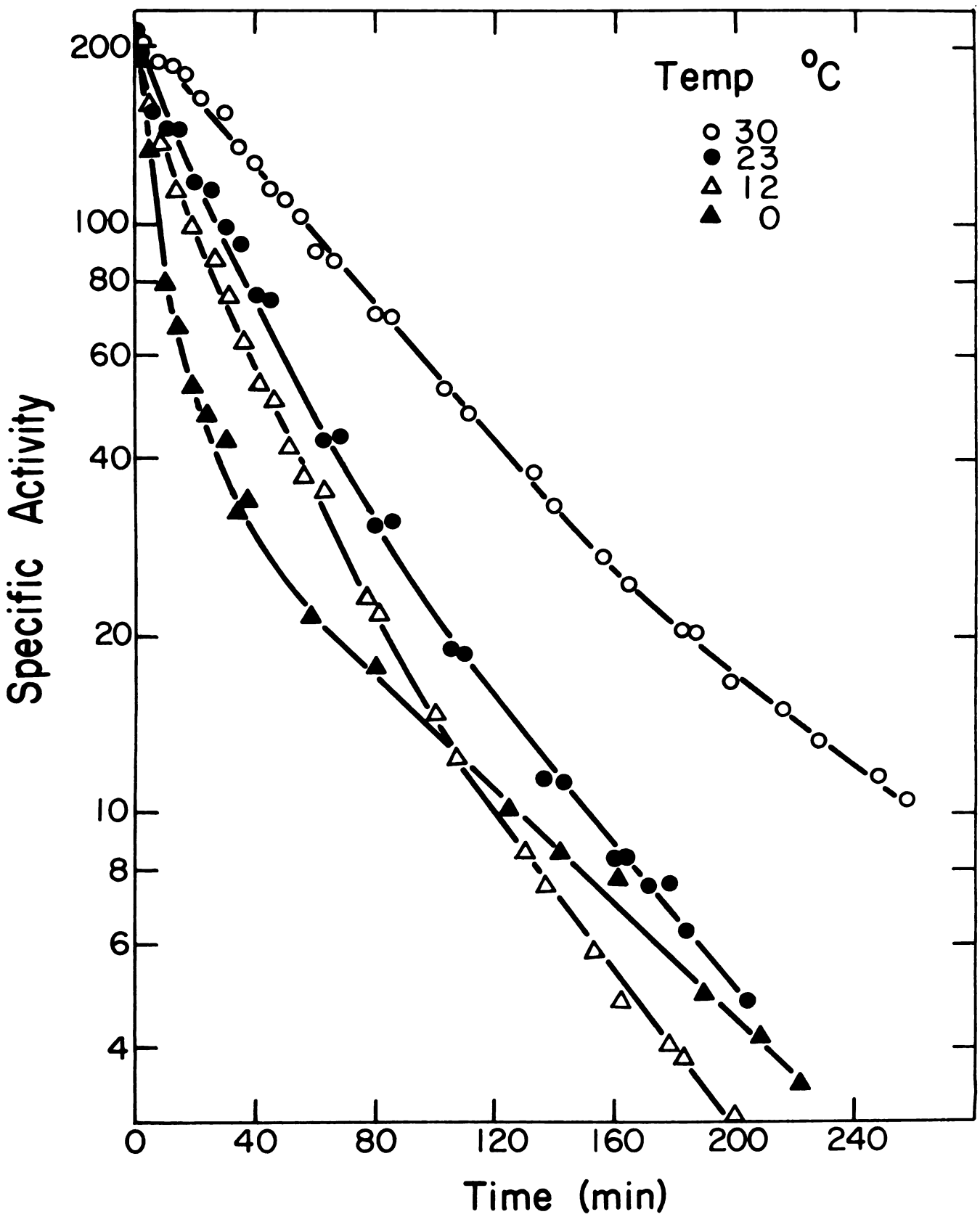
i. Dependence of the inactivation on FDP concentration

A search for compounds which might stabilize the enzyme against cold inactivation revealed that FDP, the allosteric activator of yeast PK, markedly enhanced the low temperature inactivation. Efforts were then made to characterize the mechanism by which FDP, in the absence of cations, promoted inactivation of the enzyme.

Figure 15 shows the effect of temperature on the inactivation at millimolar concentrations of FDP. The data in this Figure, as compared to Figure 1 indicate that the rate constant is increased as much as 1000-fold over the sample at 23°. (Note that the time scales on these Figures differ.) To insure that this effect was produced by FDP,

Figure 15. Effect of Temperature on the FDP-Enahnced Inactivation of Yeast Pyruvate Kinase at 0.5 mg/ml in 0.1 M Tris·HCl, pH 7.5

FDP concentration was 1.26×10^{-3} M. Each point represents an aliquot of enzyme removed from the incubation mixture and assayed at 30°.


Temp °C
30
23
12
0
Specific Activity
200
100
80
60
40
20
10
8
6
4
0
40
80
120
160
200
240
Time (min)

PK was incubated at 0° at 1.0 mg/ml under the conditions shown in Table V. Essentially two rates of inactivation were obtained: that resembling the 0° curve of Figure 1, (Samples 1, 2, and 3), and that resembling the 0° curve of Figure 15 (Samples 4, 5, and 6). The inactivation was not produced by metal contaminants of the FDP such as copper (Passeron et al., 1967) since FDP treated with Chelex and untreated FDP produced identical results. The dependence of the inactivation on FDP concentration was then measured, and the results are presented in Figure 16, for yeast PK at 0.5 mg/ml.

ii. Treatment of the data from Figure 16

The curves obtained from the semi-logarithmic plots in Figure 16 are biphasic, indicative of two pseudo-first-order inactivation steps. Under this assumption, the data shown in Figure 16 were treated as in Figure 17. Extrapolation of the data of the slow step to zero time yielded intercepts which varied from specific activity 210-39 μmoles/min per mg as FDP concentration was increased from 0 to 1.26 mM. Identical intercepts were obtained for 0.756 and 1.26 mM FDP concentrations, suggesting that the latter concentration was saturating. The difference between the intercept at saturating concentration of FDP and that obtained in the absence of FDP was equated to unity, and values, defined as

$$\alpha = \frac{(\text{intercept})_{0\ \text{FDP}} - (\text{intercept})_{X\ \text{FDP}}}{(\text{intercept})_{0\ \text{FDP}} - (\text{intercept})_{1.26\ \text{mM FDP}}} \qquad (1)$$

TABLE V

Requirement for FDP for Enhanced Inactivation of Yeast Pyruvate Kinase[a]

Sample	Additions to a Final Volume of 0.1 ml	Enhanced Inactivation[b]
1	none	-
2	1.1 ug aldolase	-
3	132 umoles DHAP	-
	120 umoles G3P	
4	132 umoles DHAP	+
	120 umoles G3P	
	1.1 ug aldolase	
5	126 umoles FDP	+
	1.1 ug aldolase	
6	126 umoles FDP	+

[a]Each sample contained yeast pyruvate kinase at 1.0 mg/ml. Samples were incubated at 0°, and activity was followed with time.

[b](-) indicates that resultant inactivation curve resembled the 0° curve of Figure 1. (+) indicates that inactivation resembled 0° curve in Figure 15, both in rate of inactivation, and in extent.

Figure 16. Effect of FDP Concentration on the Stability of Yeast Pyruvate Kinase at 0° in 0.1 M Tris·HCl, pH 7.5.

Final protein concentration was 0.50 mg/ml. All samples had an initial specific activity of 210 units/mg.

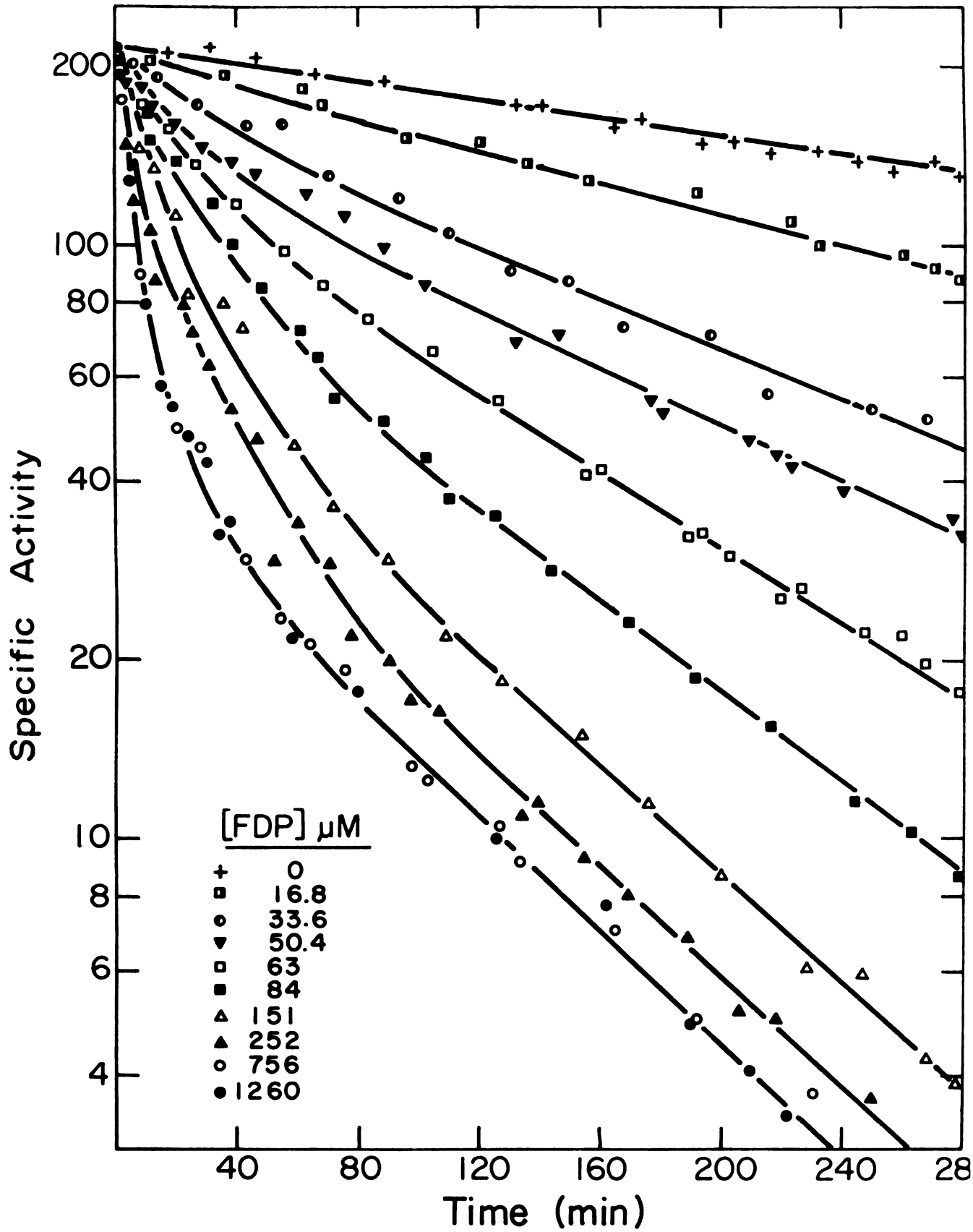
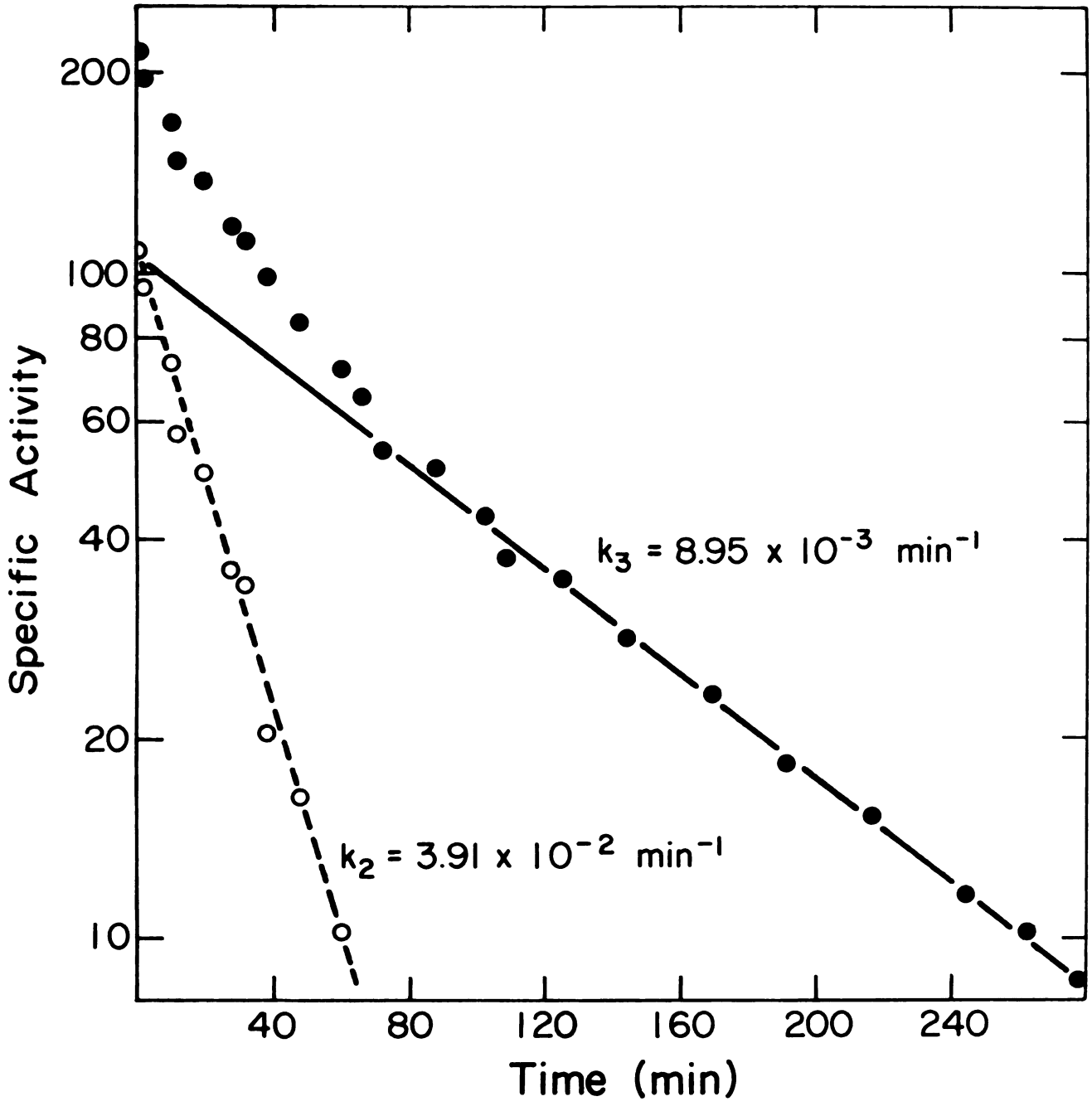

