REVERSIBLE DISSOCIATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF ADENOSINE TRIPHOSPHATE

Thesis for the Degree to Ph. D.
MICHIGAN STATE UNIVERSITY
GEORGE MICHAEL STANCEL
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This is to certify that the

thesis entitled

REVERSIBLE DISSOCIATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF ATP

presented by

George Michael Stancel

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biochemistry

William C. Leal, Jr.
Major professor

Date December 18, 1970

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ABSTRACT

REVERSIBLE DISSOCIATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF ADENOSINE TRIPHOSPHATE

By

George Michael Stancel

Tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase undergoes a time-dependent inactivation at 0° in the presence of adenosine 5'-triphosphate as a result of dissociation into subunits. The dissociation has been studied at 0° in 0.2 M Tris buffer, pH 8.0, with varying amounts of adenosine 5'-triphosphate. Inactivation studies which measure the loss of catalytic activity indicate that an adenosine 5'-triphosphate concentration of 0.5 mM produces the half-maximal effect. An ultracentrifugal analysis of the dissociation reveals in addition to the native tetramer a 3.0 S subunit (produced by incubation times less than 12 hrs), a 1.6 S subunit (24 hr incubation) and a 0.9 S species (3-7 day incubation). No dimer is observed in the analysis. Structural changes are also indicated by the appearance of an electrophoretic band with a mobility less than that of the native enzyme. The 3.0 S subunits can be reassociated to the native 7.5 S tetramer.

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George Michael Stancel

Tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase undergoes a time-dependent inactivation in the presence of adenosine 5'-triphosphate as a result of dissociation into monomeric subunits. Optimal conditions, which yield complete inactivation in 5 hours are: (1) 1-2 mM adenosine 5'-triphosphate, (2) 0°, (3) protein concentrations of 0.03-0.1 mg per ml, (4) pH 9.0, and (5) 0.1 M β-mercaptoethanol. Transition points (half-maximal loss of activity in 5 hours) are: (1) 0.5 mM adenosine 5'-triphosphate, (2) 12°, (3) 0.5 mg per ml and (4) pH 8.6. Adenosine 5'-monophosphate and adenosine 3',5'-monophosphate do not dissociate the enzyme. They, and all the substrates of the reaction, partially protect the enzyme from dissociation. Dissociation and inactivation are completely reversed by warming to 17°. Reassembly is greatly stimulated by adenosine 5'-triphosphate and by 10% sucrose. Optimal reassembly conditions are: (1) 0.04 mg per ml protein, (2) pH 7.0, (3) 1-2 mM adenosine 5'-triphosphate, (4) 17°, (5) 10% sucrose and (6) 0.1 M β-mercaptoethanol. Inactivation and dissociation apparently result from electrostatic repulsion. The results are discussed in terms of a possible role for this enzyme in the regulation of glycolysis. Since this dissociation produces fairly compact subunits, association of folded monomers to tetramers may be studied independently of the polypeptide folding.

A detailed kinetic analysis of the dissociation of tetrameric yeast GAPD to monomers at 0° in ATP has been

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performed by following the loss of catalytic activity. At pH 8.0 in 0.02 M Tris and 0.075 M β-mercaptoethanol the process is biphasic. There is a fast initial step followed by a slow second step.

The fast step can be described by a rapid reversible equilibrium between tetramers and dimers. The calculated equilibrium constant (K_{eq} = 10.2 x 10^{-8} M) for this initial dissociation yields a value of 8.9 kcal/mole for ΔG° at 0°.

The slow step exhibits psuedo-first order kinetics and a half-power dependence on protein concentration, suggesting that this step reflects the dissociation of dimers to monomers. The dependence of the dimer to monomer dissociation on ATP concentration indicates that ATP binds to the dimer in successive, reversible steps with no interaction between ATP binding sites. The average constant for binding of ATP to the dimer is 0.75 mM. The rate of dissociation of dimers to monomers is decreased as ionic strength is increased, suggesting that hydrophobic residues are exposed to the solvent medium as the dimer dissociates to monomers.

A kinetic analysis of the reversal of dissociation at 23° in the presence of 10% sucrose reveals that the reversal process is second order with respect to protein at low protein concentrations and first order at high concentrations. These results suggest that the final step in the reassembly of monomers to tetramers is a first order

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process involving a conformational change. The half-life for the reassembly process is 8.5 min at 0.20 mg/ml and 16.0 min at 0.010 mg/ml.

Examination of yeast cell extracts indicated the presence of a protein which caused an apparent inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase. Initial experiments indicated that this inhibitor protein was not a protease, and that the inhibitor was present in intact yeast cells. The inhibitor protein was purified and its molecular weight (58,000) and amino acid composition were determined. Maximal inhibition was then shown to occur when the inhibitor protein and glyceraldehyde-3-phosphate dehydrogenase were mixed in a 1:1 molar ratio, however, several physical tests failed to reveal the formation of a complex between the two proteins. The inhibitor protein was subsequently identified as triosephosphate isomerase. Nevertheless, this identification of the inhibitor protein as triosephosphate isomerase is not sufficient to clearly explain the observed inhibition, since (1) the inhibition is not completely overcome by glyceraldehyde-3-phosphate, (2) the inhibition depends on the total protein concentration as well as the ratio of the two proteins, and (3) triosephosphate isomerase seems to activate glyceraldehyde-3-phosphate dehydrogenase under certain conditions.

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By

George Michael Stancel

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ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. W. C. Deal, Jr., for his guidance and assistance throughout the course of this work. The author also thanks the members of his guidance committee, Drs. Wells, Kindel, Karabatsos and Tulinsky, for their time and efforts.

The helpful discussions, criticisms and encouragement of Drs. Suelter and Kuczenski are also greatly appreciated.

The author would also like to thank Dr. William Hare, Mr. Gregory Cook, Miss Shirley Ann Williams and Miss Mary Kuhn for their assistance in preparing various portions of this thesis.

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DHP	dihydr
ECA	(ethyl
FP	fructo
GP	D-glyc
GDO	D-glyc NAD ox (EC 1.
LP	L-lact (EC 1.
PP	pyrido
TP	trichl
TMV	tobacc
TPI	triose phosph

LIST OF ABBREVIATIONS

DHAP	dihydroxyacetone phosphate
EDTA	(ethylenedinitrilo) tetraacetic acid
FDP	fructose-1,6-diphosphate
GAP	D-glyceraldehyde-3-phosphate
GAPD	D-glyceraldehyde-3-phosphate dehydrogenase: NAD oxidoreductase (phosphorylating) (EC 1.2.1.12)
LDH	L-lactate dehydrogenase: NAD oxidoreductase (EC 1.1.1.27)
PLP	pyridoxal-5'-phosphate
TCA	trichloroacetic acid
TMV	tobacco mosaic virus
TPI	triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol isomerase) (EC 5.3.1.1)

LIST OF TABLES

Figure

1. Activity
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LIST OF FIGURES

CHAPTER ONE

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ORGANIZATION OF THE THESIS

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

Portions of Chapter Two have already been published under the title "Metabolic Control and structure of Glycolytic Enzymes. V. Dissociation of Yeast Glyceraldehyde-3-Phosphate Dehydrogenase into Subunits by ATP," by George M. Stancel and William C. Deal, Jr., (1968), Biochemical and Biophysical Research Communications 31 398.

Chapter Three has already been published under the title "Reversible Dissociation of Yeast Glyceraldehyde-3-Phosphate Dehydrogenase by Adenosine Triphosphate," by George M. Stancel and W. C. Deal, Jr. (1969), Biochemistry 8 4005. A preliminary report of the data in Chapter Three had previously been presented at a meeting of the Federation of American Societies for Experimental Biology (George M. Stancel (1969), Federation Proceedings 28 490).

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Chapter Four is being prepared for submission to Biochemistry, and it is hoped that some of the unpublished results in Chapters Two and Five will provide a stimulus for further study, and hopefully publication.

APPROACH TO THE RESEARCH

This research began with the observation that glyceraldehyde-3-phosphate dehydrogenase exhibited a time dependent loss of enzymatic activity when incubated with ATP at 0°.

The time dependence of the loss of activity suggested that structural changes might be occurring. The ultracentrifugal analysis of the enzyme at 0° in the presence of ATP was therefore undertaken (Chapter Two). This study revealed that the tetrameric enzyme was dissociated into subunits at 0° (3.0 S and 1.6 S) in the presence of ATP, the subunit species obtained depending on the length of the incubation period. Further studies revealed that the 3.0 S monomer could be reassembled to the native tetrameric 7.5 S species.

Later structural studies revealed that very long incubations at 0° in ATP (3-7 days) led to the appearance of a 0.9 S species with an apparent molecular weight lower than the expected value of 35,000 (based on the reported tetramer molecular weight of 145,000, see Jaenicke, et al., 1968).

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After the observation of this reversible dissociation, a systematic study of the variables influencing both the dissociation and reassociation processes was undertaken (Chapter Three). There were many incentives for characterizing both these processes. The study of the dissociation could yield valuable information concerning:

(1) the nature and strengths of the chemical forces involved in subunit interactions,

(2) the binding of nucleotides to the enzyme,

(3) the stability and possible activity of individual subpolymers of a polymeric enzyme, and

(4) the possible biochemical significance with respect to metabolic control of the interaction of glyceraldehyde-3-phosphate dehydrogenase with ATP.

Similarly, a study of the reassociation was expected to yield information concerning the exact sequence of events leading to the formation of the biologically active tetramers from inactive monomers. This information should be important for considerations of the in vivo assembly process, since the polypeptide chains of polymeric enzymes must fold and associate after they are synthesized on the ribosomes. This was especially interesting since another metabolite, NAD, has been shown to be required for the refolding of yeast glyceraldehyde-3-phosphate dehydrogenase (dissociated in 8 M urea), and it has been postulated that

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NAD might control the synthesis of this enzyme *in vivo* by controlling a folding reaction (Deal, 1969).

Once the systematic study of the variables affecting the dissociation and reassociation processes was completed, a detailed kinetic analysis of both processes was undertaken (Chapter Four). The kinetic study was performed in order to obtain a more thorough understanding of the mechanisms of dissociation and reassociation and to provide an explanation for these processes on theoretical grounds.

The results in Chapters Two, Three and Four did not suggest any physiological significance for the dissociation of glyceraldehyde-3-phosphate dehydrogenase into subunits by ATP, since the major effects occurred at low temperatures (0°) and low protein concentrations, conditions not likely to be encountered *in vivo*. This work did provide a stimulus, however, for other research in our laboratory. Thus, Dr. S. T. Yang, a member of our research group, discovered that very low concentrations of ATP (less than 1 mM) greatly increased the susceptibility of yeast glyceraldehyde-3-phosphate dehydrogenase to chymotrypic digestion *at 23°* (Yang and Deal, 1969b). This result prompted us to investigate our earlier suggestion (Stancel and Deal, 1968--see Chapter Two) that the physiological significance of the destabilization of glyceraldehyde-3-phosphate dehydrogenase by ATP might be an increased susceptibility of the enzyme to proteolysis, rather than a complete dissociation into subunits.

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The experiments reported in Chapter Five were initially undertaken to determine if yeast cells contained a protease(s) which would degrade yeast glyceraldehyde-3-phosphate dehydrogenase. If such a protease were found, the effect of ATP on the rate of proteolysis could then be examined. While the studies in Chapter Five did not fulfill this original intent, they provided a number of interesting observations which will hopefully form a basis for further research in Dr. Deal's laboratory.

LITERATURE REVIEW: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

INTRODUCTION. Since the crystallization of yeast GAPD in 1939 (Warburg and Christian, 1939), the properties of the enzyme have been extensively studied because of an intrinsic interest in the enzyme, because of the availability of large quantities of the enzyme and because of the nature of the catalytic reaction. A homogeneous preparation of the enzyme, which is present in yeast in large amounts [20% of the total soluble protein (Krebs et al., 1953)], is readily obtained in crystalline from (Krebs, 1955), thus providing ample material for extensive investigations. Furthermore, the enzyme catalyzes the only oxidation step in glycolysis. This catalytic reaction is a substrate level oxidative phosphorylation, and numerous mechanistic studies have been performed because it was hoped this reaction would provide a useful model for mitochondrial

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oxidative phosphorylation (Racker, 1965). Several reviews of studies concerning GAPD are available (Velick and Furfine, 1963; Racker, 1965; Krebs, 1955).

REACTION MECHANISM OF THE OXIDATION OF GLYCERALDEHYDE-3-PHOSPHATE. GAPD catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate in the presence of inorganic phosphate and NAD. If arsenate is used instead of phosphate unstable arsenophosphoglycerate is produced and this spontaneously decomposes to 3-phosphoglycerate, and this is commonly used in assaying the enzyme.

The mechanism of the reaction involves the oxidative formation of an S-acyl-enzyme intermediate between a sulfhydryl group at the enzyme active site and the aldehyde function of the substrate (Racker, 1965). This was most clearly shown by the isolation and crystallization of a stable acyl-enzyme formed by acylation of the rabbit muscle enzyme with acetyl phosphate or 1,3-diphosphoglycerate (Krimsky and Racker, 1955). It has also been shown that the active-site cysteine is more reactive toward sulfhydryl reagents than other cysteine residues in the GAPD molecule, and that this reactive cysteine is situated in the same octapeptide in the yeast, rabbit muscle and pig muscle enzymes (Harris et al., 1963; Perham and Harris, 1963). The oxidative step is then followed by the phosphoryl transfer step to yield 1,3-diphosphoglycerate.

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OTHER REACTIONS CATALYZED BY GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. In addition to the oxidation of glyceraldehyde-3-phosphate, GAPD catalyzes a number of other reactions (Taylor et al., 1963). A list of these other reactions is presented in Table I. The involvement of NAD and the requirement for free sulfhydryl groups of these reactions is also noted. The transferase activity listed refers to the transfer of the acetyl group of acetyl phosphate to a number of receptors (Taylor et al., 1963), while the diaphorase activity refers to the transfer of electrons from NADH to 2,6-dichlorophenolindophenol (Rafter and Colowick, 1957). The other activities are self-explanatory. The significance of these various reactions has been discussed by Park (1966). The variety of reactions catalyzed by the GAPD may suggest that the enzyme has multiple in vivo functions.

SUBSTRATE BINDING: NAD. Numerous studies concerning the binding of the cofactor, NAD, to GAPD from both rabbit muscle and yeast have been performed (Velick and Furfine, 1963; Racker, 1965), and it is clear that NAD is bound very tightly. Yang and Deal (1969a) recently determined a K_m for the yeast enzyme of 0.18 mM NAD. Other studies have measured dissociation constants much smaller, and the reasons for such discrepancies have been previously discussed (Velick and Furfine, 1963).

TABLE I. SUMMARY OF ACTIVITIES OF 3-PHOSPHOGLYCERALDEHYDE DEHYDROGENASE

	NAD or NADH Involved in Reaction	-SH Groups on Enzyme Required
1. Oxidation and phosphorylation		

TABLE I. SUMMARY OF ACTIVITIES OF 3-PHOSPHOGLYCERALDEHYDE DEHYDROGENASE

	NAD or NADH Involved in Reaction	-SH Groups on Enzyme Required
1. Oxidation and phosphorylation	+	+
2. Transferase, P^{32} exchange, or arsenolysis	+	+
3. Hydrolysis of acetyl phosphate	+	O*
4. Hydrolysis of p-nitrophenyl acetate	O†	+
5. NADH - NADH-X	+	+
6. Diaphorase	+	+
7. Transphosphorylase ATP + IMP - ADP + IDP	+	+

*No effect of iodoacetic acid.

†NAD inhibits.

(Taken from Taylor et al., 1963).

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Pyridine-3-aldehyde NAD is a potent inhibitor of the enzyme (Kaplan et al., 1956) and more recent studies have shown that adenine nucleotides are also inhibitors (Yang and Deal, 1969a). It is the adenine protein of the NAD molecule which appears essential for binding, and the 6-amino group and 2'-hydroxyl group of the adenine moeity appear to be critical (Yang and Deal, 1969a). Stockell (1959) had earlier suggested the importance of the 6-amino group, and Windmueller and Kaplan (1961) further suggested the importance of the 1 position of adenine.

Data has also been presented (Racker, 1965) that indicates that the 4 position of the pyridine ring may be important for binding, but there are other possible explanations for these observations (see Yang and Deal, 1969a).

As previously mentioned there have been a number of discrepancies regarding the number of moles of NAD bound per mole of enzyme as well as the magnitude of the binding constants (Velick and Furfine, 1963). Quite recently, it has been suggested for both the rabbit muscle (Conway and Koshland, 1968) and yeast (Cook and Koshland, 1970) enzymes that the binding of NAD is a cooperative phenomena. Kirschener et al., (1966) obtained weakly sigmoidal NAD binding curves at 40° C for the yeast enzyme, but the binding curves were hyperbolic at 20° C. Stopped-flow experiments have further revealed that the binding of the first mole of NAD to the rabbit muscle enzyme is faster

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than the binding of subsequent moles of NAD (De Vijider and Slater, 1968).

It seems clear that the binding of NAD is a complex process which involves conformational changes in the enzyme. For example, NAD enhances the rate of carboxymethylation of the enzyme by iodoacetate, while ATP decreases the rate in the absence of NAD, but has no effect in the presence of NAD (Fenselau, 1970). Similarly, ATP enhances the rate of chymotryptic digestion of yeast GAPD, and NAD prevents the effect (Yang and Deal, 1969b). Kirtley and Koshland (1970) have also shown that the addition of NAD or ATP to rabbit muscle GAPD, labelled at the active site with 2-acetamide-4-nitrophenol, produces large difference spectra.

SUBSTRATE BINDING: GLYCERALDEHYDE-3-PHOSPHATE. GAPD utilizes only the free aldehyde form of the triosephosphate substrate glyceraldehyde-3-phosphate in the catalytic reaction (Trentham et al., 1969). Glyceraldehyde-3-phosphate, however, exists in two forms in aqueous solution, since the aldehyde may be hydrated to yield a diol (Trentham et al., 1969). At equilibrium, 96% of the glyceraldehyde-3-phosphate exists as the non-utilizable diol (Trentham et al., 1969). The free aldehyde is also the form of glyceraldehyde-3-phosphate utilized by aldolase and triosephosphate isomerase (Trentham et al., 1969).

SUBUNIT STRUCTURE OF NATIVE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. While the molecular weight of yeast and

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rabbit muscle GAPD has been examined in a number of studies, there does not seem to be any general agreement on the molecular weight of the native enzymes. This has been previously pointed out in a review of the data by Elias et al., (1960). Table II summarizes the molecular weight values obtained in a number of studies on the rabbit muscle enzyme. Similarly, for the yeast enzyme Jaenicke et al., (1968) have reported values of 143,000--148,000, while Deal and Holleman (1964) and Taylor and Lowry (1956) have reported 116,000. Harrington and Karr (1965) have also reported values for the pig muscle enzyme of 139,000--156,000.

There is agreement, however, that both rabbit muscle (Deal and Holleman, 1964; Harrington and Karr, 1965) and yeast (Deal and Holleman, 1964) GAPD subunits, produced by a variety of treatments, have a molecular weight of 35,000--38,000. It is clear from these and other studies (Harris and Perham, 1965; Meighen and Schachman, 1970) that the native enzyme is composed of four polypeptide chains, which are thought to be identical. Harris and Perham (1965) have shown with the rabbit muscle enzyme that the amino acid analysis, end-group analysis and peptide maps obtained after tryptic digestion are consistent with a tetramer of four identical subunits. Meighen and Schachman (1970) have further shown that hybridization of succinylated and native rabbit muscle GAPD produced five hybrids which

TABLE II. REPORTED MOLECULAR WEIGHTS FOR RABBIT MUSCLE GAPD

Experimental Technique	Reported Value[a]	Reference
Light Scattering	139,000-148,000	Dandliker and Fox, 1955
Light Scattering	143,000	Jaenicke et al., 1968

TABLE II. REPORTED MOLECULAR WEIGHTS FOR RABBIT MUSCLE GAPD

Experimental Technique	Reported Value[a]	Reference
Light Scattering	139,000-148,000	Dandliker and Fox, 1955
Light Scattering	143,000	Jaenicke et al., 1968
Approach to Sedimentation Equilibrium (Archibald)	118,000	Elias et al., 1960
Sedimentation/Diffusion	143,000	Elodi, 1958
Sedimentation/Diffusion	145,000	Jaenicke et al., 1968
Sedimentation/Diffusion	120,000	Elias et al., 1960
Sedimentation/Diffusion	137,000	Fox and Dandliker, 1956
Sedimentation/Diffusion	118,000	Taylor, 1950
Sedimentation/Diffusion	120,000	Taylor et al., 1965
Sedimentation Equilibrium	142,000	Jaenicke et al., 1968
Osmometry	149,000	Jaenicke et al., 1968
High Speed Equilibrium (Yphantis Method)	134,000-147,000	Harrington and Karr, 1965
High Speed Equilibrium (Yphantis Method)	144,000[b]	Hoagland and Teller, 1969
	72,000	

[a]In most of the studies, this is the value obtained upon the extrapolation to the limit of infinite dilution.

[b]The value of 72,000 was obtained by extrapolation to the meniscus (concentration approaching zero), and the value of 144,000 was the limiting value at the cell bottom.

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could be electrophoretically distinguished. This result demonstrates quite clearly that the native enzyme is composed of four polypeptide chains.

The most reasonable explanation for the discrepancies in the molecular weight data is that the yeast and muscle enzymes undergo some type of association-dissociation phenomena. This has been clearly shown for the rabbit muscle enzyme, which can be reversibly dissociated to either dimers or monomers (depending on the choice of experimental conditions) in dilute salt solutions at low temperatures (Constantinides and Deal, 1970). Similar behavior has also been suggested from other studies (Hoagland and Teller, 1969; Meighen and Schachman, 1970). The rabbit muscle enzyme is also reversibly dissociated in the presence of ATP to dimers, or monomers, the species obtained depending on experimental conditions such as protein concentration (Constantinides and Deal, 1969).

In addition to the dissociation produced by low temperatures and dilute salts or ATP, the GAPD from yeast (Deal and Holleman, 1964; Deal, 1969) and rabbit muscle (Deal and Holleman, 1964) can be reversibly dissociated into 36,000 molecular weight subunits by exposure to 8 M urea solutions. In both cases, the cofactor, NAD, is required for the reversal of dissociation. The implications of these findings have been discussed in detail (Deal, 1969).

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The subunit structure of GAPD from a number of sources is very likely quite similar to that of the yeast and muscle enzymes, since comparative studies have indicated that the structure of the native enzyme from a number of sources is quite similar. Allison and Kaplan (1964) have purified the native GAPD's from rabbit muscle, beef muscle, human muscle, chicken muscle, turkey muscle, pheasant muscle, halibut muscle, sturgeon muscle, lobster muscle, yeast and E. Coli. The corn, wheat, oat and barley enzymes have also been studied (Hageman and Waygood, 1959). The spinach leaf enzyme is different in that it uses NADP and has a higher molecular weight (Yonuschot et al., 1970). The gross structure of most of these enzymes seems quite similar. For example, the enzymes studied by Allison and Kaplan (1964) all have sedimentation coefficients, $s^{\circ}_{20,w}$, within the range 7.44 $\pm$ 0.15 S. Because of this structural similarity, it is expected that the studies reported in this work with the yeast enzyme may have application to other systems.

It is also interesting to note that the subunits of the lobster GAPD, which is quite similar to both the yeast and rabbit muscle enzymes (Allison and Kaplan, 1964), appear to be geometrically arranged at the vertices of a tetrahedron, since this arrangement is most consistent with the D_2 dihedral symmetry observed by X-ray diffraction (Watson and Banaszak, 1964). At least four other tetrameric

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proteins, isocitrate lyase, aldolase, hemoglobin and lactic dehydrogenase possess similar symmetry (Klotz et al., 1970). Kirschener (1968) has pointed out that the X-ray studies indicate that the over-all structure of the native tetramer resembles a dimer of dimers more closely than four equivalent monomers.

LITERATURE REVIEW: SUBUNIT ASSOCIATION

In a recent review, Klotz et al., (1970) have listed 109 different proteins which are composed of subunits. This is indicative of the great interest in the subunit structure of enzymes. In large part this interest stems from the suggested relationships between subunit interactions and regulatory properties of polymeric enzymes (Monod et al., 1963; Koshland and Neet, 1968; Atkinson, 1966).

A large number of polymeric enzymes undergo reversible association phenomena of the type $nP = P_n$. For the systems in which the equilibrium constants for such processes have been obtained, it has been possible to evaluate the free energy change per monomer, ΔG°_m, where ΔG°_m is defined as $\Delta G^\circ/n$, with n being the stoichiometric coefficient of the reactant in the reaction $nP = P_n$ (Klotz et al., 1970; Langerman and Klotz, 1969). For all such cases, values of ΔG°_m between -2.1 and -6.3 kcal/mole subunit have been obtained (Klotz et al., 1970).

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LITERATURE REVIEW: COLD LABILITY

Historically, it has been a common practice in enzymology to perform studies at or near 0° C whenever permitted by the experimental approach being used. This has often been a successful procedure, since many enzymes lose activity at higher temperatures for a variety of reasons, e. g., heat denaturation, oxidation of sulfhydryl groups and proteolytic degradation. A number of recent reports have nevertheless demonstrated that some enzymes tend to lose activity more readily at 0° C than at room temperatures.

One of the earliest discoveries of such a cold-labile enzyme was Jack bean urease (Hofstee, 1949). Shortly thereafter, von Hippel and Waugh (1955) demonstrated that casein undergoes a temperature-dependent dissociation to monomers of molecular weight 15,000 as the temperature is lowered to 1.5° C. They suggested that this behavior might arise from the unusually large number of non-polar amino acid residues in the casein molecule.

Harrington and Schachman (1956), working with alkaline-treated tobacco mosaic virus (TMV) protein demonstrated the dramatic effect of temperature on aggregation phenomenon. Thus, a 133 S TMV protein particle, originally obtained as an alkaline degradation product of a 194 S particle at 25°, dimerized to a 170 S species at 25°, while at 0° the same 133 S particle dissociated to a 94 S species.

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Lauffer (1962) has also investigated the temperature-dependent polymerization of TMV protein, and shown that the pH of unbuffered solutions of TMV protein shows a temperature dependent pH change. It is not clear, however, if this change is the cause of, or result of, the polymerization.

An interesting study of glutamine dehydrogenase from Neurospora crassa was reported by Fincham (1957). In this study a mutant enzyme was shown to undergo a reversible temperature dependent inactivation while the wild-type enzyme did not exhibit this behavior. It thus seemed that minor changes in primary structure, possibly produced by a single point mutation, could affect the temperature sensitivity of the enzyme.

More recently, Kuczenski (1970) and Jarabek et al., (1966) have tabulated a number of proteins which are inactivated at 0°. A partial list of these enzymes is presented in Table III. It is readily seen that a variety of enzymes from many different sources undergo cold inactivation. In many of these cases, inactivation is affected by metabolites.

Most of these enzymes are made up of subunits, and for the examples marked with an asterisk in the preceeding table, the cold inactivation is known to be accompanied by dissociation. Kuczenski (1970) has further pointed out that in most cases inactivation (measured as a

TABLE III. ENZYMES WHICH UNDERGO COLD-INACTIVATION

Enzyme	Source	Effect of Metabolites on Inactivation
Pyruvate kinase[a,*]	Baker's yeast	FDP enhances
FDPase[b,*]	Candida utilis	AMP required, FDP retards
Carbamoylphosphate synthase[c,*]	Rat liver	ATP retards, N-acetyl-L-glutamate enhances
ATPase[d,*]	Beef heart mitochondria	- -
17-β-hydroxysteroid dehydrogenase[e]	Human placenta	NADH retards, 17-β-estradiol retards
Carbamoylphosphate synthetase[f,*]	Frog liver	Acetylglutamate required
Glucose-6-phosphate dehydrogenase[g,*]	Human erythrocytes	NADPH and AMP retard
Pyruvate carboxylase[h,*]	Chicken liver	Acetyl CoA, pyruvate, ATP, HCO_3 retard
Glycogen phosphorylase a[i]	Rabbit muscle	Glycogen, PLP, and AMP retard
Arginnosuccinase[j,*]	Steer liver	Arginine and argininosuccinate retard
N_2-fixing enzyme[k]	Clostridium Pasteurianum	- -
Glutamic acid decarboxylase[l,*]	E. coli	PLP retards
β-hydroxybutyric acid dehydrogenase[m]	Rhodospirillum rubrum	NAD retards
Glyceraldehyde-3-phosphate dehydrogenase[n,*]	Rabbit muscle	ATP enhances, NAD prevents
ATPase[o]	Yeast	- -
Fatty acid synthetase[p,*]	Chicken liver	- -
UDP-galactose-4-epimerase[q]	Bovine mammary gland	- -
Prolyl tRNA synthetase[r,*]	E. coli	- -
Threonine deaminase[s]	B. subtilis	PLP enhances

[a]Kuczenski and Suelter, 1970.
[b]Rosen et al., 1967.
[c]Guthohrlein and Knappe, 1968.
[d]Pullman et al., 1960; Penefsky and Warner, 1965.
[e]Jarabak et al., 1966.
[f]Raijman and Grisola, 1961.
[g]Kirkman and Hendrickson, 1962.
[h]Scrutton and Utter, 1965.
[i]Graves et al., 1965.
[j]Havir et al., 1965.
[k]Dua and Burris, 1963.
[l]Shukuya and Schwert, 1960.
[m]Shuster and Doudoroff, 1962.
[n]Constantinides and Deal, 1969.
[o]Racker et al., 1963.
[p]Hsu and Yun, 1970.
[q]Tsai et al., 1970.
[r]Lee and Muench, 1970.
[s]Hatfield and Umbarger, 1970.

*Inactivation is accompanied by dissociation into subunits.

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function of time) appears to be a bi-phasic process, in which the second step is frequently irreversible. Furthermore, in many cases the cold inactivation and accompanying dissociation are reversed by raising the temperature above 20°.

In a classic paper, Kauzmann (1959) pointed out that decreased temperatures could lessen the strength of hydrophobic interactions involved in maintaining proteins in their native configuration.

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CHAPTER TWO

STRUCTURAL STUDIES OF THE DISSOCIATION AND REASSOCIATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF ATP

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ABSTRACT

Tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase undergoes a time-dependent inactivation at 0° in the presence of adenosine 5'-triphosphate as a result of dissociation into subunits. The dissociation has been studied at 0° in 0.2 M Tris buffer, pH 8.0, with varying amounts of adenosine 5'-triphosphate. Inactivation studies which measure the loss of catalytic activity indicate that an adenosine 5'-triphosphate concentration of 0.5 mM produces the half-maximal effect. An ultracentrifugal analysis of the dissociation reveals in addition to the native tetramer a 3.0 S subunit (produced by incubation times less than 12 hrs), a 1.6 S subunit (24 hr incubation) and a 0.9 S species (3-7 day incubation). No dimer is observed in the analysis. Structural changes are also indicated by the appearance of an electrophoretic band with a mobility less than that of the native enzyme. The 3.0 S subunits can be reassociated to the native 7.5 S tetramer.

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INTRODUCTION

Numerous examples of the effects of adenine nucleotides on the regulation of carbohydrate metabolism have been reported (for recent reviews, see Atkinson, 1965, 1966; Stadtman, 1966; and Wood, 1966). Great emphasis has been placed on the influence of ATP, ADP, AMP and 3',5'-AMP on the activity of phosphorylase, phosphofructokinase and fructose diphosphatase and their potential ability to produce major fluctuations in the rate of glycolysis (Krebs and Fischer, 1962; Cori, 1942, Mansour and Mansour, 1962, Passonneau and Lowry, 1962, Williamson, 1965; Taketa and Pogell, 1963, Bonsignore et al., 1963). The possibility of similar (although perhaps lesser in magnitude) effects of these adenine nucleotides on other glycolytic enzymes has largely been ignored. We have, therefore, undertaken a study of the effects of metabolites on the control of several important glycolytic enzymes, including the glyceraldehyde-3-phosphate dehydrogenases (GAPD) from yeast, rabbit muscle, and liver. Our initial studies have shown that ATP produces two separate, major effects with yeast GAPD, both of which affect its activity. One effect, which is due to slow structural change and which leads to loss of activity, is described in this communication. The other effect, which is due to ATP competition with NAD for binding sites, and which leads to instantaneous inhibition of activity is to be described elsewhere (Yang and Deal, 1969a).

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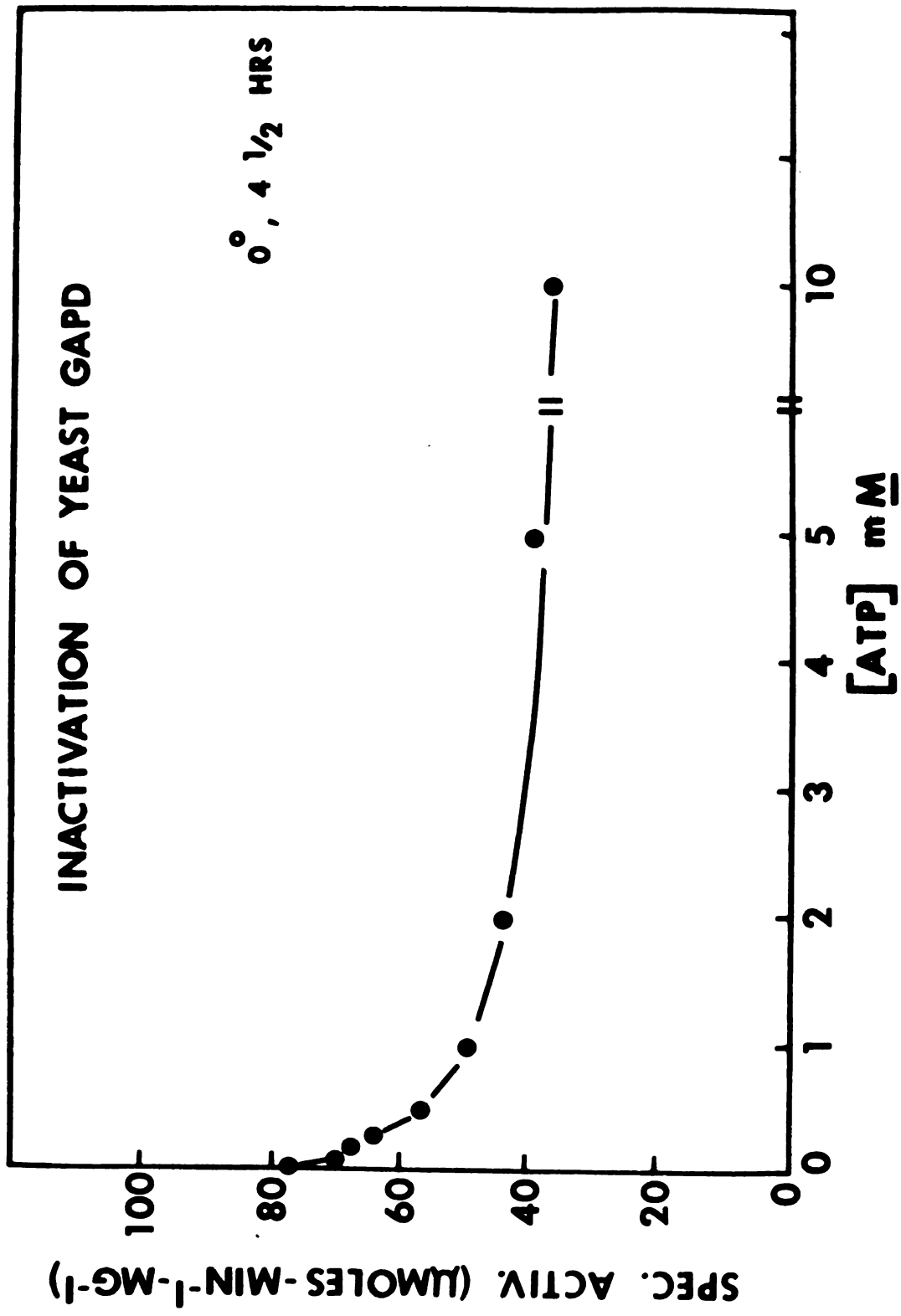
The main purpose of this paper is to report the phenomenon of destabilization of yeast GAPD, as evidenced by *in vitro* inactivation and dissociation of the enzyme into 1.6 S subunits. One strong incentive for characterizing the process was the possibility that the destabilization and presumed increased susceptibility to proteolytic degradation might be a mechanism for control *in vivo*.

RESULTS

LOSS OF ACTIVITY UPON INCUBATION WITH ATP AND PRODUCTION OF A SLOWER-MOVING COMPONENT IN POLYACRYLAMIDE DISC ELECTROPHORESIS. The initial tests for inhibition of yeast GAPD by ATP revealed that after incubation with ATP, the enzyme showed a marked loss of activity (assayed in the absence of ATP). The fact that the loss of activity of GAPD increased significantly with time of incubation with ATP suggested that marked structural changes were occurring with time. To define the structural change, initially a study of the effect of ATP concentration was conducted with an arbitrarily selected incubation time of 4-1/2 hours. Protein samples (0.2 mg/ml) were incubated at 0° in 0.2 M tris (HCl) buffer, pH 8.0, with varying amounts of ATP. After 4-1/2 hours aliquots were removed and assayed in the standard assay. Since all but a negligible residual amount of ATP was diluted out, any activity loss should have been due to structural changes which were not immediately reversible. As seen in Figure 1,

Figure 1. Activity of yeast glyceraldehyde-3-phosphate dehydrogenase after 4 1/2 hr incubation with ATP. See Text.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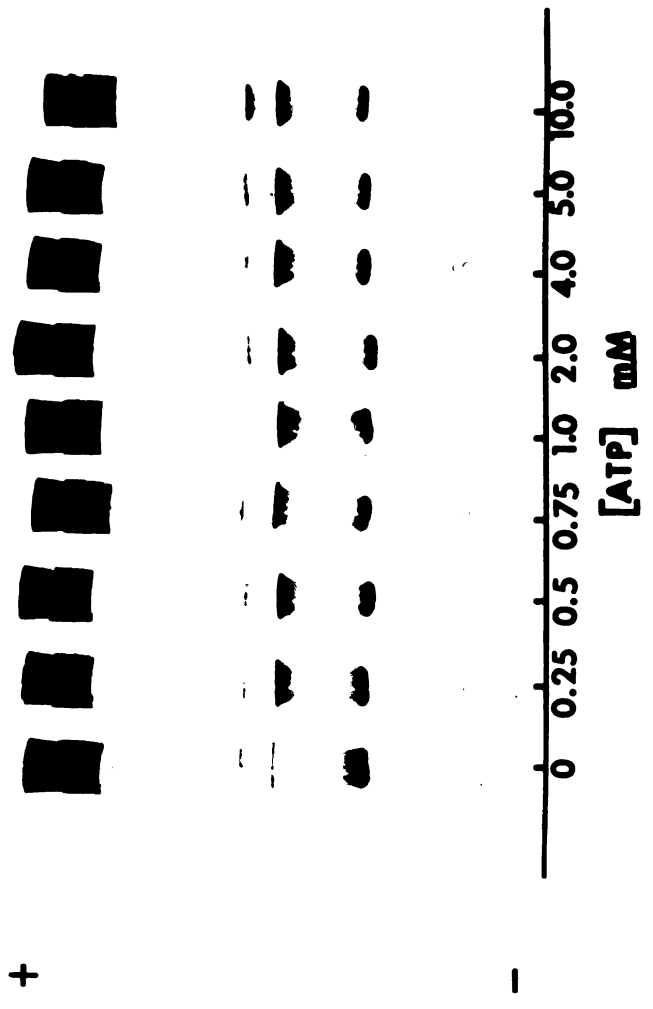
After activity measurements were performed as above, aliquots were removed from the tubes and subjected to polyacrylamide (6-1/2%) disc electrophoresis at pH 8.3. The sample without ATP showed only a single native band (Figure 2). All other samples showed an additional, slower-moving band whose concentration apparently was greater at higher concentrations of ATP (Figure 2). Again the maximum change appeared to be exerted by all concentrations greater than 2 mM ATP. In contrast, incubation of GAPD with an NAD concentration equal to that of the ATP prevented, to a large extent, the loss of activity and the appearance of the more slowly moving band upon electrophoresis.

This set of experiments provided the first direct evidence that ATP produced a marked structural change in the enzyme. A tempting explanation of the data was that ATP might cause dissociation of the enzyme into subunits, since this enzyme has been shown to be dissociated into subunits by urea (Deal, 1963; Deal and Holleman, 1964; Deal, 1969). However, the alternative possibility that the slower-moving species was an aggregated product of the native enzyme could not be ruled out.

Figure 2. Electrophoretic patterns of yeast glyceraldehyde-3-phosphate dehydrogenase after 4 1/2 hr incubation with various amounts of ATP. See Text.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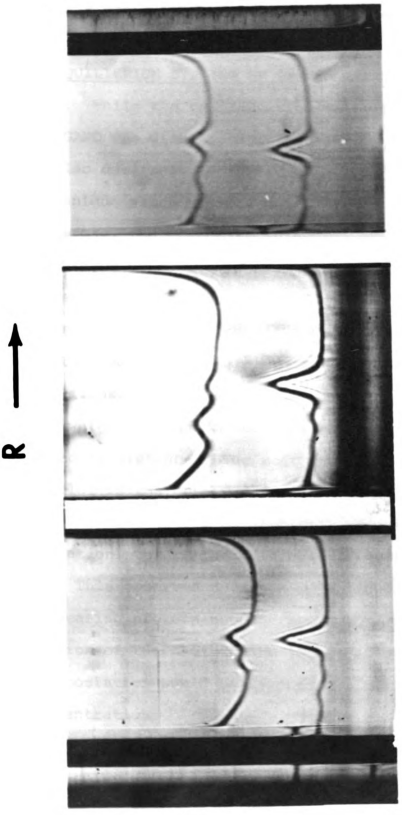
PRODUCTION OF A 3.0 AND A 1.6 S SUBUNIT. To decide between these possibilities, sedimentation velocity experiments were performed. It was desirable for the sedimentation experiments to increase the protein concentration to 2.5 mg/ml. The concentration of the dissociating agent, ATP, was made higher to help compensate for the unfavorable effect of increased protein concentration on dissociation. Samples of GAPD (2.5 mg/ml) were dialyzed at 0° for 4-1/2 or 24 hours against a solvent containing 10 mM ATP, 0.15 M KCl and 0.02 M imidazole, pH 7.5. Native enzyme, treated in a similar manner, but without ATP, served as a control. As seen in Figure 3A and 3B, the ATP-treated samples showed, in addition to the usual 7.5 S native peak, a slower-moving peak, suggesting a dissociation into subunits. It should be noted that the sedimentation coefficients of the slower moving peaks were $s_{20,w}$ = 3.0 S and 1.6 S, respectively, for the samples incubated 4-1/2 hours and 24 hours.

To determine whether this might be a physiologically significant process, two conditions used in the previous experiments were changed to more closely approximate in vivo conditions. The ATP concentration was lowered to 2 mM and the experiment was run at 15-16°. As seen in Figure 3C, incubation of the enzyme with ATP under these conditions for about 48 hours produced about 20-25% dissociation. Although, the dissociation at 15° with 2 mM

Figure 3. Sedimentation velocity patterns of yeast GAPD after incubation with ATP under various conditions. Pattern A: 4 hr dialysis at 0° in 10 mM ATP. Pattern B: 24 hr dialysis at 0° in 10 mM ATP. Pattern C: 48 hr dialysis at 15° in 2 mM ATP. The protein concentration was 2.5 mg/ml for each sample, and the centrifuge runs were performed at temperatures close to those of the incubations.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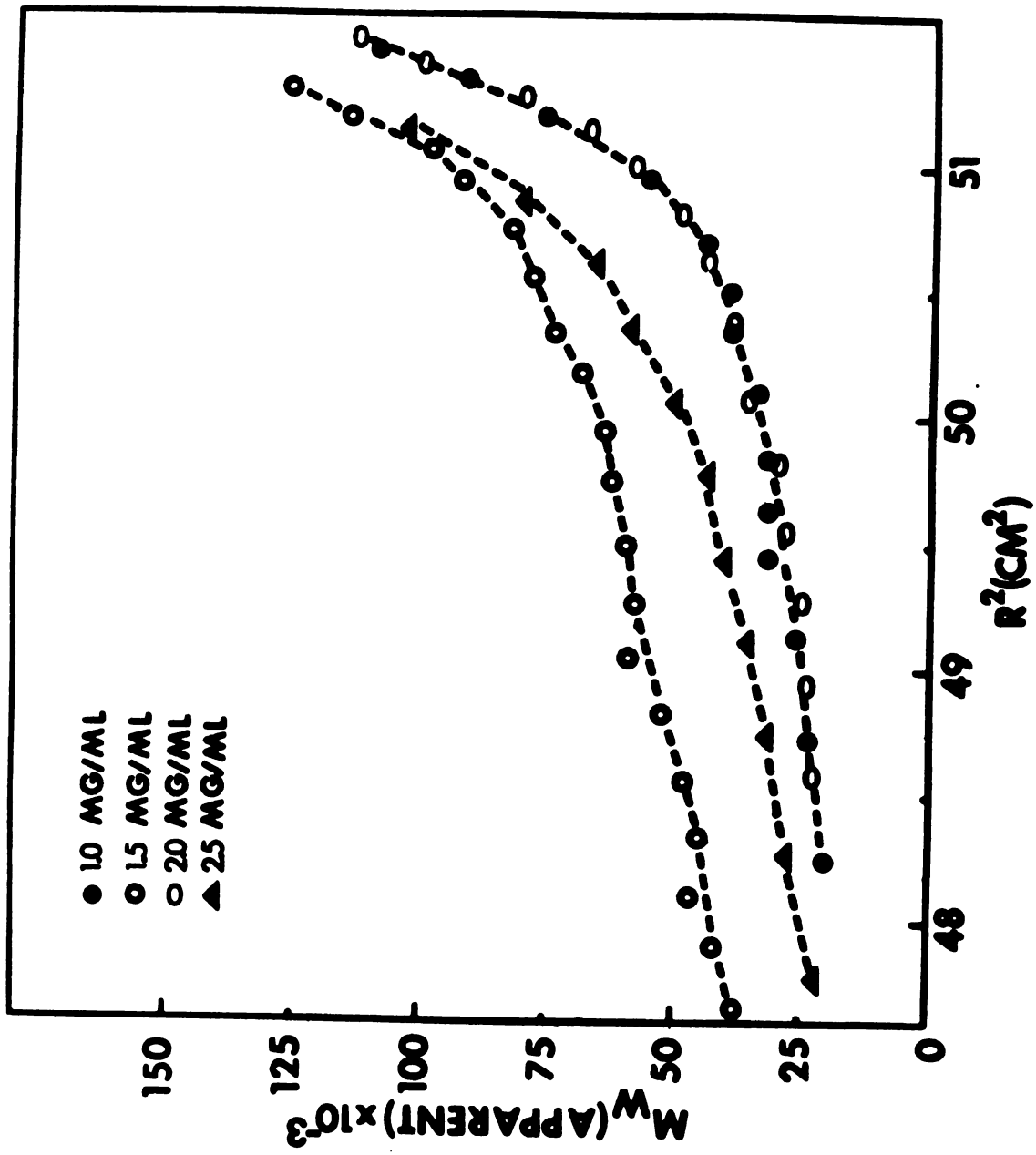
SEDIMENTATION EQUILIBRIUM STUDIES OF YEAST GAPD IN THE PRESENCE OF ATP. While the sedimentation velocity studies indicated that GAPD was dissociating into subunits in ATP at 0°, it was also desirable to investigate this structural change by a technique which yielded molecular weight values directly. Therefore, the molecular weight of GAPD samples at 2° in the presence of ATP was determined by conventional low speed sedimentation equilibrium.

The molecular weight values of four GAPD samples (initial concentrations of 1.0 mg/ml to 2.5 mg/ml) at 2° in 5 mM ATP are illustrated in Figure 4, which is a plot of the apparent weight-average molecular weight vs r^2, the square of the radial distance. The most distinctive feature of the plot is the observed decrease in molecular weight in going from the bottom of each cell (highest protein concentration) toward the meniscus (lowest protein concentration). This observed decrease in molecular weight with decreasing protein concentration is consistent with a dissociation of the native GAPD tetramer into subunits, since dissociation would be favored by lowering the protein concentration.

The most striking feature of this data, however, was that the molecular weights observed at the meniscus

Figure 4. Molecular weight analysis of yeast glyceraldehyde-3-phosphate dehydrogenase in the presence of ATP. The sedimentation equilibrium run was performed at 2° and a speed of 9341 RPM; solvent was 0.02 M Tris, 0.02 M dithiothreitol and 0.005 M ATP, pH 8.0.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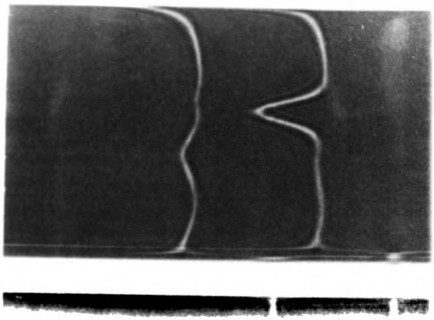
(20,000 to 35,000) were considerably lower than the minimum molecular weights expected for the monomer of GAPD (35,000), since the molecular weight of the native tetramer is 145,000 (Jaenicke et al., 1968). These results raised the possibility that the subunits of 35,000 molecular weight were somehow being converted to a lower molecular weight species in ATP.

To test this possibility, a sedimentation velocity experiment was performed with the stock ATP-GAPD solution (10 mg/ml), aliquots of which had been diluted to prepare the samples for the sedimentation equilibrium experiments. Since this stock solution had remained in the cold room during the equilibrium run, and while the equilibrium calculations were being performed, the GAPD solution (10 mg/ml) had remained at 2° in 5 mM ATP for 7 days prior to the sedimentation velocity experiment described in the next section.

PRODUCTION OF A 0.9 S SUBUNIT. A sedimentation velocity experiment using the GAPD sample which had been incubated for 7 days with ATP at 0° revealed only two peaks, which had sedimentation coefficients ($s_{20,w}$) of 7.7 S and 0.85 S (Figure 5). The value of 7.7 S for the faster sedimenting peak is the expected value for the native enzyme, but the value of 0.85 S is considerably lower than the value of 1.6 S observed in 8 M urea for subunits of 35,000 molecular weight (Deal and Holleman, 1964). One explanation for

Figure 5. Sedimentation velocity patterns of yeast GAPD (10 mg/ml) after incubation for 7 days with (upper pattern) or without (lower pattern) 5 mM ATP. Both the incubation and centrifuge run with performed at 2°. In the upper pattern (with ATP) the sedimentation coefficients ($s_{20,w}$) are 7.7 S and 0.85 S for the leading and trailing peaks respectively. The peak in the lower pattern (minus ATP) has a sedimentation coefficient of 7.7 S. See text for further details.



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this observation and for the results from the preceding molecular weight analysis was that in the presence of ATP the 35,000 molecular weight subunits were being converted to a lower molecular weight species by some unknown mechanism. Alternatively, the sedimentation coefficient of 0.85 S could suggest a more unfolded conformation than that observed in 8 M urea, but this would not explain molecular weight values of 20,000 obtained in the previous sedimentation equilibrium experiments.

At this point, however, there was another possible explanation for the observation of the 0.85 S protein species, based on the conditions under which the preceding experiments had been performed. While ultra-centrifugal studies are routinely performed with solvents containing 0.10 M to 0.15 M salt (usually KCl or NaCl) to "swamp out" any electrostatic effects, no additional salt had been added to the buffered solvent used in the preceding sedimentation equilibrium and sedimentation velocity experiments. Salt had been purposely omitted in these experiments because other studies, which are fully described in Chapter Four of this thesis, had indicated that increasing ionic strength inhibits the over-all dissociation of tetramers to monomers.[1] Therefore, it was possible that

[1]Since this inhibition of dissociation by ionic strength was not discovered until after the initial sedimentation velocity studies, i.e., the studies which demonstrated the production of the 3.0 S and 1.6 S species, 0.15 M KCl had been present in those experiments. Therefore, the observation of the 3.0 S and 1.6 S species is not subject to this criticism.

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the observed sedimentation coefficient of 0.85 S arose from a slowing of the sedimentation of 35,000 molecular weight subunits due to the formation of an electrostatic gradient in the centrifuge cell as the protein molecules sedimented. This criticism also applied to the sedimentation equilbrium studies, which were also performed in the absence of salt. To test this possibility, sedimentation velocity experiments were performed in the presence of 0.1 M KCl.

When further sedimentation velocity experiments were performed with GAPD samples which had been incubated at 0° in 5 mM ATP and 0.1 M KCl for 3-7 days, a species with a sedimentation coefficient ($s_{20,w}$) of 0.95 S was observed, in addition to the native enzyme. This result indicated that the observed sedimentation coefficient of approximately 0.9 S was not an artifact resulting from electrostatic effects.

Control samples of native GAPD minus ATP, which were incubated at 0° for 3-7 days, sedimentated predominantly as the native tetramer but also contained a small amount of a more slowly sedimenting species. The concentration of the slowly sedimenting species in these control samples (minus ATP) was much lower than in the GAPD samples with ATP. The concentration was so small that the sedimentation coefficient of the slowly moving species in these controls (minus ATP) could not be accurately measured. However, experiments in which two GAPD samples (with and without ATP) were run in

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separate cells in the same rotor suggested that the slowly sedimenting peak in the controls corresponded to the 0.9 S peak in the ATP sample. Therefore, it is clear that ATP is in some way responsible for the production (or at least for an increased rate of production) of the 0.9 S material. At present, the exact nature of the 0.9 S species and the mechanism of its formation are not clear, but further molecular weight studies may provide a more precise explanation.

REVERSAL OF DISSOCIATION: PRODUCTION OF A 7.5 S TETRAMER FROM A 3.0 S MONOMER. One major question was whether the structural changes, i.e., dissociation into subunits by incubation of GAPD at 0°, produced in the presence of ATP were reversible. Initial attempts to reverse the dissociation by removal of the ATP or by raising the temperature to 23° were unsuccessful, since catalytic activity was not recovered. Further studies revealed, however, that 3.0 S subunits could be reassembled to yield a fully active tetrameric enzyme, under the proper conditions. However, both dissociation and reassociation processes are discussed in great detail in the following chapters of this thesis, and therefore only a minimum amount of data necessary to demonstrate that 3.0 S subunits can be reassembled to 7.5 S tetramers is presented in this section.

While the sedimentation velocity studies reported in the preceeding sections were performed at protein

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concentrations above 2.0 mg/ml, reassembly experiments were performed at concentrations an order of magnitude lower. Lower concentrations were used for these studies because Deal (1969) demonstrated that reversal of dissociation of GAPD, from 8 M urea, decreases at protein concentrations above 0.04 mg/ml. Therefore, a sample of GAPD (0.2 mg/ml) was completely dissociated[2] by incubation at 0° in 5 mM ATP (0.2 M Tris, pH 8.5 and 0.1 M β-mercaptoethanol) for 6 hrs.

When a solution of GAPD dissociated at 0° by this procedure was (1) made 7.5% in sucrose[3] (by the addition of solid sucrose at 0°), (2) warmed to 23° for 2 hrs, (3) diluted 1:1 with the above buffer, and (4) layered on a sucrose gradient, the GAPD activity sedimented as a 7.5 S species. This is illustrated in Figure 6. It is seen in Figure 6 that the GAPD activity coincided with the 7.5 S activity peak of the lactic dehydrogenase (LDH) marker (Kaplan, 1964). For comparison, the sedimentation profile of a GAPD control sample treated in a similar manner, but minus ATP, is shown in Figure 7. This experiment proved that inactive 3.0 S monomers could be reassembled to yield

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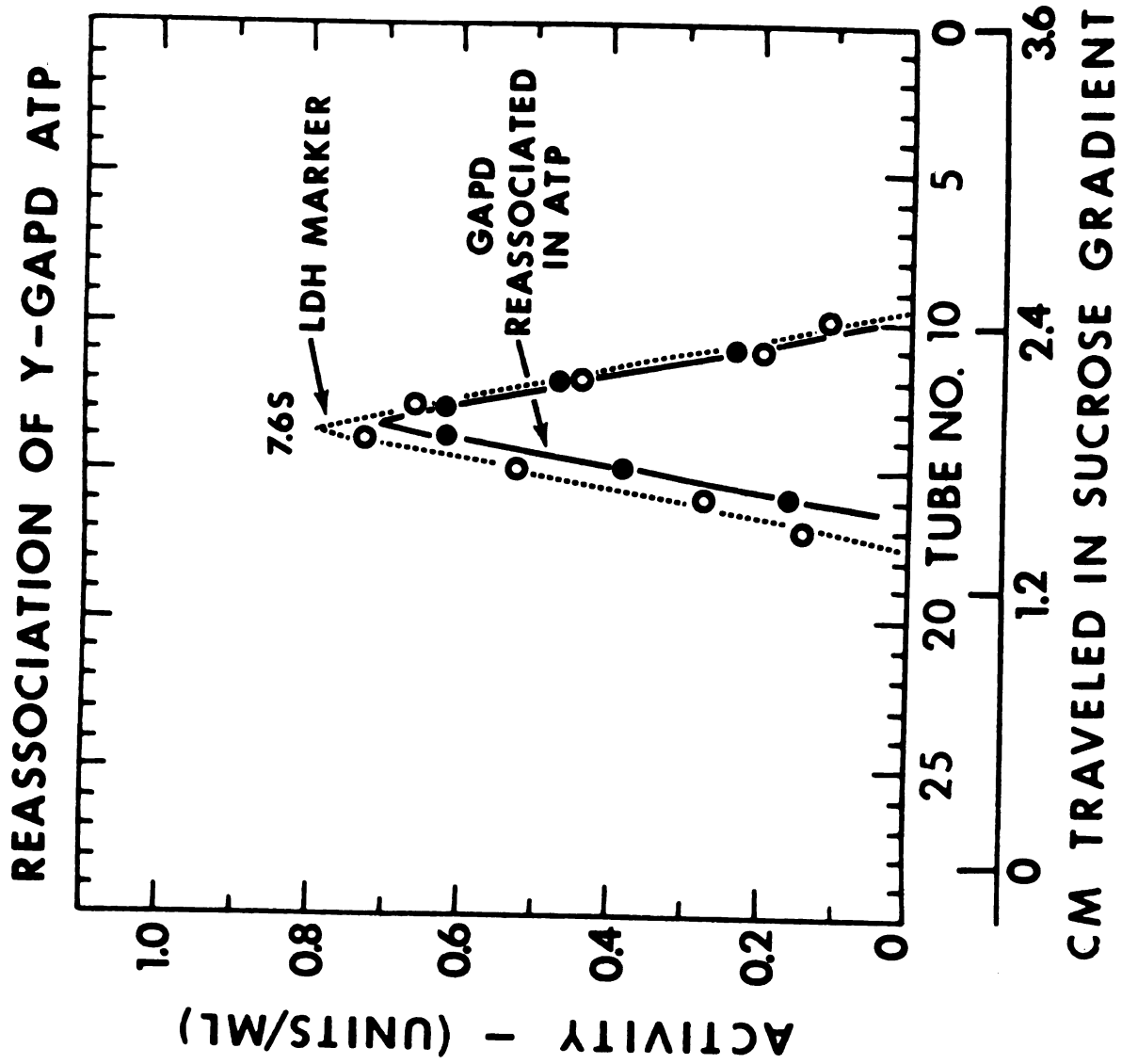
[2]This sample had lost all activity after 6 hrs and the dissociation was therefore considered complete.

[3]Sucrose was found to be required for reassembly. The effect of sucrose on reassembly is described more fully in Chapter Three, and the theoretical implications of this effect are discussed in Chapter Four.

Figure 6. Sucrose density gradient centrifugation pattern of glyceraldehyde-3-phosphate dehydrogenase dissociated in ATP and reassociated by warming to 23° in the presence of sucrose before centrifugation. See Text.

REASSOCIATION OF X GAPD


REASSOCIATION OF Y-GAPD ATP
7.6S
LDH MARKER
GAPD
REASSOCIATED
IN ATP
ACTIVITY - (UNITS/ML)
1.0
0.8
0.6
0.4
0.2
0
25
20
TUBE NO.
10
5
0
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

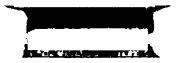
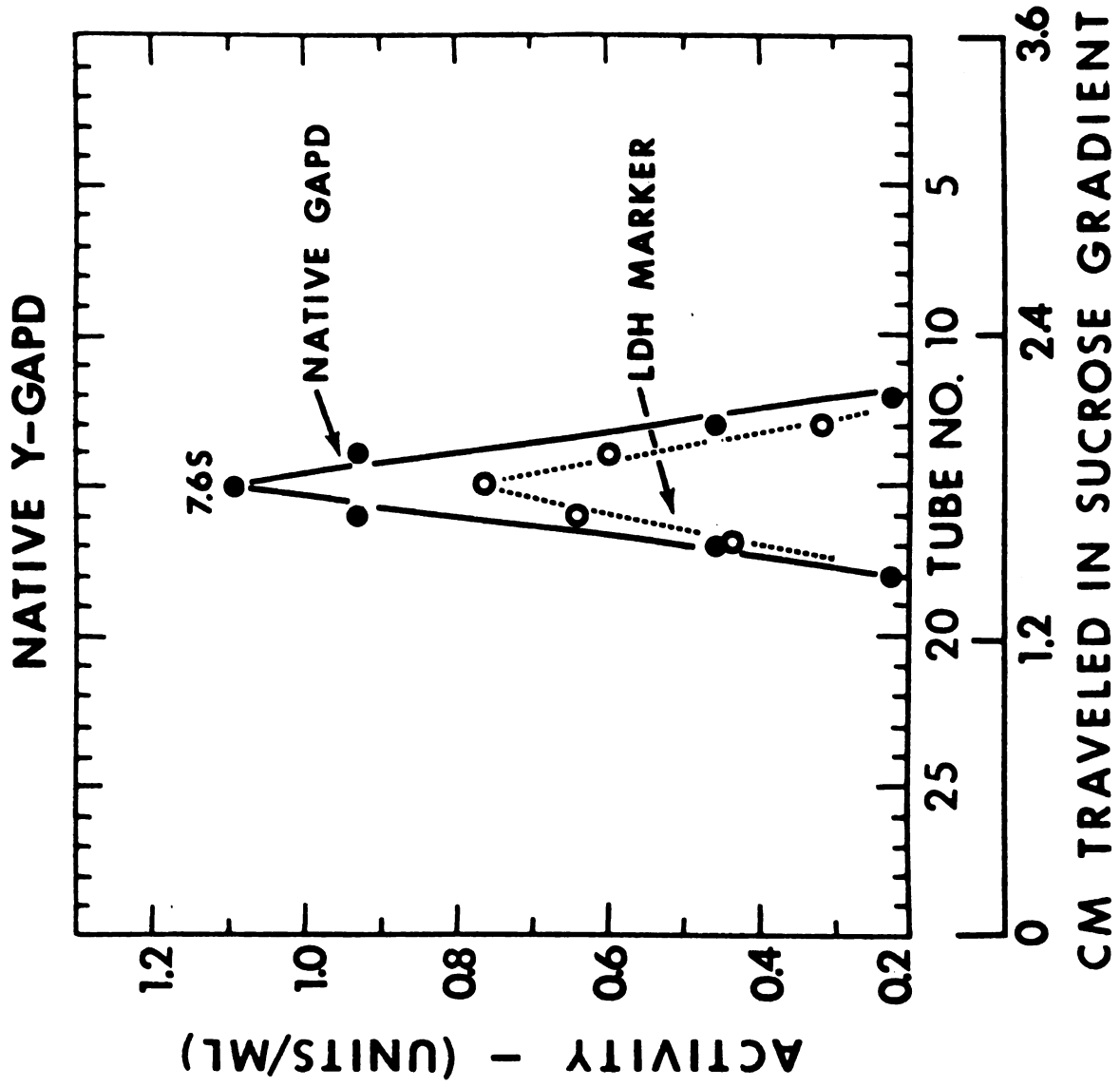


Figure 7. Sucrose density gradient centrifugation pattern of native tetrameric glyceraldehyde-3-phosphate dehydrogenase. See Text.


NATIVE Y-GAPD
NATIVE GAPD
LDH MARKER
7.6S
ACTIVITY - (UNITS/ML)
1.2
1.0
0.8
0.6
0.4
0.2
25
20
TUBE NO. 10
5
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

an active 7.5 S tetramer. To date, it has not been possible to reassemble either the 1.6 S or 0.9 S species obtained in ATP to the native tetramer. Deal (1969), however, has been able to reassemble subunits of GAPD (presumably 1.6 S) produced by dissociation in 8 M urea.

Other experiments demonstrated that after warming of dissociated GAPD subunits (3.0 S) to 23° in the presence of sucrose, the slower-moving electrophoretic band disappeared and only the native electrophoretic band was observed (see Figure 2). These experiments also illustrated the reversal of the structural changes produced by ATP at 0°.

DISCUSSION

PRODUCTION OF A 3.0 S AND A 1.6 S SUBUNIT. One explanation for the observation of both the 3.0 S and 1.6 S subunits is that the enzyme (1) first undergoes only a limited unfolding, (2) then dissociates, and (3) then unfolds rather extensively to yield the 1.6 S subunit. It is interesting that the sedimentation coefficient of 3.0 S is consistent with that expected for a folded monomeric subunit of molecular weight about 35,000 and the value of 1.6 S is identical to that found for unfolded subunits in urea (Deal, 1963; Deal and Holleman, 1964; Deal, 1969). For this reason, the 3.0 S subunits will be referred to as folded or globular subunits throughout this thesis.

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One interesting aspect of these structural studies is that a dimer intermediate is not observed in ultracentrifugal analysis of the dissociation process. This aspect of the dissociation process is discussed more fully in Chapter Four of this thesis.

REVERSAL OF DISSOCIATION. The discovery that 3.0 S monomers could be reassembled to 7.5 S tetramers was exciting because it provided a system ideally suited to investigate the assembly of folded or globular subunits to native enzyme. This is in contrast to most dissociation systems (e. g., urea or acid treatment) which produce extensive unfolding of the individual polypeptide chains as well as dissociation into subunits. The discovery that folded GAPD monomers could be reassembled to tetrameric enzyme was especially exciting, since Deal (1969) had reassembled highly unfolded subunits (produced in 8 M urea) of GAPD to the native tetramer. Thus, a comparison of the reassembly processes for the two types of subunits should allow one to separate folding and association effects for the overall assembly of tetrameric enzyme from individual, unfolded polypeptide chains. Since the individual polypeptide chains must fold and assemble in vivo after they are synthesized on polysomes, it is hoped that the studies of the reassociation of folded subunits presented in this thesis will provide an insight into the overall process of protein synthesis, folding and association which occurs in vivo.

MECHANISM OF DISSOCIATION AND THE OVERALL CONTROL OF GAPD. Because of structural similarities it seems reasonable to suspect that ATP is binding at an NAD site, and that the additional phosphate and additional negative charge on the terminal phosphate of ATP may be responsible for the destabilization.

The conditions of ATP concentration (Betz and Moore, 1967), salt, temperature, pH and protein concentration expected in vivo in yeast are resonably close to those described in this work. Furthermore, since only a small structural change may be necessary to increase GAPD susceptibility to yeast proteolytic enzymes, the destabilization by ATP could be a physiologically significant process. Studies concerning the role that this process might play in the metabolism of yeast have led to results presented in Chapter Five of this thesis.

For comparison, it is interesting to note that rabbit muscle GAPD shows dissociation by ATP (Constantinides and Deal, 1969), by urea (Deal and Holleman, 1964; Deal, 1969), and by low temperatures at low protein concentrations (Constantinides and Deal, 1967; 1970) and all of these dissociation processes are inhibited by NAD.

The stabilization by NAD of yeast GAPD against ATP-induced destabilization and dissociation provides a mechanism involving NAD in control of degradation of this enzyme. These effects, together with the results (Deal, 1967; 1969; Constantinides and Deal, 1967) suggesting a

role for NAD in accelerating the rate of synthesis of the enzyme, provide mechanisms for a logical, systematic, and effective control of both synthesis and degradation of GAPD by NAD, with the degradative control being shared antagonistically with ATP.

MATERIALS AND METHODS

ULTRACENTRIFUGAL ANALYSIS. All experiments were performed in a Spinco Model E ultracentrifuge equipped with either phase-plate schlieren optics or Rayleigh interference optics. The exact temperatures were obtained with a calibrated rotor and temperature indicator control (RTIC) unit. Photographic plates were measured with a Bausch and Lomb microcomparator.

SEDIMENTATION VELOCITY ANALYSIS. Sedimentation velocity experiments were performed at 56,000 RPM in an An-D Rotor using wedge and plane single-sector capillary synthetic-boundary cells. The sample volumes were approximately 0.35 ml, and approximately 0.15 ml were used in the solvent chamber. After the boundary formation, the total volume was therefore approximately 0.50 ml. Sedimentation coefficients were calculated from the expression:

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

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

SEDIMENTATION EQUILIBRIUM ANALYSIS. Rayleigh double sector interference cells were used for these studies with an An-G or an An-D rotor. The molecular weight values were calculated using the equation:

$$M = \frac{2\ RT}{(1-\bar{v}\rho)\ w^2} \quad \frac{2.303\ (d\ \log\ c)}{(dr^2)}$$

where T is the absolute temperature, R is the gas constant of 8.314×10^7 ergs/mole/degree, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solution, w is the angular velocity of the rotor in radians/sec, and d log c/d (r^2) is the slope of a plot of concentration as a function or radial distance, r, squared (semi-log plot).

ELECTROPHORESIS. Electrophoresis was performed in 7% polyacrylamide at pH 8.3 (Tris/glycine buffer). A 5% polyacrylamide stacking gel was also used. Protein samples were applied in 10% sucrose. Approximately 50 µg of protein was applied per gel. The protein bands were stained with 0.5% amido-schwartz and de-stained with 7.5% acetic acid.

ENZYME PREPARATION AND ASSAY. The enzyme assay and preparation is completely described in Methods and Materials, Chapter Three of this thesis.

CHAPTER THREE

SYSTEMATIC STUDY OF THE VARIABLES AFFECTING DISSOCIATION AND REASSOCIATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AT O° IN THE PRESENCE OF ATP

ABSTRACT

Tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase undergoes a time-dependent inactivation in the presence of adenosine 5'-triphosphate as a result of dissociation into monomeric subunits. Optimal conditions, which yield complete inactivation in 5 hours are: (1) 1-2 m$\underline{M}$ adenosine 5'-triphosphate, (2) 0°, (3) protein concentrations of 0.03-0.1 mg per ml, (4) pH 9.0, and (5) 0.1 $\underline{M}$ β-mercaptoethanol. Transition points (half-maximal loss of activity in 5 hours) are: (1) 0.5 m$\underline{M}$ adenosine 5'-triphosphate, (2) 12°, (3) 0.5 mg per ml and (4) pH 8.6. Adenosine 5'-monophosphate and adenosine 3',5'-monophosphate do not dissociate the enzyme. They, and all the substrates of the reaction, partially protect the enzyme from dissociation. Dissociation and inactivation are completely reversed by warming to 17°. Reassembly is greatly stimulated by adenosine 5'-triphosphate and by 10% sucrose. Optimal reassembly conditions are: (1) 0.04 mg per ml protein, (2) pH 7.0, (3) 1-2 m$\underline{M}$ adenosine 5'-triphosphate, (4) 17°, (5) 10% sucrose and (6) 0.1 $\underline{M}$ β-mercaptoethanol. Inactivation and dissociation apparently result from electrostatic repulsion. The results are discussed in terms of a possible role for this enzyme in the regulation

of glycolysis. Since this dissociation produces fairly compact subunits, association of folded monomers to tetramers may be studied independently of the polypeptide folding.

INTRODUCTION

Numerous examples of the effects of adenine nucleotides on the structure and activity of various enzymes of carbohydrate metabolism have previously been reported (Atkinson, 1965, 1966; Wood, 1966; Stadtman, 1966; Scutton and Utter, 1968). Recent investigations in this laboratory have demonstrated that ATP and other adenine-containing compounds have pronounced effects upon the structure and catalytic activity of yeast glyceraldehyde-3-phosphate dehydrogenase (GAPD). Glyceraldehyde-3-phosphate dehydrogenase is inactivated by ATP as a result of dissociation of the native tetrameric enzyme into monomeric subunits (Stancel and Deal, 1968; Stancel, 1969). ATP and other adenine nucleotides also produce an instantaneous inhibition of the catalytic activity of yeast glyceraldehyde-3-phosphate dehydrogenase (Yang and Deal, 1969a), which is measured before any appreciable dissociation occurs.

This paper reports the reversal of the dissociation of yeast glyceraldehyde-3-phosphate dehydrogenase by ATP (Stancel and Deal, 1968) and a detailed characterization of the dissociation and reassembly processes (Stancel, 1969). Other papers (Constantinides and Deal, 1969) from this laboratory show that rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is also reversibly dissociated into subunits by ATP and that the characteristics of that system are greatly different from the yeast system.

There were several strong incentives for characterizing the dissociation of yeast glyceraldehyde-3-phosphate dehydrogenase by ATP. First, the process might be involved in metabolic control; other work in this laboratory has shown that incubation of glyceraldehyde-3-phosphate dehydrogenase with ATP greatly increases the susceptibility of the enzyme to digestion and inactivation by chymotrypsin (Yang and Deal, 1969b). Also, Williamson (1965, 1967) has shown that in rat liver and heart, the glyceraldehyde-3-phosphate dehydrogenase reaction is far from equilibrium and pointed out that it is a potential control point for glycolysis. If, as expected, the reaction is also not in equilibrium in yeast and rabbit muscle, then a remarkable situation exists, because glyceraldehyde-3-phosphate dehydrogenase constitutes 20% of the total soluble protein in yeast (Krebs et al., 1953), and about 10% in rabbit muscle (Cori et al., 1948).

In contrast to most dissociating agents, which extensively unfold the polypeptide chains of yeast glyceraldehyde-3-phosphate dehydrogenase, ATP produces a fairly compact subunit. This system seems ideal to investigate several important biochemical questions: (1) Can individual subunits exhibit catalytic activity? (2) Do subunits associate after synthesis or during synthesis? (3) Do metabolites affect the association of subunits?

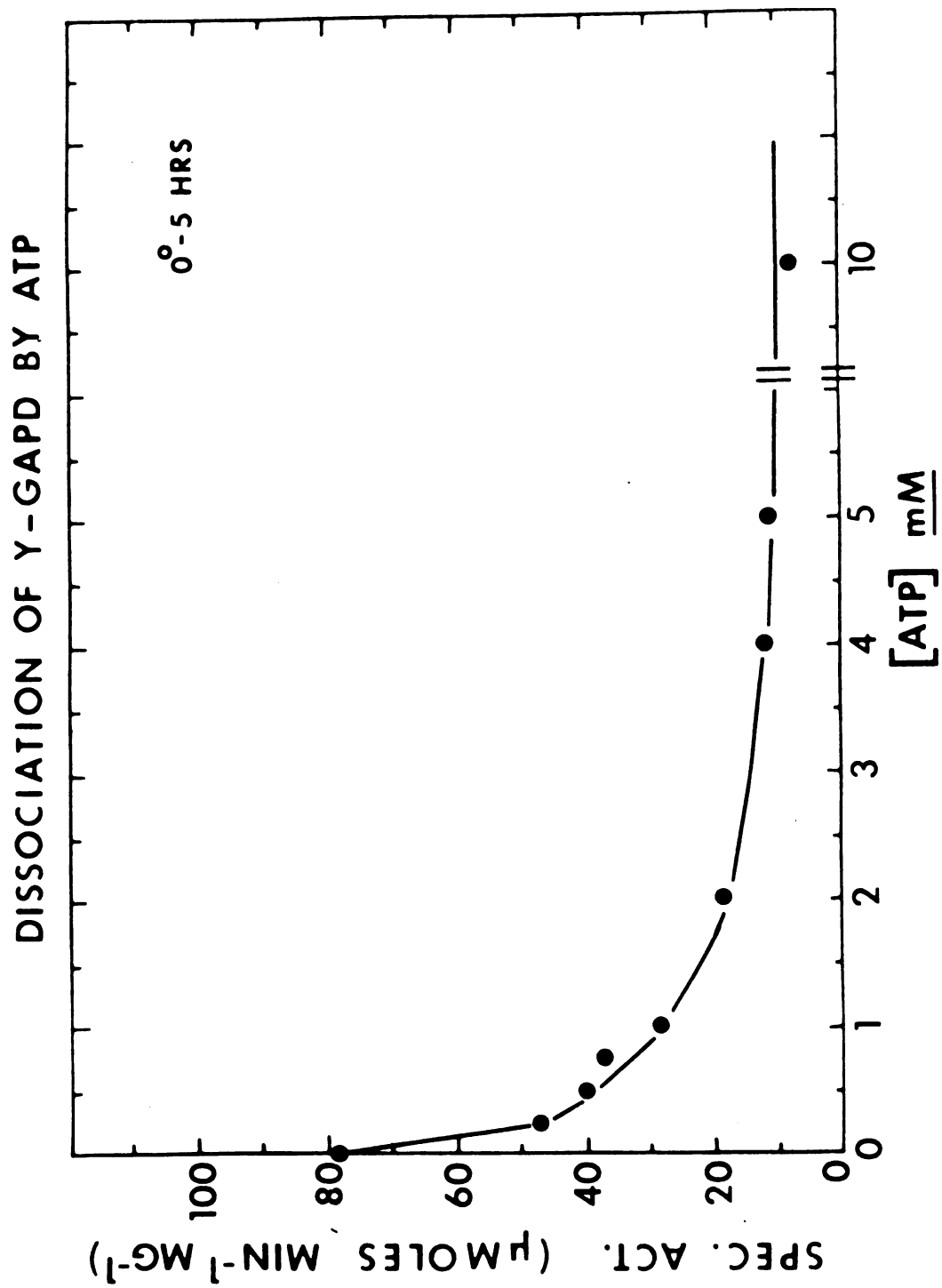
RESULTS

Except for the particular variable under study, all experiments in this section used the following procedures and conditions. The enzyme (0.2 mg per ml) was inactivated and dissociated at 0° for 5 hr in solutions containing 0.1 M Tris (HCl), pH 8.5, 0.1 M β-mercaptoethanol, and 4 mM ATP. Control enzyme samples were treated identically, except ATP was omitted.

EFFECT OF ATP CONCENTRATION ON INACTIVATION AND DISSOCIATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. Yeast glyceraldehyde-3-phosphate dehydrogenase showed a time-dependent loss of activity upon incubation with ATP. As seen in Figure 8, at ATP concentrations of 2 mM or higher, about 75% of the activity was lost after 5 hr. At higher concentrations the curve leveled off. Half-maximal loss of activity occurred at an ATP concentration of 0.4-0.5 mM, a value which may be taken as the approximate dissociation constant for the enzyme-ATP complex. The residual ATP added into the assay with the enzyme was too low to inhibit the enzyme (Yang and Deal, 1969a); therefore, the activity loss was due to structural changes which were not immediately reversible.

The inactivation by ATP was accompanied by dissociation of the enzyme into a 2.8 S species, while the enzyme without ATP sedimented as the tetrameric 7.6 S species. This is shown by the sucrose density gradient

Figure 8. Activity of glyceraldehyde-3-phosphate dehydrogenase (0.2 mg/ml) after incubation for 5 hr at 0° with ATP in 0.1 M Tris, pH 8.5, and 0.1 M β-mercaptoethanol. An ammonium sulfate suspension of the enzyme was centrifuged, the pellet was taken up in 0.1 M Tris, pH 8.5, and 0.1 M β-mercaptoethanol, and the enzyme was diluted into a series of samples with varying amounts of ATP.


DISSOCIATION OF Y-GAPD BY ATP
0°-5 HRS
SPEC. ACT. (μMOLES MIN⁻¹ MG⁻¹)
0
20
40
60
80
100
0
1
2
3
4
5
10
[ATP] mM

pattern given in Figure 9. It should be noted that since the 2.8 S species was enzymatically inactive, a reassembly process (see later section) was used to detect the otherwise inactive enzyme in the gradient; the fractions collected after centrifugation were warmed to 23°.

EFFECT OF PROTEIN CONCENTRATION. Dissociating-associating systems are expected to exhibit a strong dependence on protein concentration. The results shown in Figure 10 indicate that the inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase by ATP increased as the protein concentration was lowered. Inactivation was complete at protein concentrations lower then 0.1 mg per ml, and inversely related to protein concentration in the concentration range 0.1 to 1.0 mg per ml. In contrast, the control enzyme with no ATP was inactivated only slightly at lower protein concentrations. These data provided further evidence that the inactivation involved dissociation into subunits.

EFFECT OF TEMPERATURE. The dissociation showed a marked dependence on temperature as illustrated in Figure 11; the specific activity of the control samples remained essentially constant over the temperature range studied. Although no activity was lost at 23°, a conformational change occurred as indicated by a difference spectrum.

EFFECT OF pH. Enzyme samples were incubated for 5 hr at various pH values in 0.1 M Tris buffers. As shown in

Figure 9. Sucrose density gradient centrifugation of glyceraldehyde-3-phosphate dehydrogenase (0.25 mg/ml) after 9 hr incubation at 0° with or without 5 mM ATP in the standard dissociation medium (Figure 8). The samples were then centrifuged for 22 hr at 0° in the sucrose gradients with the same composition. The fractions were collected at 0°, warmed to 23° for 2 hr and assayed. Beef heart lactic dehydrogenase was used as a marker. See text for further details.

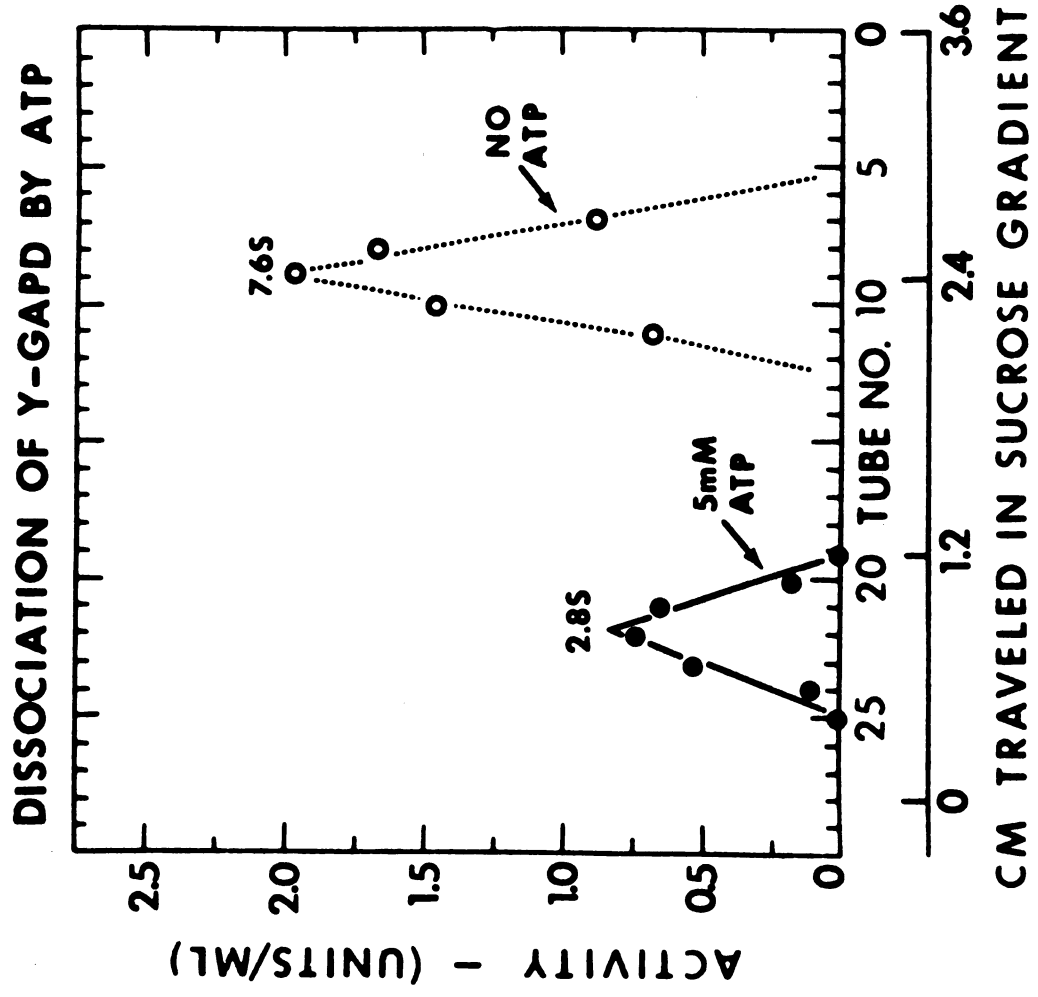

DISSOCIATION OF Y-GAPD BY ATP
ACTIVITY - (UNITS/ML)
2.5
2.0
1.5
1.0
0.5
0
7.6S
NO ATP
2.8S
5mM ATP
0
5
10
20
25
TUBE NO.
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

Figure 10. Effect of protein concentration on inactivation of glyceraldehyde-3-phosphate dehydrogenase by 4 mM ATP. See text for further details.

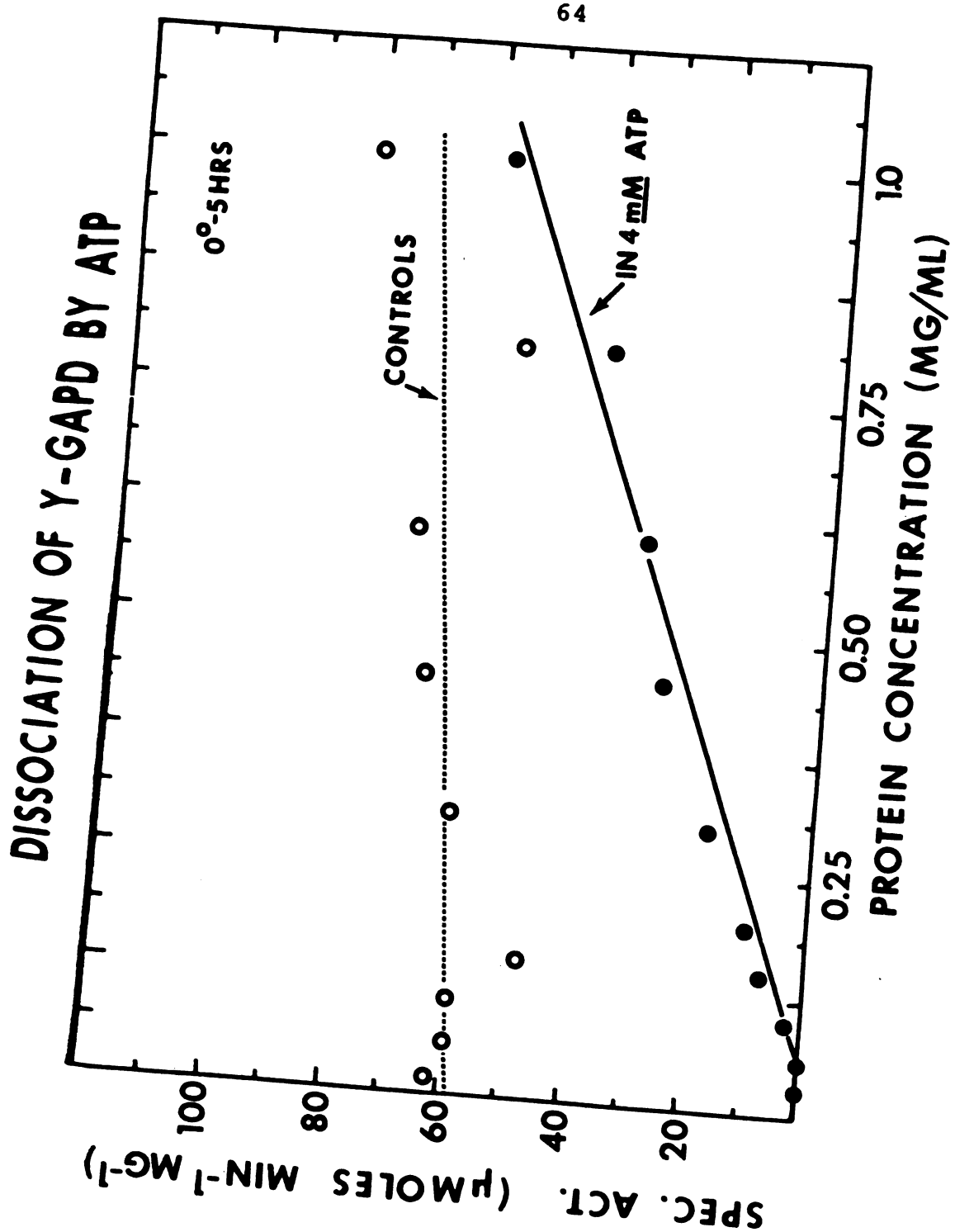

DISSOCIATION OF Y-GAPD BY ATP
0°-5HRS
CONTROLS
IN 4 mM ATP
SPEC. ACT. (μMOLES MIN-1 MG-1)
100
80
60
40
20
0.25
0.50
0.75
1.0
PROTEIN CONCENTRATION (MG/ML)

Figure 11. Effect of temperature of incubation on inactivation of glyceraldehyde-3-phosphate dehydrogenase by 4 mM ATP. See text and legend of Figure 8.