Specific Activity
Time (min)
200
100
80
60
40
20
10
8
6
4
40
80
120
160
200
240
28
[FDP] μM
0
16.8
33.6
50.4
63
84
151
252
756
1260

Figure 17. Example of the Method of Treatment of Data from Figure 16

The sample above was inactivated in the presence of 84 μM FDP in 0.1 M Tris·HCl, pH 7.5 at 0.5 mg/ml of pyruvate kinase. Inactivation was allowed to continue until the slow process, labeled k_3, became linear. This rate was extrapolated to zero time, and specific activity values along this line were subtracted from the experimental points (closed circles). The open circles represent the differences so obtained.



ata fro

sence of

f pyruvate

l the slow

extrapo-

long this

(closed

ces so

were

log

succ

and

or

where

fract

Figur

avera

treat

there

incre

enzym

FDP is

inacti

(defin

were calculated for each FDP concentration and plotted vs. log FDP in Figure 18. If the binding is represented by the successive equilibria

$$E + FDP \rightleftharpoons EFDP_I \qquad K_I = \frac{[E][FDP]}{[EFDP_1]}$$

$$EFDP_1 + FDP \rightleftharpoons EFDP_{II} \quad K_{II} = \frac{[EFDP_1][FDP]}{[EFDP_2]}$$

$$EFDP_{n-1} + FDP \rightleftharpoons EFDP_n \quad K_n = \frac{[EFDP_{n-1}][FDP]}{[EFDP_n]}$$

and if $K_I \gg K_{II} \gg K_n$, then

$$pK_I + pK_{II} \ldots + pK_n - np[FDP] = \log \frac{\alpha}{1-\alpha}$$

or

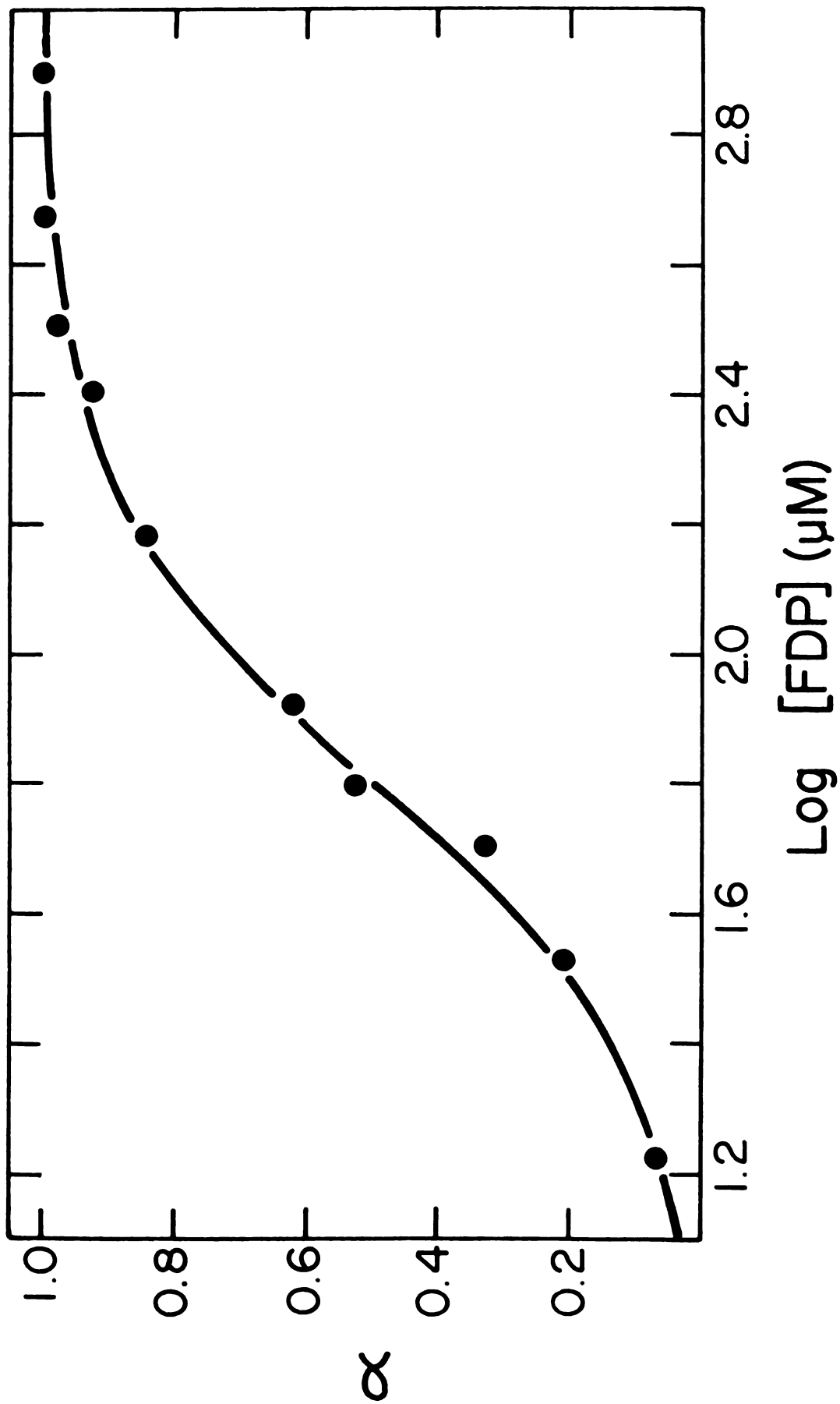
$$n(pK_{av} - p[FDP]) = \log \frac{\alpha}{1-\alpha} \qquad (2)$$

where E is enzyme, and α, defined in eq 1, represents the fraction of enzyme-FDP complex. The theoretical curve in Figure 18 was calculated assuming $n = 2$ and the geometrical average dissociation constant, $K_D = 63$ μM. For similar treatments of data, see Suelter et al. (1966) and references therein.

If the variation in extent of the first reaction with increasing FDP concentrations reflects an equilibrium of enzyme with FDP, and if the fraction of enzyme reacting with FDP is equal to α, the remaining unreacted enzyme would become inactivated at the rate observed in the absence of FDP (defined as k_1 and obtained from Figure 16 at zero concentration).

Figure 18. Determination of the Apparent K_D at 0° of FDP for Yeast Pyruvate Kinase from Inactivation Data in Figure 16

The data are plotted as α, defined in the text in eq 1, *vs.* log FDP. The solid line is calculated assuming $n = 2$, and $K_D = 63$ uM.


1.0
0.8
0.6
0.4
0.2
α
1.2
1.6
2.0
2.4
2.8
Log [FDP] (μM)

Based on this assumption, theoretical $\underline{k}_T$'s (slow step) were calculated for each FDP concentration with the use of

$$e^{-\underline{k}_T} = \alpha e^{-\underline{k}_3} + (1 - \alpha)e^{-\underline{k}_1} \qquad (3)$$

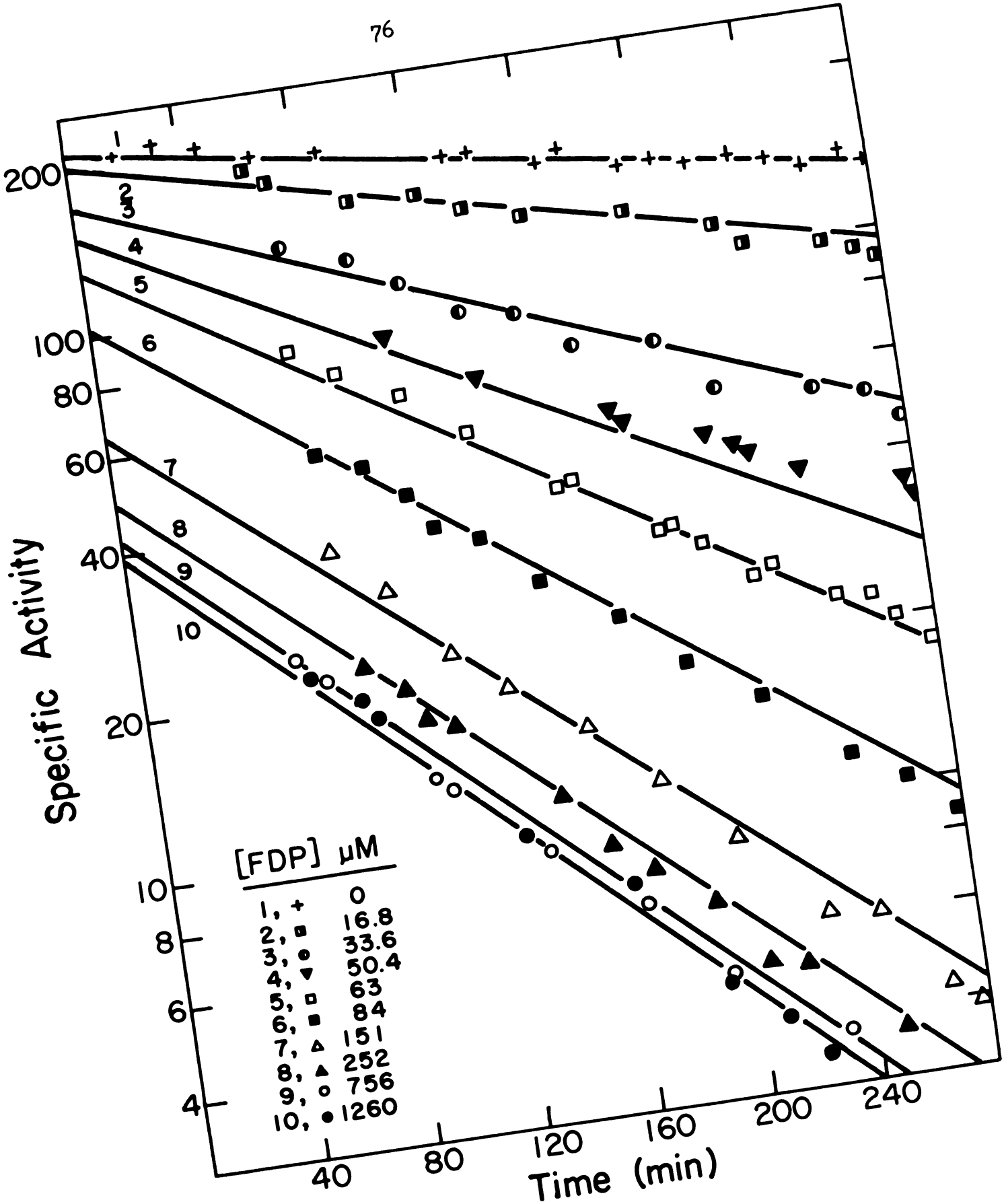
where $\underline{k}_T$ represents the sum of the rates of slow inactivation, $\underline{k}_1 + \underline{k}_3$, α is the theoretical fraction of enzyme-FDP complex derived from the solid line in Figure 18, $1 - \alpha$ is the fraction of free enzyme, $\underline{k}_3$ is the rate constant of inactivation for the enzyme-FDP complex, and $\underline{k}_1$ represents the rate constant of inactivation of free enzyme. The theoretical inactivation curves obtained in this manner are plotted as solid lines in Figure 19, along with the experimental points for each FDP concentration as obtained from Figure 16. The theoretical intercepts for 0.756 and 1.26 mM FDP (Figure 19) reflect 98.2 and 99.7% saturation of enzyme with FDP and thus are not distinguishable in the experimental data.

The rate constants for the fast inactivation, $\underline{k}_2$, were treated by the half-life method (Frost and Pearson, 1961) to obtain the order of the reaction with respect of FDP. For any rate expression of the type $d\underline{x}/d\underline{t} = \underline{k}(\underline{a} - \underline{x})^{\underline{n}}$, the half-life may be defined for all values of $\underline{n}$ as $\underline{t}_{\frac{1}{2}} = \underline{f}(\underline{n},\underline{k})/\underline{a}^{\underline{n}-1}$, where $\underline{f}$ is some function of $\underline{n}$ and $\underline{k}$, and $\underline{a}$ is defined as the initial concentration of the reactant. Placing this equation in logarithmic form yields

$$\log \underline{t}_{\frac{1}{2}} = \log \underline{f} - (\underline{n} - 1) \log \underline{a} \qquad (4)$$

Figure 19. Comparison of Experimental Data with Theoretical Determinations of the Zero Time Intercept and Calculated Rate Constants

Points are taken from the final half of Figure 16. Intercepts at zero time were calculated using experimental FDP concentrations, and the theoretical curve in Figure 18. Solid lines were drawn using calculated rate constants as described in the text using eq 3.