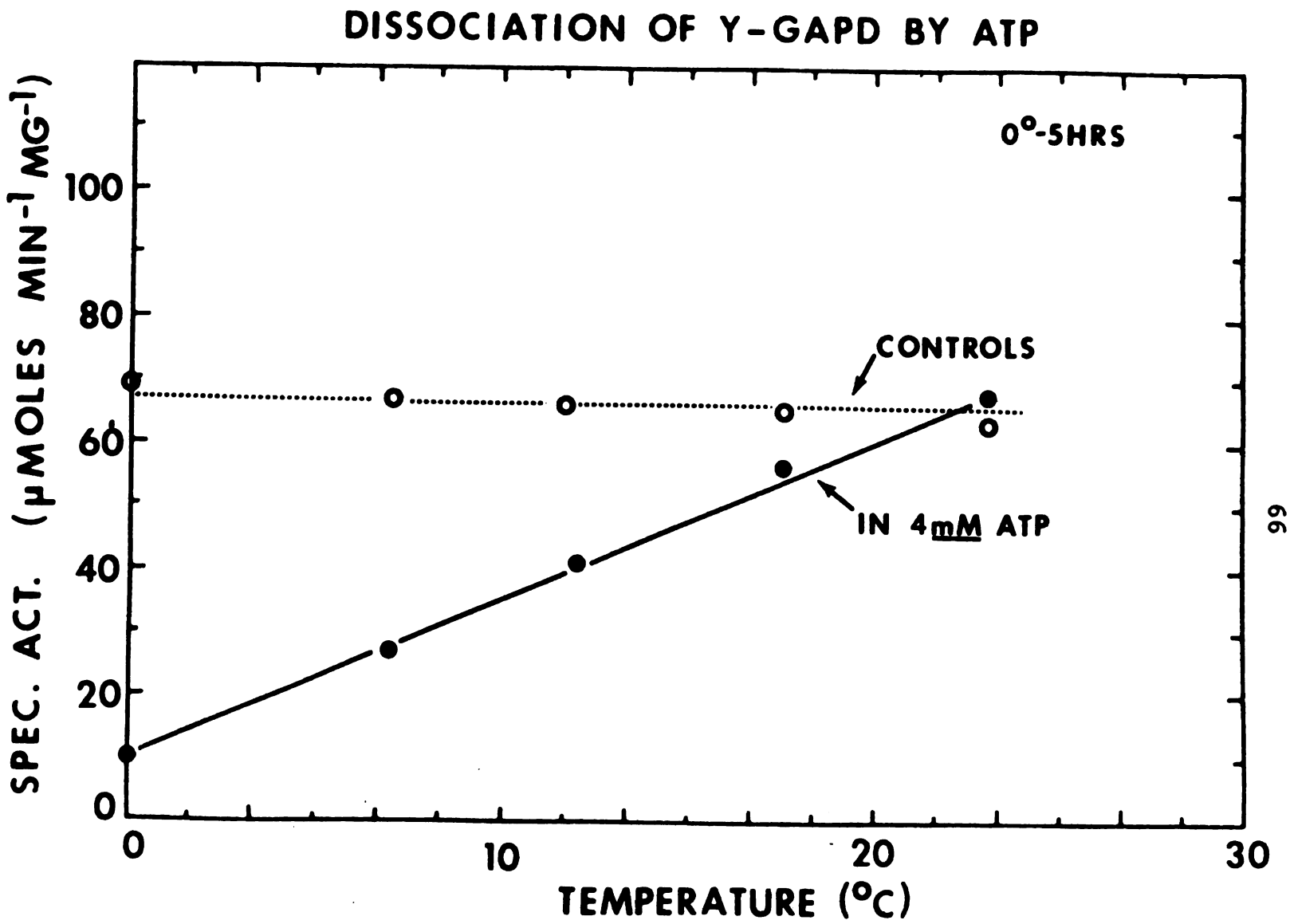

DISSOCIATION OF Y-GAPD BY ATP
0°-5HRS
CONTROLS
IN 4mM ATP
SPEC. ACT. (μMOLES MIN-1 MG-1)
100
80
60
40
20
0
0
10
20
30
TEMPERATURE (°C)

Figure 12, inactivation by ATP was favored at high pH; the control samples exhibited constant activity over the pH range examined.

The pH-dependent transition is centered at about pH 8.6 (Figure 12). The pH dependence was not a result of titration of the Tris buffer, since samples in Tris buffers of fixed pH (8.5), but varying concentrations (0.02-0.2 M), gave similar results, as did other buffers.

SPECIFICITY OF THE DISSOCIATION PROCESS FOR ATP. Several competitive inhibitors (Yang and Deal, 1969a), including ATP, ADP, AMP and 3',5'-AMP were examined for dissociating ability. Samples of glyceraldehyde-3-phosphate dehydrogenase (0.2 mg per ml) were incubated at 0° for 5 hr in 0.1 M Tris buffer, pH 8.1, and 0.1 M β-mercaptoethanol with 1 mM concentrations of either ATP, ADP, AMP, or 3',5'-AMP. The control was treated similarly but no nucleotide was added. As shown in Figure 13, AMP and 3',5'-AMP had no appreciable effect. The ADP and ATP samples lost 20% and 60%, respectively, of the control activity. These results indicated that the additional phosphoryl groups on the ATP molecule were responsible for the dissociation.

PROTECTIVE EFFECTS OF 3',5'-AMP, AMP, ADP, AND SUBSTRATES. In light of the findings in the preceding section and the discovery that ATP and other adenine nucleotides are competitive inhibitors with respect to NAD (Yang and Deal, 1969a), it was of interest to determine whether the

Figure 12. Effect of pH of Tris buffer on inactivation of glyceraldehyde-3-phosphate dehydrogenase by 4 mM ATP. See text and legend of Figure 8.

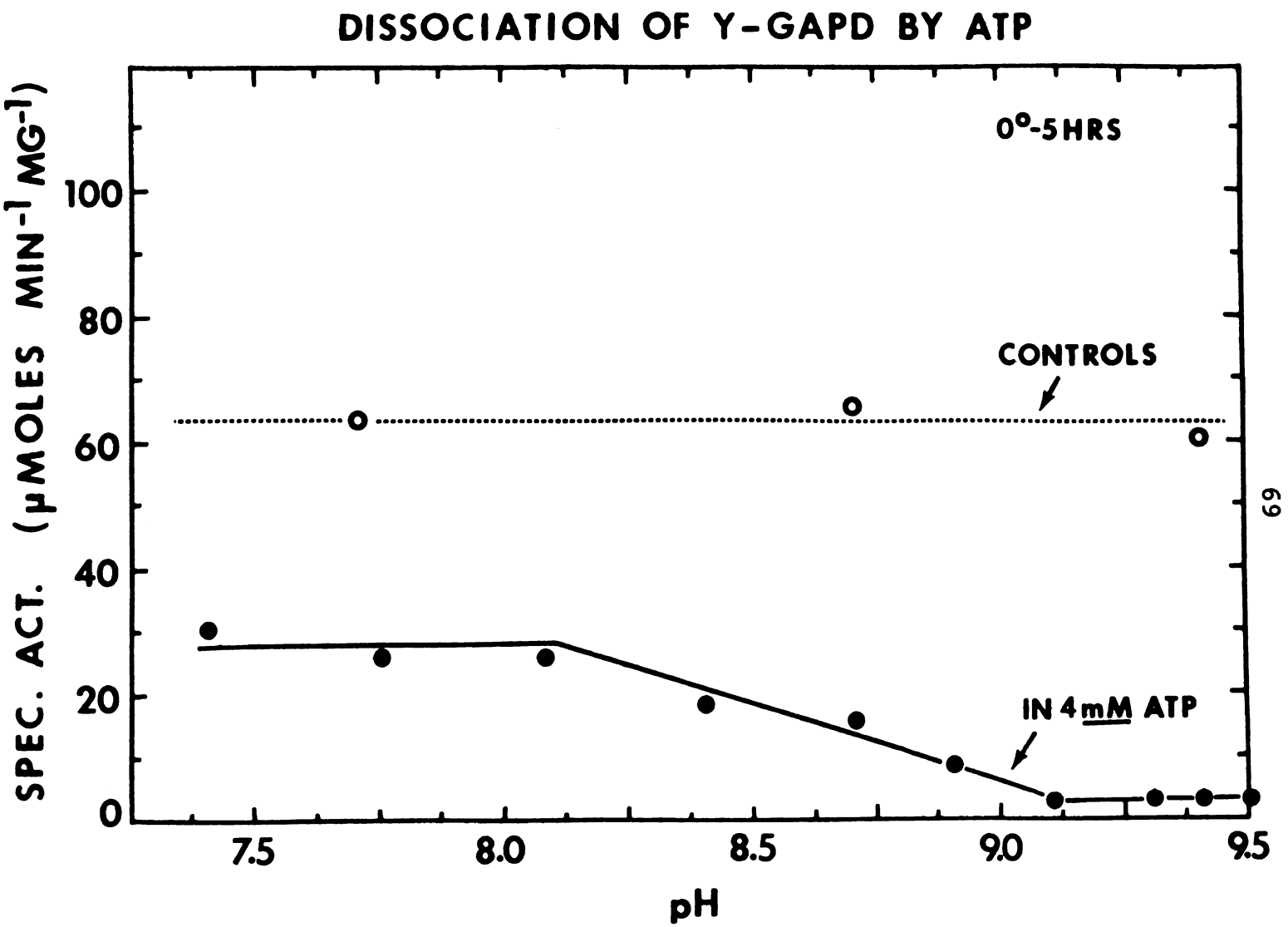
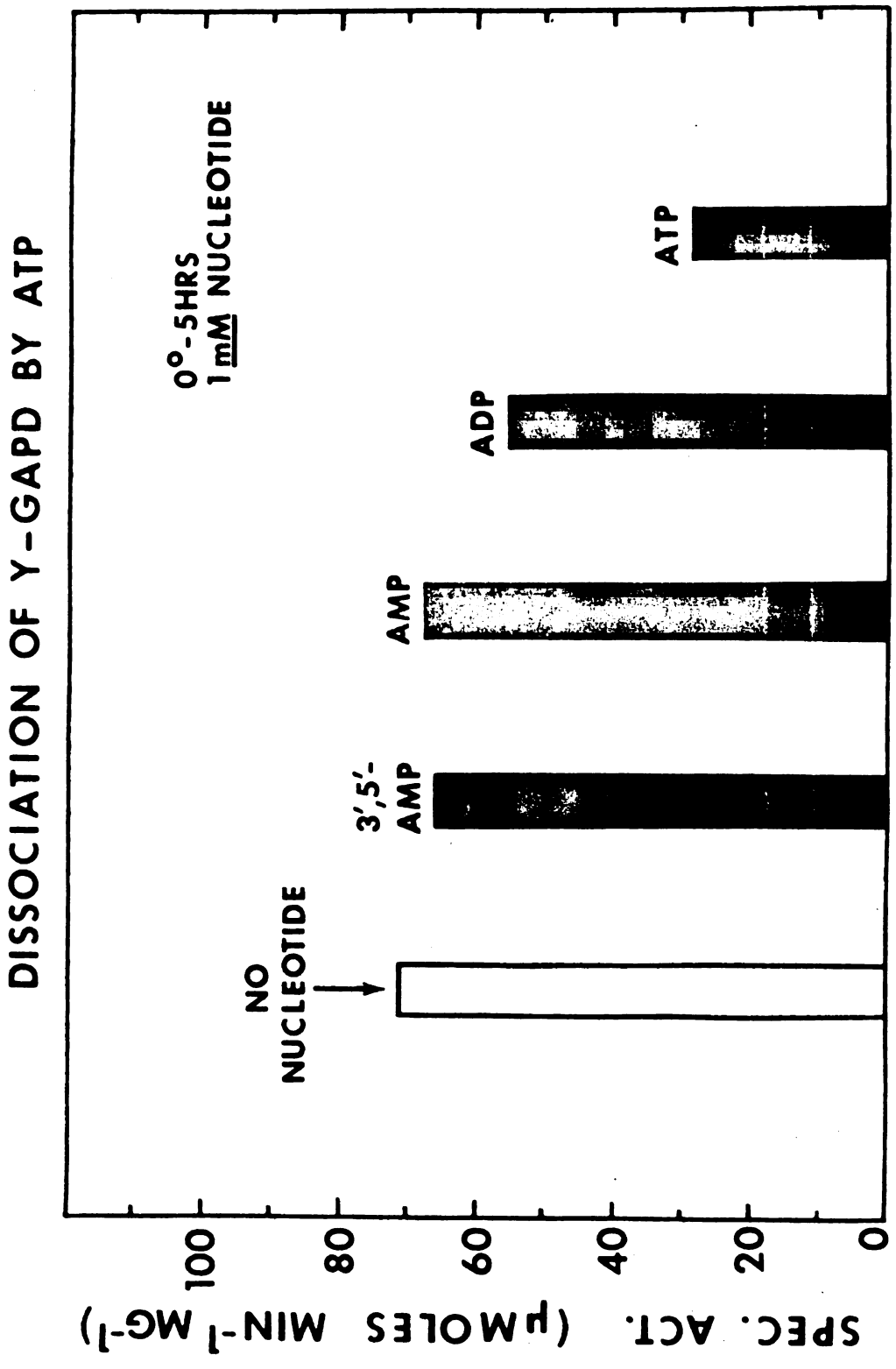

DISSOCIATION OF Y-GAPD BY ATP
0°-5HRS
CONTROLS
IN 4 mM ATP
SPEC. ACT. (μMOLES MIN-1 MG-1)
100
80
60
40
20
0
7.5
8.0
8.5
9.0
9.5
pH

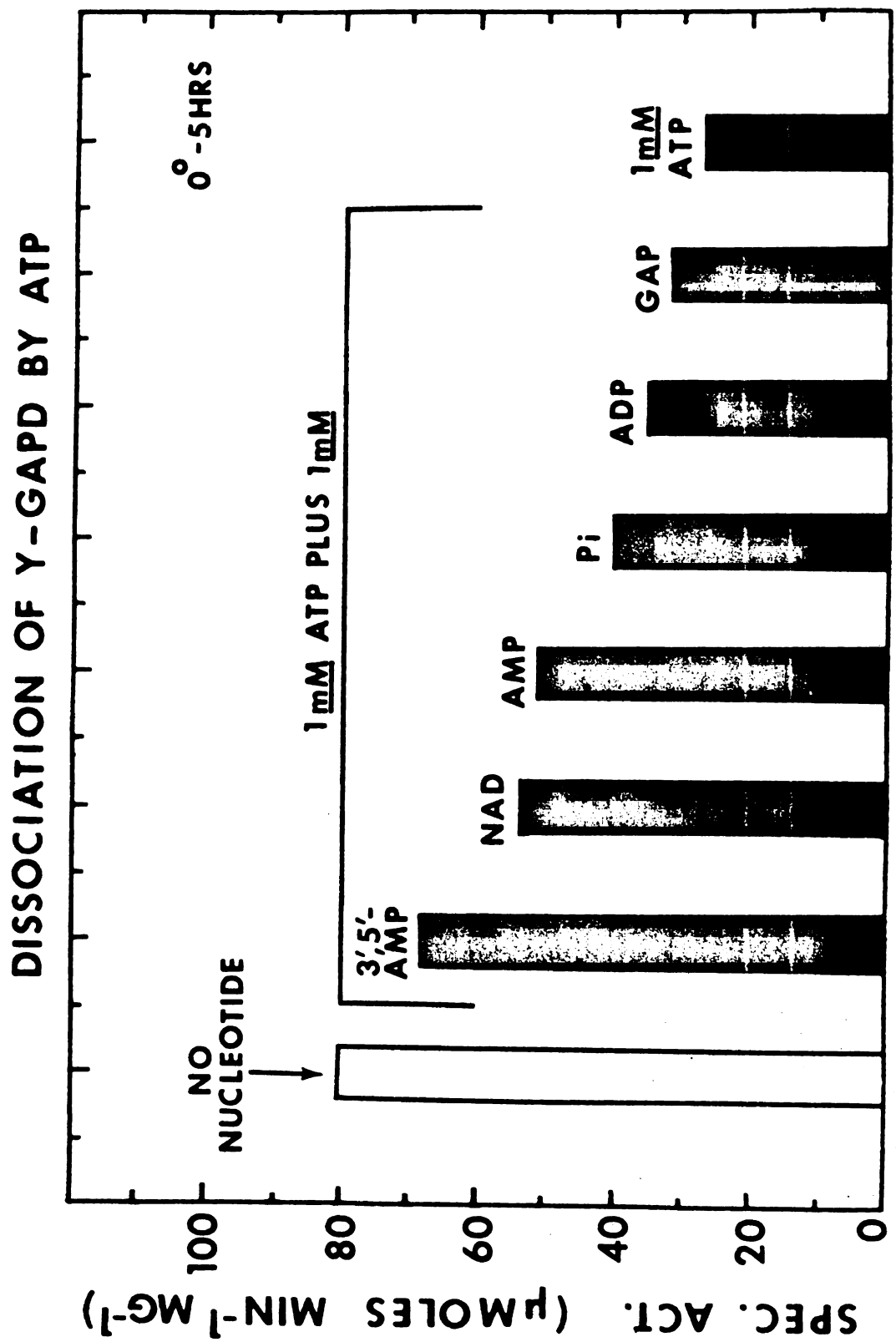
Figure 13. Activity of glyceraldehyde-3-phosphate dehydrogenase (0.2 mg/ml) after incubation at 0° for 5 hr with 1 mM adenine nucleotides in 0.1 M Tris, pH 8.1, and 0.1 M β-mercaptoethanol.


DISSOCIATION OF Y-GAPD BY ATP
0°-5HRS
1 mM NUCLEOTIDE
NO NUCLEOTIDE
3',5'-AMP
AMP
ADP
ATP
SPEC. ACT. (μMOLES MIN-1 MG-1)
0
20
40
60
80
100

substrates and above compounds protected glyceraldehyde-3-phosphate dehydrogenase from dissociation by ATP. As in the previous experiment, samples of glyceraldehyde-3-phosphate dehydrogenase (0.2 mg per ml) were incubated with or without added nucleotides. One control contained no ATP; a second control contained only 1 mM ATP. The test samples all contained 1 mM ATP plus 1 mM additional nucleotide (or substrate), except for the 10 mM phosphate sample. All these compounds protected the enzyme from inactivation (Figure 14). The order of effectiveness was 3',5'-AMP > NAD > AMP > P_i > ADP > glyceraldehyde-3-phosphate.

REVERSAL OF THE INACTIVATION AND DISSOCIATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. The temperature dependence of the dissociation process (Figure 11) suggested a temperature-dependent equilibrium existed and that activity might be regained by warming the dissociated enzyme. The initial reassembly studies yielded only about 30% of the control value upon warming the samples to 23°. With 10% sucrose, there was essentially complete recovery. The sucrose effect is nonspecific, since maltose, fructose, or glucose also increased recovery of activity. Studies with different concentrations of sucrose indicated the maximal effect was achieved with about 7.5-10% sucrose. A sucrose concentration of 10% was used to provide a safe margin of error. Other experiments indicated that the

Figure 14. Protective effects of adenine nucleotides and substrates against inactivation of glyceraldehyde-3-phosphate dehydrogenase by ATP. See text and legend of Figure 13.


DISSOCIATION OF Y-GAPD BY ATP
SPEC. ACT. (μMOLES MIN-1 MG-1)
100
80
60
40
20
0
0°-5HRS
NO NUCLEOTIDE
1mM ATP PLUS 1mM
3',5'-AMP
NAD
AMP
Pi
ADP
GAP
1mM ATP

reassembled enzyme had the same K_m, sedimentation coefficient, and electrophoretic mobility on polyacrylamide disc electrophoresis as the native enzyme.

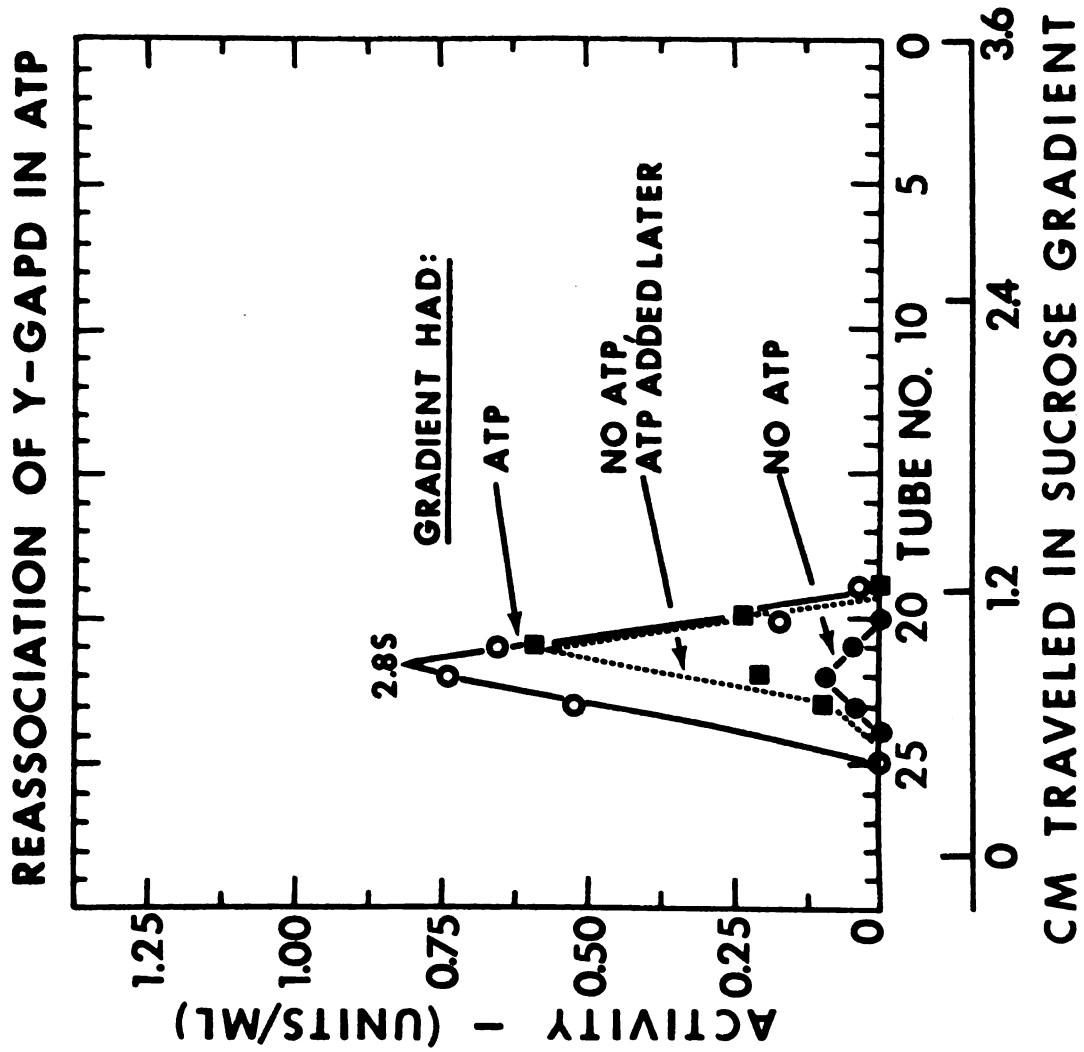
This surprising observation that the dissociation was completely reversed, even in the presence of the dissociating agent, raised the question of what role, if any, ATP played in the reassembly process.

To answer this question a sample of glyceraldehyde-3-phosphate dehydrogenase (0.25 mg per ml) was completely dissociated by a 9 hr incubation at 0° in 5 m<u>M</u> ATP, and 3 aliquots were withdrawn. One aliquot was layered on a sucrose gradient without ATP. If ATP were <u>not</u> required for reassembly, the collected fractions from this aliquot should regain enzymatic activity if warmed to 23° before being assayed. This was <u>not</u> observed; only a trace of activity was recoverable in the monomer position, as indicated by the lower solid curve in Figure 15.

A second aliquot of the original dissociated enzyme was layered on a gradient that contained 5 m<u>M</u> ATP. When the fractions collected from this sample were warmed and assayed, substantial activity was recovered (Figure 15, upper solid curve).

The third aliquot was layered on a gradient without ATP, but concentrated ATP was added to the collected fractions (to a final concentration of 5 m<u>M</u>) before they were

Figure 15. Effect of ATP on reactivation of inactive 2.8 S monomers. The samples were dissociated at 0° for 9 hr in the standard dissociation medium (Figure 8) but with 5 mM ATP. They were centrifuged for 22 hr at 0° in sucrose gradients with the same composition, except ATP was omitted where indicated. The fractions were collected at 0° and then incubated for 2 hr at 23°. See text for further details.


REASSOCIATION OF Y-GAPD IN ATP
ACTIVITY - (UNITS/ML)
1.25
1.00
0.75
0.50
0.25
0
2.8S
GRADIENT HAD:
ATP
NO ATP, ATP ADDED LATER
NO ATP
0
5
10
20
25
TUBE NO.
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

warmed to 23°. The dashed line (Figure 15) shows that ATP greatly increased activity recovery over that in its absence.

Thus ATP did not inhibit the reassembly and both ATP and 10% sucrose had to be present in the reassembly solution to achieve maximal recovery of activity.

For all the following reassembly studies, the standard dissociation procedure was changed to a 12 to 14 hr incubation at 0° in 10 m$\underline{M}$ ATP, 0.02 $\underline{M}$ Tris (pH 8.5), 0.1 $\underline{M}$ β-mercaptoethanol with 0.4 mg per ml protein. The dissociated enzyme samples were then diluted into a reassembly mixture at 0°. The higher concentrations of ATP and protein in the dissociation mixture compensated for the dilution, so that the final concentrations under which reassembly occurred were the same as those for the previously described dissociation experiments. The standard final reassembly conditions were: 0.2 mg per ml glyceraldehyde-3-phosphate dehydrogenase, 0.1 $\underline{M}$ β-mercaptoethanol, 5 m$\underline{M}$ ATP, 10% sucrose and 0.2 $\underline{M}$ Tris, pH 7.0.

The activity recovery in the following experiments was only 80-85% of that previously observed (Figure 11), presumably owing to the longer incubation times (12-14 hr), since the subunits appeared to undergo a slow, irreversible side reaction (half-time approximately 24 hr) at 0°. The longer incubation times were required to obtain complete dissociation at the higher protein concentrations used.

EFFECT OF pH ON REASSEMBLY. After incubation at 0°, aliquots of the inactive monomers were diluted 1:1 into a series of reversal mixtures at 0° containing 0.4 M Tris buffers of varying pH. The samples were warmed at 23° for 2 hr and assayed. As shown in Figure 16, there was a slight dependence of the reassembly on pH in the range of 7.0-8.5 and at higher pH values the activity recovery decreased even more. Other experiments showed that activity recovery was less in imidazole than in Tris buffers at given values of pH.

EFFECT OF TEMPERATURE ON REASSEMBLY. Inactive monomers were diluted into the reassembly mixture at 0° and warmed to various temperatures for 1 hr. There was a sharp dependence of activity recovery on temperature (Figure 17). These samples were also assayed after 3 and 4 hr at the indicated temperatures and equilibrium appeared to be established within 2-3 hr in the temperature range of 0-12°. Presumably tetramers, dimers and monomers are all present in this equilibrium mixture in this temperature range. Since incubation at 17° appeared to yield maximum reassembly under these conditions (Figure 17) it was used as the optimal reassembly temperature. This is very near the optimal temperature for reassembly of the urea-dissociated subunits (Deal, 1969).

EFFECT OF PROTEIN CONCENTRATION ON REASSEMBLY. Aliquots were removed from a stock solution of inactive monomers (0.4 mg per ml) and diluted with various amounts of

Figure 16. Effect of pH (0.2 M Tris buffers) on reassociation of glyceraldehyde-3-phosphate dehydrogenase subunits (0.2 mg/ml) by warming to 23° for 2 hr. The samples also contained 5 mM ATP, 0.1 M β-mercaptoethanol and 10% sucrose, as described in Methods.

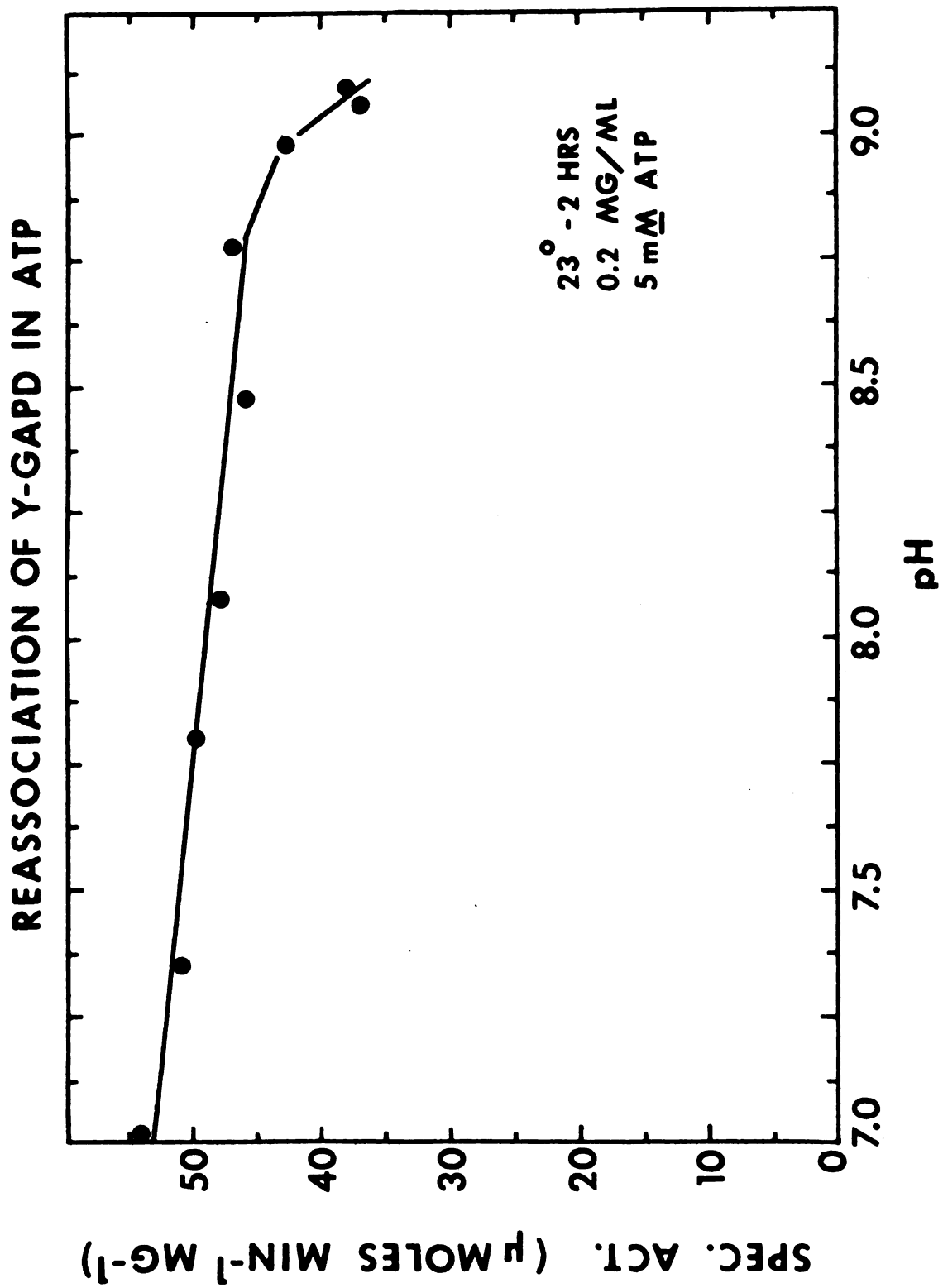
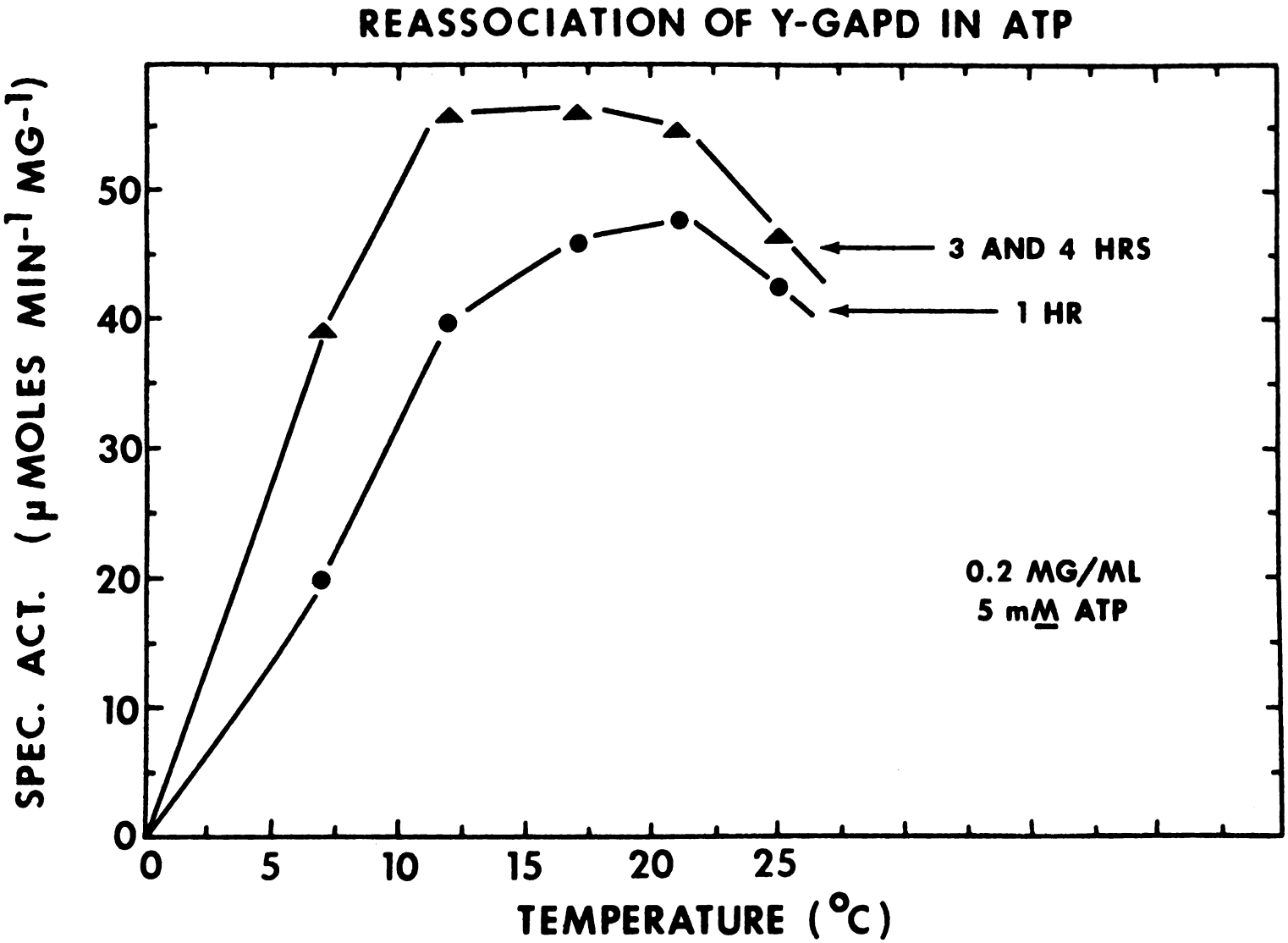

REASSOCIATION OF Y-GAPD IN ATP
23° -2 HRS
0.2 MG/ML
5 mM ATP
SPEC. ACT. (μ MOLES MIN-1 MG-1)
0
10
20
30
40
50
pH
7.0
7.5
8.0
8.5
9.0

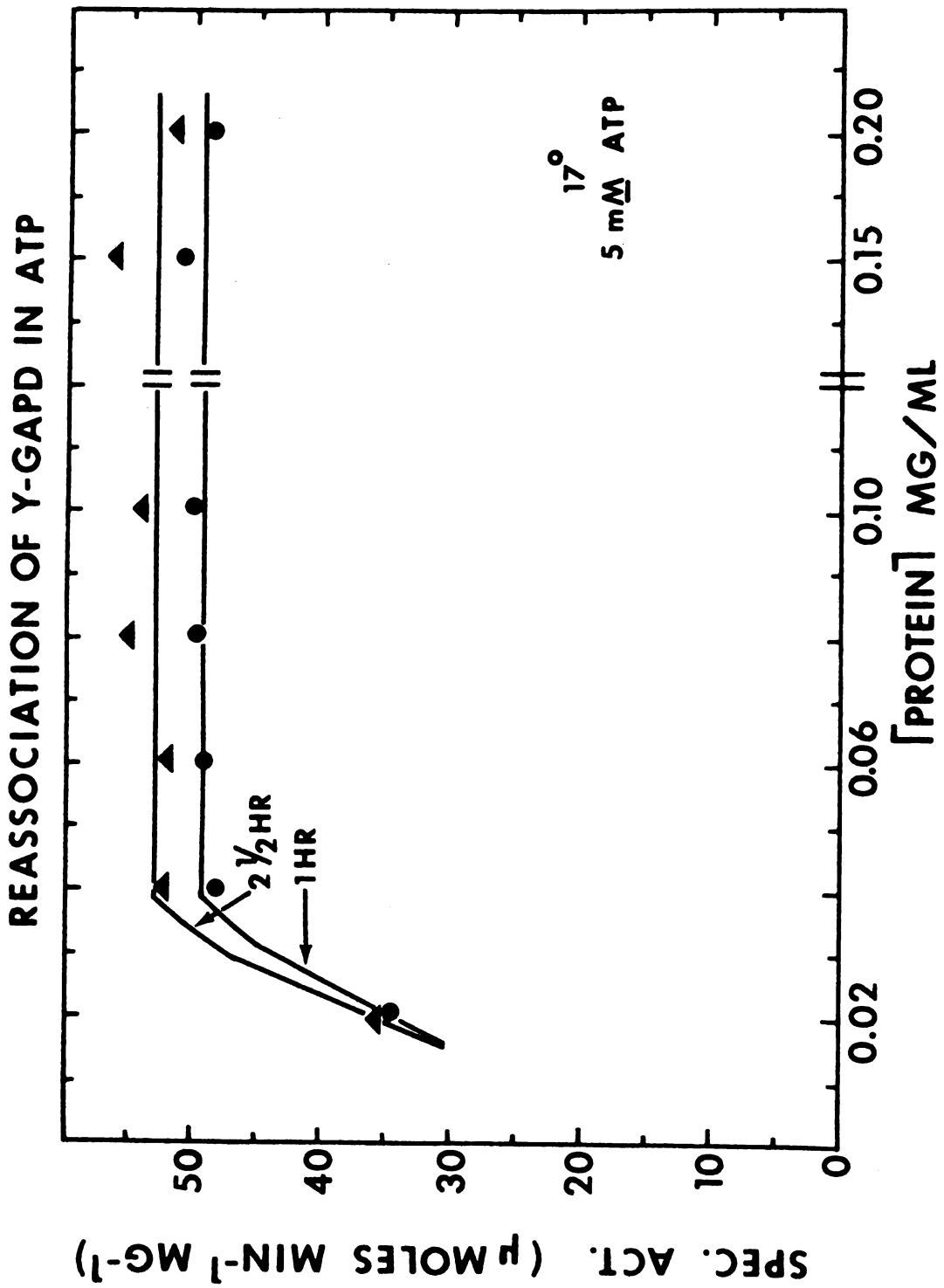
Figure 17. Effect of temperature on reassociation of glyceraldehyde-3-phosphate dehydrogenase subunits. Only one curve is drawn for the 3 and 4 hr incubations, since they were identical. See legend of Figure 16.


REASSOCIATION OF Y-GAPD IN ATP
SPEC. ACT. (μ MOLES MIN-1 MG-1)
50
40
30
20
10
0
0
5
10
15
20
25
TEMPERATURE (°C)
3 AND 4 HRS
1 HR
0.2 MG/ML
5 mM ATP

dissociation medium at 0° before the 1:1 dilution into the reversal mixture. This kept all parameters except protein concentration constant. After the dilution into the reassembly mixture, the samples were placed at 17° and assayed after 1 hr and 2 1/2 hr. The results indicated a requirement for a minimum protein concentration, 0.04 mg per ml, for maximal reassembly (Figure 18). The same value was obtained for the reassembly of yeast glyceraldehyde-3-phosphate dehydrogenase dissociated by 8 M urea (Deal, 1969). This probably reflects the concentration dependence of a dissociation-reassociation equilibrium.

EFFECT OF ATP CONCENTRATION ON THE REASSEMBLY. For this experiment, the ATP concentration in the dissociation medium was lowered, since we wished to study reassembly at much lower ATP concentrations in order to obtain larger differences in reassembly. Enzyme samples were incubated at 0° with 2 mM ATP for 14 hr. Aliquots were withdrawn and first diluted five-fold at 0° into a series of solutions which were similar to the dissociation medium, but contained varying amounts of ATP. A second dilution (1:1) of these solutions at 0° with the reassembly mixture then yielded a series of similar solutions containing 0.04 mg per ml glyceraldehyde-3-phosphate dehydrogenase and various amounts of ATP. The samples were then incubated at 17°. Maximal activity was recovered with 1-2 mM ATP; half-maximal activity was recovered with 0.3 to 0.4 mM ATP

Figure 18. Effect of protein concentration on reassociation of glyceraldehyde-3-phosphate dehydrogenase subunits. See legend of Figure 16 and Methods.


REASSOCIATION OF Y-GAPD IN ATP
17°
5 mM ATP
2½HR
1HR
SPEC. ACT. (μ MOLES MIN⁻¹ MG⁻¹)
[PROTEIN] MG/ML
0
10
20
30
40
50
0.02
0.06
0.10
0.15
0.20

(Figure 19). These values are in excellent agreement with those obtained for the dependence of the dissociation process on ATP concentration (Figure 8).

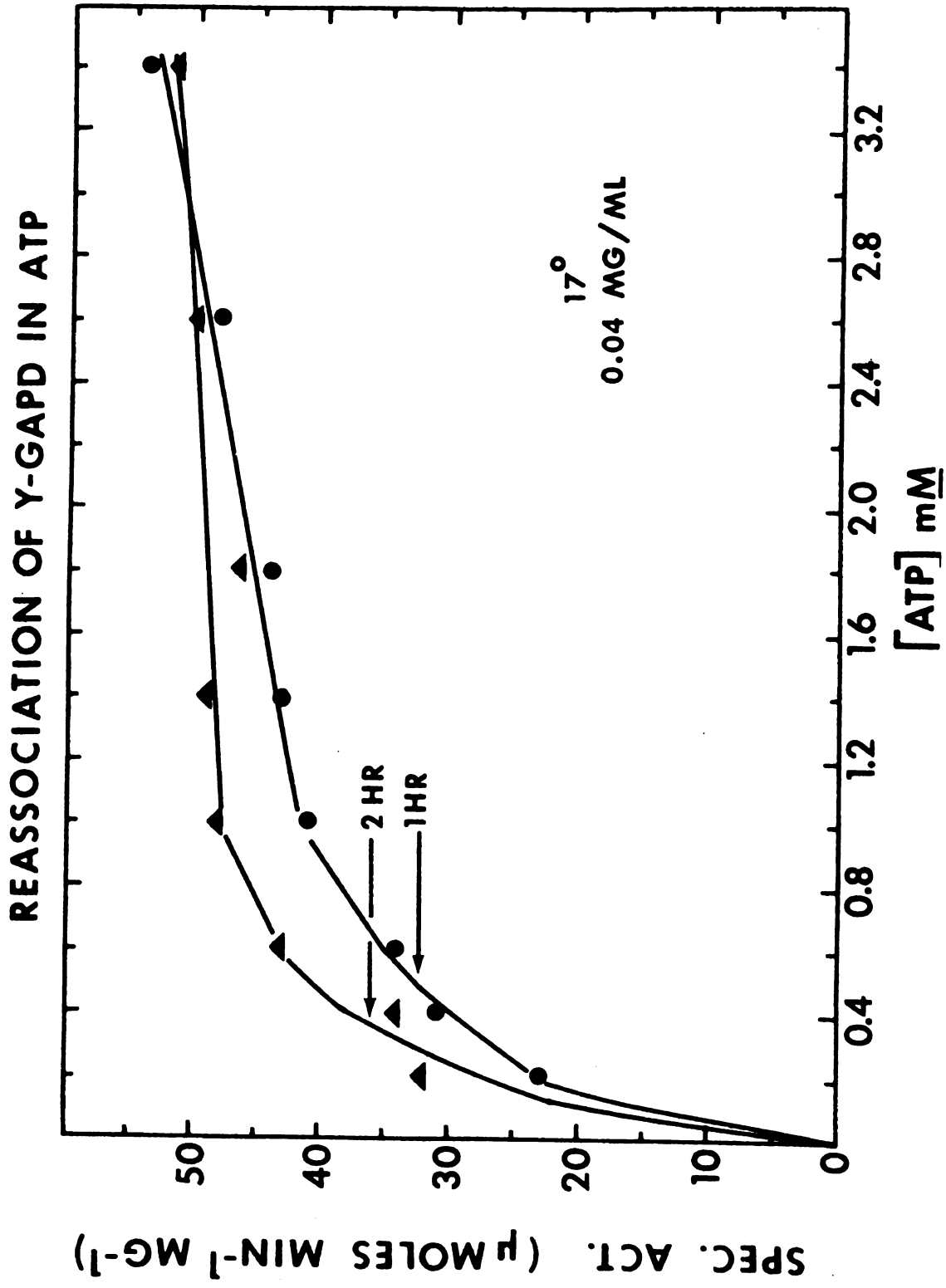
DISCUSSION

MODE OF ACTION OF ATP. Native glyceraldehyde-3-phosphate dehydrogenase does not undergo any appreciable dissociation when incubated at 0° under the conditions employed in the work reported here. Likewise, at 23° ATP does not significantly dissociate yeast glyceraldehyde-3-phosphate dehydrogenase. However, it does cause a conformational change since an ultraviolet difference spectrum is observed and the rate of degradation of the enzyme by chymotrypsin is greatly increased (Yang and Deal, 1969b). Thus, neither ATP alone nor low temperature alone can shift the equilibrium of yeast glyceraldehyde-3-phosphate dehydrogenase toward dissociation, but together they do, under proper conditions.