Specific Activity
200
100
80
60
40
20
10
8
6
4
Time (min)
40
80
120
160
200
240
[FDP] μM
1, + 0
2, ■ 16.8
3, ● 33.6
4, ▼ 50.4
5, □ 63
6, ■ 84
7, △ 151
8, ▲ 252
9, ○ 756
10, ● 1260

A log-log plot of $t_{\frac{1}{2}}$ *vs.* *a* should yield a straight line with slope (1 - *n*). Figure 20 is a log-log plot of the $t_{\frac{1}{2}}$'s obtained from the experimental k_2's *vs.* FDP concentration from which a value for *n* = 1.4 was calculated.

iii. Effect of protein concentration on the inactivation

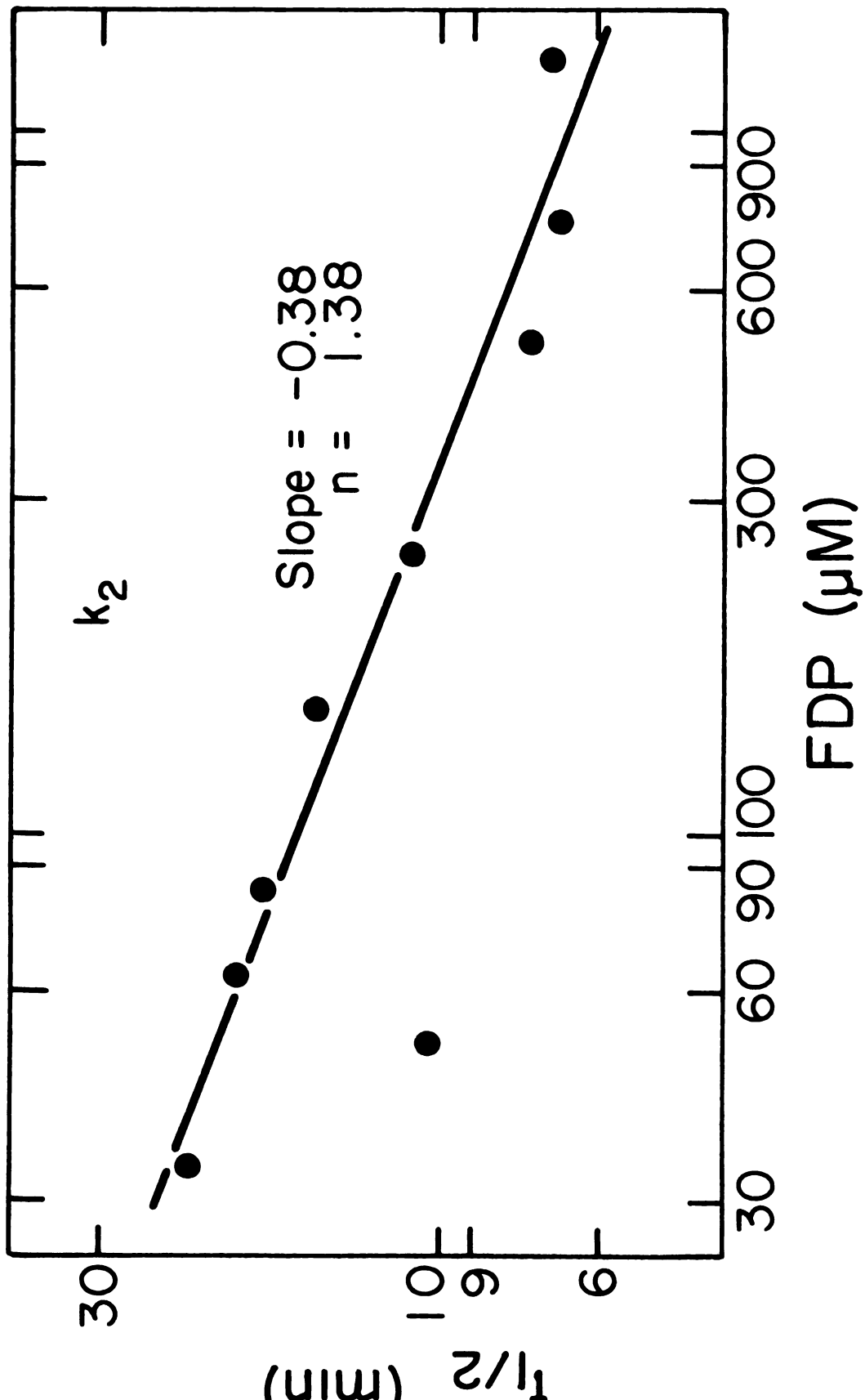
Inactivation was examined as a function of five different protein concentrations (Figure 21), each in the presence of 1.26 mM FDP. Again inactivation was biphasic except at very low protein concentrations, where a single-step inactivation was obtained to the extent that the inactivation could be followed. Log-log plots of $t_{\frac{1}{2}}$ *vs.* protein concentration are shown in Figure 22a for k_2's (fast rate) and in Figure 22b for k_3's (slow rate). It should be noted at this point that 1.0 mg/ml of bovine serum albumin in the 0° reaction vessel containing PK at 0.20 mg/ml and FDP at 1.26 mM had no effect on either k_2 or k_3.

iv. Sedimentation coefficient of inactivated enzyme

The effect of FDP-enhanced inactivation on the sedimentation coefficient of yeast PK was also examined at 0.8 mg/ml after incubation in the presence or absence of 1.26 mM FDP at 3.6°. Aliquots of protein to be assayed were removed from the incubation mixtures before loading the centrifuge cell and maintained at 3.6°. Activity remaining was determined at the time that the first picture of the sedimenting species was taken. Enzyme minus FDP (95% activity) sedimented as a single peak (8.6 S). In the presence of FDP, with 30%

Figure 20. Determination of the Kinetic Interaction Constant Between FDP and Protein Using Eq 4

The line is a least-squares plot, eliminating the point at 50.4 μM FDP. Details in text.


$t_{1/2}$ (min)
30
10
9
6
FDP (μM)
30
60
90
100
300
600
900
k_2
Slope = −0.38
n = 1.38

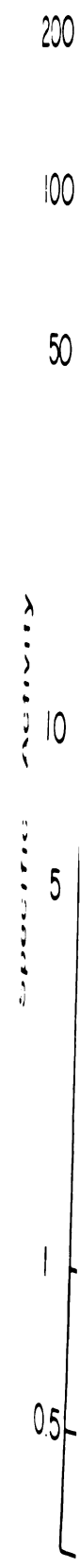


Figure 21. Effect of Protein Concentration on the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 0° in 0.1 M Tris·HCl, pH 7.5

FDP concentration in all cases was 1.26×10^{-3} M.

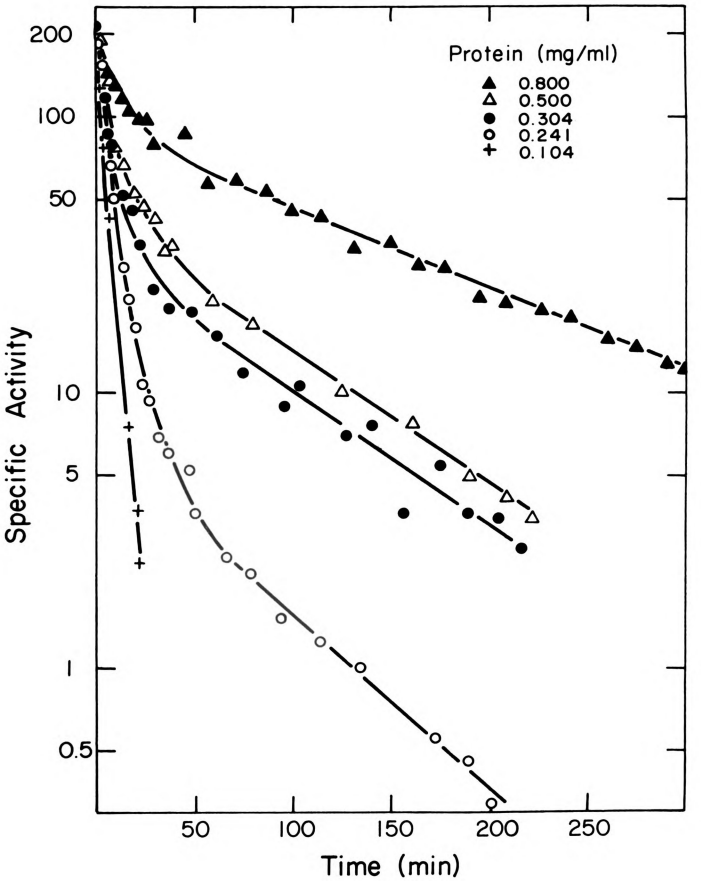
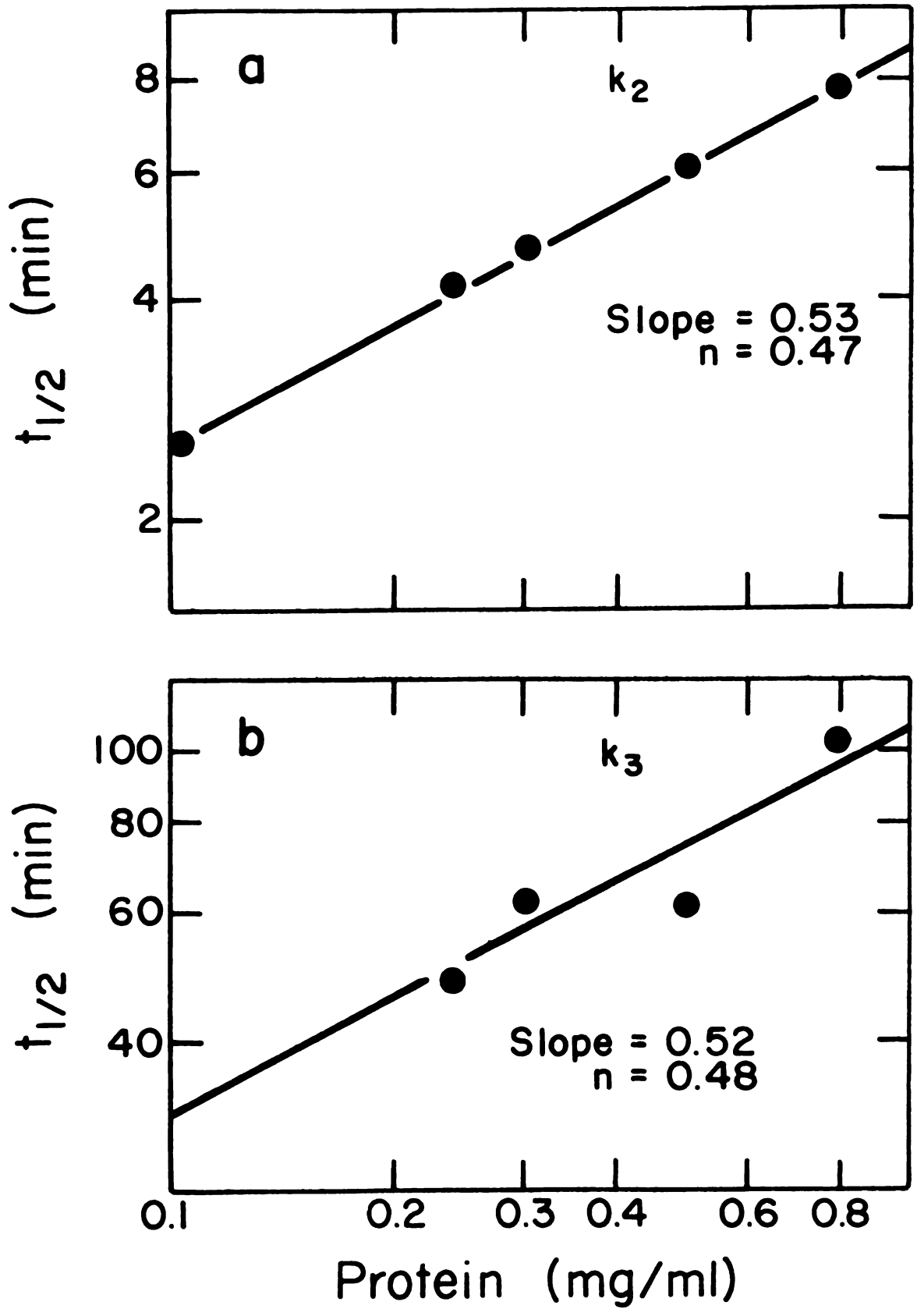

Protein (mg/ml)
0.800
0.500
0.304
0.241
0.104
Specific Activity
200
100
50
10
5
1
0.5
50
100
150
200
250
Time (min)

Figure 22. Determination of the Order with Respect to Protein of the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 0°

Both lines are least-squares plots. Rate constants were determined from the data in Figure 21, as described in Figure 17. The contribution to the rate of inactivation by k_3 at 0.104 mg/ml was assumed to be negligible, and the k_2 in part a at this concentration was assumed to be the initial rate of inactivation.


a
k_2
Slope = 0.53
n = 0.47
$t_{1/2}$ (min)
8
6
4
2
b
k_3
Slope = 0.52
n = 0.48
$t_{1/2}$ (min)
100
80
60
40
0.1
0.2
0.3
0.4
0.6
0.8
Protein (mg/ml)

activity remaining, two peaks could be observed with $\underline{s}$ values of 3 and 8 S in a ratio of about 80:20. With less than 0.1% activity remaining, in the presence or absence of FDP, a single peak was observed with an $\underline{s}$ value of 1.7 S.

2. Inactivation at 23°

i. Dependence of the inactivation on FDP concentration

Although yeast PK is considerably more stable at 23° than at 0° in the absence of FDP (half-lives differ by nearly an order of magnitude) the addition of FDP to the enzyme in saturating amounts induces an essentially identical lack of stability at the two temperatures. Data obtained at 23° showing the effect of increasing FDP concentration from 0 to 2.68 mM on the stability of PK at 0.25 mg/ml are presented in Figure 23.

ii. Treatment of data from Figure 23

The curves obtained from the semi-logarithmic plot of specific activity $\underline{vs}$. time are again biphasic as was seen at 0°. Under this assumption, the data shown in Figure 23 were also treated as in Figure 17. Extrapolation of the data from the slow step ($\underline{k}_3$) to zero time yields essentially identical intercepts of specific activity 110.7 ± 6.5 units/mg representing 52% of the initial activity. This is in contrast to the results obtained at 0° where the intercepts varied as a function of the FDP concentration. As will be shown, increasing the FDP concentration above 2.68 mM has no further effect on the fast step ($\underline{k}_2$), suggesting that this concentration of FDP was saturating. The $\underline{k}_2$'s obtained from

Figure 23. Effect of FDP Concentration on the Stability of Yeast Pyruvate Kinase at 23° in 0.1 M Tris·HCl, pH 7.5

Protein concentration was 0.25 mg/ml. All samples had an initial specific activity of 211 units/mg. Each point represents an assay at 30° of an aliquot removed from the incubation mixture.

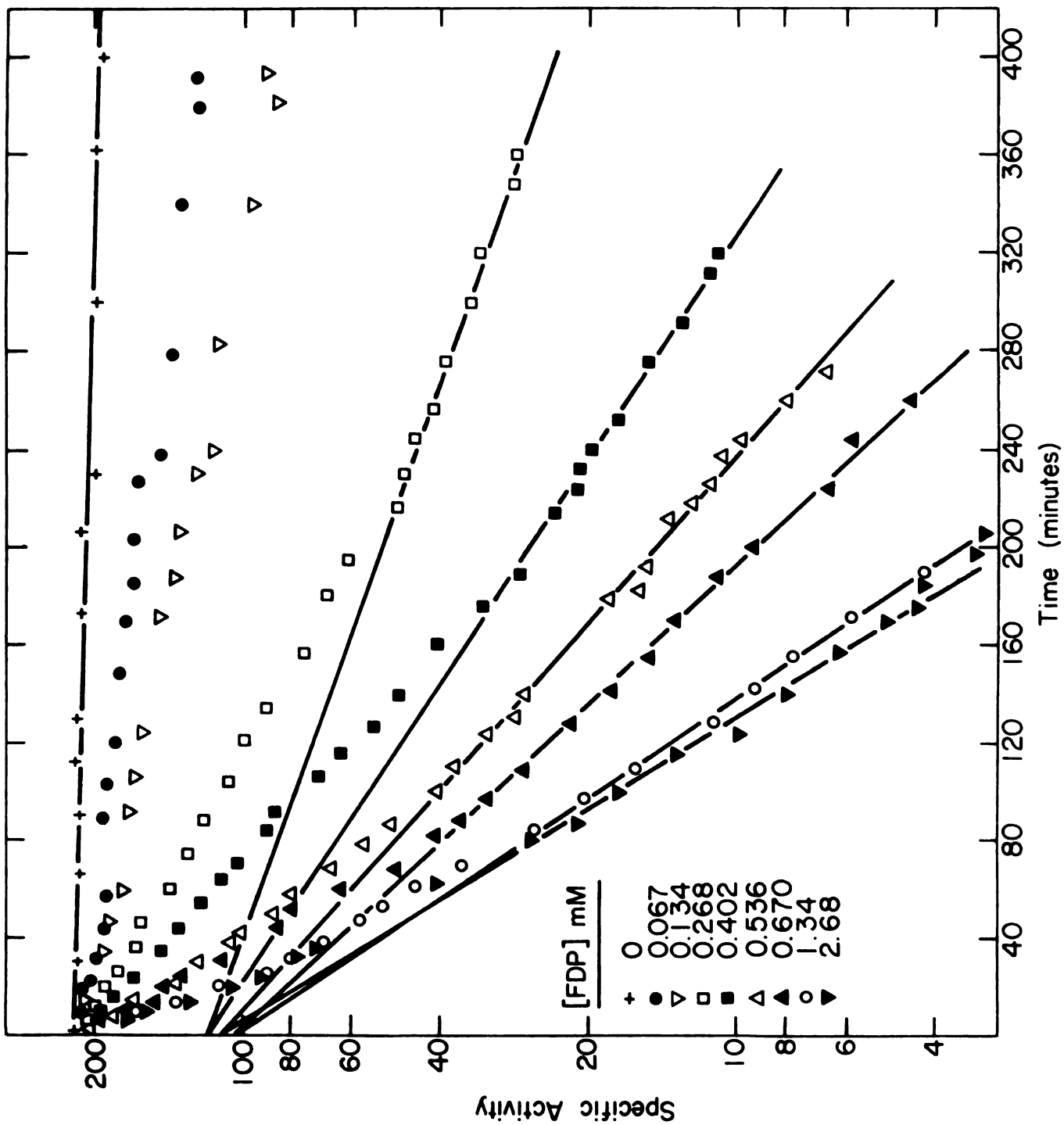

Specific Activity
200
100
80
60
40
20
10
8
6
4
40
80
120
160
200
240
280
320
360
400
Time (minutes)
[FDP] mM
0
0.067
0.134
0.268
0.402
0.536
0.670
1.34
2.68

Figure 23 were treated according to equation 5,

$$\alpha = \frac{\underline{k}_2(\text{x FDP}) - \underline{k}_1}{\underline{k}_2(2.68 \text{ mM FDP}) - \underline{k}_1} \qquad (5)$$

where $\underline{k}_1$ represents the observed rate of inactivation in the absence of FDP (Figure 23); α values were plotted *vs.* log FDP in Figure 24 as described in the section for 0° inactivation. The theoretical line in Figure 24 was calculated assuming $\underline{n} = 2$ and the geometrical average $K_D = 0.66$ mM.

The rate constants for the slow step ($\underline{k}_3$) were plotted in Figure 25 as $1/\underline{k}_3$ *vs.* $1/\text{FDP}^{\underline{n}}$ with $\underline{n} = 2.1$ giving a straight line which best fit the data. The K_D obtained for the slow step was 0.52 mM, with a maximum $\underline{k}_3 = 1.9 \times 10^{-2}$ min^{-1}. The maximal rates of inactivation in the presence and absence of FDP at 0° and 23° are summarized in Table VI.

TABLE VI

Rates of Inactivation of Yeast Pyruvate Kinase[a]

Temperature	Rate Constants ($\text{min}^{-1} \times 10^3$)		
	$\underline{k}_1$(-FDP)	$\underline{k}_2$(+FDP)	$\underline{k}_3$(+FDP)
0°	1.81	102[b]	10.6[b]
23°	0.235	63.4[c]	19[c]

[a]Rate constants were determined in 0.1 M Tris·HCl, pH 7.5, at 0.5 mg/ml (0°), and 0.25 mg/ml (23°).

[b]Saturating FDP = 1.26 mM.

[c]Saturating FDP = 2.68 mM.

Figure 24. Determination of the Apparent K_D at 23° of FDP for Yeast Pyruvate Kinase from the Fast-Step Inactivation Data

The data are plotted as α, obtained from eq 5, *vs.* log FDP. The solid line is calculated assuming $n = 2$ and the apparent K_D = 0.66 mM. The contribution to the rate of inactivation by k_3 at 0.067 mM and 0.134 mM FDP was assumed to be negligable; k_2 at these concentrations was assumed to be the initial rate of inactivation.

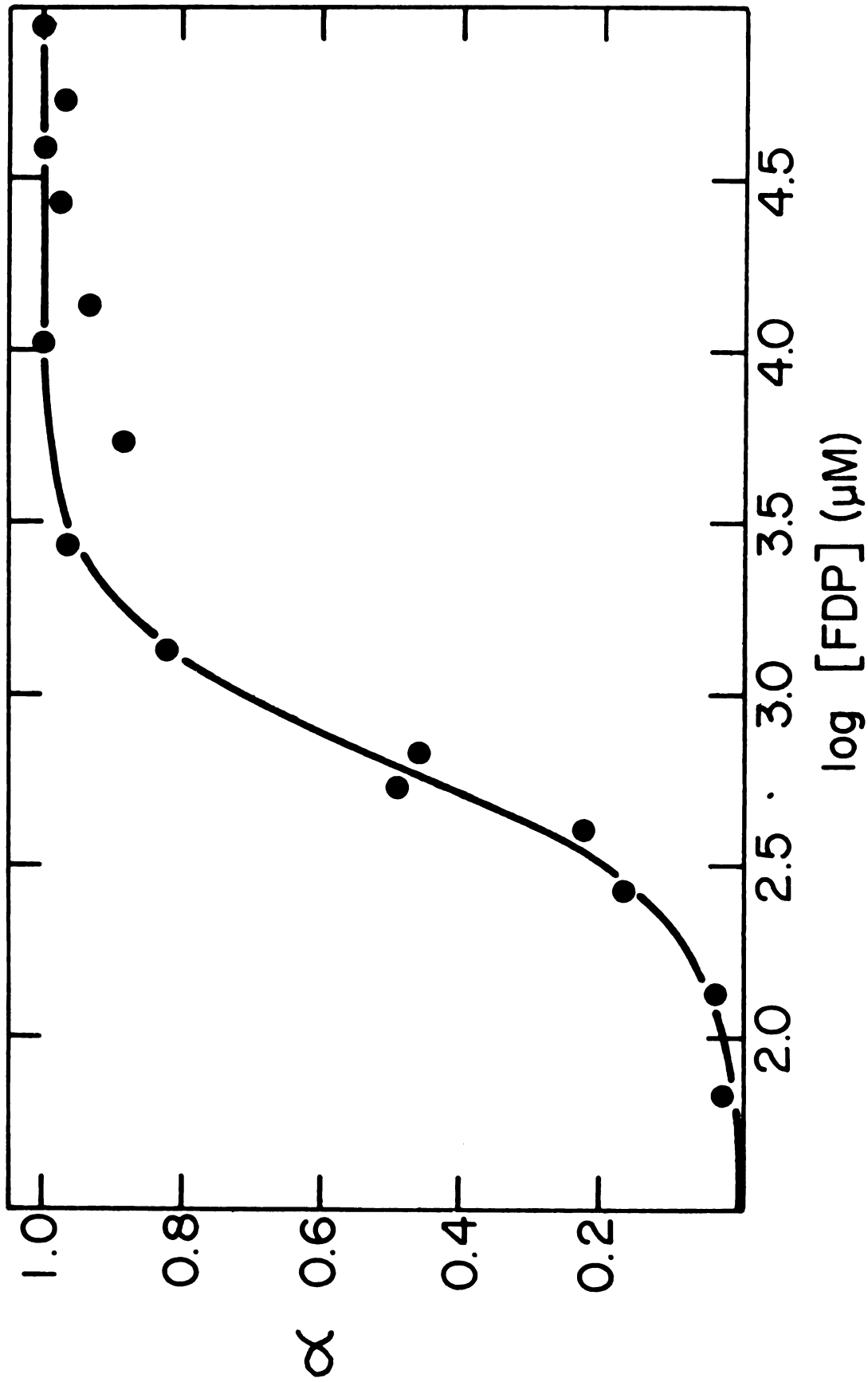
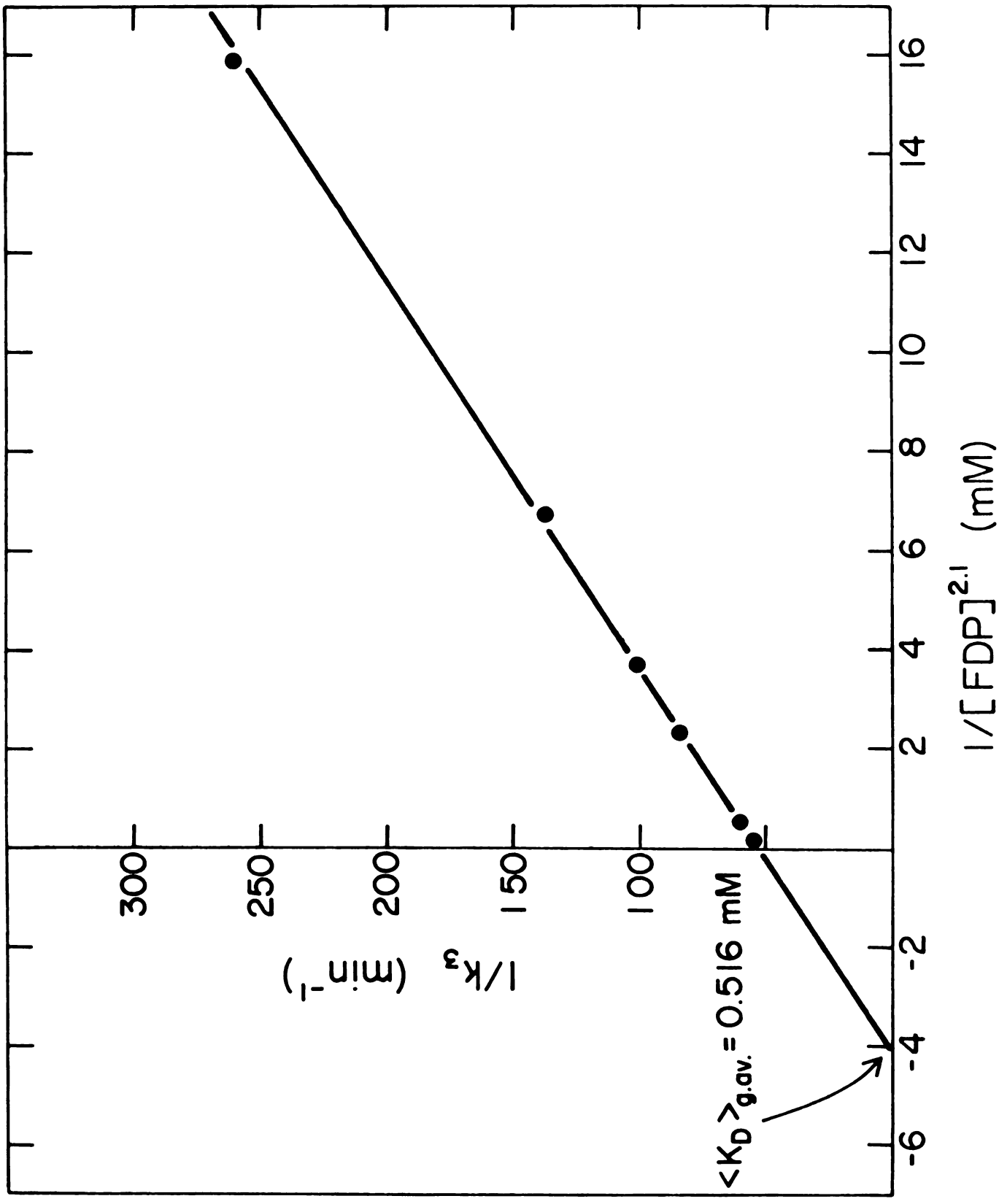

1.0
0.8
0.6
0.4
0.2
α
2.0
2.5
3.0
3.5
4.0
4.5
log [FDP] (μM)

Figure 25. Plot of the Slow-Step Rate Constants from Figure 23 as $1/k_3$ *vs.* $1/FDP^{2.1}$

The apparent dissociation constant is 0.516 mM, and the k_{3max} = 1.9×10^{-2} min^{-1}.


1/k3 (min-1)
300
250
200
150
100
-6
-4
-2
2
4
6
8
10
12
14
16
1/[FDP]2.1 (mM)
<KD>g.av. = 0.516 mM

iii. Effect of protein concentration on the inactivation

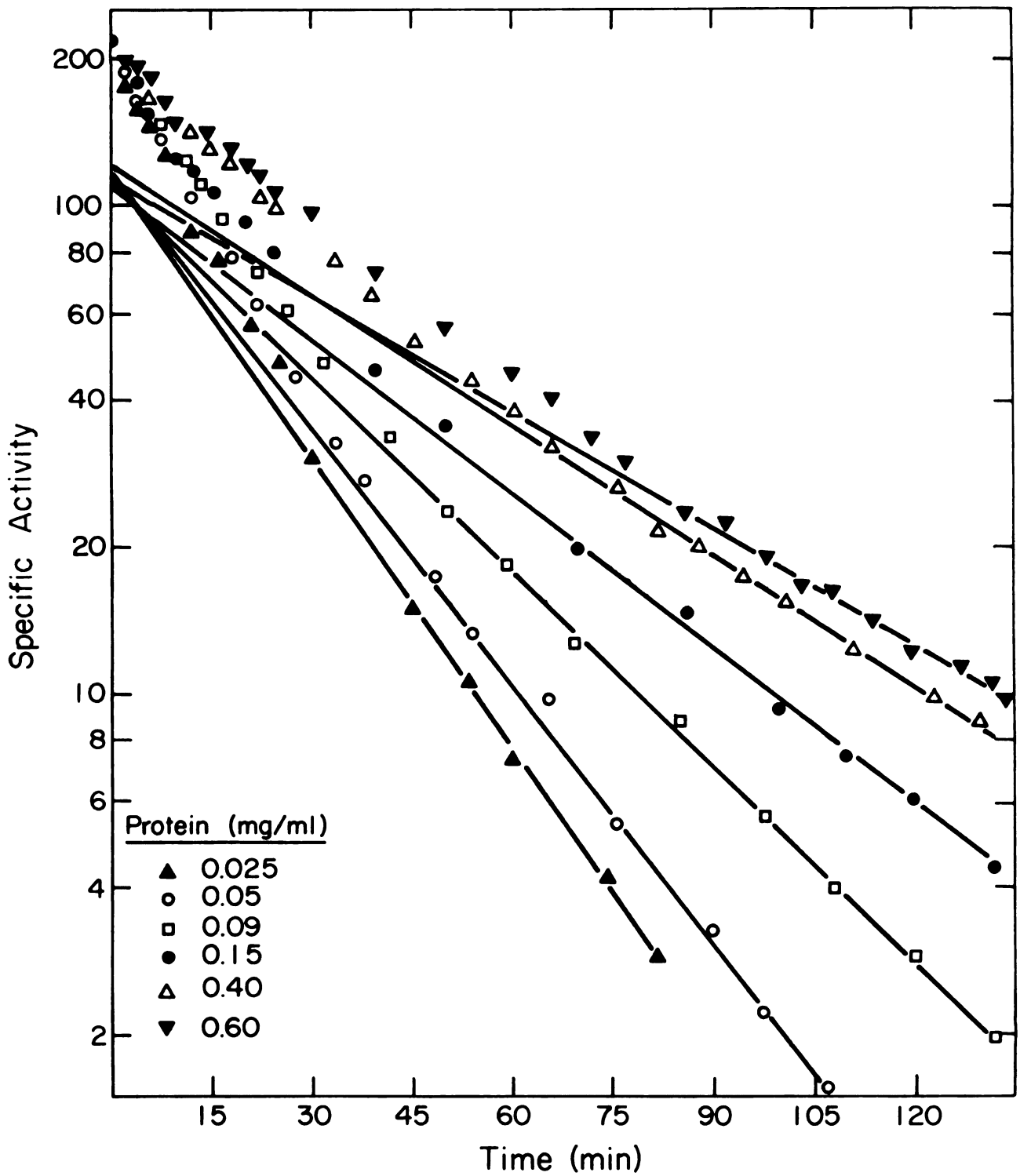
Inactivation at 23° was studied at six different protein concentrations, in addition to 0.25 mg/ml used in Figure 23, each in the presence of 2.68 mM FDP. The data are presented in Figure 26. Again, inactivation was biphasic, and the extrapolation to zero time of the slow step ($\underline{k}_3$) gave values which, within experimental error, were independent of protein concentration and identical to the values obtained in Figure 23. As at 0° both the fast-step and the slow-step rate constants increased as the protein concentration decreased. The data were plotted according to the half-life method described earlier as log $\underline{t}_{\frac{1}{2}}$ *vs.* log protein in Figure 27.