Two major questions are: (1) How does the binding of ATP by the enzyme alter the folding of the polypeptide chains? (2) What types of forces involved in subunit bonding would be sufficiently decreased by lowering the temperature to lead to dissociation?

Electrostatic repulsion between the negatively charged phosphate portion of ATP and negatively charged groups at the active center of the glyceraldehyde-3-phosphate dehydrogenase molecule appear to be responsible

Figure 19. Effect of ATP concentration on reassociation of glyceraldehyde-3-phosphate dehydrogenase subunits. See legend of Figure 16 and Methods.


REASSOCIATION OF Y-GAPD IN ATP
17°
0.04 MG/ML
2 HR
1HR
SPEC. ACT. (μMOLES MIN-1 MG-1)
[ATP] mM
0
10
20
30
40
50
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2

for conformational changes produced by ATP as indicated by the following: (1) reassembly experiments with urea dissociated yeast glyceraldehyde-3-phosphate dehydrogenase indicate that it contains a region of electrostatic charge near its active center which prevents proper refolding unless neutralized by NAD or high salt (Deal, 1969). (2) The order of the strength of binding of the adenine nucleotides to glyceraldehyde-3-phosphate dehydrogenase (Yang and Deal, 1969a) is 3',5'-AMP > AMP > ADP ~ ATP, but their net charge and dissociation ability is exactly opposite. (3) Mg^{2+} ion, which chelates with the negatively charged phosphate portion of ATP, prevents inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase by ATP in yeast glyceraldehyde-3-phosphate dehydrogenase solutions containing both ATP and chymotrypsin (Yang and Deal, 1969b). (4) Dissociation is favored at higher pH, with a transition point at pH 8.6.

This transition point suggests the involvement of a cysteine residue in the dissociation process. A sulfhydryl group has been reported to be involved in the binding of NAD (Racker, 1965), and ATP is a competitive inhibitor with respect to this substrate (Yang and Deal, 1969a). Also, a different sulfhydryl group forms a covalent acyl thioester with the substrate in the oxidation reaction (Racker, 1965).

It appears that the loss of critical hydrophobic bonds at 0° is the factor which, coupled with the ATP-induced electrostatic repulsion, causes dissociation of the enzyme into subunits. Kauzmann (1959) has discussed the reasons for expecting hydrophobic bonds to be more stable at room temperature than at 0°. A less likely explanation for the temperature-dependence is that one of the ionizable groups in the enzyme undergoes a temperature-dependent change in pK value.

Thus, dissociation appears to proceed as follows: (1) conformational changes occur in the individual subunit polypeptide chains as a result of electrostatic repulsions when ATP is bound. (2) This change in the folding of the individual polypeptide chains alters the contact sites between subunits in such a manner that hydrophobic interactions assume a more critical responsibility for maintaining the native tetrameric structure. (3) Lowering the temperature to 0° is then sufficient to weaken hydrophobic interactions at the contact site between subunits to such an extent that dissociation occurs.

It is also interesting that the temperature-dependence of reassociation (Figure 17) does not exactly parallel that of dissociation (Figure 11). It thus appears that the presence of 10% sucrose in the reassociation mixture alters the temperature-dependence of the equilibrium. This may result from an effect of the sucrose on the water

structure, since alterations in water structure would be expected to affect hydrophobic interactions.

Throughout this discussion we have implicitly assumed the interaction of ATP and glyceraldehyde-3-phosphate dehydrogenase to involve binding, followed by dissociation. Tanford (1964) has pointed out, however, that in some cases the process may be reversed; i.e., dissociation may occur first, followed by binding of the denaturing agent. In such a case, the denaturing agent binds preferentially to the dissociated product, thereby shifting the equilibrium of the dissociation process by removing the product.

It seems unlikely that this occurs in this system, since ATP seems to bind to the native enzyme as tightly as it does to the subunits. This is based on the mid-points of graphs as a function of ATP concentration for (1) chymotryptic inactivation at pH 7.0 and 25° (Yang and Deal, 1969b), (2) dissociation at pH 8.5 and 25° and (3) reassembly at pH 7.0 and 17°.

It is difficult at present to calculate an equilibrium constant for this process, since dimers are almost certainly involved in the conversion of the inactive monomers to active tetramers and it is not known whether the dimers are catalytically active or not. However, if the dimer is assumed to be inactive, the equilibrium constants at 5 1/2° (and the conditions of Figure 17) for a dimer-tetramer and

a monomer-tetramer equilibrium are 0.1 and 0.001 mg per ml respectively.

It is interesting that ATP serves as a catalyst in the reassembly reaction at 17° and perhaps in the dissociation reaction at 0°; this is in addition to whatever effect it may have on the equilibrium of the dissociation. Further work is in progress on the mechanism by which ATP stimulates the reactivation and reassociation processes.

As discussed in a previous paper (Stancel and Deal, 1968), the 2.8-3.0 S dissociation product appears to be a fairly compact monomer, since that sedimentation coefficient is appropriate for the molecular weight of 38,000 obtained from subunit analysis using urea (Deal, 1963; Deal and Holleman, 1964; Deal, W. C., Jr., in preparation). The sedimentation coefficient seems too low for a dimer, since it would have to be extremely unfolded to exhibit such a small sedimentation coefficient. It is interesting that a dimer has not been detected in the dissociation. This suggests that the monomer-monomer interaction is somewhat dependent on the dimer-dimer interaction for stability and is immediately broken when the dimer-dimer interaction is broken.

The effect of ATP on yeast glyceraldehyde-3-phosphate dehydrogenase appears to be similar to the effect of oxygen on hemoglobin. The subunits of tetrameric hemoglobin undergo a marked re-orientation with respect to each other

when oxygen is bound (Muirhead and Perutz, 1963), apparently due to conformational changes in individual subunits upon oxygenation (Edsall, 1968). The binding of oxygen also increases the susceptibility of hemoglobin to proteolytic degradation (Zito et al., 1963).

BIOLOGICAL SIGNIFICANCE OF THE DISSOCIATION PROCESS FOR METABOLIC CONTROL. Since the in vivo ATP concentration of yeast is 1-2 mM (Betz and Moore, 1967), it seems likely that the interaction of ATP and glyceraldehyde-3-phosphate dehydrogenase may be physiologically significant. However, the dissociation of yeast glyceraldehyde-3-phosphate dehydrogenase into subunits by ATP occurs at low temperatures and occurs most readily at higher pH values. Also, rapid but subtle conformational changes occur when ATP is bound, but dissociation occurs much more slowly. The rapid subtle conformational changes increase the rate of inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase by chymotrypsin at 23° and neutral pH (Yang and Deal, 1969b). This effect seems to have the greatest potential as a control mechanism, and offers an additional mechanism other than induction-repression or activation-inhibition mechanisms to control metabolic processes. It is also very significant that 3',5'-AMP and 5'-AMP alone do not dissociate yeast glyceraldehyde-3-phosphate dehydrogenase and, in fact, protect it from dissociation by ATP. The possible physiological significance of these and other

effects is discussed in more detail elsewhere (Yang and Deal, 1969b).

If we assume that the 2.8 S component is a folded monomer, then the results from the reassociation experiments suggest that folded monomers can associate to a tetrameric enzyme. This would be consistent with, but would not prove, that in vivo synthesis of this polymeric enzyme may involve two processes: the assembly and folding of the individual polypeptide chains followed by a separate step, the association of folded monomers to a catalytically active polymeric enzyme. A more thorough discussion of this topic has recently been presented elsewhere (Deal, 1969).

There are very pronounced differences between the effect of ATP on yeast glyceraldehyde-3-phosphate dehydrogenase. The rabbit muscle enzyme is dissociated to a much greater extent at high protein concentrations (10 mg per ml) and is much more specific for ATP (Constantinides and Deal, 1969).

MATERIALS AND METHODS

ENZYME PREPARATION AND ASSAY. The materials and methods for preparation and assay of yeast glyceraldehyde-3-phosphate dehydrogenase were those described previously (Deal, 1969), with the following minor modifications. A stock assay solution was prepared by mixing the following (final assay concentrations in parentheses): (1) 12 ml of 0.2 M Tris, pH 8.0 (100 mM); (2) 1.2 ml of 0.2 M sodium

arsenate (10 m$\underline{M}$); (3) 2.4 ml of 0.01 $\underline{M}$ NAD (1 m$\underline{M}$); (4) 3.6 ml of 0.06 $\underline{M}$ cysteine hydrochloride (9 m$\underline{M}$); (5) 1.2 ml of 3.0 $\underline{M}$ KCl (150 m$\underline{M}$) and (6) 1.2 ml of H_2O. The final pH was 7.8. For assay, 0.36 was added to a cuvette, then 0.01 ml of enzyme solution (0.2 mg per ml) and the contents were mixed. The enzyme reaction was then initiated by addition of 0.03 ml of a stock glyceraldehyde-3-phosphate solution (0.02 $\underline{M}$), to give a final volume of 0.4 ml.

REAGENTS. Reagent grade β-mercaptoethanol was obtained from Eastman. ATP (sodium salt) was obtained from Sigma and stored dessicated under a vacuum at -20°. Stock solutions were prepared at 0.04-0.05 $\underline{M}$ concentrations, adjusted to pH 7.5 to 8.5, and stored frozen until used. Normally this storage period was not longer than 30-60 days. ADP, AMP and 3',5'-AMP were all obtained from Sigma (sodium salts) and were prepared as was the ATP. All other reagents used were the highest quality reagent grades commercially available.

SUCROSE DENSITY CENTRIFUGATION. Sucrose density gradient centrifugation was performed using the method of Martin and Ames (1961) with slight modifications which are described elsewhere (Constantinides and Deal, 1969). Samples were centrifuged at 40,000 rpm in a Beckman Model L preparative ultracentrifuge with either a SW 39 or a SW 50 rotor for 22 hr at 0 to 2° C.

DISSOCIATION AND REASSEMBLY. For the dissociation experiments, the enzyme (0.2 mg per ml) was incubated for 5 hr at 0° in 4 m*M* ATP, 0.1 *M* Tris buffer, pH 8.5, and 0.1 *M* β-mercaptoethanol. The samples were completely inactivated by this treatment.

Reassembly was accomplished by diluting 0.5 ml of the inactive monomers with 0.5 ml of reversal mixture at 0° and warming to 23° or 17° for 2 hr. The reversal mixture contained 0.4 *M* Tris buffer, pH 6.2, 20% sucrose and 0.1 *M* β-mercaptoethanol. After dilution, the resulting mixture contained 0.2 mg per ml glyceraldehyde-3-phosphate dehydrogenase, 0.1 *M* β-mercaptoethanol, 5 m*M* ATP, 0.21 *M* Tris, 10% sucrose, and had a pH of 7.0.

CHAPTER FOUR

KINETICS OF THE DISSOCIATION AND REASSOCIATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE PRESENCE OF ATP

ABSTRACT

A detailed kinetic analysis of the dissociation of tetrameric yeast GAPD to monomers at 0° in ATP has been performed by following the loss of catalytic activity. At pH 8.0 in 0.02 M Tris and 0.075 M β-mercaptoethanol the process is biphasic. There is a fast initial step followed by a slow second step.

The fast step can be described by a rapid reversible equilibrium between tetramers and dimers. The calculated equilibrium constant (K_{eq} = 10.2 x 10^{-8} M) for this initial dissociation yields a value of 8.9 kcal/mole for ΔG° at 0°.

The slow step exhibits psuedo-first order kinetics and a half-power dependence on protein concentration, suggesting that this step reflects the dissociation of dimers to monomers. The dependence of the dimer to monomer dissociation on ATP concentration indicates that ATP binds to the dimer in successive, reversible steps with no interaction between ATP binding sites. The average constant for binding of ATP to the dimer is 0.75 mM. The rate of dissociation of dimers to monomers is decreased as ionic strength is increased, suggesting that hydrophobic residues are exposed to the solvent medium as the dimer dissociates to monomers.

A kinetic analysis of the reversal of dissociation at 23° in the presence of 10% sucrose reveals that the reversal process is second order with respect to protein at low protein concentrations and first order at high concentrations. These results suggest that the final step in the reassembly of monomers to tetramers is a first order process involving a conformational change. The half-life for the reassembly process is 8.5 min at 0.20 mg/ml and 16.0 min at 0.010 mg/ml.

INTRODUCTION

In previous papers, we have reported the reversible dissociation of yeast and rabbit muscle glyceraldehydrogenases into unfolded subunits in urea (Deal and Holleman, 1964; Deal, 1969) and into folded subunits in ATP at 0° (Stancel and Deal, 1968, 1969; Constantinides and Deal, 1969). These provide ideal models for intensive investigations of the following properties: (1) subunit structure, (2) nature, types, and strength of subunit interactions, (3) folding and association reactions leading to formation of the polymeric enzymes, (4) the kinetic and thermodynamic properties of these reactions in vivo from the initial stages of protein synthesis until the final product, and (5) the significance of these properties with respect to control of activity and synthesis of the enzyme.

SUBUNIT STRUCTURE. The subunit structures of the two enzymes are similar in that both yeast and rabbit muscle glyceraldehydrogenases are tetramers of molecular weight 145,000 and are dissociated into unfolded subunits of molecular weight 36,000 in urea (Deal and Holleman, 1964) and guanidine hydrochloride solutions (Deal and Holleman, 1964; Harrington and Karr, 1965). The subunit structures are different in that the yeast enzyme subunits are different from the muscle enzyme subunits; however, both enzymes appear to be made up of identical subunits, one

type of subunit being found in yeast and another type found in rabbit muscle.

SUBUNIT INTERACTIONS. The nature, types, and strength of subunit interactions are also different for the two enzymes, and one purpose of this paper is to provide further information on this subject by defining the detailed reaction mechanisms and thermodynamic and kinetic properties of the reversible dissociation of yeast glyceraldehyde-3-phosphate dehydrogenase.

Both tetrameric yeast and rabbit muscle glyceraldehyde phosphate dehydrogenase are reversibly dissociated into monomers by ATP at 0° under proper conditions, but stable dimers are observed in sedimentation velocity experiments only with the rabbit muscle enzyme under proper conditions. With yeast glyceraldehyde phosphate dehydrogenase no dimers are observed over the entire range of protein concentration; only the native 7.4 S enzyme and 2.8-3.0 S monomers are observed (Stancel and Deal, 1968, 1969). At long times (several days), the folded monomer undergoes an irreversible change to a more slowly sedimenting 0.9 S form (Stancel and Deal, 1968, 1969). In contrast, rabbit muscle glyceraldehyde phosphate dehydrogenase is dissociated into 4.6 S dimers at protein concentrations as high as 10 mg/ml and the enzyme yields 4.6 S dimers in increasing relative amounts as the protein concentration is decreased to 1 mg/ml, where dimers alone exist. Below 1 mg/ml, the enzyme begins to

show dissociation into 2.8-3.2 S monomers and below 0.1 mg/ml, monomers alone are observed (Constantinides and Deal, 1969). Since the dissociation of yeast glyceraldehyde phosphate dehydrogenase by ATP at 0° must also produce a dimer (or trimer) as an intermediate step in the dissociation to monomers, one of the specific questions to be answered in this study was why no dimers are observed in the ultracentrifugal analysis of the dissociation products.

IN VIVO AND IN VITRO FOLDING AND ASSOCIATION. Another purpose of this paper was to obtain information related to the following question: "During, and after, its polypeptide chains are synthesized on the polysomes, what are the series of folding and association steps which lead to the native tetrameric glyceraldehyde phosphate dehydrogenase?" The two dissociation systems for the two glyceraldehyde-3-phosphate dehydrogenases have certain features which make them rather uniquely suited to help answer the question. With these two dissociation systems, one of which yields dissociation with unfolding (the urea system) while the other yields dissociation essentially without unfolding (the ATP dissociation system), we have the potential of studying not only the overall process but also of studying independently the two phases, folding and association, of the process in vitro.

In the previous study of the overall process of reassembly (folding and association) from unfolded subunits (the urea system), it was discovered that NAD was absolutely required for the process (Deal, 1969). A translational control model has been proposed (Deal, 1969) in which NAD may control the rate of glyceraldehyde phosphate dehydrogenase synthesis by dictating whether, or how fast, the nascent polypeptide chain folds correctly, which is postulated to be necessary to allow synthesis to proceed to completion.

In the systematic study of conditions required for the overall association process from folded subunits (the ATP-0° system), it was discovered that ATP and sucrose were necessary for the association processes. It was of interest to know which steps of the reassembly process were involved in these effects. The approach taken was to first define the reaction steps by which the native enzyme is produced from the "folded" subunits (the ATP-0° system), as well as the kinetic and thermodynamic parameters of these steps. This paper presents the results of these studies.

RESULTS

Precise kinetic measurements of the ATP-induced inactivation of yeast GAPD at low temperatures were initially difficult to perform because after dilution from stock solutions of enzyme stock ammonium sulfate suspensions

(Yang and Deal, 1969a) enzyme controls first showed an increase in activity with time followed by a decrease in activity. It was found that this problem could be avoided if the enzyme was first diluted into buffered solutions with small amounts of β-mercaptoethanol and allowed to incubate for 4-12 hrs at either 0° or 23° before the addition of ATP. Using this preincubation technique the kinetics of the ATP-induced dissociation at 0° were examined, and a model of the dissociation mechanism was proposed.

DESCRIPTION OF THE MODEL AND SUMMARY OF EVIDENCE. Because the kinetics of yeast GAPD inactivation in ATP are quite complex we will attempt to make the concepts easier to grasp by first presenting the resultant model, together with a summary of the kinetic evidence on which it is based. Then the kinetic data will be examined in detail. The model for the sequence of reactions is given below:

$$\underset{\text{FAST PROCESS}}{\underbrace{\text{TETRAMER} \xrightarrow{\text{ATP}} [\text{TETRAMER} \underset{k_{24}}{\overset{k_{42}}{\rightleftarrows}} \text{DIMERS}]}} \underset{\text{SLOW PROCESS}}{\underbrace{\xrightarrow{k_{21}} \text{MONOMERS}}} \quad \text{(1)}$$

RAPID EQUILIBRIUM

The basic idea is that the overall process involves two processes, one fast and one slow. The first step is postulated to be the fast step and to involve a change

from stable active tetramers to an equilibrium mixture of active tetramers and inactive dimers. The second step is postulated to be slow (rate-limiting) and to involve the dissociation of the inactive dimers into inactive monomers, practically at a constant rate because the dimer concentration is practically constant. This is because dimer removed is rapidly replaced by dimer produced by the very fast tetramer-dimer dissociation, the relatively constant amount of dimer being determined by the equilibrium constant for the tetramer-to-dimer dissociation. The establishment of an equilibrium is presumed to result from the fact that this dissociation into dimers is very fast but dissociation of dimers is so slow that it is rate-limiting. This model is supported by the following observations, which are amplified in succeeding sections.

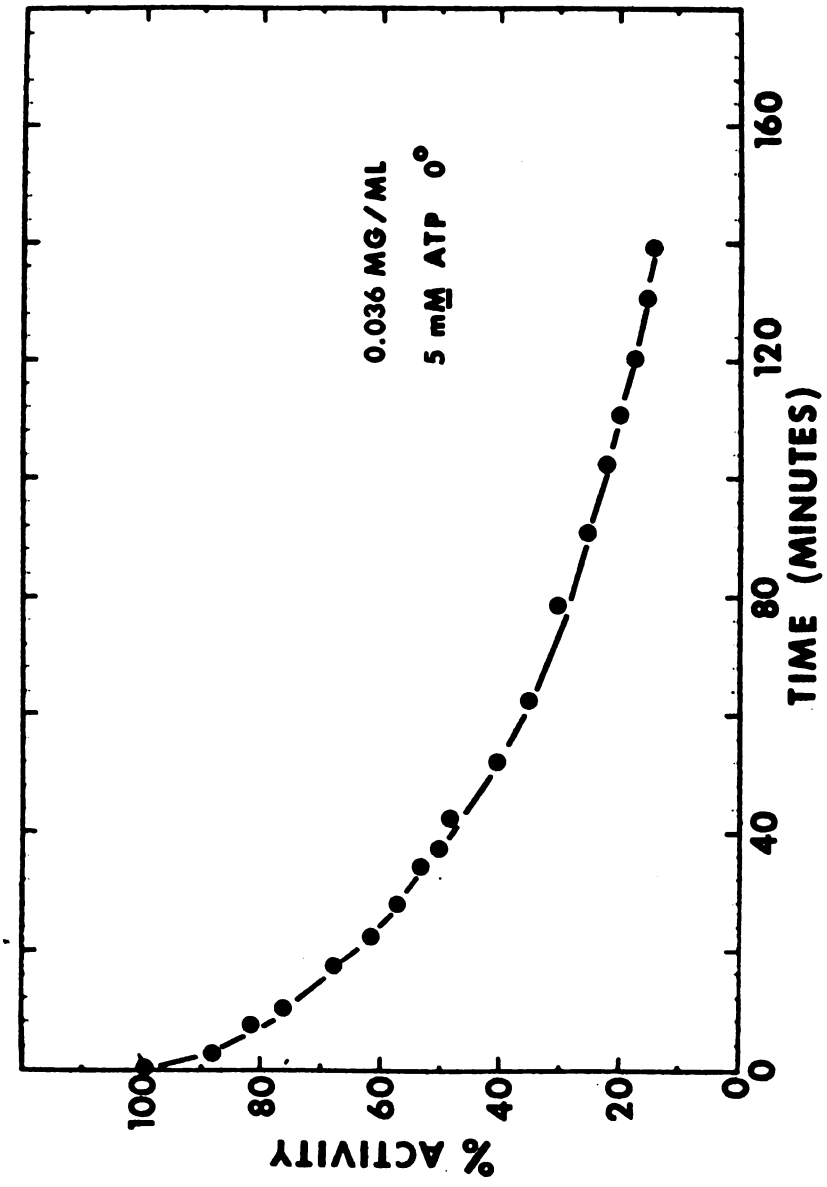
(1) The process(es) (see Figure 20) cannot be described by a single rate law.

(2) The overall process (see Figure 20) can be separated into two phases, a fast initial process and a slow process.

(3) Both processes are inversely dependent on protein concentration, suggesting both processes involve two dissociation steps.

(4) The slow process exhibits pseudo-first order kinetics and a half-power dependence on tetramer concentration, suggesting a dissociation of dimers.

Figure 20. Time-dependent loss of glyceraldehyde-3-phosphate dehydrogenase activity at 0° in ATP. The enzyme (0.04 mg/ml) was incubated overnite at 0° in 0.1 M Tris, pH 8.0, and 0.075 M β-mercaptoethanol. After incubation, 0.10 ml of 50 mM ATP (adjusted to pH 8.0) was added to 0.9 ml of the pre-incubated enzyme solution, and the activity loss was followed as a function of time. The control sample (0.9 ml of pre-incubated enzyme plus 0.10 ml of buffer) showed no activity loss during this time period.


0.036 MG/ML
5 mM ATP 0°
% ACTIVITY
100
80
60
40
20
0
0
40
80
120
160
TIME (MINUTES)

(5) The fast process can be kinetically described by a rapid, reversible dissociation of tetramers to dimers.

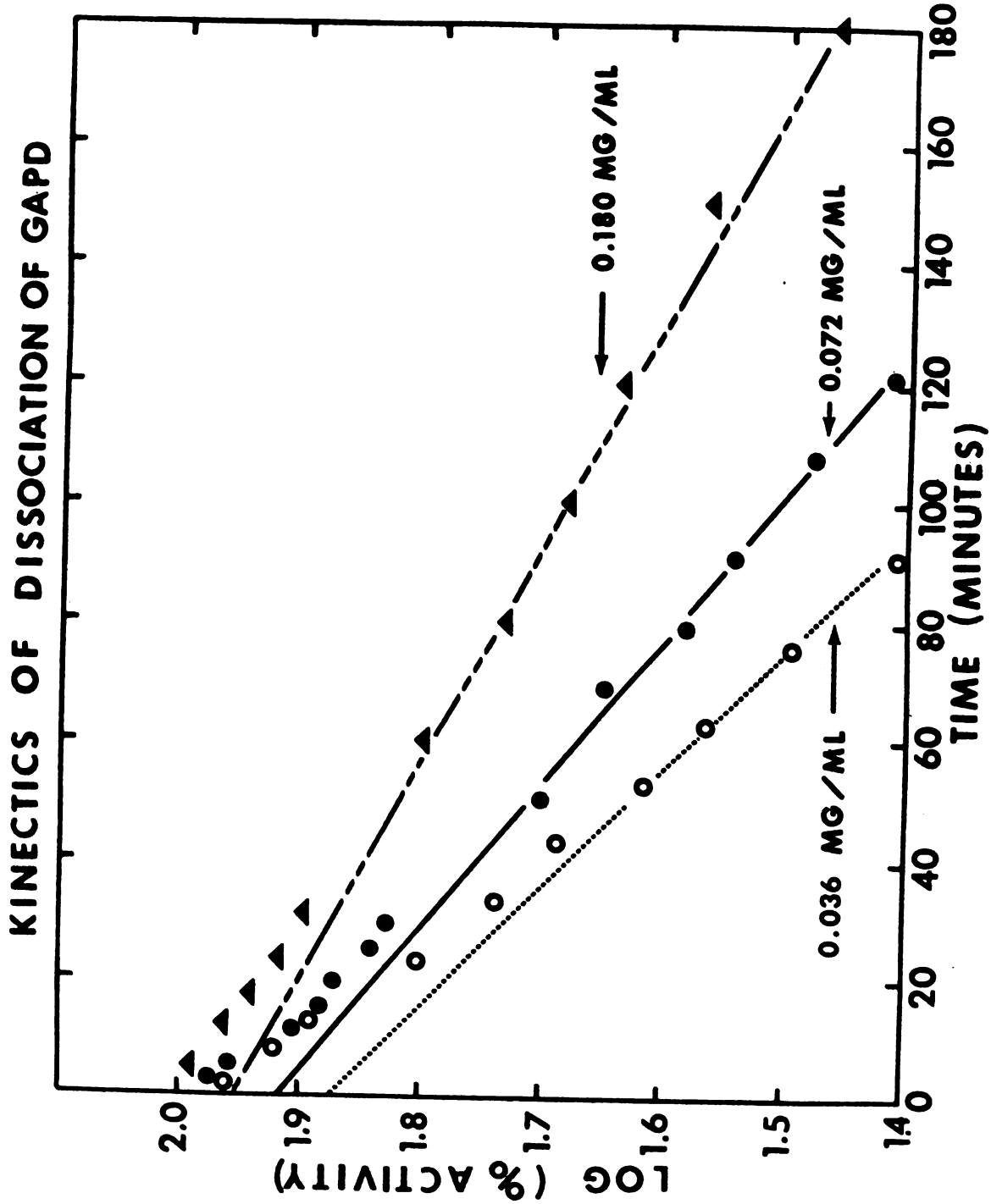
(6) The equilibrium constant calculated for the proposed tetramer-dimer equilibrium fits the data over a wide range of protein concentration.

This model assumes there is no appreciable unfolding and thus, that we are dealing only with dissociation-association reactions. The assumption is supported by the fact that the model fits the observed data.

ANALYSIS OF THE SLOW PROCESS (DISSOCIATION OF DIMERS). Since there clearly are two processes involved, we must attempt to separate the two in order to analyze the data. Thus, the logical starting point is with the slow process, since for all practical purposes, the fast reaction has already gone to completion, leaving only the slow reaction to produce the results. Figure 21 shows a semi-log plot of the loss of GAPD activity with time, for three different initial protein concentrations. It is clearly seen that the overall process is biphasic, that the slow phase does not extrapolate back to 100% activity (also indicating two processes), that both phases are inversely dependent on protein concentration (suggesting two dissociation steps), and that the slow phase is pseudo first-order.

The slow process, which is pseudo first-order, was treated by the half-life method to obtain the order of

Figure 21. Effect of protein concentration on the rate of dissociation of glyceraldehyde-3-phosphate dehydrogenase in ATP. See text and legend of Figure 20.


KINECTICS OF DISSOCIATION OF GAPD
LOG (% ACTIVITY)
TIME (MINUTES)
0.180 MG/ML
0.072 MG/ML
0.036 MG/ML
2.0
1.9
1.8
1.7
1.6
1.5
1.4
0
20
40
60
80
100
120
140
160
180

this process with respect to protein concentration (see appendix for details). As shown in Figure 22 a graph of log ($\tau_{1/2}$) vs log (initial protein concentration) yields a line with a slow process slope of 0.46, which yields an order of 0.54 with respect to protein (see appendix).

Assuming that only the tetramer is active, an interpretation of this half-power dependence is that the slow process represents dimer dissociation, preceded by the establishment of a tetramer-dimer equilibrium mixture. The following derivation shows that this interpretation is consistent with the results. From (1), the rate of monomer formation, dM/dt, is given by eqn (2).

$$+ dM/dt = k_{21} [D] \qquad (2)$$

and since the dissociation equilibrium constant, K_{eq}, is given by eqn (3),

$$K_{eq} = k_{42}/k_{24} = [D]^2/[T] \qquad (3)$$

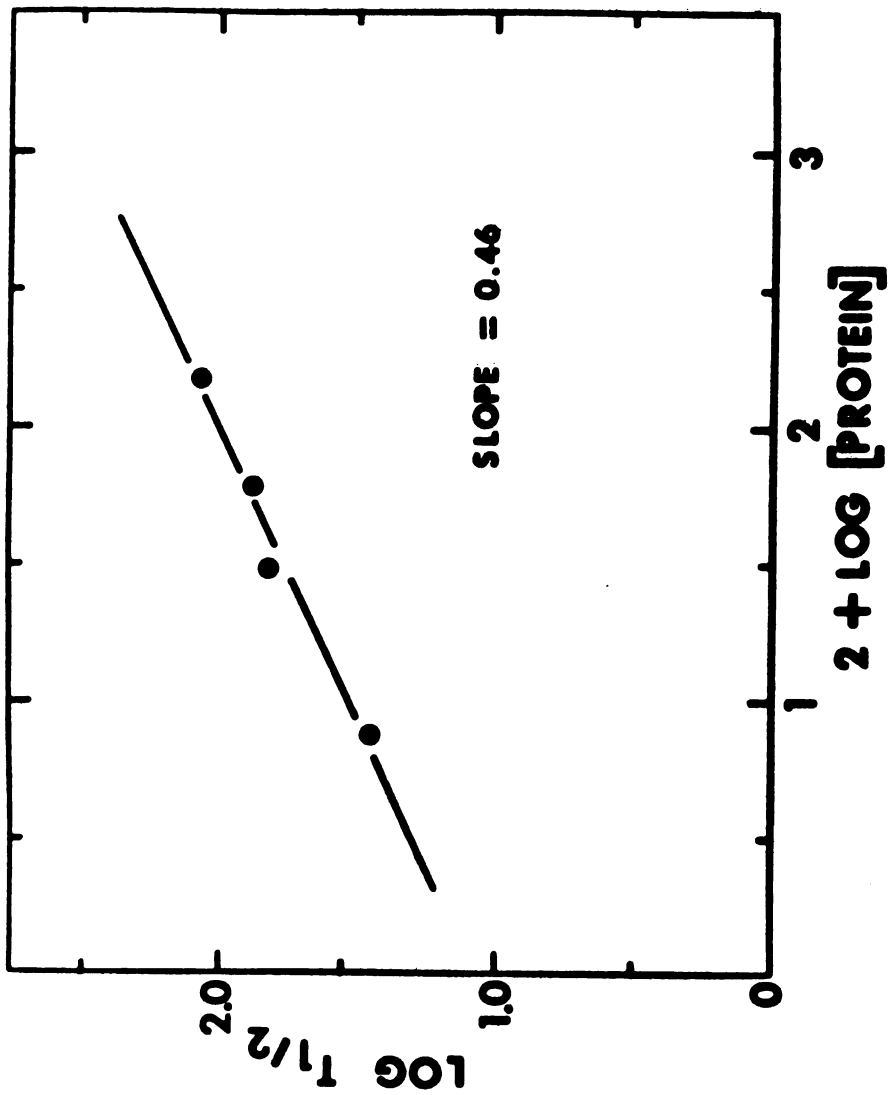
solving for D in eqn (3) and substituting into eqn (2) yields

$$+ dM/dt = k_{21} K_{eq}^{1/2} [T]^{1/2} = k'[T]^{1/2}$$

Where M, D, and T are the monomer, dimer, and tetramer concentrations, respectively; k_{42} and k_{24} are the rate constants for dissociation of tetramers to dimers and association of dimers to tetramers, respectively; k_{21} is

Figure 22. Determination of the order of the slow process (presumed dimer breakdown) by the half-life method. The slope of the line is 0.46, thus yielding an order of 0.54 (the order is equal to 1 -[observed slope]).

The half-lives are expressed in minutes and the protein concentration in mg/ml. See text.

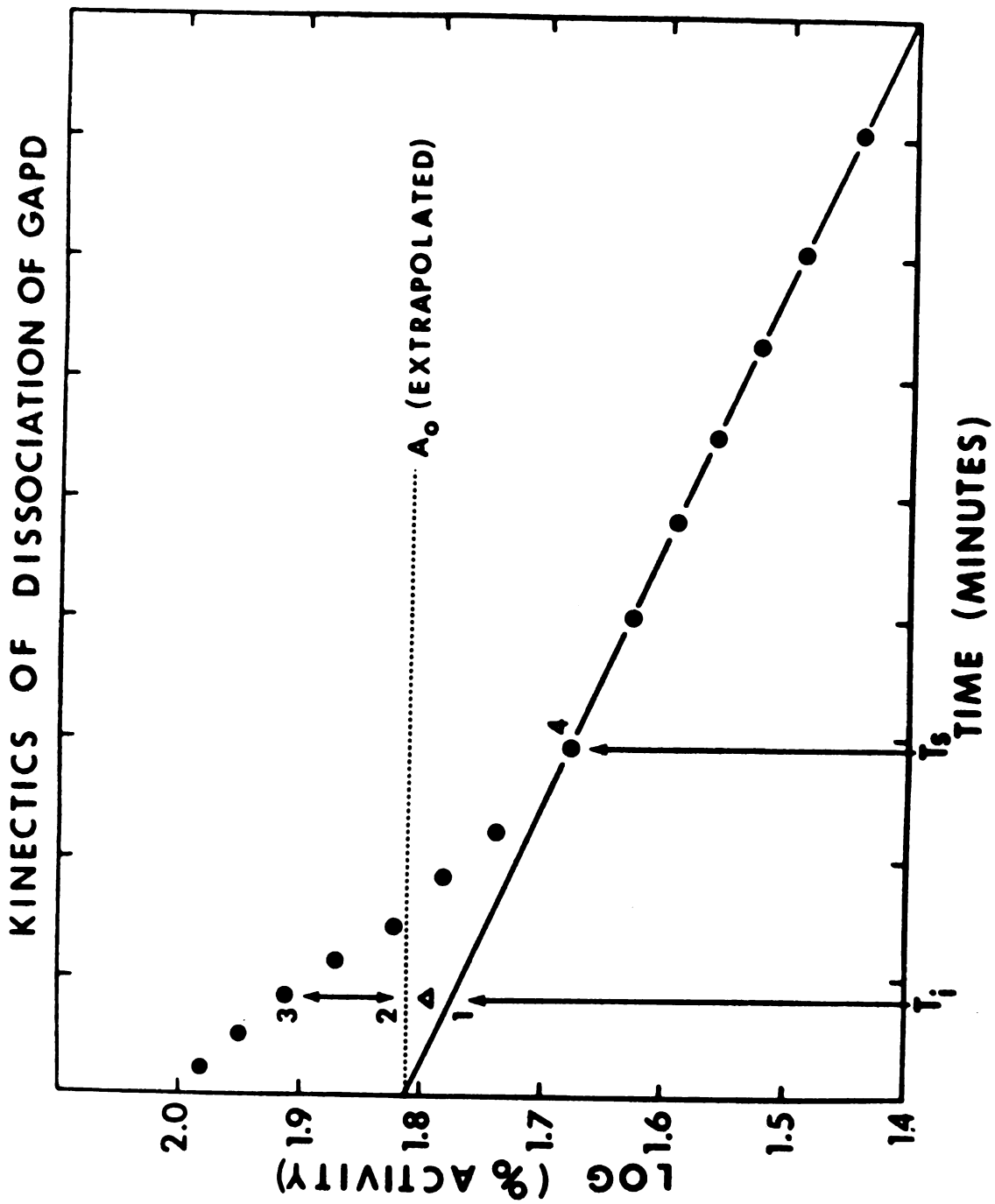

SLOPE = 0.46
LOG $T_{1/2}$
2.0
1.0
0
1
2
3
2 + LOG [PROTEIN]

the rate constant for dissociation of dimers to monomers, and k' is $k_{21} K_{eq}^{1/2}$. We consider this explanation to be the correct one because we are unable to find another feasible possible explanation for this dependence.

SEPARATION OF THE FAST PROCESS FROM THE SLOW PROCESS. In order to elucidate the nature of the fast process, a method was devised to separate the rates of the slow process and fast process. As seen in Figure 21, the data points at short times lay above the extrapolated rate of the slow process, so the data for the slow process do not extrapolate back to 100% activity but to a point A_o. Now, A_o is the activity which would be observed at equilibrium if only the first process, tetramer to dimer, were occurring (i.e., at t_s).

The points which lie above the slow process were corrected for the rate of the slow process as illustrated in Figure 23. After the extrapolation of the slow process to a zero time value, A_o, the difference in activity between A_o (point 2) and a given point (point 1) on the extrapolated line was added to the value of the experimentally measured activity (point 3) at the same time (Figure 23). With respect to the fast reaction, this amount added was equal to, and therefore corrected for, the excess loss of activity due to excess dimer formation over that dictated by the equilibrium constant for the tetramer to dimer equilibrium. All points between time

Figure 23. Synthetic example of the treatment of kinetic data used to separate the fast process (presumed tetramer-dimer equilibrium) from the slow process (presumed dimer breakdown). See text for further details.


KINECTICS OF DISSOCIATION OF GAPD
A_o (EXTRAPOLATED)
LOG (% ACTIVITY)
TIME (MINUTES)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1
2
3
4
T_i
T_s

zero and the time at which the activity points coincide with the slow process line (point 4, time t_s) were corrected in this way. Note that the first experimental point (4) on the slow process line would have a corrected value of A_o.

ANALYSIS OF THE FAST PROCESS (TETRAMER-DIMER EQUILIBRIUM). The corrected points could not be fit to a psuedo first-order rate law,[1] but did fit an integrated rate equation expected for a rapid equilibrium between active tetramers and inactive dimers. This is shown in Figure 24, which is a plot of log $\underline{x}$ vs time for the 0.036 mg/ml sample depicted in Figure 2, where $\underline{x}$ represents the quantity

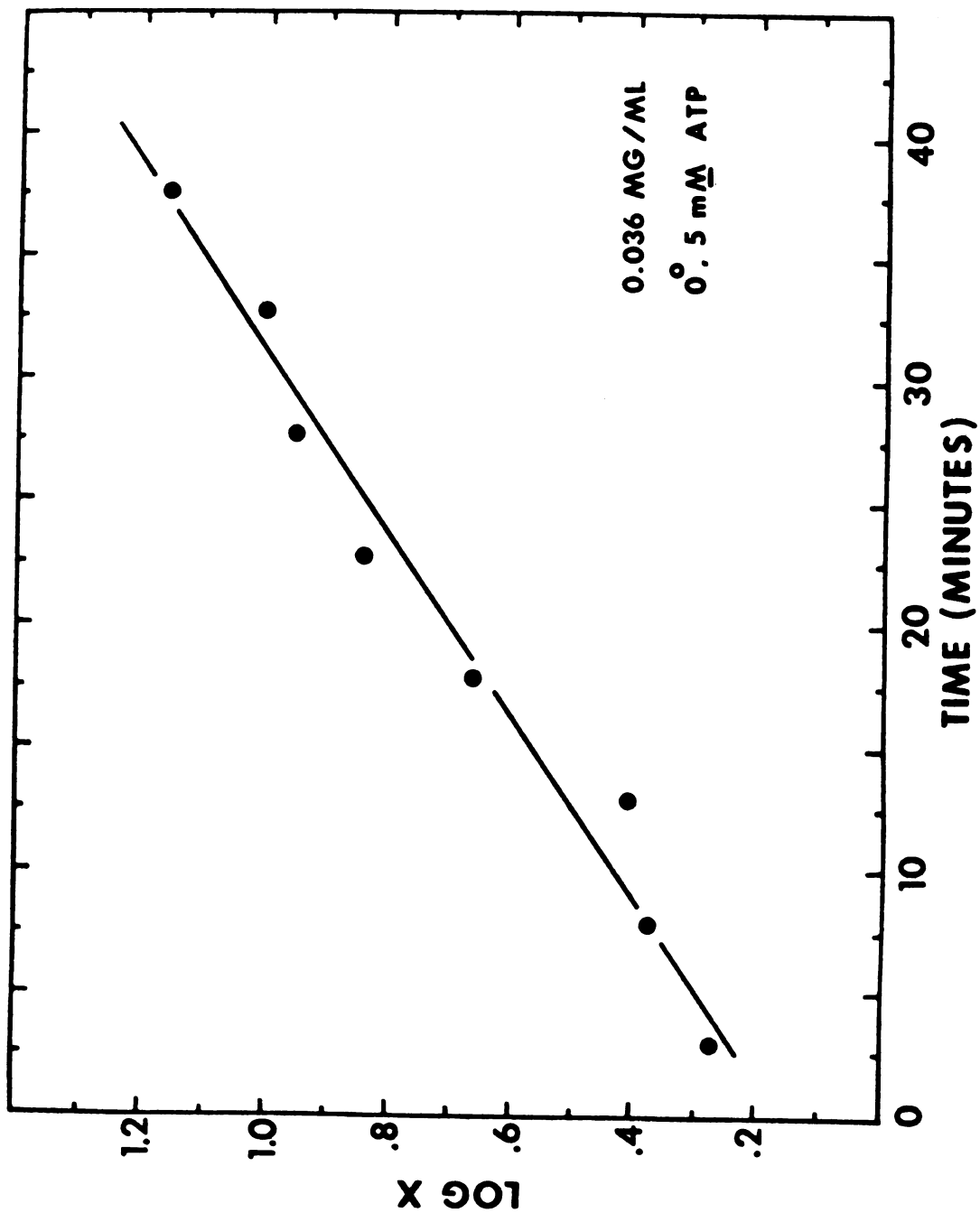
$$\frac{(-A_o - A_c) - (A_o - A_c) (T)}{(-A_o - A_c) + (A_o - A_c) (T)} \qquad (5)$$

In this term A_o is the extrapolated activity at time zero and A_c is the corrected activity at a given time (see Figure 23). For a given protein concentration T is a constant which depends on the initial and equilibrium activity values (see appendix for a complete derivation). Similar fits were obtained at other protein and ATP concentrations. This behavior is consistent with a tetramer-dimer equilibrium.

It is also possible to test for a tetramer-dimer equilibrium by calculating equilibrium constants at

[1]The data also could not be fitted to a 1st order plot when differences between experimental points and the extrapolated lines were plotted directly.

Figure 24. Kinetic fit of the corrected data points for the fast process (presumed tetramer-dimer equilibrium) to the integrated rate equation for a rapid equilibrium between active tetramers and inactive dimers. See text for a definition of the quantity x and for other details.