iv. Effect of ionic strength on the inactivation

During efforts to determine the maximal rate of inactivation as a function of FDP concentration (Figure 23), a decrease in the rate of the slow step ($\underline{k}_3$) was observed at concentrations of FDP above 5.36 mM. Increasing concentrations of FDP up to 97.2 mM are presented in Figure 28. Identical rates of inactivation were observed at 2.68 mM (Figure 23) and 5.36 mM (Figure 28) FDP, consistent with the argument that FDP saturation was achieved with respect to inactivation before stabilization began. Although the rate of the second, slow step, is markedly decreased by high FDP concentrations, no effect on the first step is observed. (Values for $\underline{k}_2$ at the high FDP concentrations

Figure 26. Effect of Protein Concentration on the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 23° in 0.1 M Tris·HCl, pH 7.5

FDP concentration in all cases was 2.68 mM.


Specific Activity
200
100
80
60
40
20
10
8
6
4
2
Protein (mg/ml)
0.025
0.05
0.09
0.15
0.40
0.60
15
30
45
60
75
90
105
120
Time (min)

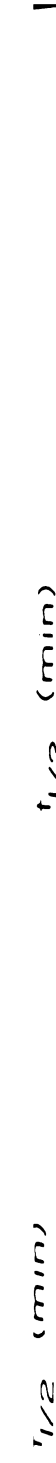


Figure 27. Determination of the Order with Respect to Protein of the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 23°

Both lines are least-squares plots. Rate constants were determined from the data in Figure 26 as described in Figure 17.

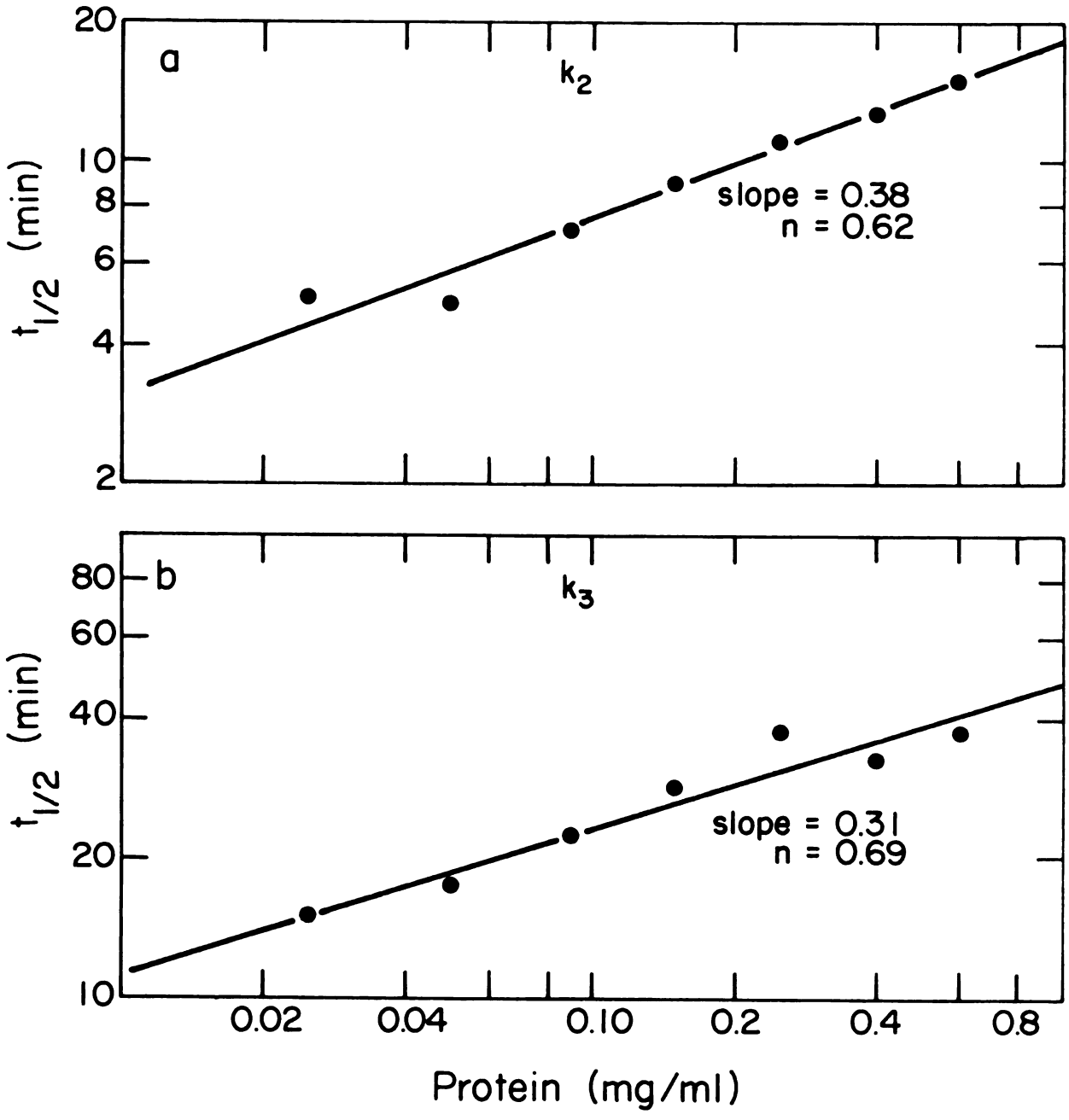
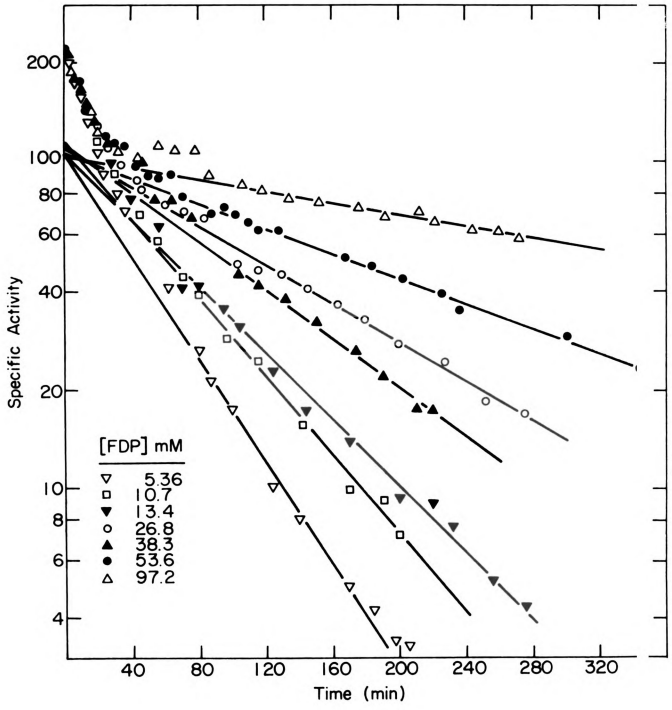

a
k_2
slope = 0.38
n = 0.62
$t_{1/2}$ (min)
20
10
8
6
4
2
b
k_3
slope = 0.31
n = 0.69
80
60
40
20
10
0.02
0.04
0.10
0.2
0.4
0.8
Protein (mg/ml)

Figure 28. Stabilizing Effect on k_3 of FDP Concentrations Above 5.36 mM for Yeast Pyruvate Kinase at 23°

Enzyme was incubated in 0.1 M Tris·HCl, pH 7.5, at 0.25 mg/ml.


Specific Activity
200
100
80
60
40
20
10
8
6
4
40
80
120
160
200
240
280
320
Time (min)
[FDP] mM
5.36
10.7
13.4
26.8
38.3
53.6
97.2

are incorporated in Figure 24.)

Since high concentrations of FDP, with a net charge near 4 at pH 7.5 (Dawson et al., 1969) markedly increases the ionic strength of the solution over the contribution of the buffer, and since it had been shown that the conformational change induced by FDP as measured by fluorescence of PK is inhibited by increasing the ionic strength of the solution, the possibility that a similar ionic strength effect, arising from the highly charged FDP molecule itself, was considered as a potential source of the apparent stabilization. The addition of 0.23 M KCl or $(CH_3)_4NCl$ in the presence of saturating (2.68 mM) FDP markedly stabilizes the enzyme (Figure 29), although K^+ apparently has a much greater effect. Included in the Figure are data for the inactivation at 26.8 mM FDP in the absence of added cation, a concentration which represents the same ionic strength as 0.23 M monovalent salt plus 2.68 mM FDP.

In an effort to determine whether the effect of ionic strength was mediated through the rate of inactivation, or through the binding constant for FDP, the effect of increasing FDP concentrations at constant ionic strength was measured (Figure 30). Ionic strength was maintained with $(CH_3)_4NCl$ at 0.934 μ, the value at 97.2 mM FDP (uppermost curve, Figure 28). Rates for both the fast step (k_2) and the slow step (k_3) at 53.6 mM FDP (Figure 30) are identical as those obtained in Figure 28 at 97.2 mM FDP.

Figure 29. Effect of Monovalent Cations on the FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 23°

Enzyme was incubated in 0.1 M Tris·HCl, pH 7.5, at 0.25 mg/ml. 0.23 M KCl or $(CH_3)_4NCl$ in the presence of 2.68 mM FDP represents the same ionic strength as 26.8 mM FDP.

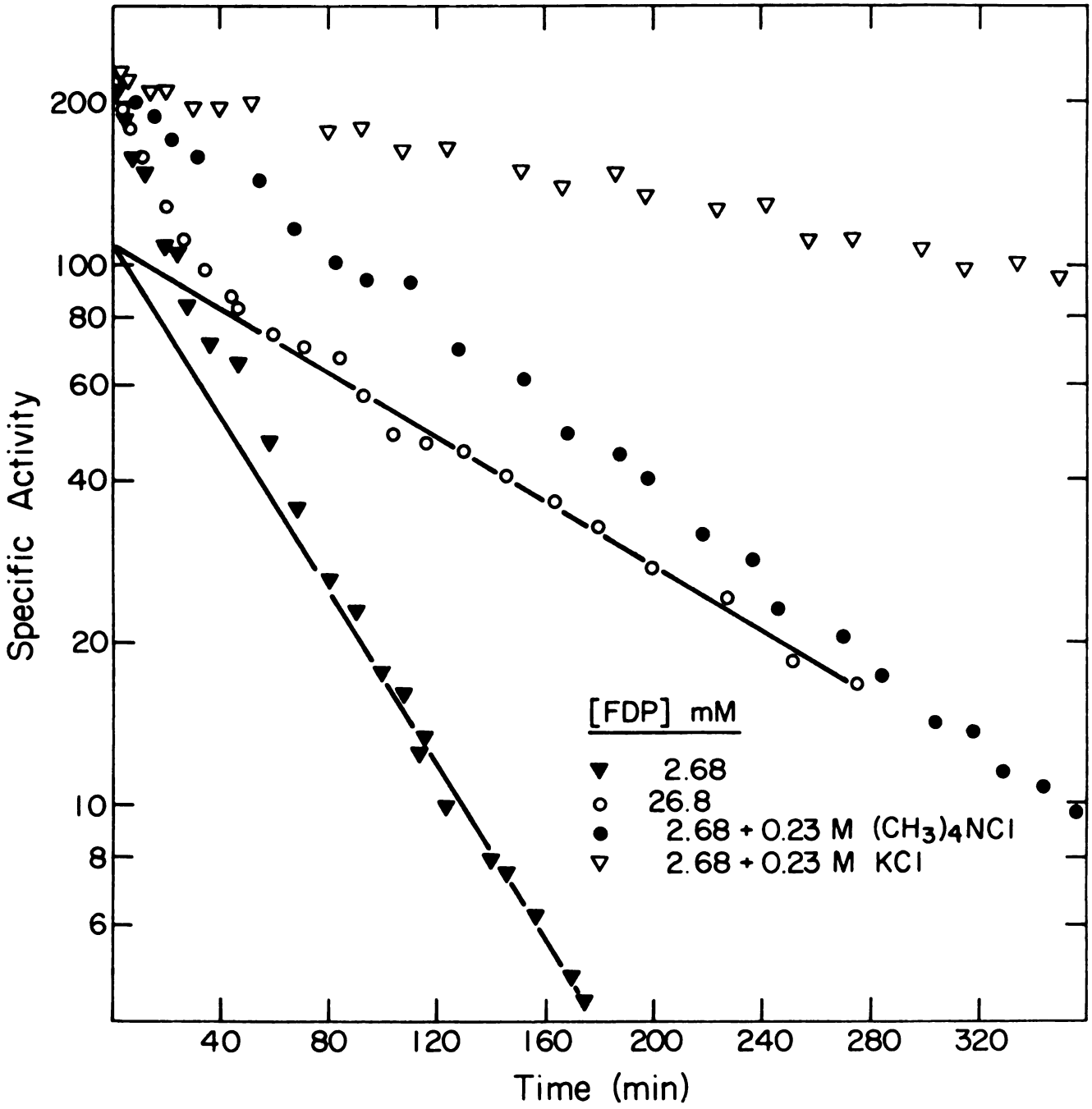
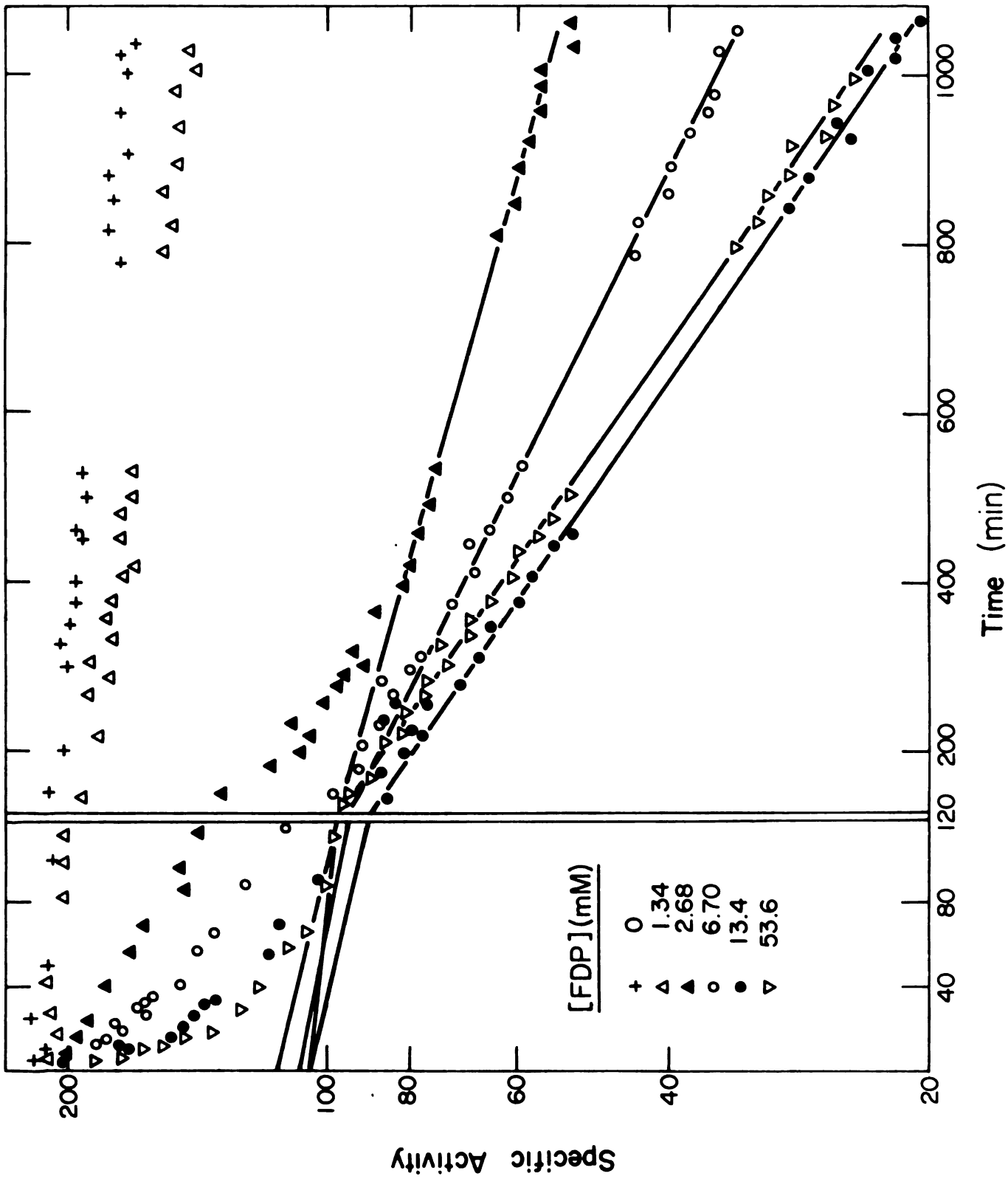

200
100
80
60
40
20
10
8
6
Specific Activity
40
80
120
160
200
240
280
320
Time (min)
[FDP] mM
2.68
26.8
2.68 + 0.23 M (CH3)4NCl
2.68 + 0.23 M KCl

Figure 30. FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at Constant Ionic Strength (0.934 μ)

Conditions were: 0.1 M Tris·HCl, pH 7.5, 0.25 mg/ml enzyme, 23°. The ionic strength was maintained with $(CH_3)_4NCl$. The lines extending to the ordinate on the left side of the Figure are extensions of the extrapolations of the slow step on the right side of the Figure.


Specific Activity
200
100
80
60
40
20
Time (min)
40
80
120
200
400
600
800
1000
[FDP](mM)
0
1.34
2.68
6.70
13.4
53.6

v. Sedimentation coefficient of inactivated enzyme

To examine the effect of FDP-enhanced inactivation on the molecular weight of the enzyme, the following sedimentation experiments were performed. Enzyme was chromatographed on a Sephadex column equilibrated with 0.1 M Tris·HCl, pH 7.5, and 0.5 M $(CH_3)_4NCl$, and then allowed to stand for three hours at 23°. The enzyme solution was next divided in two parts. FDP, to give a final concentration of 45.2 mM was added to the first part, maintaining protein at 5 mg/ml. The second part was adjusted to 5 mg/ml, while maintaining the ionic strength with $(CH_3)_4NCl$ equal to that of the first part. Aliquots of the two protein samples were introduced into single sector capillary-type synthetic boundary cells and mounted in the centrifuge, all at 23°. After about 30 min, the centrifuge was started, and at the time of the first picture, the remaining portions of the enzyme samples were assayed. The sample in the absence of FDP, with 98% of the original activity remaining, contained a major component (85%) with an $\underline{s}_{20,w}$ = 8.5 S, and minor components (10% and 5%) with sedimentation coefficients of 12.6 S and 8.5 S, respectively. On the other hand, the sample in the presence of FDP, with 65% of the original activity remaining, contained two species with $\underline{s}_{20,w}$ values of 8.1 S (25-30%) and 4.0 S (70-75%).

To determine whether the 4 S species could be stabilized or possibly reversed to the 8 S species by removal of the FDP, enzyme was prepared as in the previous

experiment, and activity was measured as a function of time after addition of FDP. When activity reached a level of 110 units/mg, an aliquot was removed and chromatographed on a Sephadex column into the Tris buffer containing 0.5 M $(CH_3)_4NCl$ to remove the FDP. Enzyme in the absence of FDP was similarly treated. Fractions with the highest protein concentration (2 mg/ml) were immediately introduced into the centrifuge cells, and sedimentation coefficients were determined. Enzyme not treated with FDP again contained species with $\underline{s}_{20,w}$ values of 8.3 S (90%) and 12.9 S (10%). On the other hand, enzyme treated with FDP contained species with $\underline{s}_{20,w}$ values of 4.2 S (50%) and 2.3 S (50%). Specific activity at the time of the first picture was only 40 units/mg, and continued to decrease linearly.

vi. Attempted reversal of the FDP-enhanced inactivation of yeast pyruvate kinase

Several attempts were made to reverse the inactivation produced in the presence of FDP. These included $(NH_4)_2SO_4$ precipitation of the enzyme at various stages of inactivation; rewarming the enzyme solution in the presence of glycerol, Mg^{2+}, Mn^{2+}, and combinations of the substrates and other effectors of yeast PK. All of these attempts were unsuccessful.

In addition, aliquots of enzyme at various stages of inactivation at 23° in the Tris buffer, were diluted into solutions whose composition was known (M. Tobes and C. H. Suelter, unpublished observation) to yield about 70%

reactivation of yeast PK which had previously been dissociated to subunits with 6 M GuHCl containing 0.15 M 2-mercaptoethanol. Although the diluted aliquots were stable to further inactivation for at least 12 hr, in no case was any activity recovered.

DISCUSSION

The primary goal of this research was the elucidation of the relationship of structural and conformational transitions of yeast pyruvate kinase to the interaction of the enzyme with substrates and effectors as first observed by the kinetic studies described by Hunsley and Suelter (1969b). Fundamental for an analysis of such changes is a characterization of the molecular weight of the enzyme and determination of the size and number of subunits associated therewith.

From sedimentation and diffusion data extrapolated to zero protein concentration under two different solvent conditions (Figures 3 and 4; Figures 5 and 6), a weight-average molecular weight near 167,000 for native yeast pyruvate kinase was calculated using the Svedberg equation. A molecular weight near 165,000 was also determined under a third solvent condition using the high-speed equilibrium technique (Figure 7) developed by Yphantis (1964). Since the molecular weight of the subunits is near 42,000 as determined for the dissociated enzyme in 6 M GuHCl containing 0.15 M 2-mercaptoethanol, and after maleylation, it is concluded that yeast PK, like muscle PK (Steinmetz and Deal, 1966), is composed of four subunits. That these subunits are very similar in size is consistent with the following observations:

(1) maleylated enzyme sediments as a single, nearly symmetrical peak; (2) the $\underline{M}_w^o$ of 42,200 from sedimentation and diffusion data for the maleylated enzyme (Figures 10 and 11) is in close agreement with $\underline{M}_w^o$ of 41,400 obtained from sedimentation equilibrium data in GuHCl (Figure 9); (3) the homogeneous molecular weight distribution for the dissociated protein throughout the centrifuge cell (Figure 8); and (4) a single band of molecular weight near 42,000 obtained by Rose (1969) after electrophoresis of the enzyme in sodium dodecyl sulfate on sodium dodecyl sulfate-polyacrylamide gels (Shapiro et al., 1967). No evidence is available regarding the chemical identity of the subunits in this preparation of pyruvate kinase.

The plots of the molecular weight ($\underline{M}_n$) vs. protein concentration in fringes (Figure 7) shows the presence of lower molecular weight components at low protein concentrations, indicative of a protein concentration dependent dissociation. This is consistent with the demonstration that yeast PK exhibits an instability, inversely proportional to protein concentration, which involves a protein dissociation to lower molecular weight species, as will be discussed later. The pattern of $\underline{M}_n$ as a function of protein concentration (Figure 7) obtained by the meniscus depletion technique (Yphantis, 1964) provides, according to Harris et al., (1969), an adequate method for distinguishing between a protein participating in a rapid chemical equilibrium, and a mixture of nonequilibrating or slowly equilibrating species.

A protein in chemical equilibrium should have molecular weight moments which are a function only of concentration throughout the cell. Hence, graphs of molecular weight *vs.* concentration should superimpose. On the other hand, a heterogeneous system not in chemical equilibrium, or one participating in a very slow equilibrium should have a distribution of mass which depends only on distance from the center of rotation (Harris *et al.*, 1969). The data presented in Figure 7 are consistent with the latter situation. Yet the enzyme at the higher concentration appears essentially homogeneous with a molecular weight approaching 165,000 in good agreement with the molecular weights calculated from the Svedberg equation.

The unusual positive slope of the protein concentration dependence of $\underline{D}_{20,w}$ (Figure 11) is an apparent anomaly which merits further discussion. It can be rationalized on the basis of extreme charge effects. Spreading of the boundary in synthetic boundary experiments would be expected to be accelerated at higher protein concentrations by the effect of high concentration of charges. Each maleyl group bound to the protein changes the net charge by two for each ε-amino group reacted, and by one for each hydroxyl group reacted.

The data in Figure 6 suggest an unusual effect of time on $\underline{D}_{20,w}$. The sedimentation data in Section I of the Results indicate that the gross physical structure changes radically during incubation at 23° in 0.1 M Tris·HCl, pH 7.5,

after removal of ammonium sulfate by G-25 Sephadex. Before incubation at least three sedimenting species are observed, two of which sediment at a rate suggestive of high molecular weight aggregates of the "native" enzyme. After three hours of incubation, only one peak is observed. Incubation up to 18 hr leads to no further change in the sedimentation pattern. However, the results in Figure 6 indicate that the diffusion coefficient changes drastically on further incubation. A plausible conclusion would be that the protein exists as a diverse mixture of extremely aggregated species, no one of which is present in sufficient amount to be observed in the sedimentation pattern. Apparently these do not affect $\underline{s}_{20,w}$ but do affect $\underline{D}_{20,w}$. Whether storage of the enzyme as a suspension in ammonium sulfate is the cause of the aggregation is presently unknown.

Considering the wide diversity of solvent conditions under which the molecular weight studies were performed, it seems reasonable to conclude that the homotropic and heterotropic substrate and effector interactions observed kinetically for yeast PK are mediated directly through conformational transitions of the enzyme as opposed to subunit association-dissociation equilibria of the protein (Frieden, 1967; Benesch *et al.*, 1966).

The quenching of the fluorescence of the enzyme by K^+ and Mg^{2+} suggests that, like the rabbit muscle PK (Suelter, 1967), yeast PK undergoes a small conformational transition as a result of interaction with the activating cations.

And, as with the rabbit muscle enzyme, the effect is specific for K^+ as opposed to $(CH_3)_4N^+$. However, the data in Table III suggest that a more profound effect is produced by FDP than by the cations taken either separately or together. The sedimentation data in Table II, while not conclusive when taken alone, are consistent with this fluorescence data. That is, a change in sedimentation coefficient suggestive of a conformational transition is observed only under those conditions which promote a large fluorescence change. This conformational change, reflected in the changes in fluorescence and in sedimentation coefficients after addition of FDP, is analogous to the transition to a kinetically activated conformer, also induced by FDP, and characterized by a lower K_m for PEP.

The apparent antagonistic effect of K^+ on the binding of FDP (Figure 13) is totally unexpected in light of the fact that this same cation is required, at the concentrations used in the fluorescence studies, for catalytic activity both in the presence and in the absence of FDP. The effect appears to be due to both the increase in ionic strength, since $(CH_3)_4N^+$ (Figure 14) yields the same increasing trend in apparent K_D's for FDP, although it does not function kinetically, and a specific K^+ effect, since the extent of the increase in apparent K_D for FDP produced by $(CH_3)_4N^+$ is not as great as by K^+. A specific K^+ effect is also supported by the unequal stabilizing effects of the two cations during the FDP-enhanced inactivation at 23° (Figure 29). The

antagonistic effect of ionic strength on the binding of FDP is also observed in the catalytic assay using sub K_m concentrations of ADP, PEP, FDP, and K^+ (0.5, 0.087, 0.027, and 10.0 mM, respectively). Increasing the K^+ from 0.01 M to 0.23 M (in a single assay) decreases the observed activity by an order of magnitude, the rate of decrease having a half-life on the order of seconds. Addition of 0.23 M $(CH_3)_4N^+$ has a similar effect, although the decrease in activity is only about half as great as in the case with K^+. Similarly, the observation (Table III) that K^+ quenches, to a limited extent, the fluorescence of yeast PK, whereas $(CH_3)_4N^+$ does not, supports a specific cation effect in addition to the effect of ionic strength.