0.036 MG/ML
0°. 5 mM ATP
LOG X
1.2
1.0
.8
.6
.4
.2
0
10
20
30
40
TIME (MINUTES)

different protein concentrations. If an equilibrium is in fact being established, then the value A_o (obtained by extrapolation of the slow rate to time zero) should correspond to the activity of a tetramer-dimer mixture dictated by the total protein concentration. Equilibrium constants for each initial protein concentration can then be calculated as follows:

$$[T],\ \underline{M} = (\ [T]_{initial},\ \underline{M}\ )\ (\%\ Act.,\ _{extrapolated})$$

$$[D],\ \underline{M} = (\ [T]_{initial},\ \underline{M} - [T],\ \underline{M}\ )\ (2) \qquad (6)$$

$$K_{42} = [D]^2 / [T]$$

Constants calculated from experiments with different initial protein concentrations are listed in Table IV. Considering the extrapolation procedure used and the square term in the calculation, the K's seem to be constant over a wide range of protein concentration. Since the overall dissociation process is known to be a conversion of tetramers to monomers (Stancel and Deal, 1968, 1969), a mechanism consistent with all the data is the establishment of a rapid equilibrium between tetramers (active) and dimers (inactive) followed by the breakdown of dimers to monomers.

EFFECT OF ATP ON DISSOCIATION. Although the previous experiments involve dissociation in the presence of ATP, we want information about the dissociation without ATP.

TABLE IV. CALCULATED EQUILIBRIUM CONSTANTS FOR A TETRAMER-DIMER EQUILIBRIUM

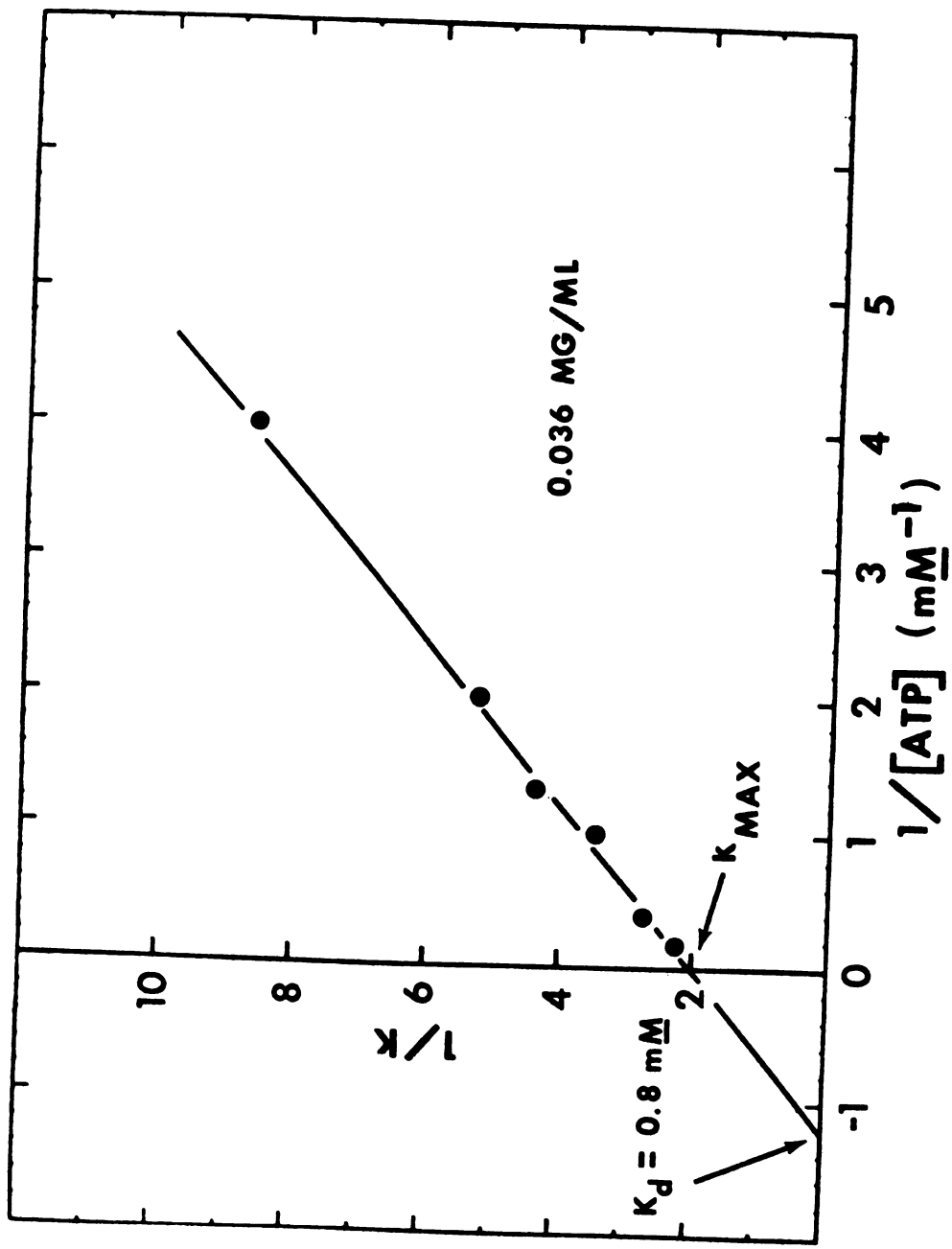
Protein Conc. (mg/ml)	$K_{eq.}$ (calc.) $\underline{M} \times 10^{8}$
0.180	8.8
0.072	14.8
0.036	11.6
0.009	5.7
	$K_{eq.}$ (AVG) = 10.2×10^{-8} $\underline{M}$

In order to obtain this, we require knowledge of the processes involving binding of ATP to the enzyme. Specifically we must know the values of the ATP-to-enzyme binding constants, the number of ATP molecules bound per molecule of enzyme and whether there is interaction between these ATP binding sites.

ATP BINDING CONSTANTS. To relate the equilibrium constants calculated in the preceding section (obtained in the presence of ATP) to the enzyme in the absence of ATP, the ATP concentration was varied at fixed protein concentrations. When experiments were performed at 0.036 mg/ml yeast GAPD at various concentrations of ATP, biphasic plots were obtained. When the data was treated as in Figure 21, the extrapolated intercepts of the slow process at time zero decreased as the ATP concentration was raised, but the changes were not sufficiently large to be treated in a quantitative manner. The rates of the slow process also increased as the ATP concentration was raised. As shown in Figure 25 these rate constants (slow process) were then plotted as 1/k vs 1/ATP to obtain the binding constant for ATP and k_{max}.

From the intercept on the horizontal axis (Figure 25) an average binding constant for ATP of 0.80 mM was obtained, as well as a value of k_{max}. The fact that a plot of the form 1/k vs $1/ATP^n$ is linear for n=1 indicates that the process appears first order with respect to ATP.

Figure 25. Determination of the ATP binding constant for the slow process. Since the slow process is presumed to be dimer breakdown, the constant determined from this plot reflects the binding of ATP to the dimer.


0.036 MG/ML
K_{MAX}
K_d = 0.8 mM
1/K
1/[ATP] (mM^{-1})
10
8
6
4
2
0
-1
1
2
3
4
5

Since these are the psuedo-first order rate constants from the presumed breakdown of dimers, this value of 0.80 m$\underline{M}$ reflects the binding of ATP to the GAPD dimer.

Because a knowledge of the binding constants for ATP was important, these experiments were repeated at a second protein concentration of 0.0125 mg/ml. When treated in a similar manner an average binding constant of 0.67 m$\underline{M}$ was obtained, as well as a value for k_{max}.

TESTS FOR INTERACTION BETWEEN ATP BINDING SITES. The rate constants obtained at different ATP concentrations can also be treated to test for any interaction between ATP binding sites. If the binding of ATP is represented by successive, reversible equilibria of the type

$$Enz(ATP)_{n-1} + ATP \rightleftarrows Enz(ATP)_n$$

$$K_n = \frac{[Enz(ATP)_{n-1}]\,[ATP]}{[Enz(ATP)_n]}$$

then

$$pK_i + \ldots\ldots + pK_n - n(p[ATP]) = \log \frac{\alpha}{1-\alpha}$$

where α is defined as the per cent saturation with ATP, and n is the interaction constant for binding of successive moles of ligand (see Suelter, 1966). In this case n is analogous to the interaction constant obtained from the slope of a Hill plot, i.e., a value of n=1 indicates no interaction between ligand binding sites.

For the dissociation of GAPD in ATP, the preceding relationship can be alternatively expressed in a form more usable for out purposes as

$$n(pK_{avg} - p([ATP]) = \log \frac{\alpha}{1-\alpha}$$

α values may be calculated by assuming that the rate of inactivation is proportional to the degree of saturation of enzyme with ATP. Therefore,

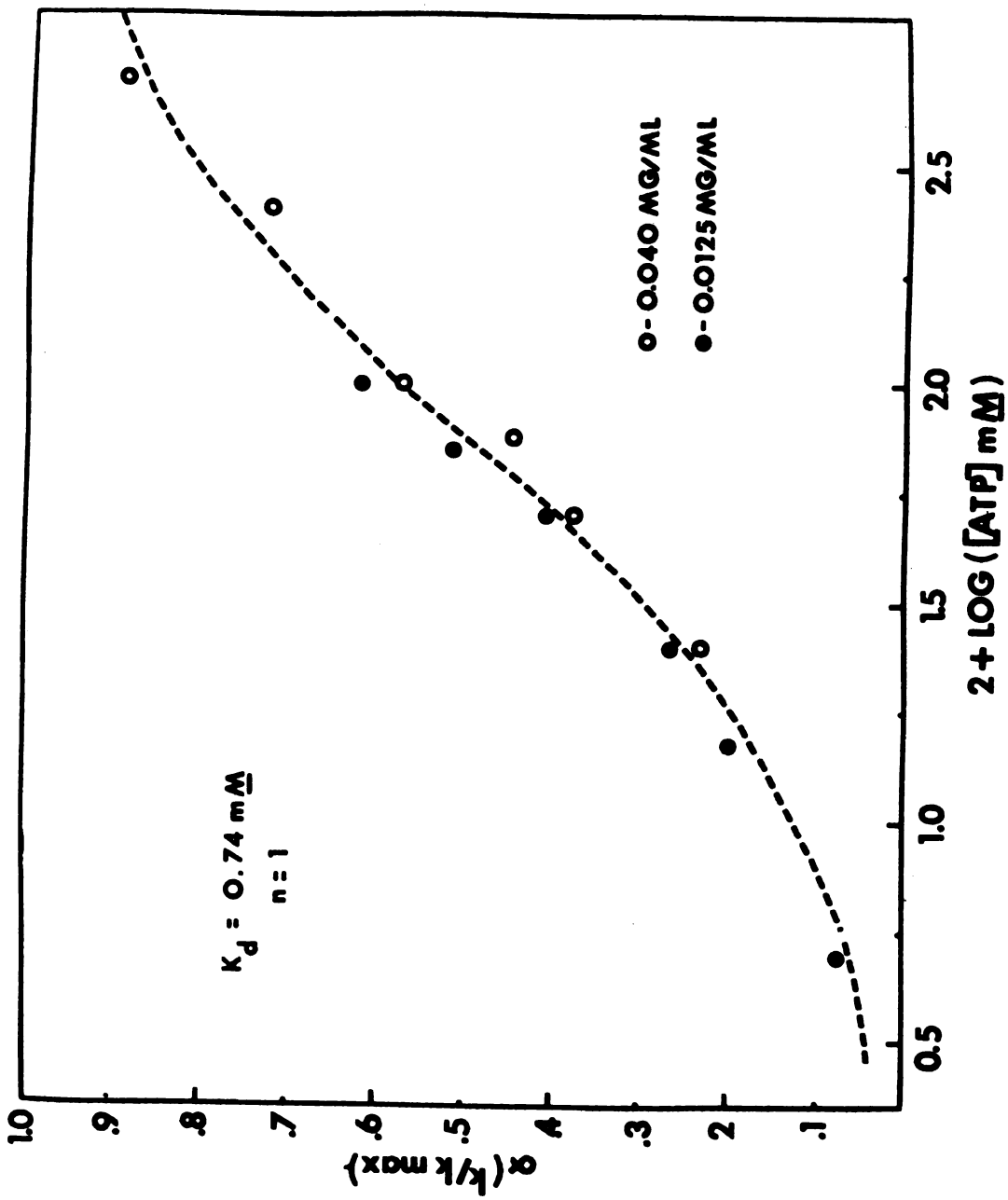
$$\alpha_{ATP,i} = \frac{(k_{ATP,i})}{(k_{max})}$$

with $k_{ATP,i}$ the experimentally determined psuedo-first order rate constant for the slow process at a given ATP concentration and k_{max} determined from a plot of 1/k vs 1/ATP (Figure 25).

To determine if there is any interaction between ATP binding sites, one can then assume various values of n and draw theoretical curves of α vs log ([ATP]) for that value of n. Figure 26 is such a plot with the theoretical line drawn for a value on n=1 and K_{avg} = 0.74 m<u>M</u> (the average of 0.80 and 0.67). The agreement between the theoretical line for n=1 and the experimental points suggests that there is no appreciable interaction between ATP binding sites.

<u>NATURE OF SUBUNIT CONTACT SITES: EFFECT OF IONIC STRENGTH ON THE DISSOCIATION PROCESS</u>. We have previously suggested

Figure 26. Test for interaction between ATP binding sites involved in the slow process. The points are the experimental points obtained at the indicated protein concentrations, and the line is the theoretical line drawn for an average dissociation constant of 0.74 mM and an interaction constant, n, equal to 1 (a value of 1 indicates no interaction between ATP binding sites).


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
α (k/k max)
0.5
1.0
1.5
2.0
2.5
2 + LOG ([ATP] mM)
K_d = 0.74 mM
n = 1
o - 0.040 MG/ML
● - 0.0125 MG/ML

that the dissociation of yeast GAPD resulted from decreased hydrophobic interactions between subunits at low temperatures in ATP (Stancel and Deal, 1969). Physically this implies that the contact sites between subunits are hydrophobic in nature, since the contact sites are presumably the major structures broken by ATP induced dissociation without unfolding

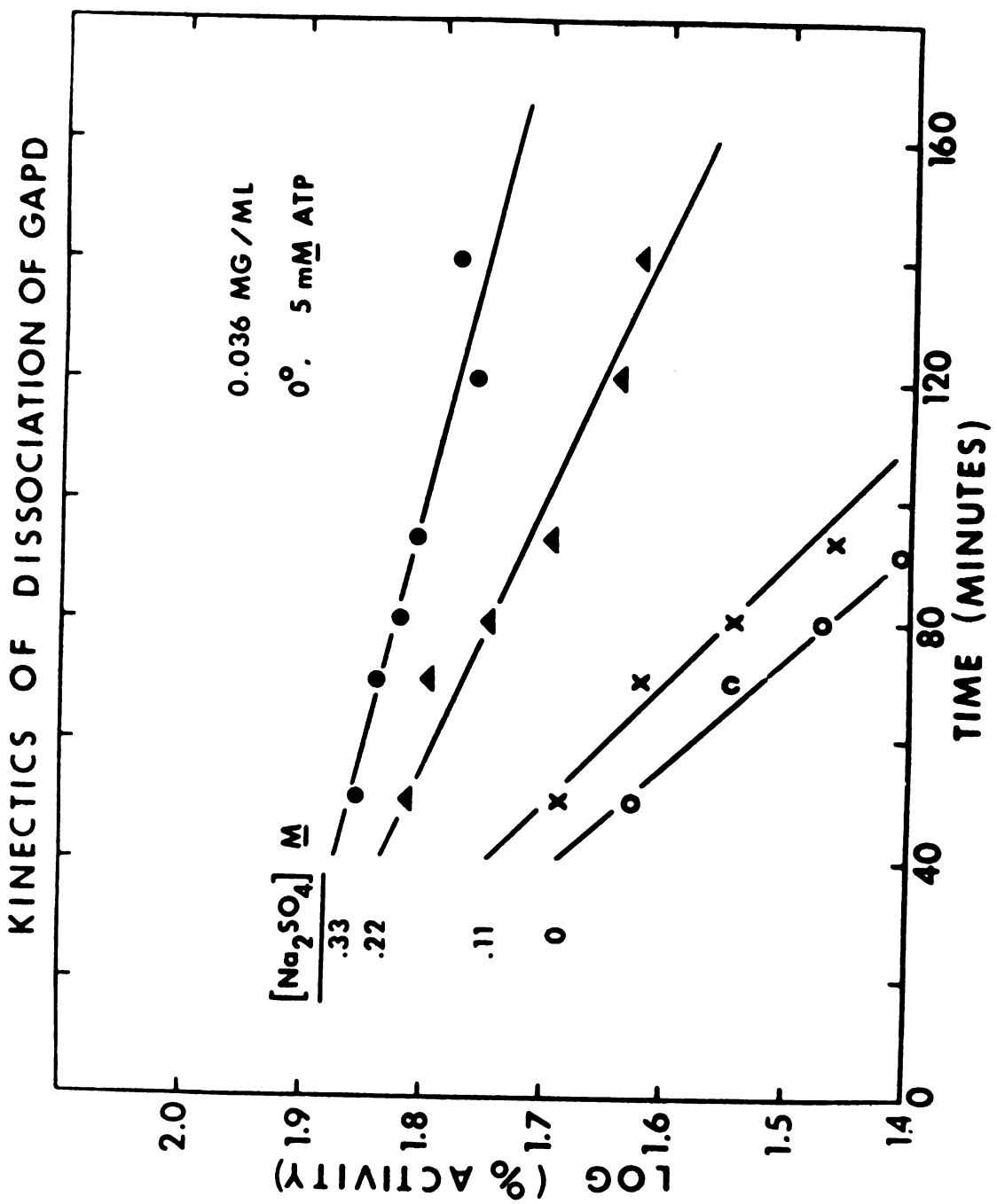
The dissociation was therefore examined as a function of Na_2SO_4 concentration (Figure 27), since increasing ionic strength should inhibit a process which exposes hydrophobic residues to the solvent medium. It is clearly seen that there is a marked inhibition of the slow process. Tests with 0.33 M NaCl also gave a noticeable inhibition (Figure 27), so the inhibition was not specific for sulfate.

The effect of Na_2SO_4 on the slow process (Figure 27) could be described by an expression of the type

$$\log (k_o/k) = S\ C_s$$

where k_o is the rate constant measured with no added salt, k is the rate constant measured at a given salt concentration, C_s (M), and S is a constant. This observation supports the hypothesis that the contact sites between subunits in the dimer involves considerable hydrophobic interactions (Tanford, 1964; von Hippel, 1969).

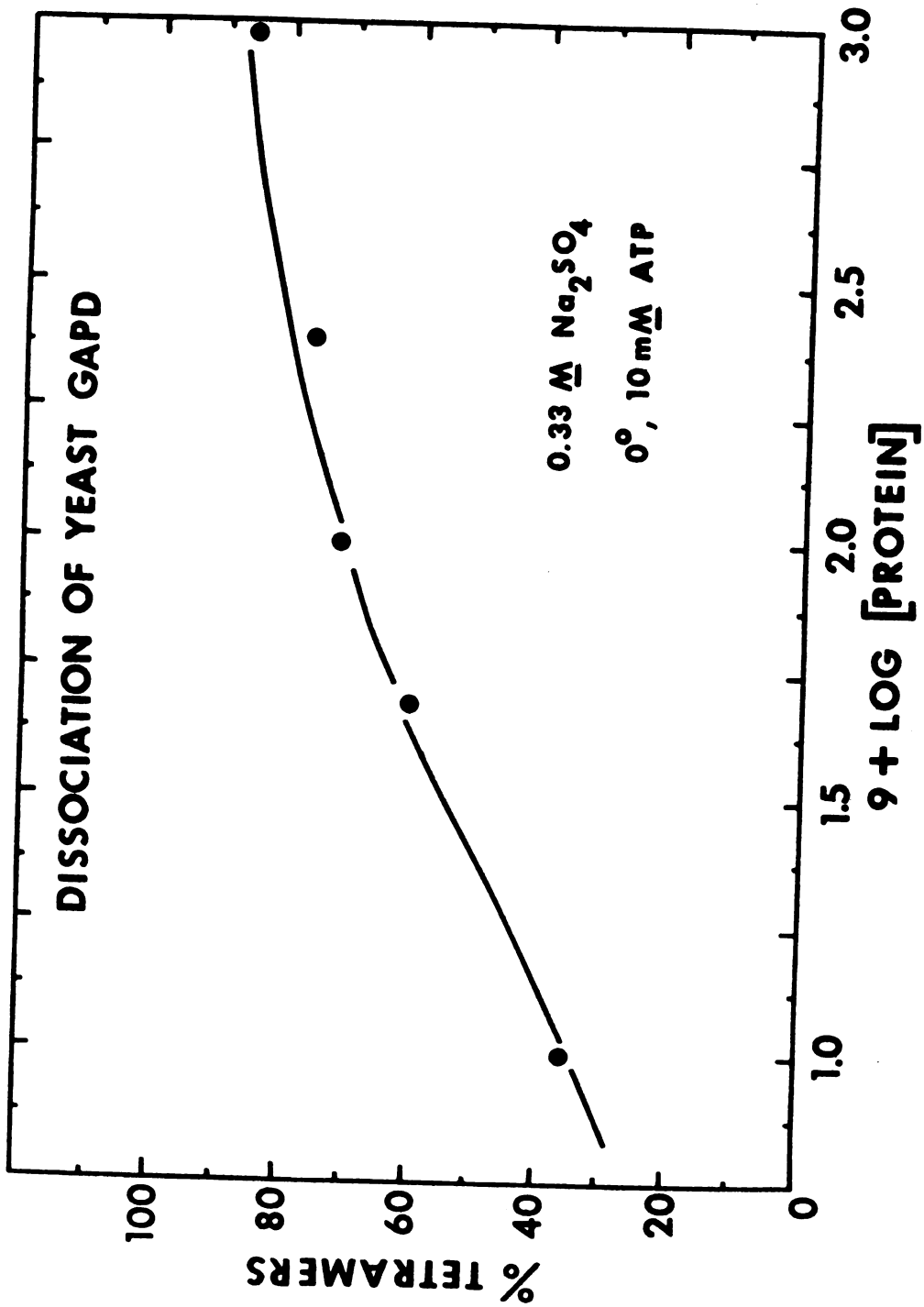
Figure 27. Inhibition of the slow process (presumed dimer breakdown) by increasing ionic strength. See text for further details.


KINECTICS OF DISSOCIATION OF GAPD
0.036 MG/ML
0°. 5 mM ATP
[Na2SO4] M
.33
.22
.11
0
LOG (% ACTIVITY)
TIME (MINUTES)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
0
40
80
120
160

To insure that the effect of the salt on the dissociation was not to alter the ATP binding constant or the mechanism of the dissociation, the binding constant for ATP and the order of the slow step were determined in the presence of 0.33 M Na_2SO_4. Using procedures similar to those in Figure 25 a binding constant of 0.5 mM was obtained in the presence of salt, and using the half-life method (Figure 22) an order of 0.4 was determined for the slow process in the presence of salt. Thus it seemed that the salt had no major effects on the ATP binding or the mechanism of dissociation.

The intercepts obtained by extrapolation of the rate of the slow process to time zero in the presence of 0.33 M Na_2SO_4 were also used to calculate equilibrium constants for a tetramer-dimer equilibrium. The average of 5 values in 0.33 M Na_2SO_4 was 4.8×10^{-8} M. The constancy of the values obtained at the different protein concentrations is illustrated in Figure 28, which is a plot of % tetramers (active) as a function of protein concentration. The line is a theoretical line drawn with the experimentally determined constant of 4.8×10^{-8} M. The points are the values of % tetramers calculated from the extrapolation of the rate of the slow process to time zero. The agreement between the points and the line suggests that the fast process may also be described as a tetramer-dimer equilibrium in the presence of salt.

Figure 28. The per cent tetramers of an equilibrium mixture of tetramers and dimers as a function of total protein concentration (expressed on a molar basis). The points are calculated from the experimentally determined equilibrium constants at each protein concentration and the line is the theoretical line drawn for an average equilibrium constant for 4.8×10^{-8} M. See text for further details.


DISSOCIATION OF YEAST GAPD
0.33 M Na2SO4
0°, 10 mM ATP
% TETRAMERS
9 + LOG [PROTEIN]
100
80
60
40
20
0
1.0
1.5
2.0
2.5
3.0

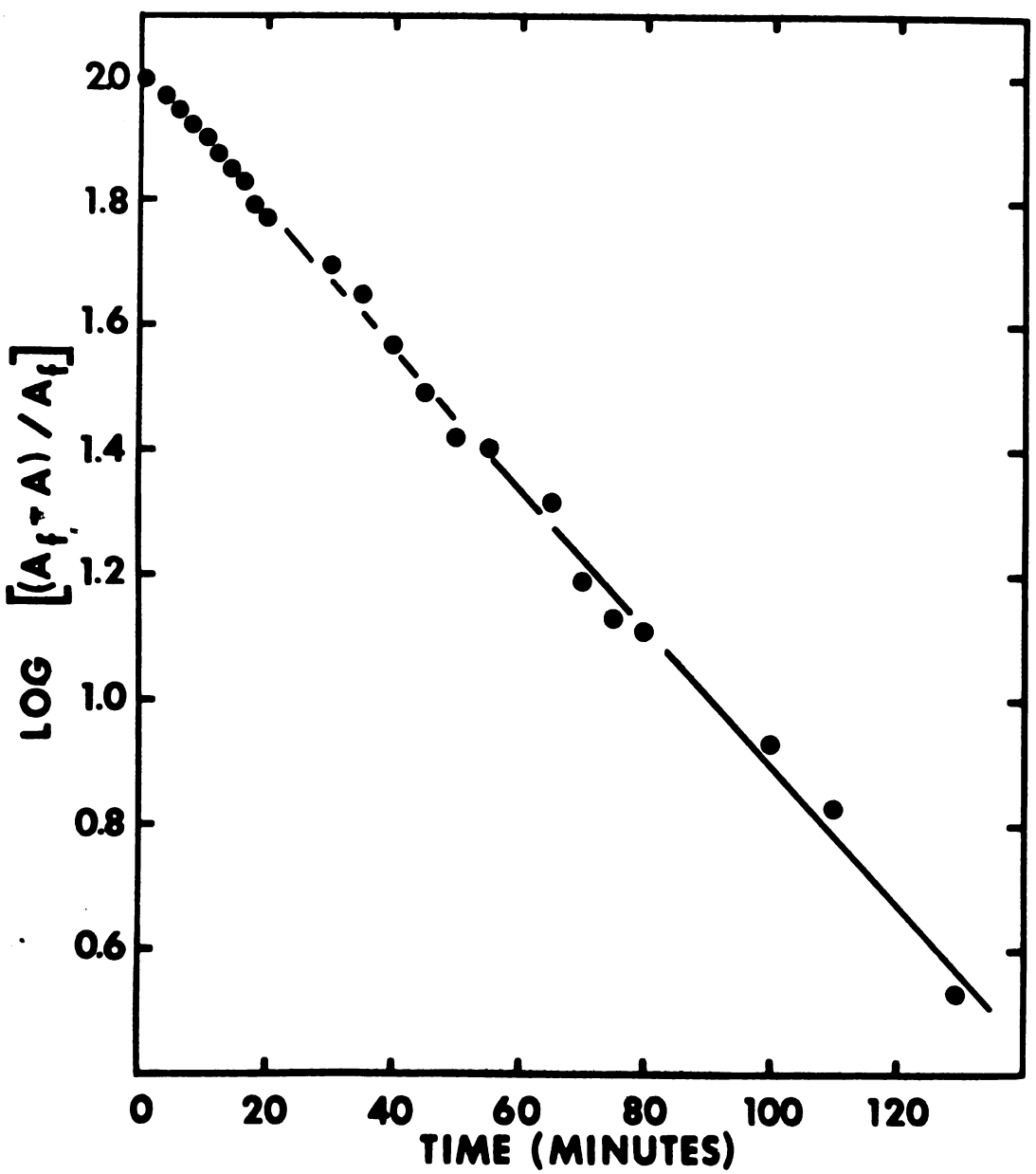
REVERSAL OF DISSOCIATION

Previous work had indicated that the dissociation could be reversed by warming the monomers to temperatures above 15° in the presence of 10% sucrose and small amounts of β-mercaptoethanol (Stancel and Deal, 1969). It was therefore of great interest to study the kinetics of the reversal process.

FIRST ORDER DEPENDENCE OF THE REASSEMBLY PROCESS AT HIGHER PROTEIN CONCENTRATION. When a completely dissociated sample of GAPD was subjected to the reversal conditions and warmed to 23°, the recovery of activity gave a very good fit to an empirical first-order rate law (Figure 29). This suggested that a first order process was the rate limiting step in the reassociation. To further test this possibility the points were replotted as the final activity recoverable (A_f) minus the activity at a given time (A_t) vs time. Tangents were drawn to the curve at various points and the logs of the slopes of these tangents were plotted vs the logs of (A_f-A_t) at the point the tangent was drawn. These points fell on a straight line with a slope of 0.96, thus indicating that the rate-limiting step was in fact first-order with respect to protein concentration under the conditions of the experiment.

Another test of a first order process is that the half-times should be independent of initial protein concentration. The dependence of the half-times for reversal

Figure 29. Semi-log plot of activity recovery observed upon warming yeast GAPD subunits to 23° as described in Methods. The protein sample (0.1 mg/ml) was reassociated in 0.21 M Tris, pH 7.0, 10% sucrose, 5 mM ATP and 0.075 M β-mercaptoethanol. See text and Methods for further details.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
LOG [(A_f - A) / A_f]
0
20
40
60
80
100
120
TIME (MINUTES)

on initial protein concentrations was thus determined under two sets of conditions, the standard condition and the standard condition plus 0.3 M KCl. This was done since a previous study of the reversal of urea-dissociated subunits employed this concentration of KCl (Deal, 1969). Results illustrated in Table V clearly indicate that the half-times in this concentration range are independent of protein concentration.

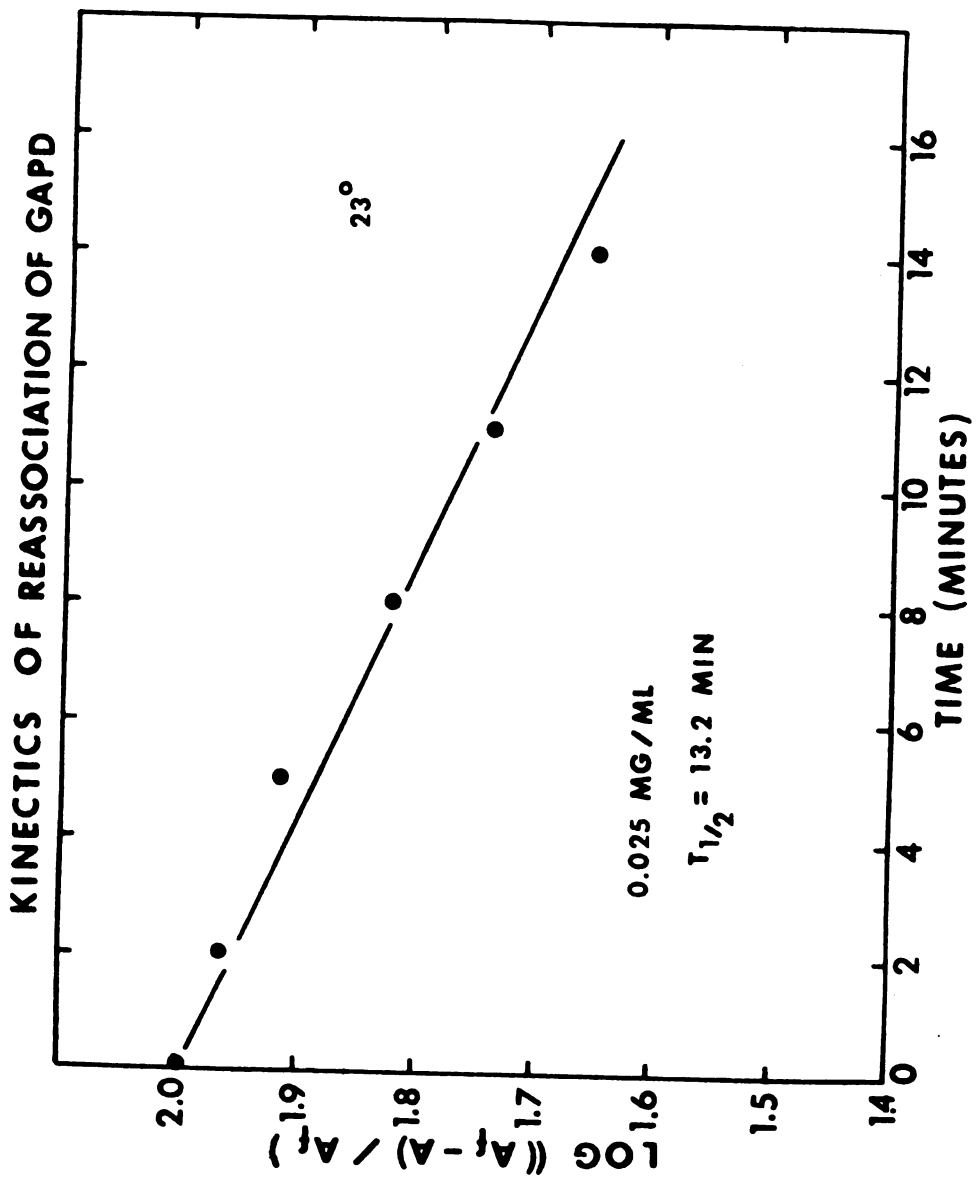
SECOND ORDER DEPENDENCE OF THE REASSEMBLY PROCESS AT LOW PROTEIN CONCENTRATION. To further insure, however, that the half-lives did not depend on concentration, these experiments were repeated at protein concentrations an order of magnitude lower than those used for the data of Table V. The results are given in semi-log form in Figure 30 (A-D), and indicate that the half-times at these lower concentrations do vary with initial protein concentration. These results suggested that at the high protein concentrations the rate-limiting step of the reversal was a first order process, but that as the protein concentration was lowered, the overall order of the reaction was increasing.

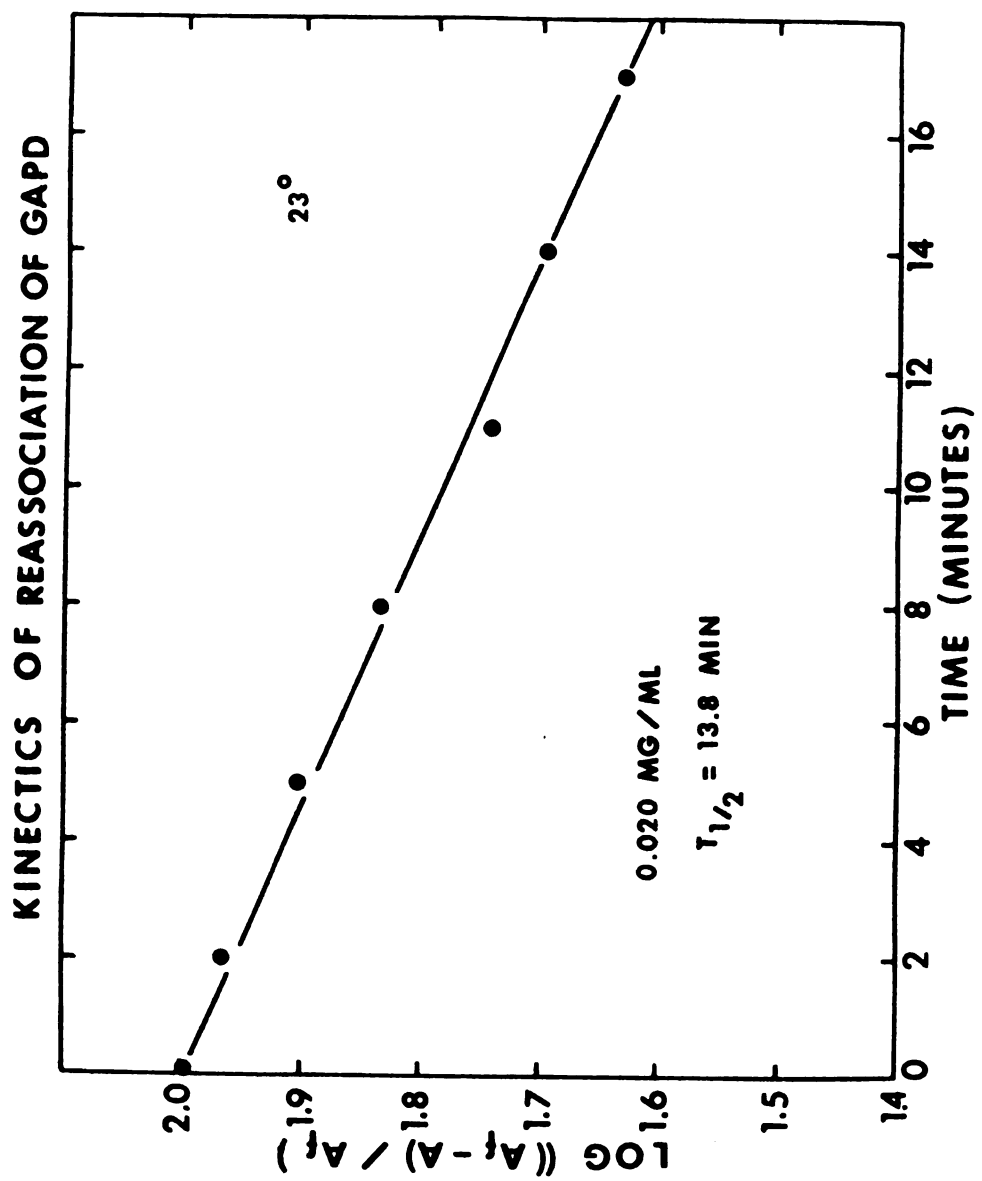
CHANGE IN ORDER WITH CHANGE IN PROTEIN CONCENTRATION. To more accurately define this apparent change in order with changes in protein concentrations, a direct differential method was employed. Various concentrations of monomers produced by dissociation at 0° were warmed at 23° in 10%

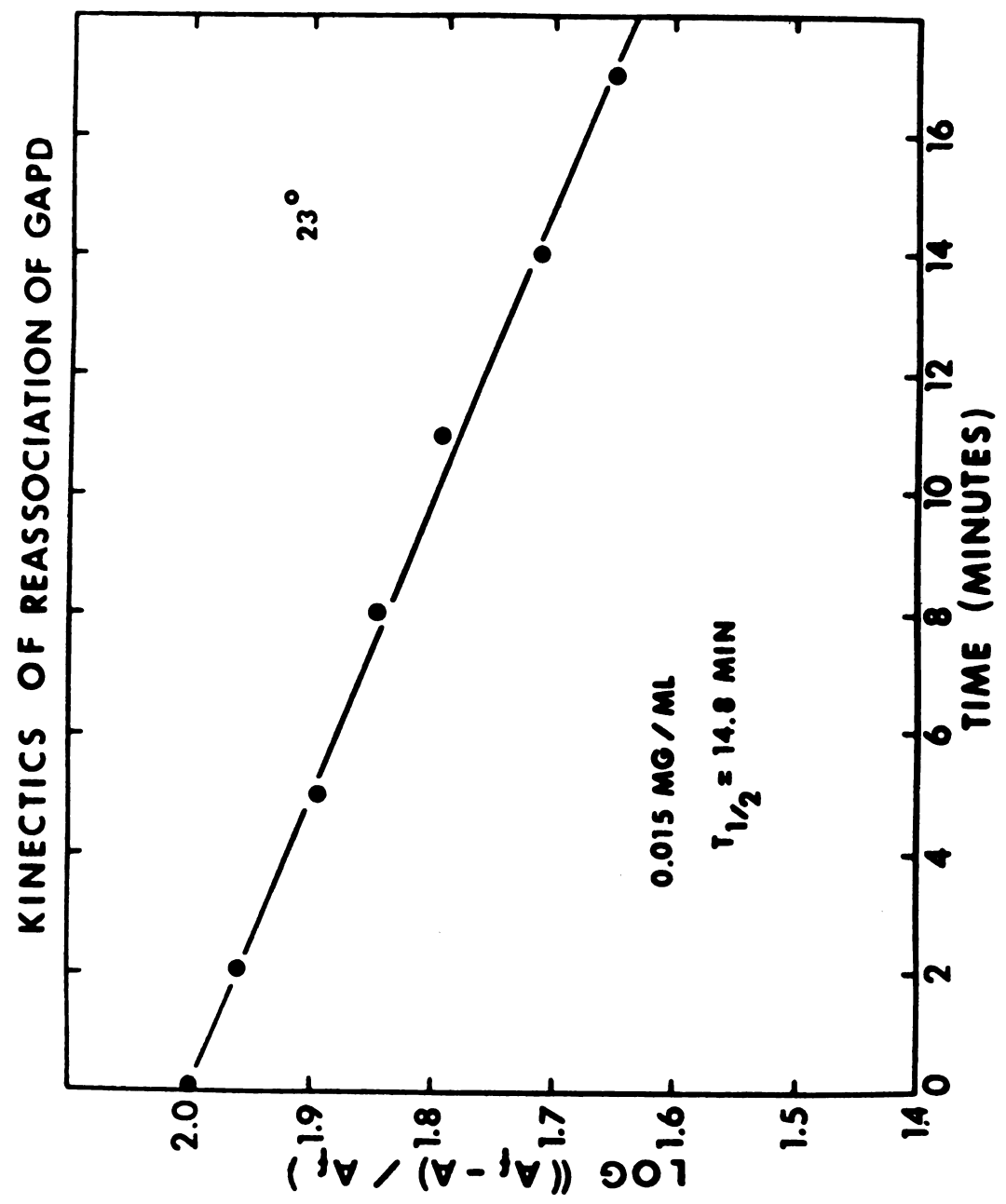
TABLE V. DEPENDENCE OF HALF-LIVES FOR REASSOCIATION ON PROTEIN CONCENTRATION

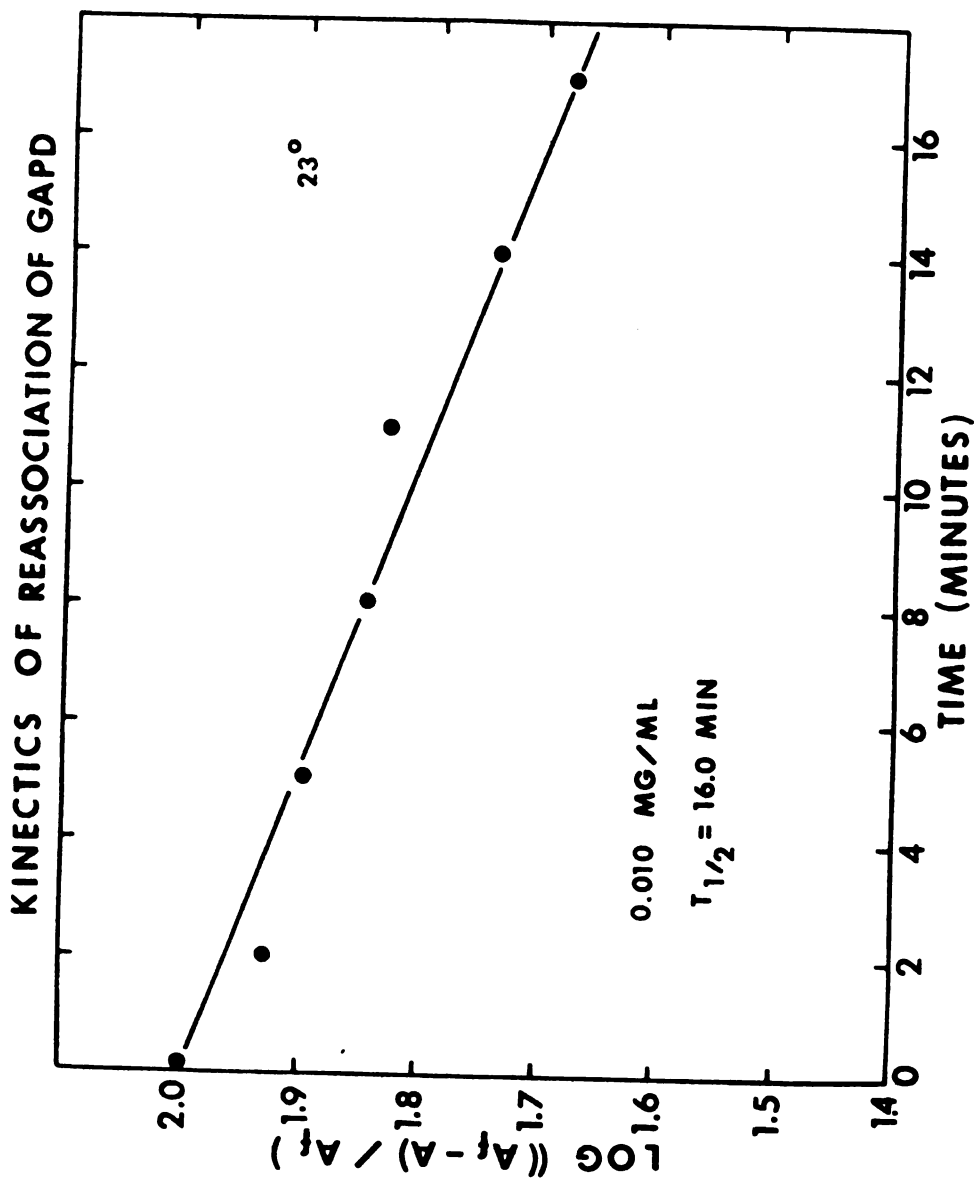
Protein Conc. (mg/ml)	No Added Salt	0.3 M KCl
	τ 1/2(min^{-1})	τ 1/2(min.)
0.063	9.0	7.6
0.10	8.7	7.5
0.20	8.5	7.5

Figure 30. Variation of the half-lives of reassociation with protein concentration, at low protein concentrations. See text for further details.