The most marked influence on the binding of FDP to yeast PK is exerted by PEP in conjunction with K^+ (Table IV), an effect which might be expected, since kinetically, the K_m's and $\underline{n}_H$'s for PEP are markedly affected by FDP (Hunsley and Suelter, 1969b). The affinity of the enzyme for FDP in the presence of PEP and K^+ is increased by almost two orders of magnitude over the affinity at the analogous K^+ concentration in the absence of PEP. On the other hand, PEP alone, and PEP in the presence of 0.23 M $(CH_3)_4N^+$ do not decrease the K_D for FDP, suggestive of a specific monovalent cation-substrate-enzyme complex involved in the FDP-induced conversion of the enzyme to its activated conformer. The implication that K^+ might bind at or very near the PEP binding site on the enzyme is consistent with

the nuclear magnetic resonance data of Kayne and Reuben (1970) using Tl^+ and rabbit muscle PK. Suelter (1970) has recently suggested that K^+-activated enzymes may involve the monovalent cation in a ternary enzyme-substrate complex per se, implying binding at the active site.

The observation that ADP in the presence of cations also affects the dissociation constant for FDP with yeast PK is surprising. Kinetically, FDP has little or no effect on the K_m for ADP and no heterotropic or homotropic cooperativity has been shown involving ADP, with the Hill slope equal to unity under all conditions (Hunsley and Suelter, 1969b). The addition of 5 mM ADP in the presence of 0.23 M K^+ or $(CH_3)_4N^+$, however, reduces the K_D for FDP, as determined by the fluorescence titrations, by an order of magnitude. Further, the Hill slopes near 2 in the absence of ADP were reduced to unity in its presence, suggesting a significant role for ADP in the determination of conformation of the enzyme, a conclusion not apparent from the kinetic data. Unlike the results obtained for PEP, the enzyme-ADP interaction which is reflected in the K_D for FDP is not mediated by monovalent cations, and occurs in the absence of both K^+ and $(CH_3)_4N^+$. Further, the effect of ADP appears to be the dominant interaction, sufficiently strong to reverse the antagonism between monovalent cation and FDP.

The substantial increase in K_D for FDP as the temperature is raised from 0 to 30° (as determined from fluorescence changes in the presence of Mg^{2+}) agrees with the

similar increase in K_D monitored by the FDP-enhanced inactivation of the enzyme in the absence of Mg^{2+} (summarized, in Table VII). These observations are consistent with a conformer of yeast PK favored by both low temperatures and FDP.

TABLE VII

Temperature Effects of K_D's for FDP and Yeast PK in 0.1 M Tris·HCl, pH 7.5 by Fluorescence Changes and Inactivation Data

Method	Temperature	K_D (mM)
(1) Fluorescence[a]	0	0.123
	23	0.487
	30	0.912
(2) Inactivation	0	0.063
	23	0.660

[a]In the presence of 0.025 M $MgCl_2$.

Although an identical V_{max} is obtained for yeast PK in the presence or absence of FDP, and both conditions require K^+ (Hunsley and Suelter, 1969b), the data in Table III indicate that 0.23 M K^+, even in the presence of Mg^{2+} and PEP, does not produce the same fluorescence change in PK as is obtained with FDP. If the fluorescence properties of the enzyme reflect its conformation, and if the spectrum in the presence of FDP reflects an activated conformer, one of two alternatives could be considered. On the one hand, the fluorescence of the enzyme in the presence of K^+, Mg^{2+},

and PEP would reflect a conformation of the enzyme which is intermediate on the path to the kinetically active conformation. In this case ADP is required to complete the conformational transition. Such a possibility cannot be excluded on the basis of kinetic data, since ADP apparently alters the structure of the enzyme in such a manner as to facilitate FDP binding, as shown in Table IV.

On the other hand, if ADP does not provide the driving force to complete the conformational transition, then it would seem necessary to argue the existence of two different active conformers controlled by FDP and K^+. These would be distinguished by their fluorescent properties, and kinetically, by differences in K_m's for PEP. This second alternative is supported by the antagonistic effect of K^+ on the binding of FDP, and further suggests a dual role for K^+: (1) as a required cation for catalysis in the presence and absence of FDP and (2) in producing an apparently unique active conformer in the absence of FDP. Such a dual role is also consistent with the observation (Hunsley and Suelter, 1969b) that Na^+ can substitute for K^+ only in the presence of FDP.

On the basis of the fluorescence data, the suggestion that FDP promotes a major conformational transition of yeast PK seems well-founded. The relationship between this structural change, observed in the presence of Mg^{2+}, and the inactivation enhanced by FDP in the absence of Mg^{2+}, depends to a considerable extent on whether the conformational

changes involved in each case are identical or at least similar, and whether FDP in each case binds at the same site on the enzyme.

It seems unlikely that two distinct sites exist on the enzyme for FDP, one site promoting an activation, and the second site promoting an inactivation. Protection by Mg^{2+} *in vivo* would require extensive compartmentalization and/or regulation of the uptake of this cation for the inactivating role of FDP to be metabolically operative. The K^{+} content of the cell should also be sufficient to protect the enzyme from inactivation. In addition, the FDP activation as a regulatory mechanism for glycolysis makes the second site alternative highly improbable. If the effects on the kinetic properties of yeast PK and on the stability of the enzyme result from ligand binding at the same site, then binding of the divalent cation in conjunction with FDP could promote a conformational change leading to an activated enzyme form. Without a divalent cation, the enzyme would respond "incorrectly" or incompletely to the bound FDP, and would dissociate.

Alternatively, Mg^{2+} may function as a stabilizing factor. Binding of FDP in the presence or absence of the metal may produce identical structural effects on PK, but the divalent cation is required to stabilize the newly induced conformation. A combination of these two alternatives seems most likely; *i.e.*, binding of FDP might induce only a partial conformational change to a highly unstable

form of the enzyme, which, in the presence of cation, would be completed to the fully active, stabilized conformer. The fact that FDP in the absence of Mg^{2+} produces a very similar though slightly smaller quenching of the fluorescence of PK, as compared to that obtained in the presence of the metal, supports this contention. In addition, the analogous effects of temperature and ionic strength (including the apparent specific and general monovalent cation effects) on the fluorescence change produced by FDP and the instability of the enzyme induced by the ligand are consistent with the implication of identical binding sites and similar conformational changes in both processes. With this as an assumption, an analysis of the inactivation mechanism may reveal information concerning the structural changes involved in the ligand binding during the catalytic process.

The sedimentation data for yeast pyruvate kinase in the presence and absence of FDP at 23°, and the inverse dependence of both steps of the inactivation on protein concentration (Figure 27) are consistent with a mechanism of inactivation involving two consecutive dissociations of the native tetramer to inactive monomers. If the 2.1 S species represents the partially unfolded monomer of molecular weight 42,000 [a folded globular protein of this molecular weight would be expected to have an $\underline{s}$ of about 2.4 S (Holleman, 1966)], the 4 S species suggests the existence of a partially unfolded dimer as an intermediate in the inactivation (eq 6a).

$$8.5\ S \longrightarrow 4\ S \longrightarrow 2.1\ S \qquad (6a)$$

Similarly, the dependence of the rapid ($\underline{k}_2$) and slow ($\underline{k}_3$) steps of the inactivation at 0° on protein concentration (Figure 22) and the sedimentation pattern of the enzyme at various stages of the inactivation, are consistent with a protein dissociation into subunits (eq 6b). The 3 S species

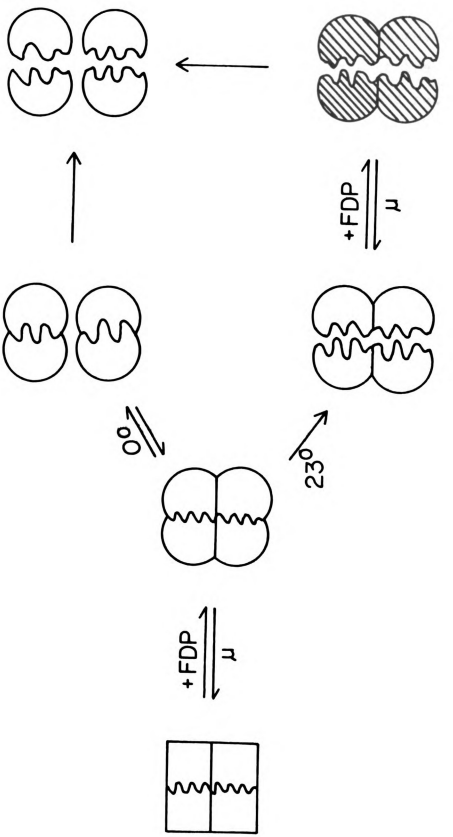
$$8.6\ S \longrightarrow 3\ S \longrightarrow 1.7\ S \qquad (6b)$$

represents either an extensively unfolded dimer, or a mixture of dimer and monomer resulting in an apparent $\underline{s}$ = 3 S, and the 1.7 S species corresponds to an unfolded monomer produced by the slow inactivation, $\underline{k}_3$. The biphasic inactivation curve obtained at 0° in the presence or absence of FDP (Figure 1 and Figure 16) and the appearance of the 1.7 S species after nearly complete cold inactivation, also in the presence or absence of FDP, suggest that similar mechanisms of inactivation are operative in both cases.

Although similar two-step protein dissociations appear operative at both 0° and 23° in the FDP-enhanced inactivation, a comparison (Figure 16 *vs.* Figure 23) of the characteristics of the effects of FDP on the course of the inactivation at each temperature is difficult to reconcile without the existence of alternate pathways from tetramer to monomer. For this reason the overall inactivation scheme as presented in Figure 31 is proposed.

That the binding of FDP to yeast PK in the absence of

Figure 31. Proposed Mechanism of FDP-Enhanced Inactivation of Yeast Pyruvate Kinase at 0 and 23°


+FDP
μ
0°
23°
+FDP
μ

Mg^{2+} promotes an alteration in quaternary structure is, of course, a conclusion reached directly from the observation that the ligand promotes a rapid inactivation of the enzyme. In the presence of saturating FDP at 0° the rapid rate of inactivation, k_2 (Figure 22a), is dependent on protein concentration, suggesting that protein dissociation is then the rate-limiting step. The data also suggest (Figure 18 and eq 2) that FDP enhances the inactivation by promoting a shift in the equilibrium between the two tetrameric conformers in Figure 31, and that a minimum of two moles of FDP bind per mole of enzyme in shifting this equilibrium. Wieker *et al.* (1969) have shown that two moles of FDP bind per mole of native enzyme consistent with this suggestion. The interaction constant of 2 for FDP (Figure 18) in defining the equilibrium between tetramer and 0° dimer following the rapid inactivation is also consistent with the above model. The kinetic order of 1.4 for interaction of FDP at 0° (Figure 20) suggests a complex series of steps involved before establishment of the inactivation equilibrium. However, the interaction constant of 2 in Figure 18 suggests that the concentration of the intermediate complexes with FDP which might occur during the inactivation are insignificant after the equilibrium is established. Such a mechanism is analogous to the dissociation of a diprotic acid in which two protons dissociate with no detectable amount of an intermediate singly dissociated form in the equilibrium mixture (Suelter *et al.*, 1966, and references therein).

While the data at 0° are consistent with the establishment of an inactivation equilibrium between tetramer and dimer as reflected in the dependence of the rapid step of inactivation on both FDP (Figures 16 and 18) and protein concentration (Figure 21), the extent of this step at 23°, proceeding to 50% inactivation, is independent of either of these variables (Figures 23 and 26). The corresponding changes in sedimentation coefficient suggest that this activity may be attributed to the 4 S species, a conclusion consistent with the lack of any observable 8 S species obtained after removal of FDP by treatment with Sephadex, even though approximately 25% of the initial activity remained. Although the dimer obtained at 0° could be partially active, attributing a specific activity of 50% of the native enzyme to the 0° dimer would not be consistent with the data, and suggests that the dimers obtained at 0° and at 23° are different.

The argument for two different dimers after the first inactivation step at the two temperatures is also consistent with the divergent nature of their formation. The extent of dimer formation at 23° is independent of FDP and protein concentration (Figures 23 and 26), that is, the first step of inactivation at 23° appears essentially irreversible. At 0° the extent of dimer formation is dependent on both FDP and protein concentration (Figures 16 and 21). The dependence of the rate of the second step of inactivation on FDP concentration (Figure 25) suggests a conformational transition of the dimer. Since the removal of FDP after completion

of the first step of inactivation at 23° led to an inactivation of the dimer, at 0.5 μ, much faster than the rate observed at the comparable ionic strength maintained with FDP (53.6 mM FDP, Figure 28), but slower than the maximum rate at saturating FDP (Figure 23), it would appear that FDP at high ionic strengths contributes some stability to the enzyme. Such an effect would suggest a second alteration in the conformation of the 23° dimer, perhaps by a mechanism consistent with a dissociation of the bound FDP followed by a rebinding of the ligand.

The unusual effect of high concentrations of FDP on $\underline{k}_3$ at 23° (Figure 28) is also consistent, however, with an inhibition by ionic strength of both the binding of FDP to the dimer as well as the dissociation of the dimer to inactive monomers. The first effect is suggested by the data in both Figures 29 and 30, and the observation that increased ionic strength markedly inhibits the binding of FDP as measured by changes in the enzyme fluorescence. When ionic strength is maintained at 0.934 μ (Figure 30), the apparent $\underline{K}_D$ for FDP increased from 0.66 mM to approximately 5 mM as reflected in $\underline{k}_2$, and from 0.56 mM to about 4 mM as reflected in $\underline{k}_3$. However, the maximum rate obtained for $\underline{k}_2$ at saturating FDP is independent of ionic strength, whereas $\underline{k}_3$ at saturating FDP is markedly decreased in the medium of high ionic strength. The invariance of $\underline{k}_2$ with ionic strength (Figure 28) does not contradict the above conclusions, but rather suggests that the FDP concentration always remained

sufficiently high to out weigh the expected effect of ionic strength on the dissociation constant for FDP with enzyme.

If the inactivation from tetramer to monomer, facilitated by the presence of FDP, proceeds by alternate pathways of cleavage of the tetramer at the two temperatures examined, as depicted in Figure 31, heterologous interactions between the subunits of yeast pyruvate kinase would seem likely. The temperature dependence of inactivation in the absence of FDP (Figure 1) suggests a break in the stability curves between 6 and 0°, reminiscent of similar sharp changes in stability of cold labile enzymes (for example, pyruvate carboxylase, Scrutton and Utter, 1965; 17-β-hydroxysteroid dehydrogenase, Jarabak et al., 1966). Possibly the increased structuring of water, which occurs near 4°, may be influential in creating the sudden instability of this enzyme in this temperature region.