KINECTICS OF REASSOCIATION OF GAPD
23°
0.025 MG/ML
$T_{1/2}$ = 13.2 MIN
TIME (MINUTES)
LOG $((A_t - A_\infty) / A_\infty)$
2.0
1.9
1.8
1.7
1.6
1.5
0
2
4
6
8
10
12
14
16


KINECTICS OF REASSOCIATION OF GAPD
23°
0.020 MG/ML
$T_{1/2}$ = 13.8 MIN
TIME (MINUTES)
LOG $((A_f - A) / A_f)$
2.0
1.9
1.8
1.7
1.6
1.5
1.4
0
2
4
6
8
10
12
14
16


KINECTICS OF REASSOCIATION OF GAPD
23°
0.015 MG/ML
$T_{1/2}$ = 14.8 MIN
$\log ((A_\infty - A) / A_\infty)$
TIME (MINUTES)
2.0
1.9
1.8
1.7
1.6
1.5
0
2
4
6
8
10
12
14
16


KINECTICS OF REASSOCIATION OF GAPD
23°
0.010 MG/ML
$T_{1/2}$ = 16.0 MIN
LOG $((A_f - A) / A_f)$
TIME (MINUTES)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
0
2
4
6
8
10
12
14
16

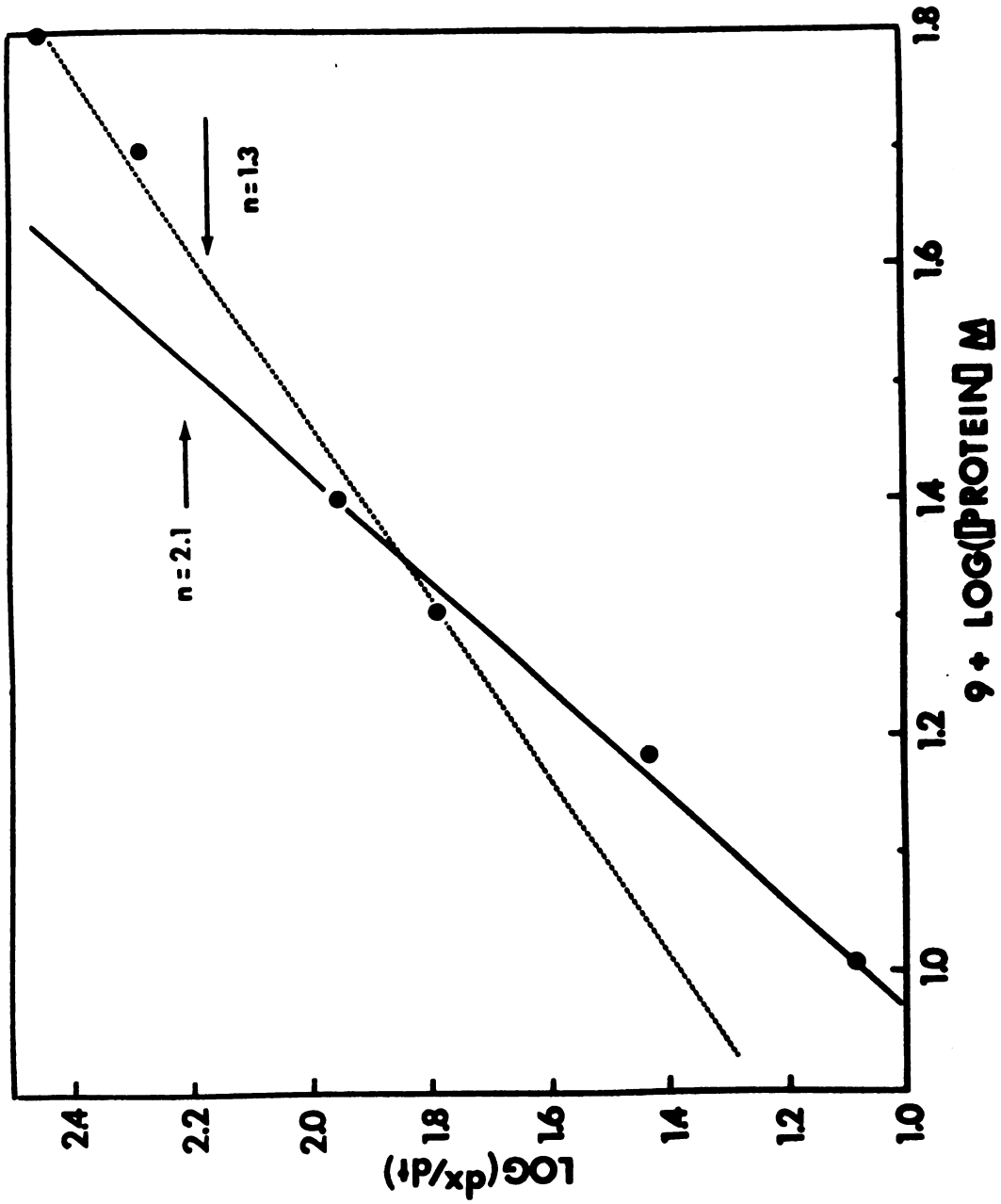
sucrose and the rate of increase of activity was measured. The data was then plotted directly as activity (μmoles substrate/min.) vs time, and an extrapolation was made to time zero to obtain the initial rate at each protein concentration. The logs of the initial rates obtained in this manner were then plotted vs the logs of the initial protein concentrations (Figure 31). The slope of such a plot yields the order of the reaction directly.

As seen in Figure 31 there is a break in the plot, indicating that the apparent order is dependent upon protein concentration. The slopes at the two ends of the plot yielded orders of 2.1 and 1.3 for the low and high protein concentrations, respectively. This experiment was repeated four times with essentially identical results. The values of the reaction order ranged from 1.9 to 2.1 at low concentrations, and from 1.2 to 1.5 at the high concentrations. Thus, the only type of mechanism consistent with this data and the previous half-times must involve a second order rate-limiting step at low protein concentrations and a first order rate-limiting step at higher protein concentrations. The interpretation of these observations is considered further in the discussion.

DISCUSSION

MECHANISM OF DISSOCIATION. The simplest mechanism of dissociation most consistent with our kinetic evidence is as follows:

Figure 31. Change in order of the reassociation process with change in the protein concentration. dx/dt is the initial rate at each concentration (obtained from extrapolation to time zero) plotted directly as activity (μmoles substrate/min.). The slopes of the lines yield the order with respect to protein directly.


n=1.3
n=2.1
LOG(dx/dt)
9 + LOG([PROTEIN] M)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
1.0
1.2
1.4
1.6
1.8

RAPID EQUILIBRIUM

$$\text{TETRAMER} \underset{-\text{ ATP}}{\overset{+\text{ ATP}}{\rightleftharpoons}} [(\text{Tetramer}).\text{ATP}_{n_1} \rightleftharpoons 2(\text{Dimers}).$$

$$.\text{ATP}_{n_2}] \longrightarrow 4(\text{Monomers}).\text{ATP}_{n_3}$$

This mechanism is supported by the bi-phasic nature of the activity loss with time, by the inverse relationship between protein concentration and rates of both steps of the observed biphasic process, by the fit of the first step (short times) to the expected integrated rate equation for a reversible dissociation and the fit of the second step (long times) to a pseudo-first order rate law. The mechanism is also supported by the agreement of equilibrium constants calculated from the data for the first step at various protein concentrations under two different sets of conditions (Table IV and Figure 28). The half-power dependence of the second phase of the process on protein concentration is also consistent with the mechanism (see p. 106).

Although the mechanism presented above is the simplest mechanism consistent with our kinetic data the true mechanism may involve an additional tetramer species. This possibility is discussed more fully in a following section (see Chapter Six for discussion).

ABSENCE OF A DIMER PEAK IN ULTRACENTRIFUGAL ANALYSIS OF DISSOCIATION OF YEAST GAPD BY ATP. In the proposed mechanism for the dissociation of yeast GAPD the amount of dimer should be relatively small (especially at high protein concentrations) due to the magnitude of the calculated equilibrium constant, K_{42}, for the dissociation of tetramer to dimer while the amount of monomer is still able to reach sizeable proportions due to the essential irreversibility of dimer breakdown at 0°. Consequently, physical measurements, such as sedimentation velocity, which require protein concentrations several orders of magnitude above the calculated equilibrium constant, should reveal tetramers and monomers. (At a total protein concentration [on a mg/ml basis] equal to K_{42}, one would observe equal concentrations of dimer and tetramer, but at concentrations much greater than K_{42} tetramer would be the predominant species.) However, the concentration of dimers (controlled by K_{42}) should be too low to observe, especially since the relative amount of dimer decreases with increasing concentration. This seems to be the case, since sedimentation velocity experiments of GAPD incubated with ATP reveal monomer and tetramer peaks,[3] but do not exhibit a detectable dimer peak (Stancel and Deal, 1968).

[3]What we have called the tetramer peak in the noted reference may actually be a peak representing a rapidly equilibrating mixture of tetramers and dimers. The sedimentation coefficient observed for such a mixture would be the weighted average, $s_{obs.} = (c_T s_T + c_D s_D)/(c_T + c_D)$.

Rabbit muscle GAPD also seems to be dissociated by ATP at low temperatures via a similar mechanism. In this case, however, a dimer peak is observed in ultracentrifugal analysis of the dissociation process (Constantinides and Deal, 1969), indicating that the equilibrium constant for the tetramer-dimer dissociation of the rabbit muscle enzyme must be considerably larger than that for the yeast system. This appears to be the case, since rough calculations, similar to those in this work (Table IV), based on the extrapolated activities (A_o) obtained for a tetramer-dimer equilibrium, indicate that the K_{42} for the tetramer-dimer equilibrium is of the order of 10^{-6} M for the muscle enzyme. Calculation of the equilibrium constant for the rabbit muscle tetramer-dimer equilibrium based directly on the distribution between the tetramer and dimer peaks observed in ultracentrifugal analysis (Constantinides and Deal, 1969) yields an equilibrium constant of 3×10^{-5} M. The calculated values of K_{42} for the muscle enzyme, obtained by two different methods are at least two orders of magnitude larger than the value of 10.2×10^{-8} M calculated for the yeast enzyme. This indicates that the dimer-dimer attraction in the yeast enzyme is very much stronger than that in the muscle enzyme.

However, based on the equilibrium constant calculated in this work, c_D would be very small, and the observed sedimentation coefficient would be expected to very closely approach the value for the tetramer alone.

STRENGTHS OF THE DIMER-DIMER ATTRACTION: ENERGETICS OF THE TETRAMER-DIMER EQUILIBRIUM. Since energy changes between different states are independent of the pathway between the states, the free energy change upon dissociation of tetramers to dimers in the presence of ATP may be related to the dissociation in the absence of ATP.

Using the average equilibrium constant calculated for the tetramer-dimer equilibrium (Table IV) a free energy change of 8.9 kcal/mole was obtained from

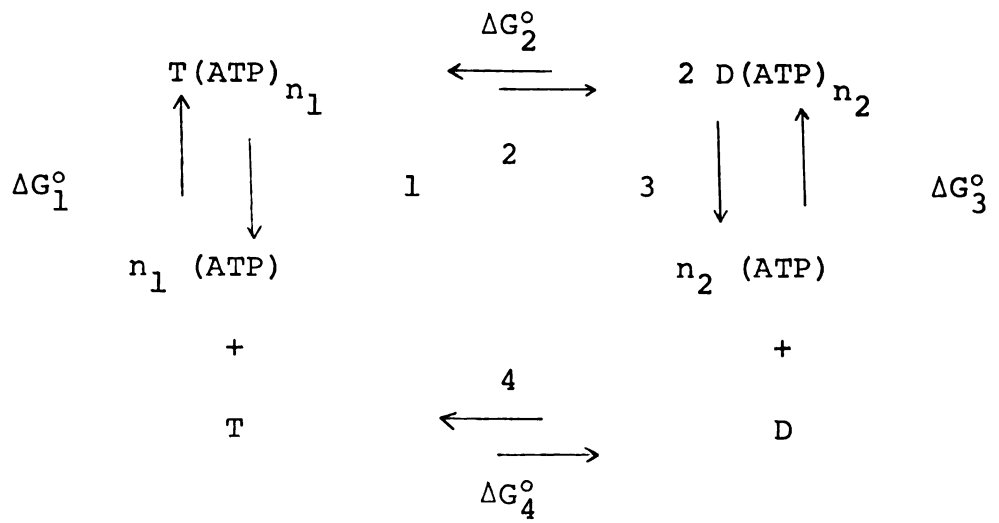
$$\Delta G^\circ = - RT \ln K_{eq}$$

Likewise, a value of 9.3 kcal/mole is calculated from the average constant obtained in the presence of 0.33 M Na_2SO_4.

These constants are obtained from experiments performed at 5-10 mM ATP and varying protein concentrations. At these ATP concentrations the tetramer may be regarded as completely saturated with ATP (Yang and Deal, 1969a; 1969b), and the dimer is also seen to be essentially saturated (Figure 25). Consequently, the calculated energy changes correspond to the process

$$T(ATP) \rightleftharpoons 2\ D(ATP)$$

If, however, the ATP binding constants for tetramers and dimers are equal, the calculated energy changes also apply to the dissociation in the absence of ATP. This is illustrated below.



For such a cycle

$$(\Delta G^\circ_1 - \Delta G^\circ_3) + (\Delta G^\circ_4 - \Delta G^\circ_2) = 0$$

Therefore, if the binding constants of ATP to the tetramer and dimer are equal (represented by steps 1 and 3 above), then $\Delta G^\circ_4 = \Delta G^\circ_2$.

The association constant for the binding of ATP to yeast GAPD dimer was determined in this work to be 0.75 mM, as illustrated in Figure 25. This value agrees well with binding constant estimated for the overall dissociation process (Stancel and Deal, 1969) and with the values for the reassembly[4] (Stancel and Deal, 1969). The value of 0.75 m<u>M</u> is also close to the values obtained for the binding of ATP to the tetramer (Yang and Deal, 1969a; 1969b) by

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[4]We have previously shown (Stancel and Deal, 1969) that ATP is required for reassociation of the subunits, as well as for dissociation.

two separate measurements. Thus it seems reasonable that $\Delta G^\circ_3 \cong \Delta G^\circ_1$, in which case $\Delta G^\circ_4 \cong \Delta G^\circ_2$.

We must stress that this calculation is only a rough approximation of ΔG° in the absence of ATP, because the binding constant of ATP to the yeast GAPD tetramer has not been precisely determined under conditions identical to those used to produce dissociation. Nevertheless, the calculation is useful and appropriate because it allows us to compare the strengths of yeast GAPD subunit interactions to the strengths of subunit interactions in other proteins (Klotz et al., 1970) and because it provides us with a rough reference point for discussing the chemical nature of the subunit contact sites.

By analogy with Langerman and Klotz (1969) for the dissociation of hemerythrin we may define a quantity ΔG°_m, such that

$$\Delta G^\circ_m = \Delta G^\circ/n$$

where n refers to the number of subunit contacts disrupted in the dissociation process. For yeast GAPD, ΔG°_m becomes approximately 4.5 kcal/mole at 0°, since two subunit contacts are disrupted in the conversion of tetramers to dimers. By way of comparison, Langerman and Klotz (1969) determined a ΔG°_m for hemerythrin of 5.8 kcal/mole at 5° in Tris/cacodylate buffer, pH 7.0, conditions very similar to those employed in this work.

Klotz et al. (1970) have also tabulated values of ΔG°_{m} for a number of proteins involved in association-dissociation reactions, and in all cases the values are between 2 kcal/mole and 8 kcal/mole.

CHEMICAL NATURE OF THE SUBUNIT CONTACT SITES. There are two major experimental findings which suggest that hydrophobic interactions between GAPD subunit contact sites play a key role in maintaining the tetrameric structure of the native enzyme. The first is that in the presence of ATP the yeast GAPD tetramer dissociates at 0°, but does not dissociate at 23° (Stancel and Deal, 1969). The second is that the breakdown of dimers to monomers is drastically inhibited by increasing the ionic strength (Figure 27).

The susceptibility of polymeric enzymes to dissociation at low temperatures indicates that hydrophobic interactions, i.e., non-covalent interactions between apolar residues, are important in maintaining the structural integrity of the enzyme (Kauzman, 1959; Scheraga et al., 1962). Studies with model systems suggest that such cold-lability of proteins may arise from unfavorable entropy losses for the interaction of apolar groups as the temperature is lowered (Kauzman, 1959). Kauzman (1959) has thoroughly discussed the phenomenon of hydrophobic interactions in a classic review. This topic has also been previously discussed for the yeast GAPD-ATP system (Stancel and Deal, 1969).

The breakdown of yeast GAPD dimers to monomers is drastically inhibited by increasing the ionic strength (Figure 27), which also suggests that the dissociation involves the exposure of hydrophobic residues to the solvent medium (Tanford, 1964; von Hippel, 1969). The inhibition of the dissociation of rabbit muscle GAPD at 0° in the presence of ATP is also inhibited by increasing ionic strength (Constantinides and Deal, 1969). Furthermore, a similar behavior is observed for the dissociation of yeast pyruvate kinase at 0° (Kuczenski and Suelter, 1970). These observations suggest that in all three cases, hydrophobic interactions play an important role at the subunit contact sites.

The requirement of sucrose (or other polyhydroxy compounds) for complete reassociation (Stancel and Deal, 1969) is also consistent with the proposed role of hydrophobic interactions. If hydrophobic residues (involved in subunit contacts) become exposed to solvent as a result of dissociation at 0°, "iceberg" structures (Frank and Evans, 1945) of water would probably form around the exposed residues to form complexes similar to clatharate structures (Kauzmann, 1959). Reassociation of subunits (at 23°) would then require that water structures which formed around exposed hydrophobic groups "melt" (Brandts, 1969) before the proper subunit contacts are formed, since water would be excluded from the hydrophobic

subunit contact sites in the tetrameric structure. Therefore, polyhydroxy compounds such as sucrose, which disrupt ordered water structures, should aid the reassociation process. Consequently, the requirement of the reassociation process for sucrose (Stancel and Deal, 1969) is consistent with the idea that the subunit contacts involve hydrophobic interactions.

A more quantitative explanation for the effect of salt on the rate of breakdown of dimers, can be obtained using transition-state theory. Thus, from transition-state theory we may write

$$D \rightleftharpoons D^* \longrightarrow M$$

The rate constant for the process at a given salt concentration, k_s, is given by (Gould, 1959)

$$k_s = [kT/h][(D^*)/(D)] = [kT/h][K^*_{eq}] \frac{(\gamma D)}{(\gamma D^*)}$$

where k and h and the Boltzmann and Planck constants, respectively, K* is the equilibrium constant for the isomerization of dimers in the ground state (D) to the transition state (D*) in the standard state (taken here to be the standard dissociation medium, minus salt), and γ has the usual significance of activity coefficient.

Since the activity coefficients in the standard state are unity, we may write

$$k_o = [kT/h][K^*]$$

and it is seen that the previous relationship reduces to

$$k_s = k_o \frac{(\gamma D)}{(\gamma D^*)}$$

Thus it is seen that

$$\log k_o - \log k_s = \log \gamma_{D^*} - \log \gamma_D$$

Since k_o is clearly greater than k_s (Figure 26), γ_{D^*} must also be greater than γ_D. This is the expected result for a process in which hydrophobic residues become exposed to the solvent medium (aqueous) in the transition state complex. It also explains the description of the salt effect on the rate by the relationship

$$\log (k_o/k_s) = S\ C_s \quad \text{(page 130)}$$

since the activity coefficients will depend on the ionic strength of the aqueous medium in which dissociation occurs.

Dissociation and reassociation studies provide valuable information about subunit contacts, but a precise determination of the chemical nature of subunit contacts in a polymeric enzyme can only be obtained from detailed X-ray analysis. At present, hemoglobin is the only polymeric protein for which X-ray analysis has reached a resolution

down to 2.8 Å (Perutz et al., 1968a; Perutz et al., 1968b). For the hemoglobin molecule contacts between unlike subunits (α and β) involve mainly apolar residues.

REASSOCIATION OF GAPD MONOMERS TO TETRAMERS. The kinetics of reassociation indicated that the rate limiting step was first order at high protein concentrations, but second order at low protein concentrations. A mechanism consistent with these observations is given below

$$\underset{\text{(inactive)}}{\text{4 MONOMERS}} \xrightarrow{1} \underset{\text{(inactive)}}{\text{2 DIMERS}} \xrightarrow{2} \underset{\text{(inactive)}}{\text{TETRAMER*}} \xrightarrow{3} \underset{\text{(active)}}{\text{TETRAMER}}$$

The kinetic analysis does not allow us to determine which of the proposed second order steps is limiting at low concentrations. Similarly, it is not possible to demonstrate that the tetramer formed in step 2 is completely inactive, but only that its activity is much less than that of the tetramer depicted as active in the proposed scheme.

As pointed out earlier (p. 90) steps 2 and 3 of the proposed mechanism should probably be written as reversible, since recombination of globular subunits produced at 0° with ATP (p. 79) and recombination of completely disordered subunits produced by exposure to 8 M urea (Deal, 1969) both show decreasing recovery of activity below protein concentrations of 0.04 mg/ml, thus suggesting that the recombination in both cases involves a concentration dependent equilibrium step.

The most interesting aspect of the proposed mechanism for reassociation is the proposed equilibrium between active and inactive tetramers (step 3). In addition to the kinetic evidence reported in this paper, two independent studies (Yang and Deal, 1969b; Kirschner, 1968) have presented data supporting an equilibrium between two different tetramers of yeast GAPD.

Yang and Deal (1969b) showed that ATP greatly increased the susceptibility of yeast GAPD to chymotryptic digestion. Since these experiments were performed under conditions which did not yield any detectable dissociation, it is clear that the ATP caused a conformational change in the tetramer sufficient to greatly increase the susceptibility of the enzyme to digestion (Yang and Deal, 1969b).

Kirschner (1968) has also shown that the tetramer of yeast GAPD undergoes an isomerization reaction at pH 8.5 and 40°. He has shown with stopped-flow techniques that the thermodynamically stable form of the tetramer is catalytically active while the second tetrameric conformation observed is practically devoid of catalytic activity (Kirschner, 1968). Interestingly, the inactive form of the tetramer can bind all of the substrates (NAD, glyceraldehyde-3-phosphate and arsenate), even though it cannot catalyze the required hydride transfer (Kirschner, 1968). These results and the results presented in this

work for the reassociation of GAPD subunits to the tetrameric enzyme compliment each other well in that they provide independent evidence for the existence of two forms of the GAPD tetramer which have greatly different catalytic properties.

COMPARISON OF THE PROPOSED MECHANISM FOR REASSOCIATION OF GAPD SUBUNITS TO OTHER ENZYMES. Steps 2 and 3 (dimer association and isomerization of an inactive to an active enzyme respectively) of the proposed mechanism for the reassociation of GAPD subunits are identical to the mechanism of recombination of aldolase dimers produced by acid treatment of the native aldolase tetramer (Blatti, 1968). Furthermore, like the recombination of GAPD subunits, recombination of aldolase monomers produced by acid dissociation of the native tetramer, also follows good first order kinetics for the over-all process under certain conditions (Deal et al., 1963). Thus it seems that in both cases, the formation of the native, active enzymes involves a conformational change as the final step which produces fully active enzyme.

The kinetics of reassociation of inactive arginnosuccinase monomers to an active dimer (Schulze et al., 1970) are similar to the kinetics of reassociation of GAPD and aldolase subpolymers in that the final step of the reassociation process appears to be the isomerization of an inactive dimer to an active dimer. For arginnosuccinase, which also undergoes cold dissociation (see p. 19),

the kinetics of reassociation are second order for reassociation in imidazole buffer and first order for reassociation in phosphate buffer (Schulze et al., 1970). Thus, for all three enzymes it appears that the final step in the reassociation process is a first order isomerization step.

MATERIALS AND METHODS

DISSOCIATION. Prior to the addition of ATP, enzyme samples were always pre-incubated for 4-12 hrs. at 0° at a protein concentration 1.11 times greater than that incubated in the text and legends for the individual experiments. After the incubation, 0.1 ml of concentrated ATP (10 times the final concentration for dissociation) previously adjusted to pH 8.0, was added at 0° to 0.9 ml of the pre-incubated enzyme solution. The time of addition of ATP was taken as time zero for the kinetic analysis. For example, to obtain the data in Figure 20, GAPD (0.04 mg/ml in 0.02 M Tris, pH 8.0 and 0.075 M β-mercaptoethanol) was incubated overnite at 0°. After this incubation, 0.1 ml of 50 mM ATP (0°) was added to 0.9 ml of the enzyme solution at 0°. Therefore, the final concentrations of GAPD and ATP were 0.036 mg/ml and 5 mM respectively. Aliquots of this solution, which were kept at 0°, were then withdrawn at various times and assayed.

Assays of catalytic activity were performed as follows. A cuvette containing 0.36 ml of the assay mix

(see Chapter Three--Methods) was placed in the sample holder of the Gilford spectrophotomer (the temperature of the sample holder was maintained at 25°). Exactly 30 sec after the cuvette was placed in the chamber, a 0.04 ml aliquot of glyceraldehyde-3-phosphate was added with a 50 µl Hamilton syringe and the mixture stirred for exactly 5 sec. with the tip of the syringe. The chart drive was turned at this time. Exactly 30 sec. after the glyceraldehyde-3-phosphate addition, an aliquot (10-25 µl) of the GAPD-ATP mixture was added with a second 50 µl Hamilton syringe and gently stirred for exactly 5 sec. with the tip of the syringe, and the monitoring of the absorbance at 340 mµ was begun as quickly as possible.

REASSOCIATION. GAPD (0.4 mg/ml in 0.02 M Tris, pH 8.0 and 0.075 M β-mercaptoethanol) was dissociated at 0° by a 12-14 hr. incubation with 10 mM ATP. The enzyme was then diluted (at 0°) with the dissociation solvent (including 10 mM ATP) to a protein concentration exactly two times that at which the reassociation kinetics were to be followed. This diluted enzyme sample was then further diluted (at 0°) with an equal volume of a solution containing 0.4 M Tris, pH 6.9, 0.075 M β-mercaptoethanol and 20% sucrose. The final conditions for reassembly were therefore 0.21 M Tris, pH 7.0, 10% sucrose, 5 mM ATP, and 0.075 M MSH. (A similar procedure in described in the

Methods of Chapter Three.) Finally, the sample in 10% sucrose (normally 1 ml) was removed from ice and placed in a large volume of water (at least 2 l) at 23° or 15° as indicated for individual experiments--this was taken as time zero in the reassembly studies. Aliquots were then removed and assayed exactly as above for the dissociation studies.

All other methods and materials have been described in Chapter Three.

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF AN INHIBITOR PROTEIN FROM YEAST WHICH INHIBITS GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND IDENTIFICATION OF THE INHIBITOR PROTEIN AS TRIOSEPHOSPHATE ISOMERASE

ABSTRACT

Examination of yeast cell extracts indicated the presence of a protein which caused an apparent inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase. Initial experiments indicated that this inhibitor protein was not a protease, and that the inhibitor was present in intact yeast cells. The inhibitor protein was purified and its molecular weight (58,000) and amino acid composition were determined. Maximal inhibition was then shown to occur when the inhibitor protein and glyceraldehyde-3-phosphate dehydrogenase were mixed in a 1:1 molar ratio, however, several physical tests failed to reveal the formation of a complex between the two proteins. The inhibitor protein was subsequently identified as triosephosphate isomerase. Nevertheless, this identification of the inhibitor protein as triosephosphate isomerase is not sufficient to clearly explain the observed inhibition, since (1) the inhibition is not completely overcome by glyceraldehyde-3-phosphate, (2) the inhibition depends on the total protein concentration as well as the ratio of the two proteins, and (3) triosephosphate isomerase seems to activate glyceraldehyde-3-phosphate dehydrogenase under certain conditions.

INTRODUCTION

One major question concerning the dissociation of yeast GAPD into subunits in ATP was whether this process had any *in vivo* significance. This seemed rather unlikely, since the major effects were produced only at low temperatures (0°) and low protein concentrations, conditions not likely to be encountered *in vivo*.

Another possibility was that the effects of ATP at higher temperatures and higher protein concentrations might be to produce minor conformational changes in the native enzyme which would render the enzyme more susceptible to proteolytic degradation. This suggestion seemed plausible for the yeast GAPD system because:

(1) Yeast contain numerous proteases with different specificities (Hata *et al.*, 1967; Lenny and Dalbec, 1967; Felix and Brouillet, 1966).

(2) ATP greatly enhances the digestion of native GAPD by purified chymotrypsin (Yang and Deal, 1969b).

(3) GAPD comprises 20% of the total soluble protein of baker's yeast (Krebs *et al.*, 1953), thus raising the possibility that GAPD, or an altered form of GAPD serves some *in vivo* function other than the catalysis of the oxidative phosphorylation of glyceraldehyde-3-phosphate.

Consequently, it was decided to examine yeast extracts to determine if a protease was present which would degrade GAPD.

Since GAPD had to be used as the substrate to assay the protease, the presence of high levels of GAPD in yeast extracts complicated the problem. Therefore, the first approach was to obtain a broad activity protease fraction from yeast using procedures reported in the literature. It was hoped that such a fraction would be relatively free of GAPD activity, and could then be tested for protease activity against purified GAPD. We shall now designate this protein fraction as either protease fraction or inhibitor protein in order to give it consistent nomenclature throughout this thesis.

RESULTS

PREPARATION OF A PROTEASE-CONTAINING FRACTION. Initial attempts at obtaining such a protease containing fraction from yeast by utilizing the procedure of Lenny and Dalbec (1967) were quite successful. One pound of baker's yeast was allowed to autolyze in 80 ml of $CHCl_3$, 550 ml of water was added, the pH was adjusted to 7.3 and the mixture centrifuged. The supernatant was adjusted to pH 4.8, and the precipatate obtained between 47 and 63% saturation with ethanol was taken up in citrate buffer, pH 5.5. The absorbance of 2.5 at 280 mμ of this preparation was taken as a crude measure of the protein content; a value of 2.5 mg/ml was estimated.

TEST FOR INACTIVATION OF GAPD AND TESTS FOR PROTEASE ACTIVITY AGAINST HEMOGLOBIN, GAPD, AND ALDOLASE. When solutions of purified yeast GAPD were incubated with aliquots of the inhibitor protein, there was a rapid loss of GAPD activity. The agent responsible for this loss of activity was a protein, since it was heat-labile, TCA-precipitable and non-dialyzable. These experiments are summarized in Table VI.

It was initially thought that this loss of activity was probably due to a protease in the inhibitor protein preparation. The inhibitor protein preparation was therefore examined for proteolytic activity using 1% hemoglobin as a substrate (Figure 32). The inhibitor protein preparation released considerable amounts of TCA soluble ninhydrin positive materials from hemoglobin and this was inhibited by 1 mM phenylmethylsulfonylfluoride, an inhibitor of proteases with serine residues at the active site (Steinman and Jakoby, 1967). This seemed to provide good evidence that the inhibitor protein preparation contained a protease which could digest GAPD.

Surprisingly, treatment of the inhibitor protein preparation with 1 mM phenylmethylsulfonylfluoride did not prevent the loss of GAPD activity upon incubation with the treated inhibitor protein preparation. This unexpected result suggested that the observed loss of GAPD activity (Table VI) was not the result of proteases present in the

TABLE VI. LOSS OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY IN THE PRESENCE OF INHIBITOR PROTEIN

Treatment of Inhibitor Protein	% GAPD Activity Remaining after 5 min. Incubation*
NONE	36
Supernatant from 0.34 N TCA precipitation[a,b]	74
100°, 5 min.[a]	91
30°, 2 1/2 hrs.	25
20 hr. dialysis	29

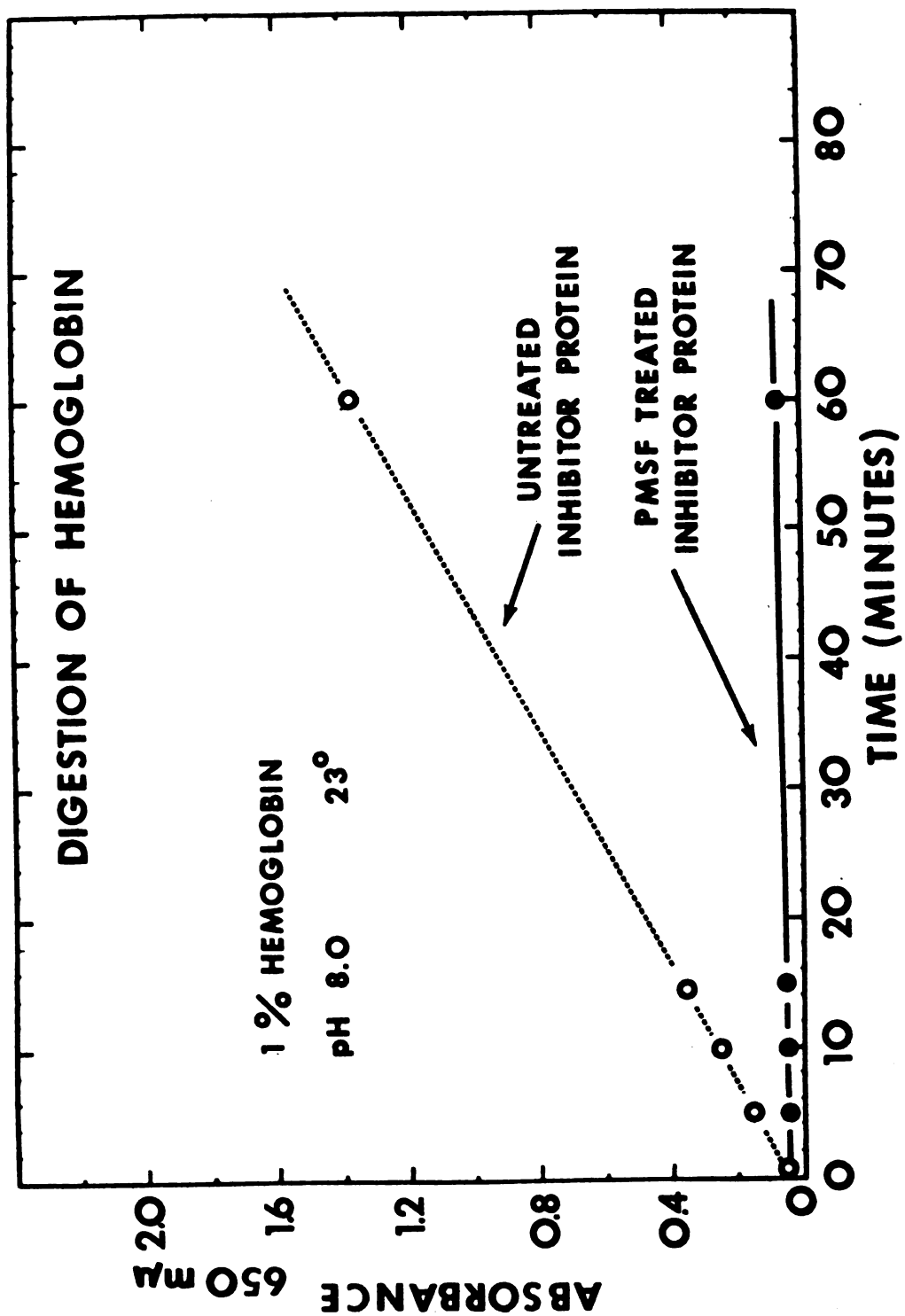
*Aliquots of the inhibitor protein preparation (0.1 ml) were added to 0.9 ml samples of GAPD (0.2 mg/ml) in 0.1 M Tris, pH 8.0 containing 0.1 M β-mercaptoethanol. After a 5 min. incubation at 23°, the GAPD activity was measured as described in Methods. % GAPD activity was calculated on the basis of the activity of a GAPD sample treated in a similar manner, minus the addition of the inhibitor protein.

[a]Precipitate which formed was discarded by centrifugation.

[b]After centrifugation, the supernatant was adjusted to neutral pH with bicarbonate.

Figure 32. Digestion of denatured hemoglobin by inhibitor protein preparation. Inhibitor protein (2.0 ml of 0.65 mg/ml in Tris, pH 8.0) was added to 6.0 ml of 1% hemoglobin (in 6.6 M urea, 0.1 M Tris, pH 8.0)-dashed line. At the given times 1 ml aliquots were removed and added to 1.25 ml of 0.34 N TCA. After filtration 2.5 ml of 0.3 N NaOH (2.9% in Na_2CO_3) was added, then 0.75 ml of Folin reagent (diluted 1:1 with water) was added. After 10 min. the absorbance at 650 mμ was read.

The phenylmethylsulfonylfluoride (PMSF) study was similar-solid line-except that the inhibitor protein was incubated for 5 min. with 10^{-3} M PMSF prior to addition to the hemoglobin solution.


DIGESTION OF HEMOGLOBIN
1% HEMOGLOBIN
pH 8.0
23°
UNTREATED INHIBITOR PROTEIN
PMSF TREATED INHIBITOR PROTEIN
ABSORBANCE 650 mμ
0
0.4
0.8
1.2
1.6
2.0
0
10
20
30
40
50
60
70
80
TIME (MINUTES)

preparation. It was therefore decided to examine the protein specificity of this inhibition by the inhibitor protein fraction. This was done by analyzing the effect of the inhibitor protein preparation on the activity of rabbit muscle aldolase, since this test would resemble the experiments with GAPD more closely than the experiments with the denatured hemoglobin.

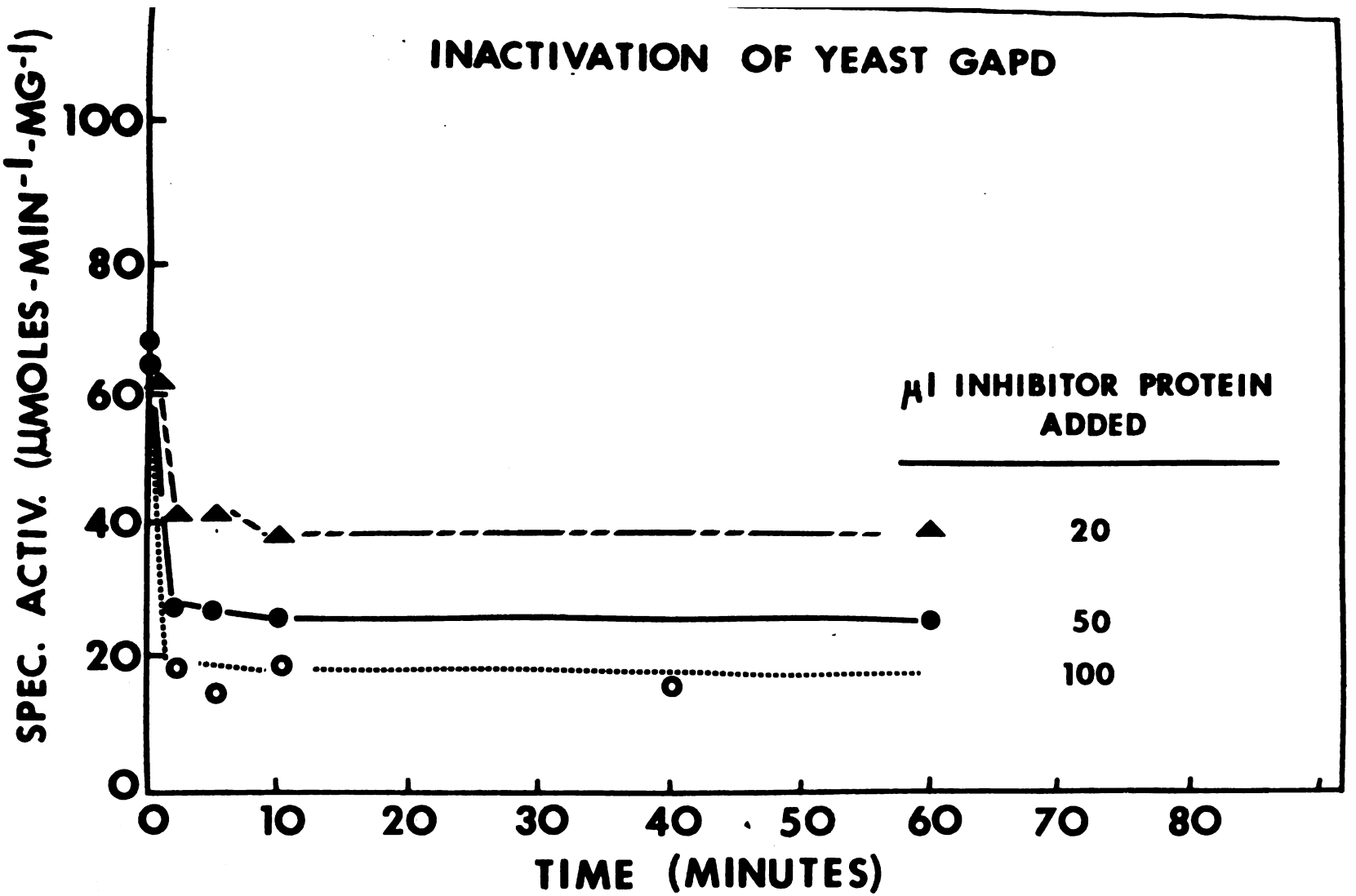
When aldolase samples were incubated with the inhibitor protein preparation at 23° all the aldolase activity was lost within 30 min. This loss of activity could not be reversed by the addition of phenylmethylsulfonylfluoride to samples of aldolase inactivated by this treatment. If however, the inhibitor protein preparation was treated with 1 mM phenylmethylsulfonylfluoride prior to incubation with the aldolase samples, the aldolase retained 75% of its original activity after a 30 min. incubation at 23°, indicating that a protease caused the loss of the aldolase activity.

EVIDENCE THAT GAPD ACTIVITY LOSS WAS NOT DUE TO A PROTEASE. The results with GAPD, hemoglobin and aldolase suggested that the inhibitor protein preparation contained a protein which caused the loss of GAPD activity by some mechanism other than proteolytic degradation, and also contained a protease(s) which degraded aldolase and hemoglobin. It is not surprising that GAPD was not degraded by the protease(s) present since Halsey and Neurath (1962) showed

that native GAPD was not degraded by carboxypeptidase, except in the presence of high concentrations of urea and Yang and Deal (1969b) showed that chymotrypsin had little effect on the enzyme in a 10 min. incubation. To further test this possibility, samples of GAPD were incubated with different amounts of the inhibitor protein preparation, and the GAPD activity was measured as a function of time. If the loss of GAPD activity were due to proteolysis, the total GAPD activity should decrease with time. This was not observed. As illustrated by the data of Figure 33 the loss of GAPD activity did not increase with time after the addition of the inhibitor protein aliquots to the GAPD samples. These results and the failure of phenylmethylsulfonylfluoride to prevent the loss of GAPD activity in the presence of the inhibitor protein preparation clearly indicated that the inactivation of GAPD was not a result of proteolysis.

If the loss of GAPD activity was not due to proteolytic degradation, it seemed likely that the activity loss might be reversible. To test this possibility a 0.5 ml sample of GAPD, specific activity of 104, was prepared (0.4 mg/ml in 0.1 M Tris, pH 8.0, and 0.1 M in β-mercaptoethanol). An aliquot of the inhibitor protein preparation (50 µl) was then added and the mixture incubated at 23° for 5 min. This treatment resulted in a decrease of specific activity from 104 to 30. When this inactivated

Figure 33. Effect of the time of incubation on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by the inhibitor protein. The indicated volumes of inhibitor protein (2.5 mg/ml) were added to glyceraldehyde-3-phosphate dehydrogenase (0.2 mg/ml) in 0.1 M Tris, pH 8.0, and 0.1 M β-mercaptoethanol. Total volume was 1.0 ml. Aliquots of the mixture of the two proteins were assayed at the indicated times.


INACTIVATION OF YEAST GAPD
SPEC. ACTIV. (μMOLES-MIN⁻¹-MG⁻¹)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
TIME (MINUTES)
μl INHIBITOR PROTEIN ADDED
20
50
100

sample was then diluted ten-fold with buffer, the specific activity increased from 30 to 108, the original value. This experiment proved conclusively that the inactivation of GAPD was not a result of proteolytic digestion.

POSSIBLE EXPLANATIONS OF THE EFFECT. There were basically three other explanations for the observed loss of GAPD activity in the presence of the inhibitor protein preparation.

(1) The inhibitor protein preparation could contain a protein which was converting one of the substrates of the GAPD reaction to some other product.

(2) The activity loss of GAPD might be an artifact of the procedure used to prepare the inhibitor protein. For example, the protease fraction might contain GAPD-like protein fragments, arising from proteolytic degradation of GAPD itself or other proteins during the preparation of the inhibitor protein, which could bind to the purified GAPD in the activity test and cause activity loss.

(3) The inhibition of GAPD could result from the reversible binding of a specific protein inhibitor present in intact yeast cells.

The first possibility, that another enzyme in the inhibitor protein preparation was rapidly removing the

substrates of the GAPD reaction, was tested by measuring GAPD activity with and without added inhibitor protein as a function of substrate concentration. Two samples of GAPD were prepared, and an aliquot of the inhibitor protein preparation was added to one of the samples. The other sample (minus the inhibitor protein) served as the control. Aliquots of both samples were withdrawn and assayed with a fixed concentration of glyceraldehyde-3-phosphate and varying concentrations of NAD; then the samples were assayed at a fixed NAD concentration and varying concentrations of glyceraldehyde-3-phosphate. The results are illustrated in Figures 34 and 35 respectively. In both cases the activity loss was not overcome by the addition of excess substrate above a concentration of about 1.5 m$\underline{M}$. Thus, it did not seem that the inactivation of GAPD resulted from decreased substrate concentrations. (However, later results disproved this conclusion--see p. 228.)

The second possibility, that the loss of activity of GAPD might be caused by the presence of GAPD-like protein fragments produced by proteolytic degradation of a protein present in the original yeast extract, was evaluated by two separate experiments. The first experiment was to see whether preparation of the protease fraction in the presence of the protease inhibitor, phenylmethylsulfonyl-fluoride, would result in the fraction possessing decreased ability to cause GAPD activity loss. The second experiment

Figure 34. Effect of NAD concentration on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by inhibitor protein. The concentration of glyceraldehyde-3-phosphate in the assays was 7.5×10^{-4} M. Glyceraldehyde-3-phosphate dehydrogenase (with and without inhibitor protein present) assayed as described in Methods. Concentration of glyceraldehyde-3-phosphate dehydrogenase was 0.2 mg/ml; concentration of inhibitor protein was 0.1 mg/ml. See text for details.

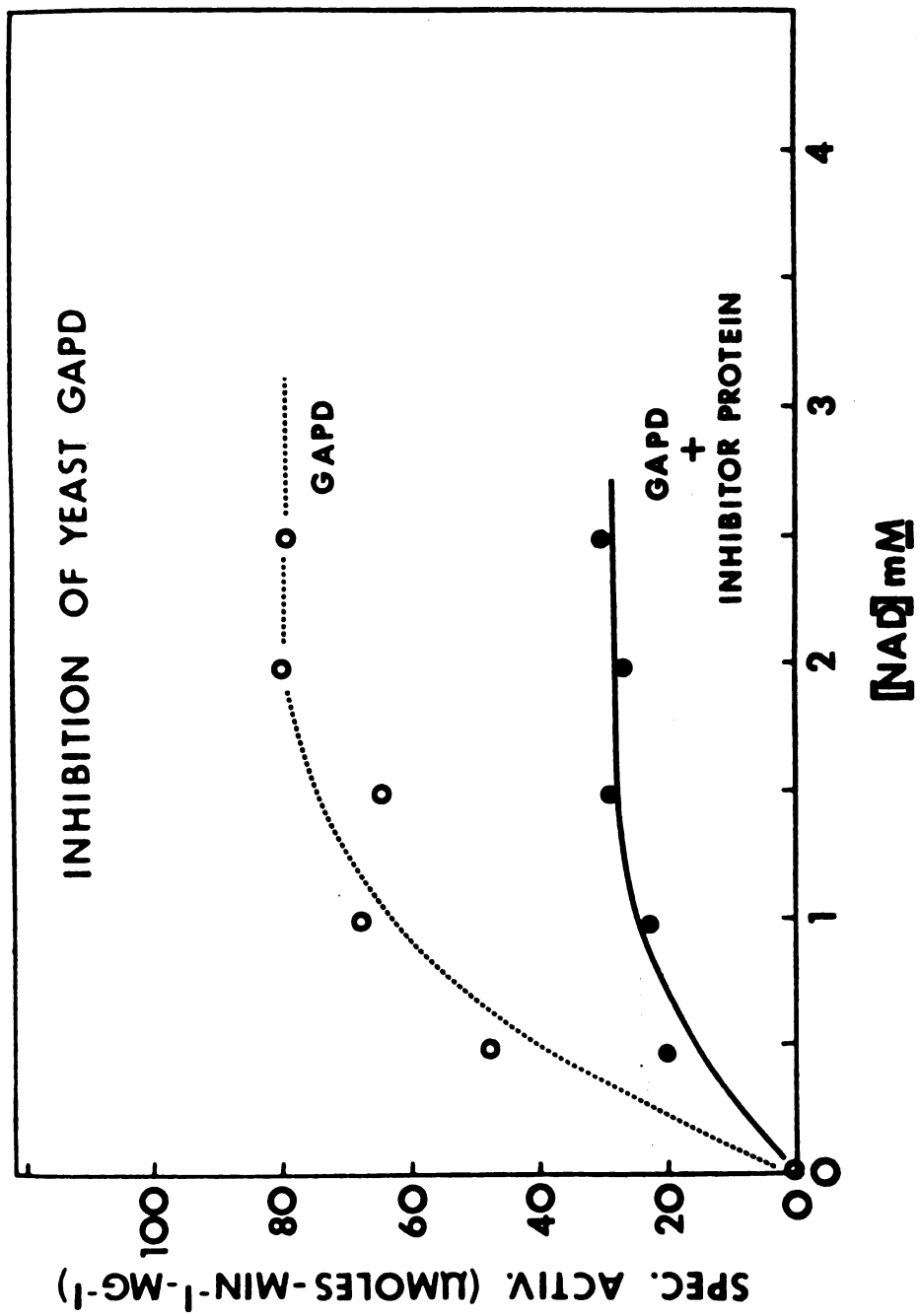
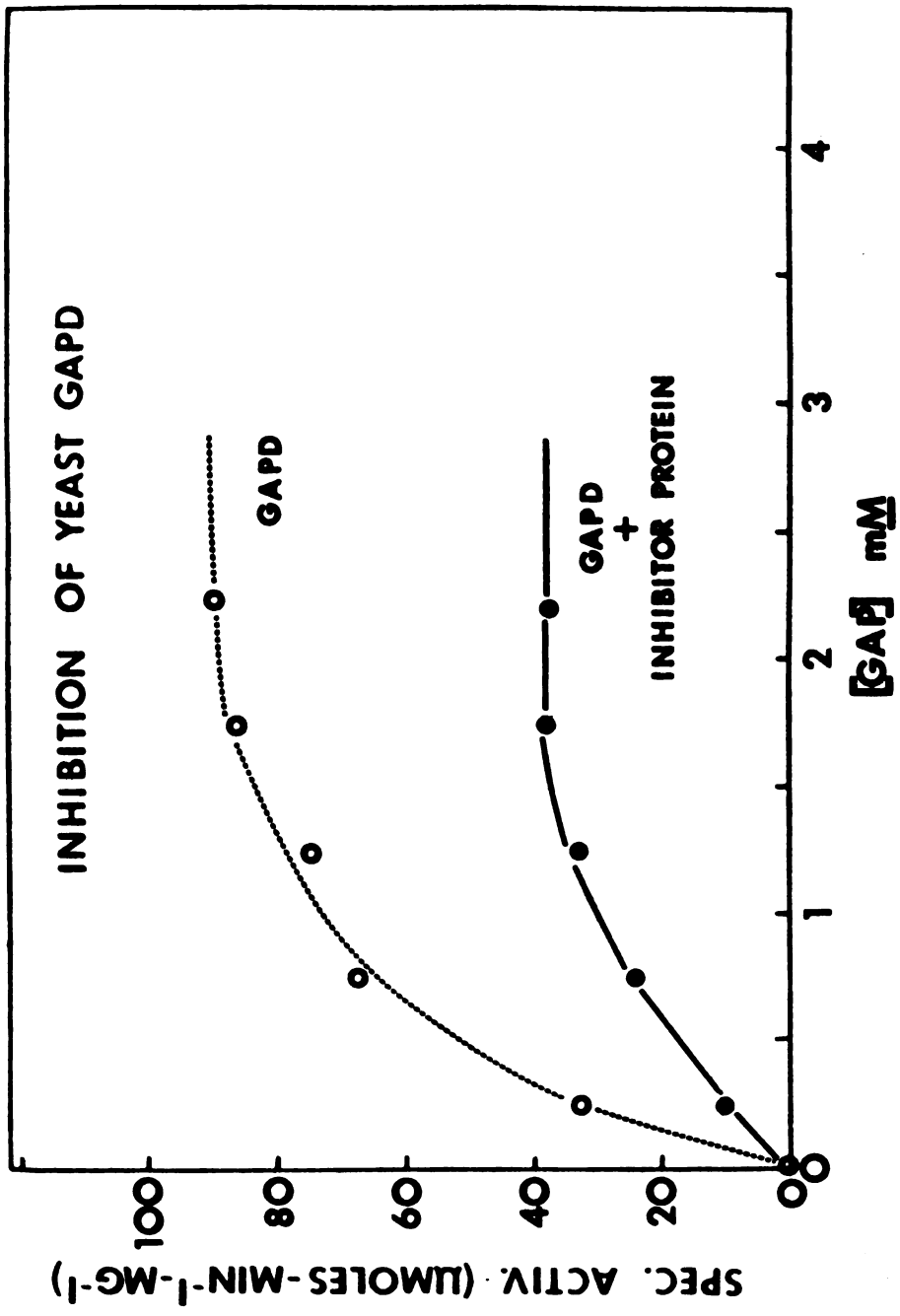

INHIBITION OF YEAST GAPD
SPEC. ACTIV. (UMOLES-MIN-1-MG-1)
100
80
60
40
20
0
0
1
2
3
4
[NAD] mM
GAPD
GAPD
+
INHIBITOR PROTEIN

Figure 35. Effect of glyceraldehyde-3-phosphate concentration on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by inhibitor protein. The concentration of NAD in the assays was 1 mM. See legend of Figure 34 and text.


INHIBITION OF YEAST GAPD
GAPD
GAPD
+
INHIBITOR PROTEIN
SPEC. ACTIV. (UMOLES-MIN-1-MG-1)
[GAP] mM
0
20
40
60
80
100
1
2
3
4

attempted to test for the production in the protease fraction of GAPD-like fragments by a protease not inhibited by phenylmethylsulfonylfluoride.

In the first experiment the inhibitor protein was prepared by two different methods. The first preparation method was as previously described. The second preparation method was similar to the first except that all aqueous solutions used contained 1 mM phenylmethylsulfonylfluoride. The protein concentrations of both the preparations were the same, and both preparations produced the same amount of activity loss when incubated with purified GAPD.

In the second experiment, a sample of baker's yeast was autolyzed in $CHCl_3$, distilled water was added, and the mixture allowed to stand at room temperature as previously described. Aliquots were then removed at various times and quickly placed in ice, since any proteolytic activity should be substantially reduced by lowering the temperature to 0°. After the last aliquot was removed and chilled in ice (23 hrs), each of the 10 aliquots were used to prepare a sample of the inhibitor protein as previously described. If the inhibitor protein was not present in intact yeast cells, but was produced as a result of proteolytic degradation during the preparation procedure, then the samples which remained at room temperature the longest should contain the highest levels of the inhibitor protein and should produce the greatest loss of GAPD activity. This

was not observed, as illustrated in Figure 36. The samples prepared from the different aliquots (which had remained at room temperature for various times) all produced the same loss of GAPD activity, and the samples all had the same protein concentration. Thus the inhibitor protein responsible for the loss of GAPD activity was present in the intact yeast cells, and was not produced by proteolysis during the preparation procedure.

If the third possibility was correct, i.e., that a specific GAPD inhibitor protein was causing the loss of GAPD activity, then a plot of the loss of GAPD activity (expressed as % inhibition) at a fixed GAPD concentration vs increasing amounts of the inhibitor protein should increase linearly when the GAPD was in excess and then level off to a plateau value when the inhibitor was in excess. The results of such an experiment (Figure 37) qualitatively seem to resemble such a titration curve, suggesting that the GAPD is being "titrated" with a protein present in the inhibitor protein preparation, since the curve (Figure 37) levels off to a plateau region at less than 100% inhibition.

PURIFICATION OF THE INHIBITOR PROTEIN. Since the preceeding results were quite exciting and seemed to offer great promise with respect to control of glycolysis, it was decided to attempt a further purification of the protein(s) responsible for the loss of GAPD activity. It was not

Figure 36. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by different inhibitor protein preparations. Inhibitor protein preparations were obtained from aliquots of crude cell extracts which had remained at 23° for different lengths of time. See text for details.

Inhibition of glyceraldehyde-3-phosphate dehydrogenase was measured by adding a 50 µl aliquot of the given inhibitor protein preparation to 0.5 ml of glyceraldehyde-3-phosphate dehydrogenase (0.2 mg/ml) in 0.1 M Tris, pH 8.0, with 0.1 M β-mercaptoethanol. See Methods for further details.

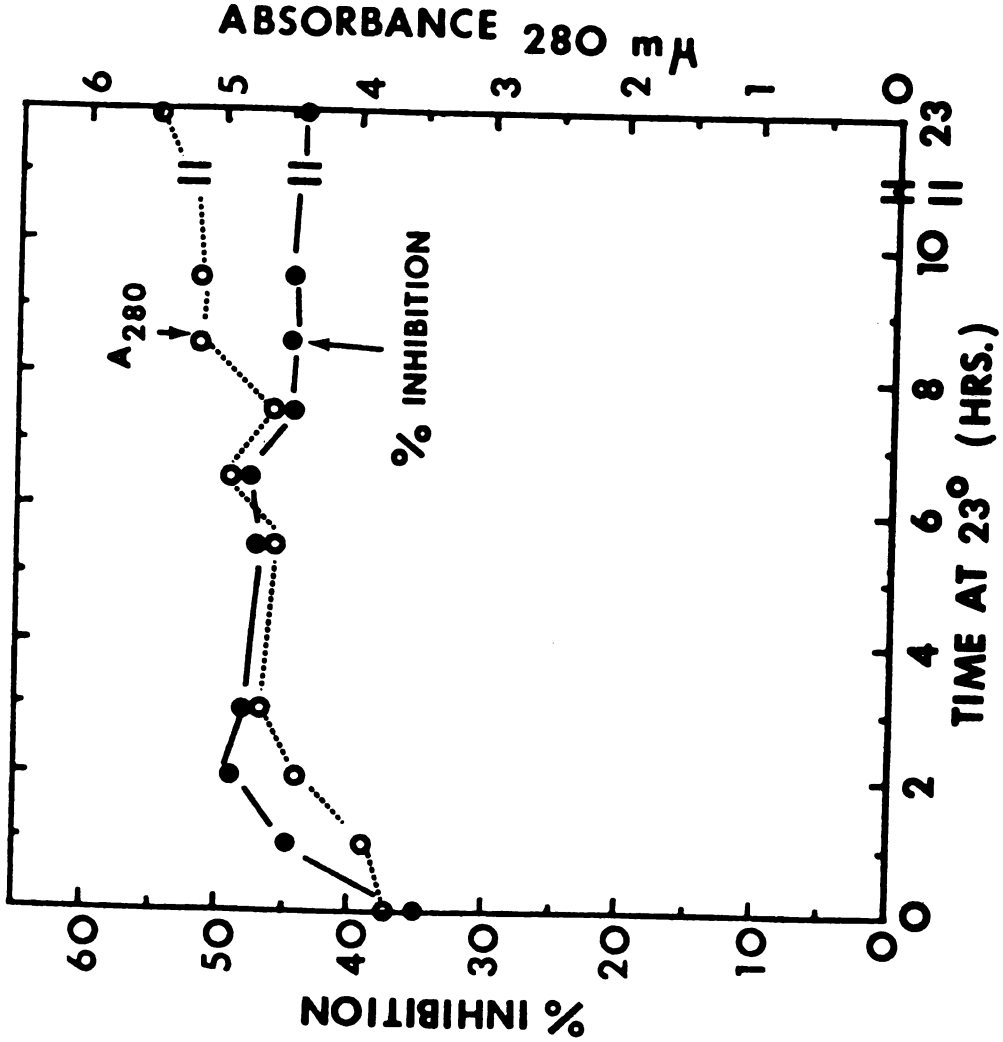
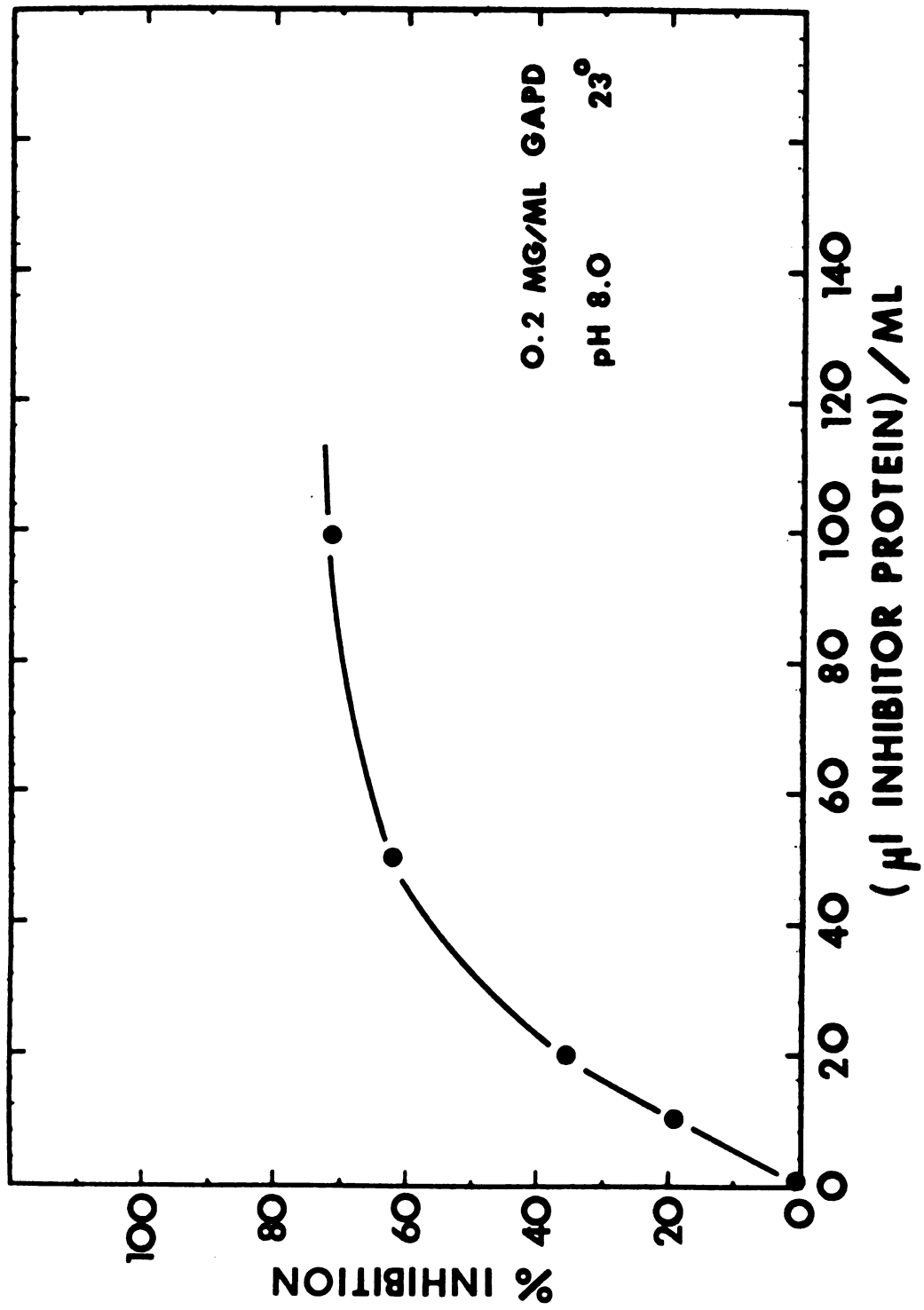

ABSORBANCE 280 mμ
6
5
4
3
2
1
0
A280
% INHIBITION
TIME AT 23° (HRS.)
0
2
4
6
8
10
23
% INHIBITION
60
50
40
30
20
10
0

Figure 37. Effect of inhibitor protein concentration on the inhibition of glyceraldehyde-3-phosphate dehydrogenase. Various amounts of the inhibitor protein preparation (2.5 mg/ml) were added to glyceraldehyde-3-phosphate dehydrogenase (0.2 mg/ml) in 0.1 M Tris, pH 8.0, with 0.1 M β-mercaptoethanol, in a total volume of 1.0 ml. An aliquot of the mixture was withdrawn and assayed as described in Methods.


0.2 MG/ML GAPD
pH 8.0
23°
% INHIBITION
0
20
40
60
80
100
0
20
40
60
80
100
120
140
(μl INHIBITOR PROTEIN)/ML

possible to attempt a purification directly from crude cell extracts because of the very high amounts of GAPD present in such extracts. The purification of the inhibitor protein was therefore begun with the previously described protease fraction obtained by the method of Lenny and Dalbec (1967).

The following section describes the purification of the inhibitor protein. The initial steps involve cell lysis, acetic acid treatment and the 47-63% alcohol fractionation and are identical to the steps in the preceeding section. Assays for the presence of the inhibitor protein, using purified GAPD to measure GAPD activity loss, were begun with the 47-63% alcohol fraction, since this was the first fraction which was relatively free of endogenous GAPD activity. Assays could not be performed at earlier steps because of the very high endogenous GAPD levels.

DEFINITION OF UNITS. One unit of activity is arbitrarily defined as the amount of inhibitor protein which produces 10% loss of GAPD activity when added to a solution containing 0.2 mg/ml of purified GAPD at pH 8.0 in 0.1 M Tris and 0.1 M β-mercaptoethanol, in a total volume of 0.5 ml. The amount of inhibitor protein added was adjusted so that the loss of activity was approximately 25-50%, since the relationship between the amount of inhibitor protein and activity loss was fairly linear in this range (see Figure 37, p. 188). Total protein was by assuming that an absorbance of 1.0 at 280 mμ corresponded to a

protein concentration of 1.0 mg/ml. A summary of the over-all purification is given in Table VII.

CELL LYSIS AND TREATMENT OF THE CRUDE EXTRACT. Two pounds of fresh Red Star Baker's yeast were crumbled into small pieces, and 160 ml of chloroform was added. The mixture was kept at room temperature and stirred occassionally for 60-90 min. until the suspension was thoroughly liquified. At this point, 1100 ml of distilled de-ionized water was added. The mixture was stirred briefly (approximately 5 min.) and the pH adjusted to 7.3 with 1 N NaOH. The mixture was then left standing at room temperature overnite.

After standing overnite, the solution was centrifuged at 1300 x g for 20 min. The precipitate was discarded and the supernatant (1475 ml) was adjusted to pH 4.8 with 3 N HOAc. As the HOAc was added, a white precipitate formed. To this mixture was added 2 ml of chloroform. The mixture was then left stirring gently overnite. If after stirring overnite, the precipitate had not completely re-dissolved, it was removed by centrifugation at 1300 x g for 10 min.

47-63% ETHANOL FRACTIONATION. The resulting solution (1475 ml) was placed in an ice bath until the temperature reached approximately 5°. At this point 1475 ml (1 volume) of 95% ethanol (-10°) was added slowly with stirring. Five minutes after the addition of ethanol was complete, the

TABLE VII. SUMMARY OF THE PURIFICATION OF THE INHIBITOR PROTEIN

Treatment	Volume	Protein Concentration	Specific Activity	Total Units
Cell lysis and HOAC treatment	1475 ml			
47-63% ethanol fractionation	158 ml	7.0 mg/ml	40	46,000
Protamine sulfate and ammonium sulfate treatment	52 ml	10.0 mg/ml	57	32,000
Ethanol fractionation	52 ml	5.2 mg/ml	100	27,000
Isoelectric focusing	41 ml	4.6 mg/ml	113	21,400

See text for details of the procedure of each treatment.

mixture was centrifuged at 1300 x g for 5 min. and the precipitate was discarded. A second 1475 ml volume of cold ethanol was added slowly with stirring. After standing for 5 min., the precipitate was collected by centrifugation at 1300 x g for 5 min. The precipitate was taken up in 150 ml of 0.01 M citrate buffer (Na), pH 5.5. After standing at 0° overnite, any insoluble material was removed by centrifugation. The total volume obtained at this point was 158 ml.

PROTAMINE SULFATE AND AMMONIUM SULFATE TREATMENT. To the fraction from the ethanol step (158 ml) 7.70 ml of protamine sulfate (20 mg/ml) was added dropwise at 0°. The dark brown precipitate which formed was removed by centrifugation.

The supernatant (162 ml) was brought to 80% saturation with ammonium sulfate by the addition of 91 g. of solid ammonium sulfate at 0°. The solution was stirred gently for 5 min. after addition was completed, and the precipitate was collected by centrifugation and re-dissolved in 60 ml of 0.01 M citrate, pH 5.5. The resulting solution (66 ml) was then brought to 70% saturation with ammonium sulfate by the addition of 31.2 g. of solid ammonium sulfate, and stirred gently for 5 min. after the addition was complete. The precipitate was collected by centrifugation and taken up in 50 ml of 0.01 M citrate, pH 5.5.

ETHANOL FRACTIONATION. At this point 90 ml (1.75 volumes) of cold (0°) ethanol (95%) was added. The precipitate which formed was suspended in 50 ml of 0.01 M citrate buffer, pH 5.5, and left at 0° overnite. Insoluble material was then removed by centrifugation.

The resulting solution (52 ml) was then dialyzed for 24 hrs. at room temperature against 2 l. of 0.01 M citrate buffer, pH 5.5, containing 0.07 M KCl. After dialysis the preparation was kept at 0° prior to the succeeding steps.

When this preparation was examined by disc gel electrophoresis one major band was observed along with 6-7 minor bands. Since the bands were quite widely separated further purification of the inhibitor protein by isoelectric focusing was attempted.

ISOELECTRIC FOCUSING. The approach was to first apply a small amount of the dialyzed protein preparation to an analytical size isoelectric focusing column utilizing a broad pH gradient (3-10) in order to determine the approximate isoelectric point of the inhibitor protein. A larger amount of protein from the dialysis step could then be applied to a preparative size isoelectric focusing column using a much narrower pH gradient, ranging about the isoelectric point; this should afford the maximum separation.

A small fraction of the solution from the dialysis step (25 mg of protein) was therefore applied to an isoelectric focusing gradient, pH 3-10, using a small (110 ml) LKB column. After 72 hrs. at 0° fractions were collected. The inhibitor protein banded at a pH of approximately 5.0.

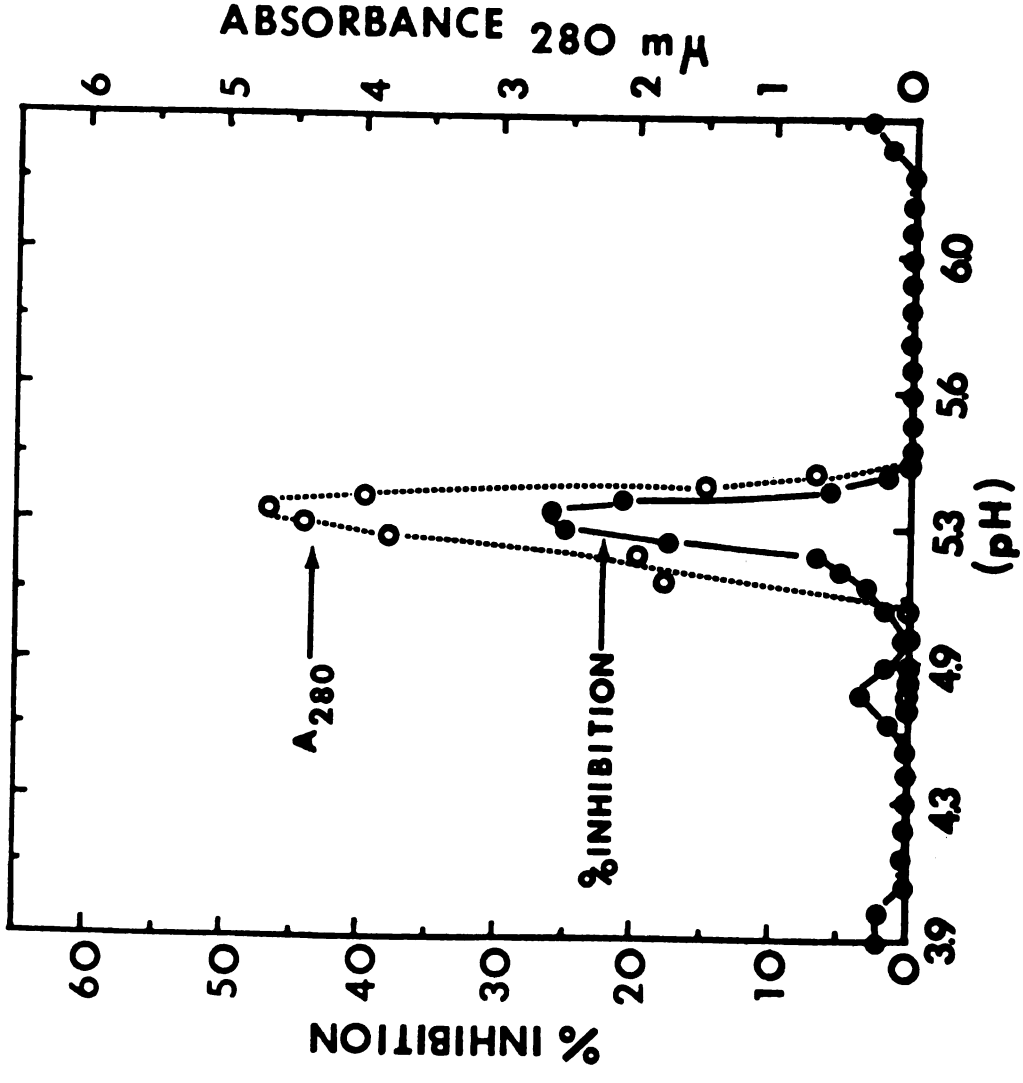
Once the approximate isoelectric point of the inhibitor protein was known, a larger fraction of protein (100 mg) from the dialysis step was applied to a larger LKB isoelectric focusing column (440 ml) utilizing a much narrower pH gradient of pH 4-6. After focusing for 72 hrs. at 0°, fractions were collected and analyzed for both protein content and inhibitor protein activity. The results are shown in Figure 38.

Small aliquots of fractions 48-54 (which contained the inhibitor protein activity) were analyzed by disc gel electrophoresis. Fractions 49-54 showed only a single band, and were therefore pooled. Fraction 48 showed a minor contaminant and was therefore discarded.

The pooled fractions (40 ml) were then brought to 90% saturation with ammonium sulfate by the addition of solid ammonium sulfate at 0°. After the addition was complete the solution was allowed to stand at 0° for 1 hr. The precipitate was then collected by centrifugation and dissolved in 10 ml of 0.01 M citrate, pH 5.5. The resulting solution was stored at 0°.

Figure 38. Purification of the inhibitor protein by isoelectric focusing. Fractions were collected from a 440 ml LKB column after focusing for 72 hr at 2°. See Methods for details of focusing procedure.

After the fractions were collected the pH was measured, the absorbance at 280 mμ was determined and the inhibition of glyceraldehyde-3-phosphate dehydrogenase activity was measured as described in Methods.


ABSORBANCE 280 mμ
6
5
4
3
2
1
0
60
5.6
5.3
(pH)
4.9
4.3
3.9
A280
%INHIBITION
60
50
40
30
20
10
0
% INHIBITION

PROOF THAT THE INHIBITOR PROTEIN WAS NOT AN ALTERED OR INCOMPLETE SUBPOLYMER OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. As previously mentioned, one possible explanation for the observed loss of activity of GAPD in the presence of the inhibitor protein was that the inhibitor protein was an altered or incomplete subunit (or dimer) of GAPD with little or no GAPD activity. Consequently, experiments had been designed to test this possibility using impure inhibitor protein (p. 177). Rigorous tests of this possibility, however, were not possible until the inhibitor protein was obtained in pure form.

A further reason for rigorously evaluating this possibility was the observation that the subsequently purified inhibitor protein showed very low levels of GAPD activity. The GAPD specific activity measured in the purified inhibitor protein preparation was approximately 1% of the specific activity of pure, native GAPD. There were two likely explanations for this observation.

The first explanation for the trace amounts of GAPD activity in the purified inhibitor solutions was that the preparation was simply contaminated with GAPD. This was a distinct possibility because of the large amount of GAPD present in yeast; GAPD comprises 18-20% of the total soluble protein of yeast (Krebs et al., 1953).

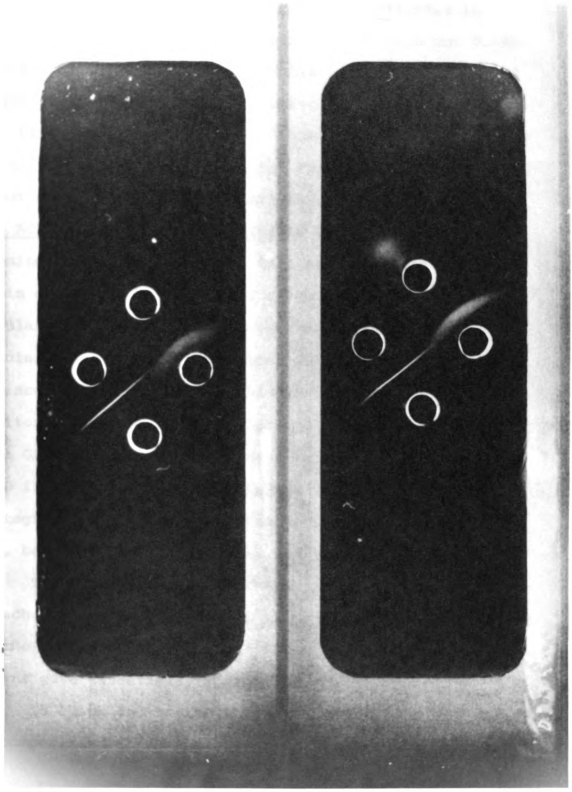
The second explanation was that the inhibitor protein was an altered or incomplete subpolymer of GAPD.

Such an altered subpolymer could possibly bind to the tetramer of yeast GAPD or could undergo an exchange reaction with a subpolymer of the native enzyme to cause the observed loss of GAPD activity. This possibility was also likely, since the native tetramer is almost certainly involved in equilibria with its subpolymers (see Literature Review).

Tests for immunological cross reaction between the two proteins provided a way to decide between these possibilities. Therefore, antibodies to both GAPD and the inhibitor protein were prepared. A second approach which could indicate whether the inhibitor protein was an altered form of a GAPD subpolymer, possibly produced by proteolytic degradation, was to determine the amino acid composition of the purified inhibitor protein and the composition of purified GAPD. Therefore, the amino acid composition of both proteins was also determined.

TESTS FOR CROSS REACTION BETWEEN THE INHIBITOR PROTEIN AND ANTI-GAPD AND BETWEEN GAPD AND ANTI-INHIBITOR PROTEIN. Antibodies to GAPD and the inhibitor protein (prepared as described in the Methods section) showed no cross reaction with purified inhibitor protein and GAPD respectively. This is clearly seen by the double diffusion patterns illustrated in Figure 39. Starting with the top well in each pattern and proceeding clockwise, the wells contain Ab_{GAPD}, inhibitor protein, $Ab_{inhibitor\ protein}$ and GAPD.

Figure 39. Immunodiffusion patterns of yeast GAPD and inhibitor protein. Starting with the top well in each pattern and proceeding clockwise, the wells contain anti-GAPD, inhibitor protein (1.0 mg/ml), anti-inhibitor protein, and GAPD (0.7 mg/ml). Both patterns pictured are identical, and were observed after diffusion overnite at 25°. See text for further details.



Both patterns depicted are identical and the concentrations of GAPD and inhibitor protein are 0.7 and 1.0 mg/ml respectively. Four other experiments were also performed in duplicate using GAPD concentrations between 0.35 and 0.035 mg/ml and inhibitor protein concentrations between 0.4 and 0.04 mg/ml. All the patterns observed were similar to those illustrated in Figure 39. These experiments provided virtually conclusive evidence that the inhibitor protein was not some type of altered GAPD subpolymer.

AMINO ACID ANALYSIS OF THE INHIBITOR PROTEIN AND GAPD. The amino acid compositions of GAPD and the inhibitor protein are given in Table VIII as residues per 69,000 molecular weight. This value was chosen since it is the molecular weight of the GAPD dimer (Jaenicke et al., 1968), and since preliminary studies had indicated that the inhibitor protein had a molecular weight close to that of the GAPD dimer (see following section). The value of 69,000 is also conveniently obtained for both proteins by integral multiplication of the limiting value in both cases, based on one tyrosine per limiting unit.

The values listed represent the average of two runs for each protein, a 24 and a 48 hr. hydrolysis. The results from the two runs were averaged for each protein, since the two runs gave essentially identical results.

To check on the accuracy of the technique the amino acid composition of GAPD as residues per 69,000 molecular

TABLE VIII. AMINO ACID COMPOSITION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND THE INHIBITOR PROTEIN[a]

AMINO ACID RESIDUE	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (Residues per 68,712)		INHIBITOR PROTEIN (Residues per 69,480)
	Literature Value	Experimental Value	Experimental Value
Asp[b]	69.2	70.6	69.1
Thr	44.3	39.9	28.3
Ser	47.1	43.7	39.4
Glu[b]	35.9	35.8	64.7
Pro	23.3	22.3	16.6
Gly	50.3	53.1	62.1
Ala	66.4	66.5	70.7
Val	61.0	66.3	68.4
Meth	12.9	11.5	NONE
Ileu	32.1	32.2	33.2
Leu	40.8	43.0	50.1
Tyr	21.6	24.0	18.0
Phe	19.6	19.9	29.9
Lys	40.1	47.8	48.8
Hist	10.8	13.2	6.4
Arg	16.6	22.8	25.9

[a]Values based on one tyrosine per limiting unit.

[b]Assuming all residues are in free acid form and not amidated.

weight was also calculated from the literature values for the amino acid composition of GAPD (Velick and Furfine, 1963). The values are also listed in Table VIII, and they agree quite well with the values for GAPD determined in this work.

If the inhibitor protein was an altered GAPD subpolymer produced, for example, by proteolytic degradation, then the amino acid composition might be expected to be similar to that of the GAPD. Examination of the data in Table VIII, however, indicates that there are four residues (underlined) that clearly occur in the inhibitor protein more frequently than in GAPD. Of these, the greatest discrepancy is noted for glutamic acid, which occurs twice as frequently in the inhibitor protein as in the GAPD. This composition seems highly unlikely if the inhibitor protein arises from digestion of a GAPD subpolymer, unless the hypothetical proteolysis selectively yielded a glutamate rich fraction.

Examination of Table VIII also reveals that methionine is absent in the inhibitor protein, while the GAPD contains approximately 12 methionine residues per 69,000 molecular weight. It thus seems inconsistent that the inhibitor protein could be a degradation product of GAPD.

The results presented in this section for the antibody experiments and the amino acid compositions proved conclusively that the inhibitor protein was not

some type of slightly altered GAPD subpolymer still possessing the ability and structure to associate with GAPD.

MOLECULAR WEIGHT OF THE INHIBITOR PROTEIN AND MOLAR STOICHIOMETRY OF THE GAPD-INHIBITOR PROTEIN INTERACTION

The most likely explanation for the inhibition of GAPD by the inhibitor protein seemed to be the formation of some type of complex formation between the two proteins. Such an explanation would be consistent with the data presented thus far (although later discoveries seemed to rule out this interpretation). Furthermore, there seemed to be some precedent for this type of complex formation, since an examination of the literature revealed several reports of inhibitor proteins for other enzymes (Bechet and Wiame, 1965; Mikola and Suolinna, 1969).

The obvious approach to the problem at this stage was to attempt to isolate a GAPD-inhibitor protein complex, or at least to demonstrate that there was a physical interaction between the two proteins. Such an attempt, however, would be greatly facilitated by a knowledge of the nature of the interaction, the molar stoichiometry involved and the molecular weight of the inhibitor protein. For these reasons the molecular weight of the inhibitor protein was determined.

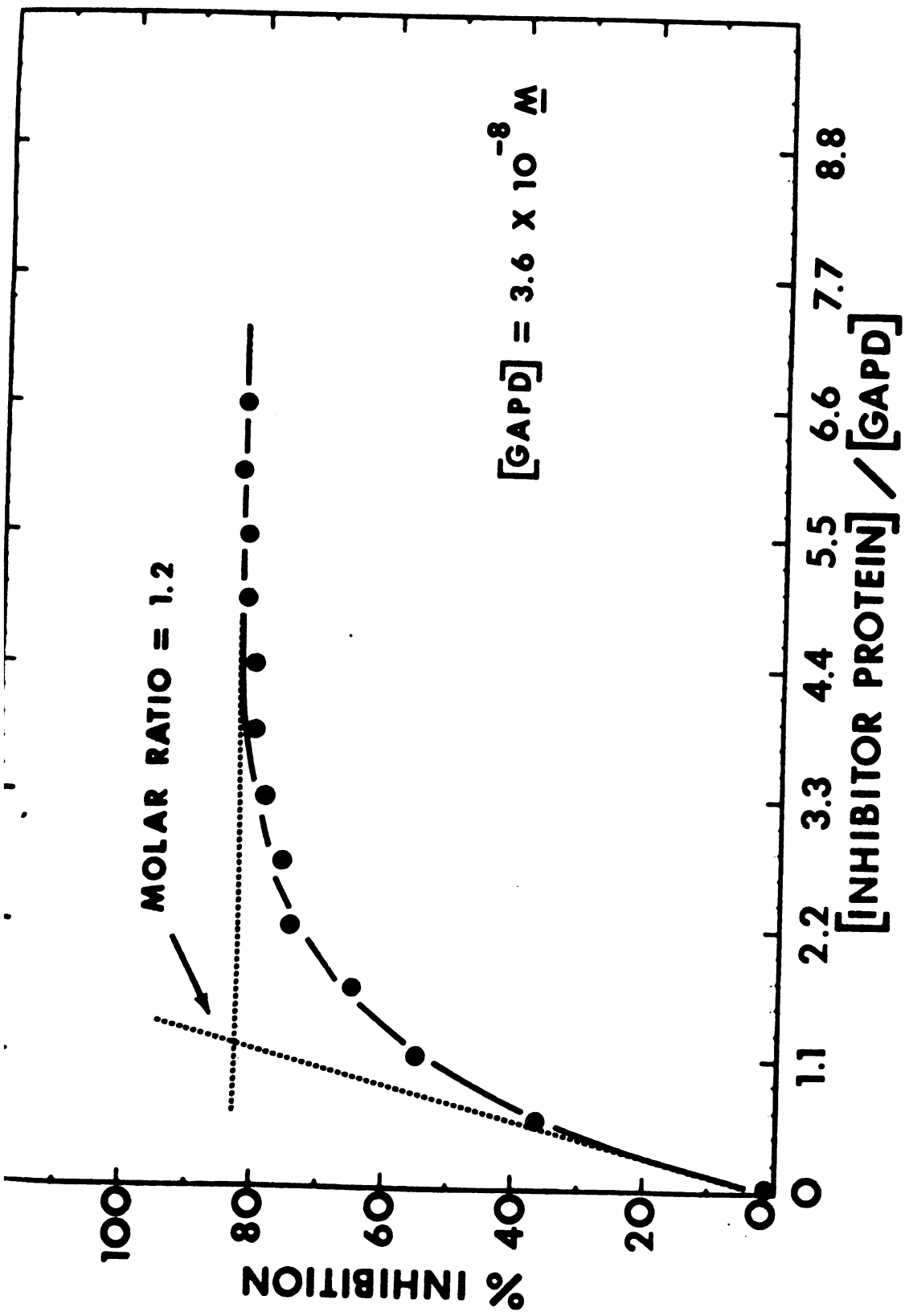
MOLECULAR WEIGHT OF THE INHIBITOR PROTEIN. The technique used to determine the molecular weight of the inhibitor

protein was high-speed sedimentation equilibrium (Yphantis, 1964), often referred to as the meniscus depletion method or Yphantis method. This technique was chosen because it provides a sensitive test for the presence of low molecular weight impurities in the sample and a test for dissociation of the sample at low protein concentrations, as well as yielding a molecular weight value.

In three separate experiments, at 17,980 RPM at pH 8.0, and 20°, with an initial protein concentration of 0.5 mg/ml, the molecular weight of the inhibitor protein determined by this technique was 56,000, 58,000 and 60,000. These values were obtained from plots of the log of the protein concentration (in fringe displacement) vs r^2, the distance from the center of rotation in cm^2. In all cases these plots were linear, indicating that no low molecular weight impurities were present and that the inhibitor protein itself was not involved in any association-dissociation reactions.