Low-temperature instability of proteins indicates that associations between apolar groups are important in these proteins, since hydrophobic bonding would be expected to be significantly weakened at low temperatures (Kauzmann, 1959; Scheraga et al., 1962). Although little can be said with certainty concerning the structure of pure water (Eisenberg and Kauzmann, 1969) thermodynamic analyses of the interaction of apolar groups with water indicate a large unfavorable entropy loss as the temperature is lowered, which may be attributed to a major change in the structuring

of water around the apolar group (Kauzmann, 1954) in some manner analogous to the "iceberg" concept of Frank and Evans (1945). Brandts (1969) has suggested that the favorable free energy change involved in removing an apolar residue from an aqueous medium is almost entirely entropic, and due to the concomitant "melting" of the "clathrate" water structure around the residue. Hence, the optimum temperature for stability of a protein should increase as the hydrophobicity of the protein increases. This holds true for chymotrypsin vs. ribonuclease. von Hippel and Waugh (1955) in describing the nature of the low temperature dissociation of casein, observed that "... casein has an unusually high number of non-polar residues ..." Hence, the effects of temperature and glycerol on the stability of yeast PK implicate water structure, and specifically hydrophobic bonding in the associations between the subunits of the enzyme.

The suggestion that different dimers are obtained during the FDP enhanced inactivation of yeast PK depending on the temperature involved, and the conclusion that heterologous subunit interactions exist in the native enzyme, implies a greater degree of hydrophobicity in the bonding between the subunits of one of the dimers. If the dissociation from tetramer to dimer at 0° favors a splitting of the enzyme along hydrophobic bonds, then it would be expected that the dissociation at 23° would lead to dimers with predominantly the same hydrophobic bonds between the subunits

of the dimer. The stabilization of the 23° dimer by high ionic strength (Figure 30 *vs.* Figure 23), which would be expected to favor association of apolar groups (von Hippel and Schleich, 1969), supports the inclusion of a relatively large number of such hydrophobic bonds between the subunits of the 23° dimer. Similarly, the lack of any effect of high ionic strength on the maximal value obtained for $\underline{k}_2$ (Figures 23, 28, and 30) might suggest that electrostatic forces play a predominant role in the association of these dimers in the native tetramer, as well as between the subunits of the 0° dimer.

The analogous effects of temperature and ionic strength on the binding of FDP to yeast PK observed both through measurement of fluorescence changes and through the inactivation phenomenon, as well as the effect of increased ionic strength on the kinetic properties of the enzyme, strongly support the argument that the structural changes observed in yeast PK during its inactivation by FDP reflect more subtle changes in the conformation of the enzyme, which yield the kinetic activation, by the same ligand, in the presence of substrates and divalent cation. A model (not meant to be unique) for kinetic activation of yeast PK by FDP consistent with the data obtained from the inactivation phenomenon, would involve a change, concomitant with the ligand binding, in the conformation of the individual subunits of the tetramer to a more compact form, with a resultant decrease in the area of contact between the sub-

units. This decreased contact between the subunits would be accompanied by exposure of the residues of the polypeptide chains, which normally interact strongly when the enzyme is in the non-activated state. Decreasing the interaction between the subunits should result in a reduction in cooperativity of ligand binding to the enzyme. This effect is obtained kinetically as the Hill slopes for all ligands in the pyruvate kinase reaction decrease in the presence of FDP (Hunsley and Suelter, 1969b).

The increase in apparent K_D for FDP observed in the fluorescence titrations as ionic strength is increased, does not necessarily reflect an inhibition of the actual binding phenomenon, but rather represents an inhibition of the structural transition of the enzyme from the non-activated state (with low affinity for FDP) to the activated state (with high affinity for FDP). If the residues which are exposed during this transition are predominantly hydrophobic in nature, high ionic strength would be expected to inhibit the transition by FDP as is observed not only in the fluorescence measurements, but also during the inactivation phenomenon, and in the kinetic assays. Similarly, glycerol, which would be expected to disrupt water structures around hydrophobic residues, and thus eliminate the unfavorable entropy change associated with the separation of apolar residues in an aqueous environment, would thus also be expected to decrease the cooperativity of protein-ligand interactions, and to decrease the kinetically activating

effects of FDP. These effects have also been observed with yeast PK (M. Ruwart and C. H. Suelter, unpublished observations). Finally, low temperatures, which would favor disruption of hydrophobic bonding by allowing for the structuring of water around the apolar residues, would be expected to also favor the conformational transition induced by FDP. This effect of temperature is reflected in the decrease in apparent K_D's for FDP as measured both by the fluorescence changes, and in the inactivation phenomenon as the temperature is lowered. It might also be expected that low temperatures would activate yeast PK somewhat like FDP activates the enzyme (see below for this type of temperature effect in PK isolated from Alaskan king crab).

The delicate interplay of forces which apparently exists in regulating the conformations of yeast pyruvate kinase may reflect a more general phenomenon particularly significant to those enzymes which exhibit regulatory properties. The exposure of hydrophobic residues, concomitant with structural transitions, would contribute significantly to the cooperativity of such transitions by introducing a significant free energy change as these apolar groups interact with the surrounding environment. The characteristics of the solvent (such as ionic strength, or the presence of polyhydroxic compounds or their physiological counterparts) thus play a major role in maintaining the subtle balance between conformational states of an enzyme.

In conclusion, it is interesting to examine other preparations of pyruvate kinase with respect to the effects of temperature on their structure and kinetic properties. Kayne and Suelter (1968) have described conformational transitions of the rabbit muscle enzyme as observed by ultraviolet difference spectra, changes in the sedimentation coefficient, and changes in optical rotatory dispersion parameters. These data indicated structural changes in the protein as the temperature was lowered. The K_m for PEP of the pyruvate kinase isolated from Trematomus bernacchii (Somero and Hochachka, 1968) decreases as the temperature is lowered to 5°. This temperature represents the habitat temperature of the arctic fish. Finally, the pyruvate kinase isolated from Alaskan king crab (Somero, 1969) has been shown to consist of two interconvertible forms, controlled by the temperature. The "cold" form exhibits hyperbolic kinetics, and has a minimal K_m for PEP at 5°. Because of the unfavorable K_m at higher temperatures, this form only functions below 10°. The "warm" form has a minimal K_m for PEP at 12°, and exhibits sigmoid kinetics. Thus, lowering the temperature induces a conformational change with effects analogous to addition of FDP to the yeast pyruvate kinase.

The biological significance of the interconversion of PK forms in the Alaskan king crab is that the organism can function equally as efficiently, at least in this enzymic reaction, throughout the entire range of habitat

temperatures which it will encounter. Its adaptation to temperature changes is thus rapid and efficient. The ability of an organism to adapt to temperature variations is essential for survival. The structural and conformational transitions described in this Discussion may suggest the involvement of subtle changes in protein structure or inter-cellular environment in the mechanism of such biological phenomena as hibernation and cold hardiness. The possibility of radical changes in the primary structure of enzymes during these phenomena cannot be discounted either. For example, Roberts (1969) has recently presented an interesting speculative review on the role of isozymic substitutions during cold hardening in plants. Examination of the enzymes and cellular compositions of organisms during these phenomena may provide the insight into the exact mechanisms involved.

LIST OF REFERENCES

Atkinson, D. (1966), Ann. Rev. Biochem. 35, 85.

Bates, S. J., and Baxter, W. P. (1929), in International Critical Tables, Washburn, E. W., Ed., 5, McGraw-Hill, New York, N. Y.

Benesch, R., Benesch, R. E., and Tyuma, I. (1966), Proc. Nat. Acad. Sci. 56, 1268.

Brandts, J. F. (1969), in Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Eds., Marcel Dekker, Inc., New York, N. Y.

Bücher, T., and Pfleiderer, G. (1955), Methods Enzymol. 1, 435.

Caravaca, J., and Grisolia, S. (1961), Biochem. Biophys. Res. Comm. 4, 262.

Chanutin, A., and Curnish, R. R. (1966), Arch. Biochem. Biophys. 113, 114.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1969), Data for Biochemical Research, Oxford Univ. Press, New York, N. Y., p. 109.

Dua, R. D., and Burris, R. H. (1963), Proc. Nat. Acad. Sci. 50, 169.

Eisenberg, D., and Kauzmann, W. (1969), The Structure and Properties of Water, Oxford Univ. Press, New York, N. Y.

Eisenkraft, B., Van Dort, J. B., and Veeger, C. (1969), Biochim. Biophys. Acta 185, 9.

Fincham, J. R. S. (1957), Biochem. J. 65, 721.

Frank, H. S., and Evans, M. W. (1945), J. Chem. Phys. 13, 507.

Freedman, M. H., Grossberg, A. L., and Pressman, D. (1968), Biochemistry 7, 1941.

Frieden, C. (1967), J. Biol. Chem. 242, 4045.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, Wiley, New York, N. Y., p. 41ff.

Graves, D. J., Sealock, R. W., and Wang, J. H. (1965), Biochemistry 4, 290.

Grisolia, S. (1964), Physiol. Rev. 44, 657.

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

Harris, C. E., Kobes, R. D., Teller, D. C., and Rutter, W. J. (1969), Biochemistry 8, 2442.

Hatfield, G. W., and Umbarger, H. E. (1970), J. Biol. Chem. 245, 1736.

Havir, E. A., Tamir, H., Ratner, S., and Warner, R. C., (1965), J. Biol. Chem. 240, 3079.

Henderson, R. F., and Henderson, T. R. (1969), Arch. Biochem. Biophys. 129, 86.

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

von Hippel, P. H., and Schleich, T. (1969), in Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Eds., Marcel Dekker, Inc., New York, N. Y.

von Hippel, P. H., and Waugh, D. F. (1955), J. Am. Chem. Soc. 77, 4311.

Hofstee, B. H. J. (1949), J. Gen. Physiol. 32, 339.

Holleman, W. (1966), Doctoral Thesis, Michigan State University, East Lansing, Michigan.

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

Horecker, B., and Kornberg, A. (1948), J. Biol. Chem. 175, 385.

Hsu, R. Y., and Yun, S. L. (1970), Biochemistry 9, 239.

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

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

Irias, J. J., Olmsted, M. R., and Utter, M. F. (1969), Biochemistry 8, 5136.

Jarabak, J., Seeds, A. E. Jr., and Talalay, P. (1966), Biochemistry 5, 1269.

Kauzmann, W. (1954), in The Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., Eds., Johns Hopkins Press, Baltimore, Maryland.

Kauzmann, W. (1959), Advan. Protein Chem. 14, 1.

Kawahara, K., Kirshner, A. G., and Tanford, C. (1965), Biochemistry 4, 1203.

Kawahara, K., and Tanford, C. (1966), J. Biol. Chem. 241, 3228.

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

Kayne, F. J., and Suelter, C. H. (1965), J. Am. Chem. Soc. 87, 897.

Kayne, F. J., and Suelter, C. H. (1968), Biochemistry 7, 1678.

Kirkman, H. N., and Hendrickson, E. M. (1962), J. Biol. Chem. 237, 2371.

Kuczenski, R. T., and Suelter, C. H. (1970a), Biochemistry 9, 939.

Kuczenski, R. T., and Suelter, C. H. (1970b), Biochemistry 9, 2043.

Lee, M., and Muench, K. H. (1969), J. Biol. Chem. 244, 223.

Lovelock, J. E., and Bishop, M. W. H. (1959), Nature 183, 1394.

Massey, V., Hofmann, T., and Palmer, G. (1962), J. Biol. Chem. 237, 3820.

Meyerhof, O., and Ohlmeyer, P. (1952), J. Biol. Chem. 195, 11.

Passeron, S., Jimenez de Asua, L., and Carminatti, H. (1967), Biochem. Biophys. Res. Comm. 27, 33.

Penefsky, H. S., and Warner, R. C. (1965), J. Biol. Chem. 240, 4694.

Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E., (1960), J. Biol. Chem. 235, 3322.

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

Racker, E., Pullman, M. E., Penefsky, H. S., and Silvermann, M. (1963), Proc. 5th Inter. Con. Biochem. 5, Macmillan, New York, N. Y.

Raijman, L., and Grisolia, S. (1961), Biochem. Biophys. Res. Comm. 4, 262.

Reynard, A., Hass, L., Jacobsen, D., and Boyer, P. (1961), J. Biol. Chem. 236, 2277.

Roberts, D. W. A. (1969), Intl. Rev. of Cytology, 26, 303.

Rose, K. (1969), Master's Thesis, Michigan State Univ., East Lansing, Michigan.

Rosen, O. M., Copeland, P. L., and Rosen, S. M. (1967), J. Biol. Chem. 242, 2760.

Rutter, W., Hunsley, J. R., Groves, W., Calder, J., Rajkumar, T., and Woodfin, B. (1966), Methods Enzymol. 9, 479.

Schachman, H. K. (1957), Methods Enzymol. 4, 32.

Scheraga, H. A., Nemethy, G., and Steinberg, I. Z. (1962), J. Biol. Chem. 237, 2506.

Shapiro, A. L., Vinuela, E., and Maizel, J. V. Jr. (1967), Biochem. Biophys. Res. Comm. 28, 815.

Shikama, K., and Yamazaki, I. (1961), Nature 190, 83.

Shukuya, R., and Schwert, G. W. (1960), J. Biol. Chem. 235, 1658.

Shuster, C. W., and Doudoroff, M. (1962), J. Biol. Chem. 237, 603.

Small, P. A. Jr., and Resnick, R. A. (1965), FORTRAN Program (PASpplC), National Institutes of Health, Bethesda, Md.

Somero, G. N. (1969), Biochem. J. 114, 237.

Somero, G. N., and Hochachka, P. W. (1968), Biochem. J. 110, 395.

Stancel, G. M., and Deal, W. C. Jr. (1969), Biochemistry 8, 4005.

Steinmetz, M., and Deal, W. C. Jr. (1966), Biochemistry 5, 1399.

Strausbauch, P. H., and Fischer, E. H. (1970), Biochemistry 9, 226.

Suelter, C. H. (1967), Biochemistry 6, 418.

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

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

Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, Oxford Univ. Press, New York, N. Y.

Tietz, A., and Ochoa, S. (1958), Arch. Biochem. Biophys. 78, 477.

Tikhonenko, A. S., Sukhareva, B. S., and Braunstein, A. E. (1968), Biochim. Biophys. Acta 167, 476.

Tsai, C. M., Holmberg, N., and Ebner, K. E. (1970), Arch. Biochem. Biophys. 136, 233.

Van Holde, K. E., and Baldwin, R. L. (1958), J. Phys. Chem. 62, 734.

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

Wieker, H. J., Johannes, K. J., and Hess, B. (1969), 6th Meeting, Fed. Europ. Biol. Soc., Madrid, Abstract #398.

Yang, S. T., and Deal, W. C. Jr. (1969), Biochemistry 8, 2814.

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