STOICHIOMETRY OF THE GAPD-INHIBITOR PROTEIN INTERACTION. The stoichiometry of the apparent interaction between the inhibitor protein and GAPD was investigated by measuring the loss of GAPD activity (at a fixed GAPD concentration) as a function of the molar concentration of the inhibitor protein. The results of such an experiment are illustrated in Figure 40. The GAPD concentration was 3.6×10^{-8} M,

Figure 40. Determination of the molar stoichiometry of the inhibitor protein-glyceraldehyde-3-phosphate dehydrogenase interaction. A fixed concentration of glyceraldehyde-3-phosphate dehydrogenase was mixed with various amounts of the inhibitor protein, and the % inhibition was measured as described in Methods.


% INHIBITION
100
80
60
40
20
0
0
1.1
2.2
3.3
4.4
5.5
6.6
7.7
8.8
[INHIBITOR PROTEIN] / [GAPD]
[GAPD] = 3.6 X 10⁻⁸ M
MOLAR RATIO = 1.2

and the inhibitor protein concentration was varied from 2×10^{-8} M to 23×10^{-8} M.

The extrapolated lines from both ends of the hyperbolic curve (Figure 40) intersect at an inhibitor protein/GAPD molar ratio of 1.2. This data suggested that the inhibitor protein might be interacting with GAPD to form a 1:1 complex, resulting in the observed inhibition.

At this point experiments were undertaken to physically test for an interaction between the inhibitor protein and GAPD.

TESTS FOR THE FORMATION OF AN INHIBITOR PROTEIN-GAPD COMPLEX. There are many possible physical techniques one might use to test directly for the interactions or formation of a complex between the two proteins. The techniques used for the inhibitor protein-GAPD system were sedimentation equilibrium, sucrose density gradient centrifugation, fluorometric titration of (GAPD-NADH) mixtures with the inhibitor protein and an immunochemical titration.

HIGH-SPEED SEDIMENTATION EQUILIBRIUM. If there is an interaction between two proteins leading to complex formation, then the observed mass distribution of a mixture of the two proteins (with initial concentrations c_1 and c_2) in a centrifugal field should differ from the sum of the mass distributions of the two proteins observed individually at identical concentrations, c_1 and c_2, in an

identical centrifugal field. Consequently, the mass distributions of the inhibitor protein and GAPD samples were determined as a function radial position in two separate centrifuge cells, and then the mass distribution of a mixture of the two (in a third cell) was determined.

Experiments with GAPD and the inhibitor protein were performed as follows, using Rayleigh interference cells with wedge centerpieces. One cell was loaded with 0.06 ml of a dialyzed GAPD solution (1.0 mg/ml) and 0.06 ml of dialyzsate. A second cell was loaded with 0.06 ml of a dialyzed inhibitor protein solution (1.0 mg/ml) plus 0.06 ml of dialyzsate. The third cell was then loaded with 0.06 ml of the GAPD solution plus 0.06 ml of the inhibitor protein solution, using the same protein solutions which were placed in the first two cells. These three cells and an interference counterbalance were then placed in a 6-hole AnG rotor.

To test for non-specific protein-protein interactions, a fourth cell was loaded with beef heart lactic dehydrogenase (LDH) and a fifth cell with an LDH-inhibitor protein mixture. The final concentration of LDH was identical to that of the GAPD, and the final concentrations of the LDH and inhibitor protein in the mixture were identical to the respective concentrations of the GAPD and the inhibitor protein in the GAPD-inhibitor protein mixture. The cells containing LDH were then placed in

the remaining two spaces in the rotor and a high-speed sedimentation equilibrium experiment was performed.

Since the cells were all in the same rotor and contained the same volumes of enzyme, minor variations normally incurred between different centrifuge runs (such as speed, temperature, vibrations and rotor expansion) may be safely ignored. Special care was also taken to insure that the radial distances measured for one cell would be comparable to those in other cells. The plate was carefully aligned and the patterns representing the GAPD, inhibitor protein and the GAPD-inhibitor protein mixture were read using the same fixed value for the reference, i.e., the plate was not re-aligned between the readings of the three different patterns, which lie in rows above one another. The plate was then re-aligned and the patterns representing the LDH, inhibitor protein and LDH-inhibitor protein mixture were read using a second fixed value for the reference. This procedure was used since the maximum vertical displacement of the comparator used to make the readings was only sufficient to read four patterns without re-aligning the plate.

The results for the inhibitor protein, GAPD and the GAPD-inhibitor protein mixture are illustrated in Figure 41, and the results for the LDH samples in Figure 42. The data is plotted as the log of the fringe displacement, (which is directly proportional to the log of

Figure 41. High-speed sedimentation equilibrium of inhibitor protein, glyceraldehyde-3-phosphate dehydrogenase and a mixture of the two proteins. The initial protein concentration of each protein was 0.50 mg/ml. The experiment was performed at 17,980 RPM and 20° in 0.02 M Tris, pH 8.0, with 0.1 M β-mercaptoethanol. Photographs were taken 24 hr after reaching speed. See text for further details.

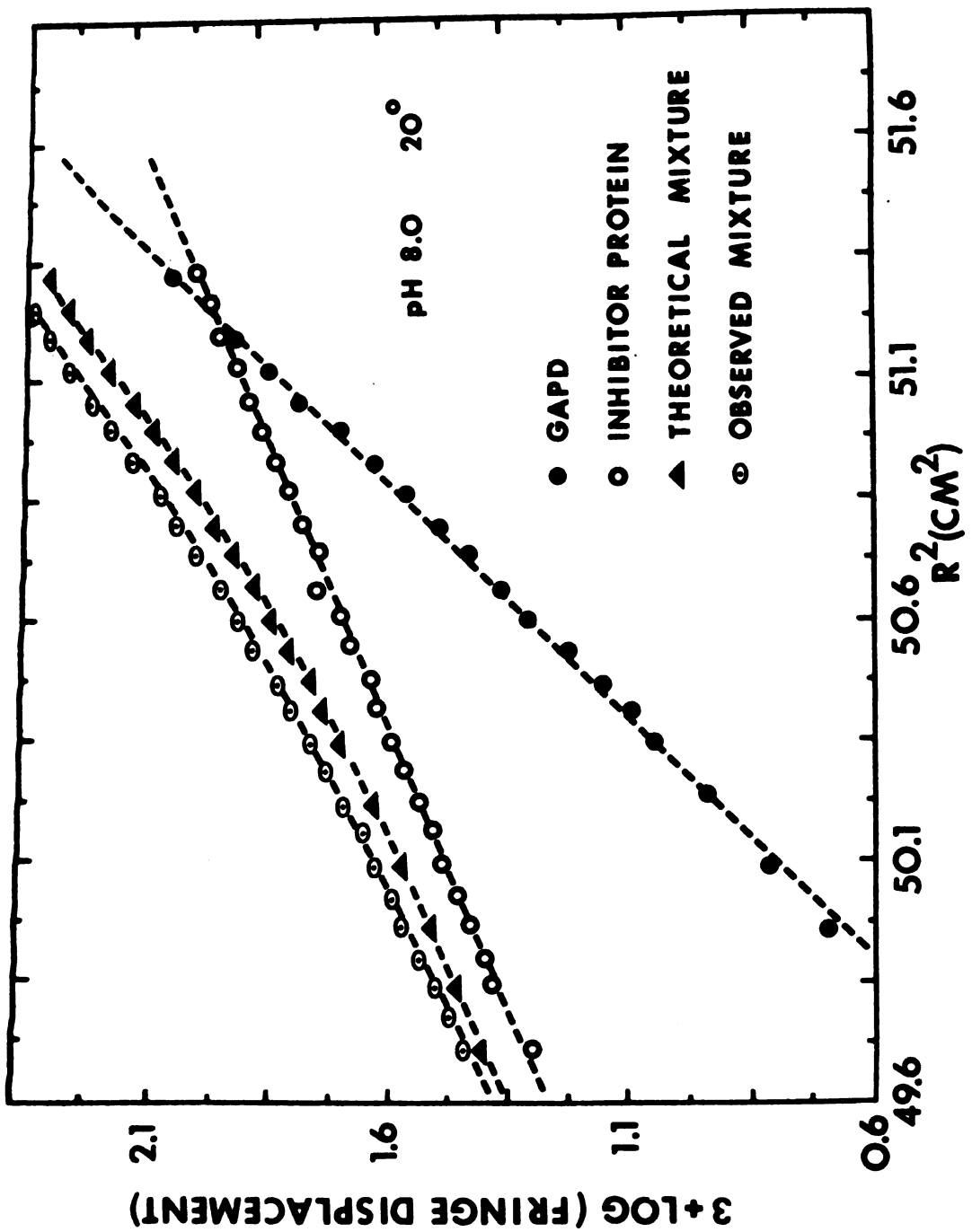
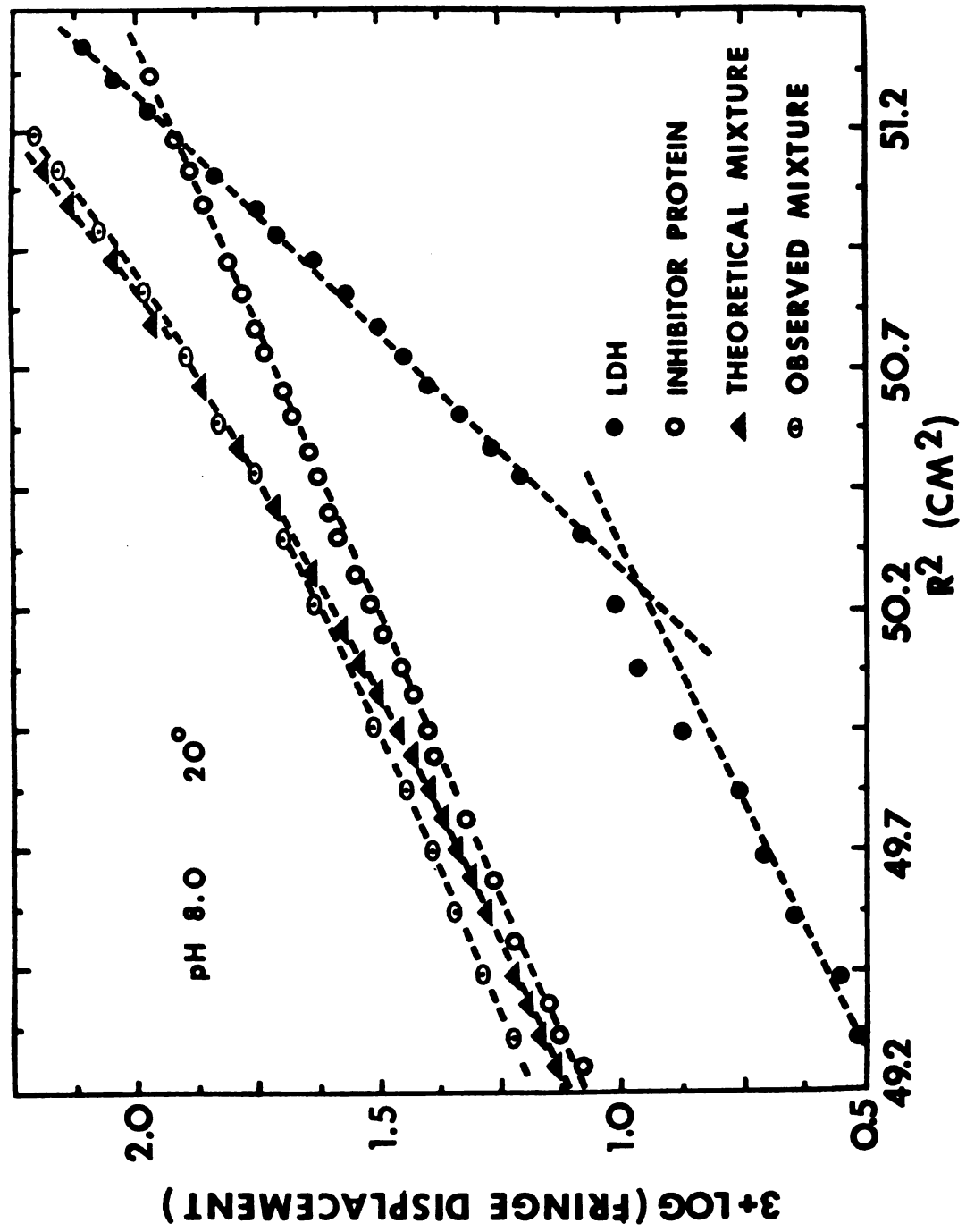

pH 8.0 20°
GAPD
INHIBITOR PROTEIN
THEORETICAL MIXTURE
OBSERVED MIXTURE
3+LOG (FRINGE DISPLACEMENT)
2.1
1.6
1.1
0.6
49.6
50.1
50.6
51.1
51.6
R²(CM²)

Figure 42. High-speed sedimentation equilibrium of inhibitor protein, lactic dehydrogenase (LDH) and a mixture of the two proteins. See legend of Figure 41 and text for further details.


3+LOG (FRINGE DISPLACEMENT)
2.0
1.5
1.0
0.5
49.2
49.7
50.2
50.7
51.2
R2 (CM2)
pH 8.0 20°
● LDH
o INHIBITOR PROTEIN
▲ THEORETICAL MIXTURE
ø OBSERVED MIXTURE

the protein concentration in mg/ml), as a function of the square of the radial distance from the center of rotation. The theoretical curves for the two mixtures were obtained by summation of the concentrations of the individual components at a given radial position.

The data of Figure 41 illustrates a small but apparently significant difference between the theoretical and observed curves for the GAPD-inhibitor protein mixture. Furthermore, the mass distribution toward the bottom of the cell should be higher for the observed mixture than for the theoretical mixture if there is an interaction between the two proteins. For the GAPD-inhibitor protein mixture this is in fact the observed direction of deviation. In contrast, the theoretical and observed curves for the LDH-inhibitor protein mixture were practically coincident. Although this data is consistent with complex formation, the magnitude of the differences (Figure 41) seems too small to offer strong evidence for complex formation. Therefore, other procedures were used to test for complex formation.

SUCROSE DENSITY GRADIENT CENTRIFUGATION. The resolving power in a sucrose density gradient experiment is not as great as the resolving power in the Model E, and a slowly equilibrating system might be resolved in the Model E, but remain in equilibrium in sucrose density gradient experiments. Thus, sucrose density gradient centrifugation

experiments are frequently useful for analyzing complex formation of the type illustrated below

$$A + B \rightleftharpoons AB$$

Depending on the magnitude of the rate constants for complex formation and breakdown, moving boundary experiments for this type of system may reveal a single boundary or two boundaries which do not separate if the equilibrium is relatively rapid compared to the rate of migration in the gradient. This technique thus offered an opportunity to test the GAPD-inhibitor protein system for possible complex formation.

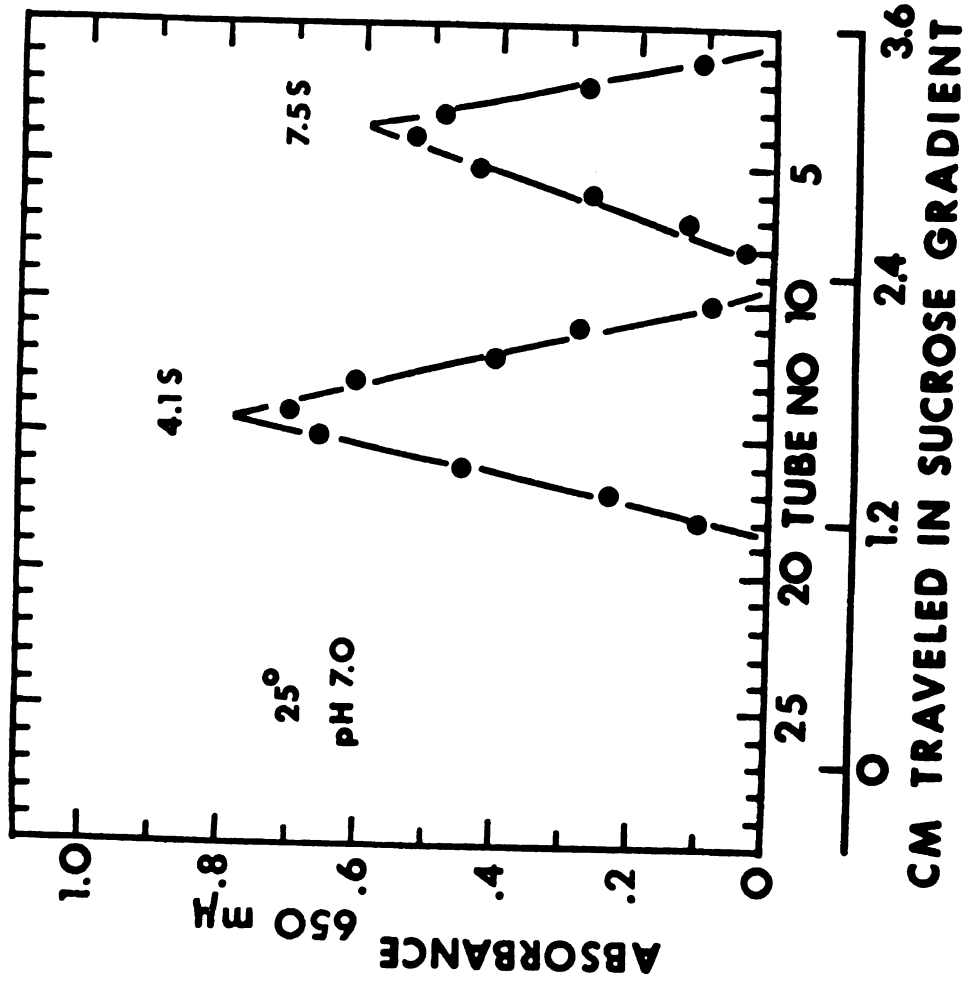
Before the sedimentation behavior of GAPD and inhibitor protein mixtures was interpreted, it was necessary to examine the sedimentation behavior of the two proteins individually. Figure 43 illustrates the individual sedimentation profiles of the inhibitor protein and the GAPD. These profiles were obtained by layering the inhibitor protein on one gradient and GAPD on a second gradient, and then running both gradients in the same rotor.[1] Under the conditions of the experiment, the GAPD and inhibitor protein peaks (run on different gradients) separated completely. Therefore, if the peaks did <u>not</u> separate

---

[1]The sedimentation coefficient of the inhibitor protein obtained from this experiment is 4.1 S (based on the value of the native GAPD), which agrees well with the mol. wt. of 58,000 which was previously obtained.

Figure 43. Sucrose density gradient centrifugation of the inhibitor protein and glyceraldehyde-3-phosphate dehydrogenase. The protein samples (0.10 ml of 0.7 mg/ml) in 0.05 M phosphate, pH 7.0, 0.15 M KCl, and 0.05 M β-mercaptoethanol, were layered on gradients of the same composition. Centrifugation was for 14 hrs at 40,000 RPM.

The protein was measured turbidimetrically (A_{650}) after precipitation with tannic acid as described in Methods.


ABSORBANCE
650 mμ
1.0
.8
.6
.4
.2
0
25°
pH 7.0
4.1S
7.5S
25
20
TUBE NO
10
5
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

completely when a mixture of the two proteins was run on a gradient together under these conditions, this would suggest complex formation.

Other gradients had been layered with mixtures of the inhibitor protein and GAPD and run in the same rotor as those shown in Figure 43. One of these gradients was identical to those used to obtain the profiles in Figure 43, and the other was identical except that the entire gradient contained 0.5 mM NAD.

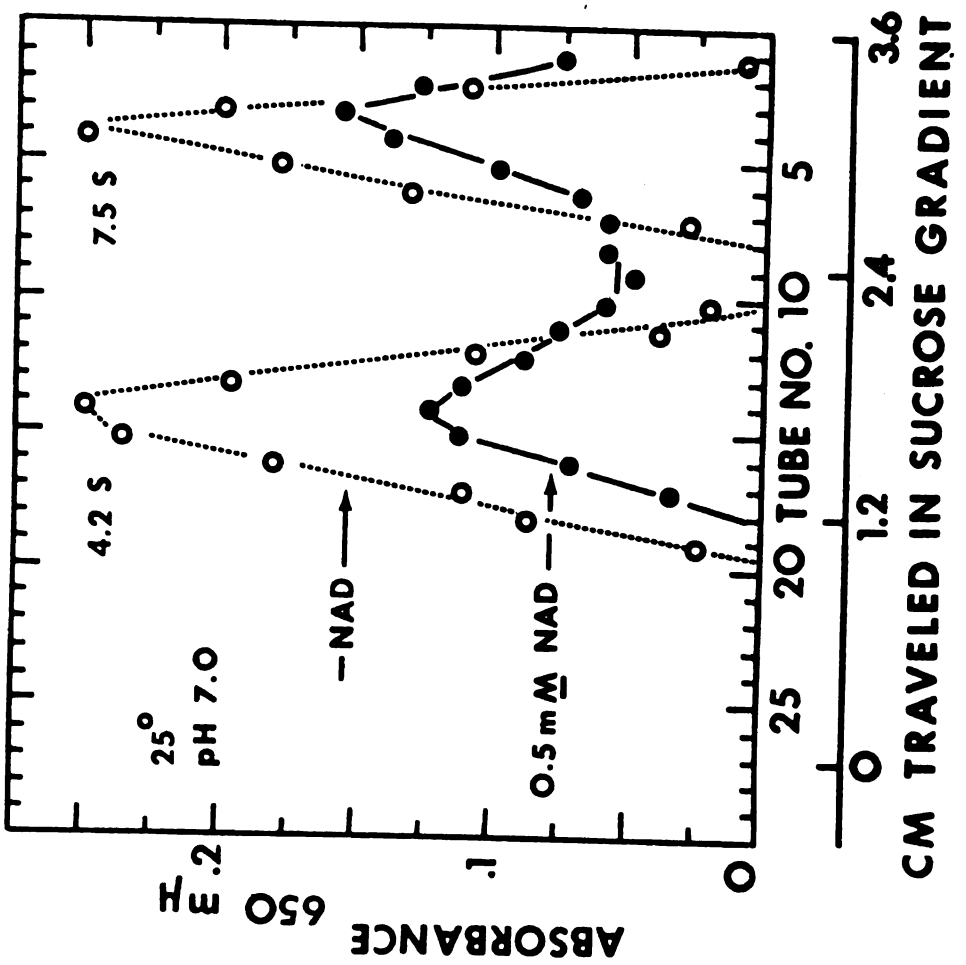
The sedimentation profiles of GAPD and inhibitor protein mixtures are illustrated in Figure 44. In the gradient without NAD there was no detectable complex formation, since the two peaks separated completely. With the NAD, the two peaks did not separate completely,[2] which seemed to indicate that some complex formation had occurred in the presence of NAD. This experiment, however, was not reproducible. Furthermore, in other experiments in which NADH, ATP and mixtures of NADH and ATP and NAD and ATP were present in the gradients, the two peaks separated completely, indicating no complex formation.

These experiments provided no evidence that the inhibitor protein and GAPD formed a complex. However, they did not exclude this possibility, since the high sucrose concentrations in the gradients may have prevented

---

[2]NAD at this concentration (0.5 mM) did not affect the sedimentation of either protein when they were run alone in separate gradients.

Figure 44. Sucrose density gradient centirfugation of mixtures of the inhibitor protein and glyceraldehyde-3-phosphate dehydrogenase with and without added NAD in the gradients. See legend of Figure 43 and text for further details.


ABSORBANCE 650 mμ
0
.1
.2
25°
pH 7.0
7.5 S
4.2 S
NAD
0.5 mM NAD
25
20
TUBE NO. 10
5
0
1.2
2.4
3.6
CM TRAVELED IN SUCROSE GRADIENT

an interaction between the inhibitor protein and the GAPD. Consequently, several other techniques were used to test for an interaction between the two proteins.

FLUOROMETRIC TITRATION OF (GAPD-NADH) WITH THE INHIBITOR PROTEIN. The fluorescence of NADH bound to GAPD is sensitive to the environment provided by the enzyme and hence to a number of factors which effect the conformation of the enzyme (Velick, 1959). Thus it might provide a useful probe to test for interactions between the inhibitor protein and GAPD. The fluorescence of bound NADH is especially useful since the characteristic absorption and emission bands of the nucleotide are far removed from any of the absorption bands of the proteins.

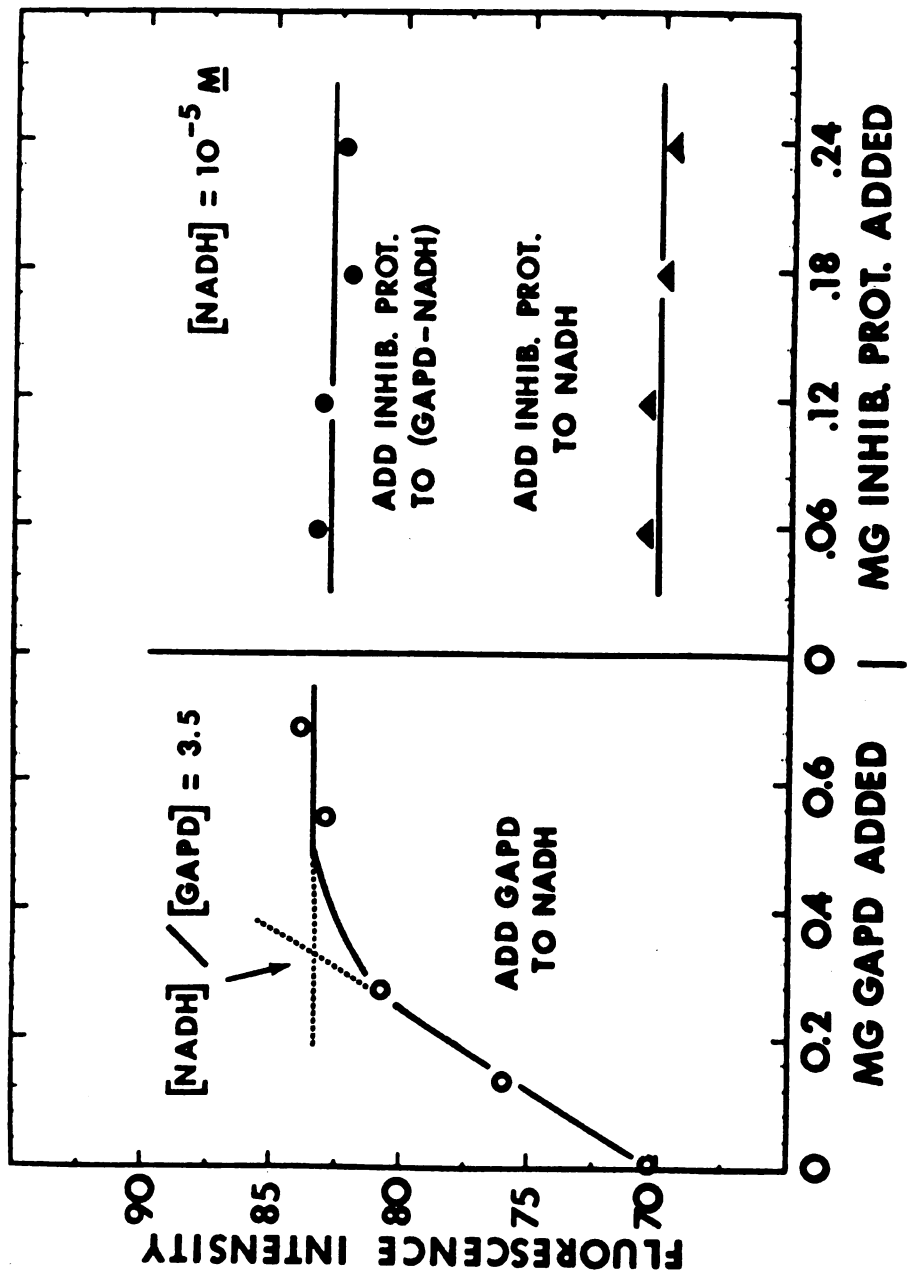
The effect of GAPD alone on NADH fluorescence is depicted by the open circles in Figure 45. As aliquots of a stock solution of GAPD are added to a solution of 10^{-5} M NADH, there is an enhancement of the fluorescence intensity which levels off to a plateau value. Extrapolation of the two ends of this curve (Figure 45) yields an equivalence point which corresponds to 3.5 moles of NADH bound per mole of GAPD.

After the fluorescence reached the plateau level, aliquots of the inhibitor protein were added directly to the same cuvette (containing NADH and GAPD) to determine the effect of the inhibitor protein on the fluorescence of the GAPD-NADH complex. As illustrated in Figure 45

Figure 45. Effect of the inhibitor on the fluorescence of NADH bound to glyceraldehyde-3-phosphate dehydrogenase. NADH was titrated with glyceraldehyde-3-phosphate dehydrogenase (open circles) until the fluorescence emission reached a plateau level; inhibitor protein was then added directly to the mixture of NADH and glyceraldehyde-3-phosphate dehydrogenase (closed circles).

The effect of the inhibitor protein on the fluorescence of NADH alone is illustrated by the triangles.

See text and Methods for further details.


FLUORESCENCE INTENSITY
90
85
80
75
70
[NADH] / [GAPD] = 3.5
ADD GAPD TO NADH
0
0.2
0.4
0.6
MG GAPD ADDED
[NADH] = 10^{-5} M
ADD INHIB. PROT. TO (GAPD-NADH)
ADD INHIB. PROT. TO NADH
0
.06
.12
.18
.24
MG INHIB. PROT. ADDED

(closed circles) the addition of the inhibitor protein did not produce any observable changes in the fluorescence of the bound nucleotide. The lower curve in Figure 45 (triangles) illustrates that the inhibitor protein alone does not alter the fluorescence of NADH. Thus, in the region of the GAPD molecule where the NADH is bound, the inhibitor protein did not sufficiently alter the structure of GAPD to produce any observable change in fluorescence.

IMMUNOCHEMICAL TITRATION OF A GAPD-INHIBITOR PROTEIN MIXTURE WITH ANTI-INHIBITOR PROTEIN. Another method to test for possible complex formation between the inhibitor protein and GAPD is to determine if antibodies to the inhibitor protein could precipitate GAPD activity when added to a mixture of GAPD and the inhibitor protein. If the inhibitor protein and GAPD formed a complex as illustrated below

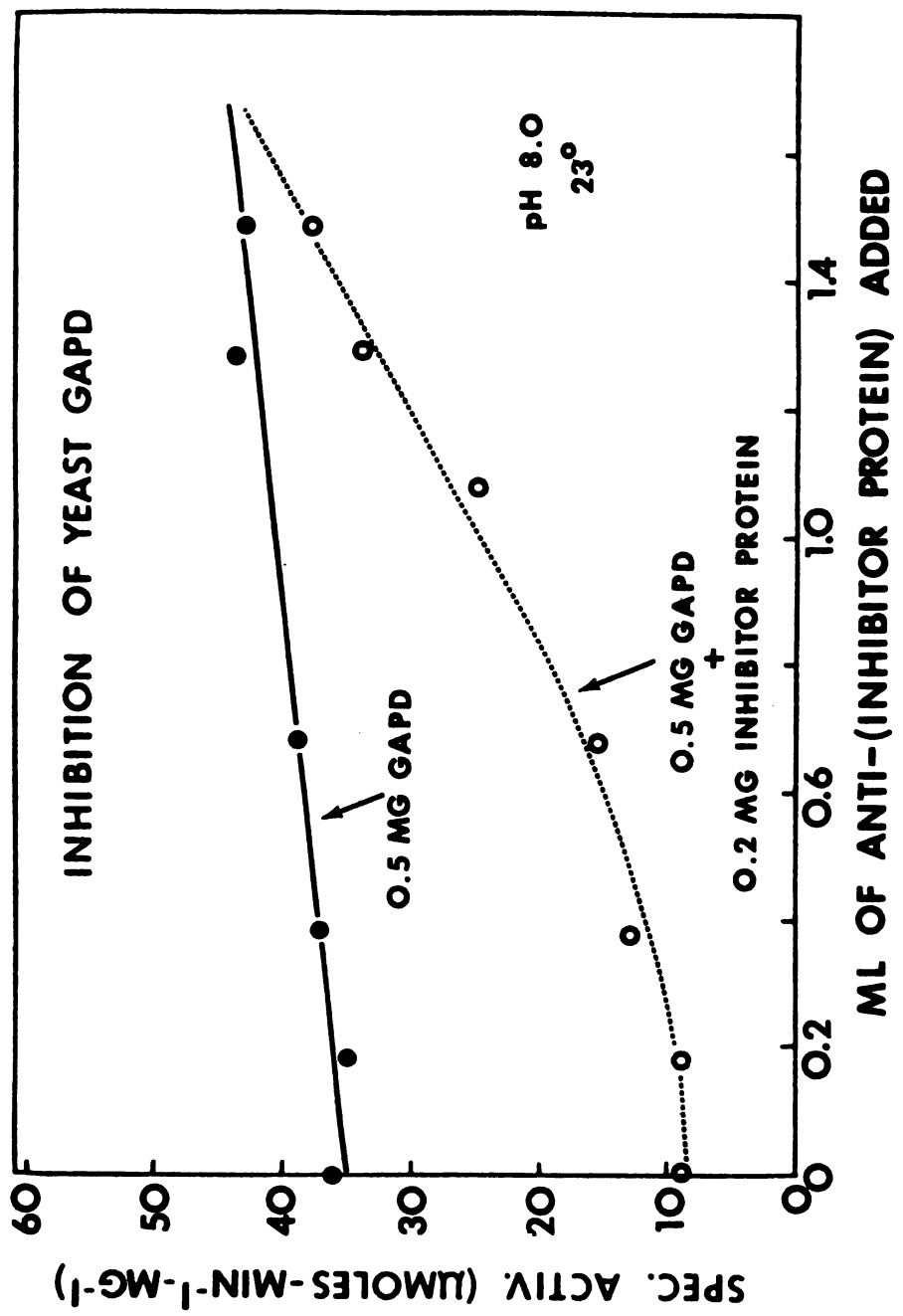
$$\text{GAPD} + \text{INHIBITOR PROTEIN} \rightleftharpoons (\text{GAPD})\text{-}(\text{INHIBITOR PROTEIN})$$

antibodies prepared to the inhibitor protein might be able to precipitate the complex as well as the free inhibitor protein.

When added to a mixture of the inhibitor protein and GAPD, antibodies to the inhibitor protein did not precipitate GAPD activity. This is illustrated in Figure 46. GAPD and the inhibitor protein were mixed in a 1:1

Figure 46. Immunochemical titration of a mixture of the inhibitor protein and glyceraldehyde-3-phosphate dehydrogenase with antibodies to the inhibitor protein. Experiments performed in 0.2 M Tris, pH 8.0, with 0.05 M β-mercaptoethanol. Initial volume was 1.0 ml.

See text and Methods for further details.


INHIBITION OF YEAST GAPD
pH 8.0
23°
0.5 MG GAPD
0.5 MG GAPD
+
0.2 MG INHIBITOR PROTEIN
SPEC. ACTIV. (μMOLES-MIN-1-MG-1)
ML OF ANTI-(INHIBITOR PROTEIN) ADDED
0
10
20
30
40
50
60
0.2
0.6
1.0
1.4

molar ratio and the GAPD activity was measured (lower curve). An aliquot of $Ab_{inhibitor\ protein}$ was added, the mixture briefly centrifuged and the GAPD activity re-measured. As the amount of the $Ab_{inhibitor\ protein}$ increased, the GAPD activity increased to the level of the control, which was a sample of pure GAPD to which $Ab_{inhibitor\ protein}$ was added. As seen in Figure 46 the addition of antibodies to the inhibitor protein did not affect the activity of the control sample of GAPD alone. Thus the results of this test for complex formation were also negative.

IDENTIFICATION OF THE INHIBITOR PROTEIN AS TRIOSEPHOSPHATE ISOMERASE. At this point there was no acceptable explanation for the inhibition of GAPD by the inhibitor protein. All the tests for complex formation in the previous section had been either negative or inconclusive, but the kinetic (inhibition) studies could best be explained by the formation of an enzyme-inhibitor protein complex. It was therefore decided to review all the data and attempt to formulate alternate hypotheses as to the origin of the observed inhibition.

One possible explanation which had previously been proposed but apparently ruled out was that the inhibitor protein was irreversibly removing one of the substrates of the GAPD catalyzed reaction. This possibility had previously been rejected because the inhibition did not seem to be overcome by increasing the substrate concentration

(p. 182) and because the total absorbance change (due to the conversion of NAD to NADH) observed at 340 mμ in the course of the GAPD assays was not noticeably affected by the addition of the inhibitor protein to the assays.

Another possibility, however, was that the inhibitor protein was reversibly converting one of the substrates of the GAPD reaction to a form that could not be directly utilized by the GAPD, that is, it might lower the effective glyceraldehyde-3-phosphate concentration at any given time but as glyceraldehyde-3-phosphate was used up, it would be replenished by reversal of the original extraneous glyceraldehyde-3-phosphate removal reaction. The most obvious example of this possibility, was that the inhibitor protein was an enzyme which could rapidly and reversibly convert one of the GAPD substrates to another chemical species. When this possibility was considered, the first enzyme which came to mind was triosephosphate isomerase.

When the inhibitor protein was assayed for triosephosphate isomerase activity, it rapidly catalyzed the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate in an assay coupled with α-glycerophosphate dehydrogenase. Further examination revealed that the inhibitor protein was in fact triosephosphate isomerase. The data which support this conclusion is as follows:

(1) When assayed for triosephosphate activity, the inhibitor protein had a specific activity

of 2000 μmoles $min.^{-1}$ mg. $protein^{-1}$, which agrees well with the literature value for this enzyme (Krietsch et al., 1970).

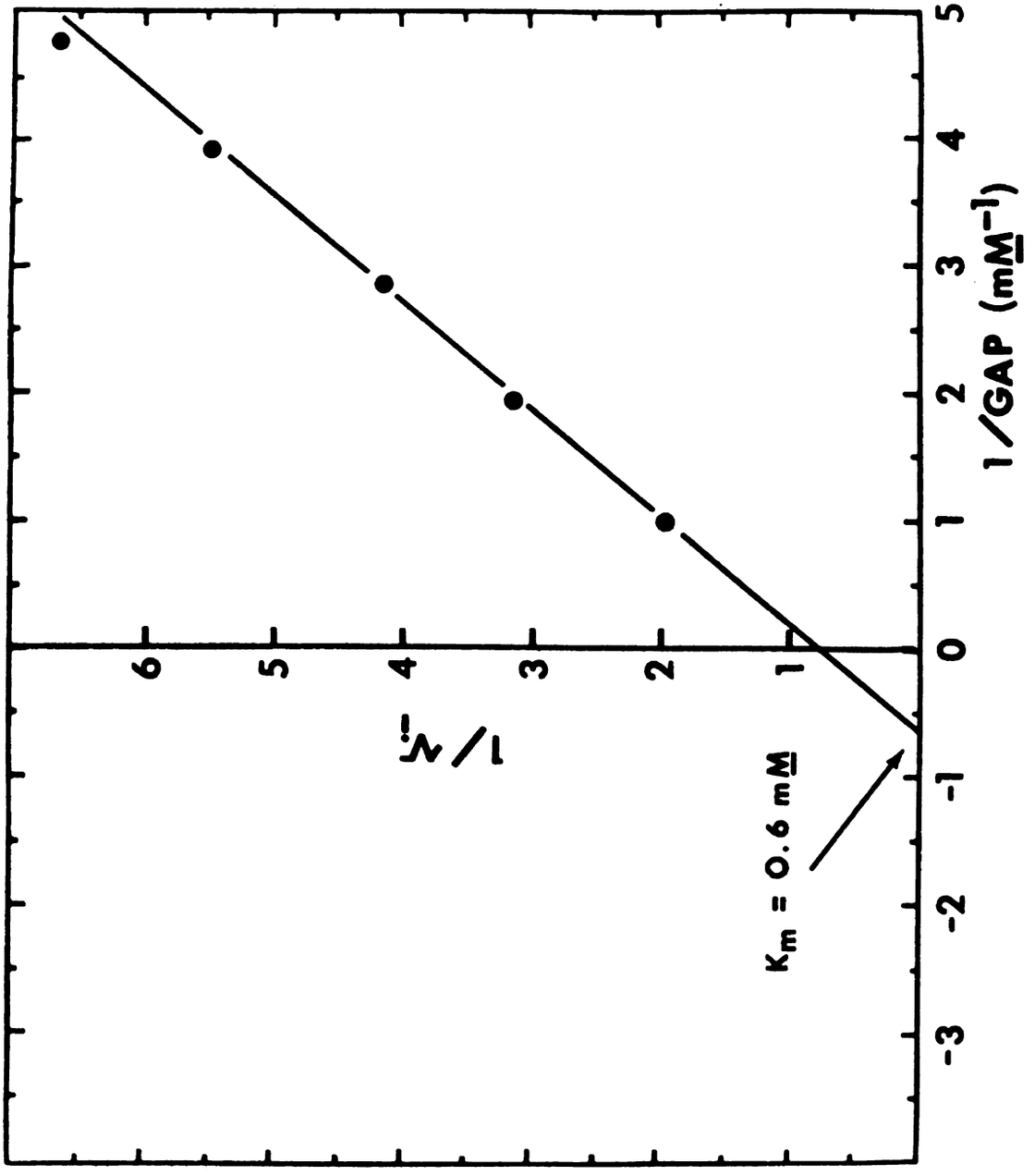
(2) The K_m for D-glyceraldehyde-3-phosphate of 0.6 mM (Figure 47) agrees with the reported literature value for yeast triosephosphate isomerase (Krietsch et al., 1970).

(3) The molecular weight of 58,000 (p. 204) agrees with the reported values of 52,000 to 60,000 for yeast triosephosphate isomerase (Krietsch et al., 1970).

(4) The amino acid composition (p. 201) is in agreement with the composition reported for yeast triosephosphate isomerase (Krietsch et al., 1970).

To insure that the inhibition of GAPD was definitely due to the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate, an inhibitor protein preparation was carried out as previously described (p. 186) and the fractions obtained at the various steps were assayed for inhibitor protein activity (as previously described) and for triosephosphate isomerase activity. This preparation was carried out up to the point of the electrofocusing step. The ratio of the specific activities of the inhibitor protein to triosephosphate isomerase remained constant through these steps, indicating that the triosephosphate isomerase activity was the cause of the inhibition.

Figure 47. Determination of the K_m of triosephosphate isomerase for D-glyceraldehyde-3-phosphate. Activity was measured in a coupled assay with α-glycerophosphate dehydrogenase as described in Methods.


1/GAP (mM⁻¹)
1/v
K_m = 0.6 mM

MECHANISM OF INHIBITION OF GAPD BY TRIOSEPHOSPHATE ISOMERASE. Initially, it seemed that the identification of the inhibitor protein as triosephosphate isomerase provided a simple explanation for the inhibition of GAPD, but a careful review of all data revealed that the following observations could not be satisfactorily explained by the triosephosphate isomerase catalyzed conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate:

(1) increasing the glyceraldehyde-3-phosphate concentration did not completely overcome the inhibition; above 1.5 mM glyceraldehyde-3-phosphate the GAPD activity showed a plateau with increasing substrate (p 182);

(2) the inhibition of GAPD in the presence of triosephosphate isomerase was reversible by dilution (p. 173), e.g., samples diluted from a stock solution mixture of GAPD and inhibitor protein showed a decreasing % inhibition as the total concentration of the mixture was decreased;

(3) at a definite molar stoichiometry of 1:1 (GAPD:triosephosphate isomerase, see p. 204) a maximal inhibition was observed; this suggests an interaction between enzymes rather than a competition for substrate, since a coincidental ratio of 1:1 seems highly unlikely in view of

the fact that the specific activity of the triosephosphate isomerase is 10 to 20 times that of GAPD.

Therefore, further experimentation was undertaken to determine if the observed inhibition could be explained solely by the triosephosphate isomerase catalyzed conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate.

EFFECT OF TRIOSEPHOSPHATE ISOMERASE ON THE GAPD CATALYZED HYDROLYSIS OF p-NITROPHENYL ACETATE. If the inhibition of GAPD by triosephosphate isomerase resulted only from the conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate, then the triosephosphate isomerase should have no effect on other reactions that GAPD catalyzes. Besides the oxidation of glyceraldehyde-3-phosphate, GAPD also catalyzes the hydrolysis of p-nitrophenyl acetate (Taylor et al., 1963). The effect of triosephosphate isomerase on this GAPD-catalyzed reaction was thus investigated.

The results of these experiments are presented in Table IX, and it is readily seen that the triosephosphate isomerase has no measurable effect on the hydrolysis of p-nitrophenyl acetate by GAPD under several different conditions. These experiments were performed by measuring the esterase activity of GAPD and triosephosphate isomerase separately and comparing the sum of these rates to the rate observed when the two proteins were mixed and assayed in the same cuvette. Comparison of the rates, which were

TABLE IX. HYDROLYSIS OF para-NITROPHENYL ACETATE BY GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND TRIOSEPHOSPHATE ISOMERASE

SOLVENT	(1)	(2)	(3)	(4)
	*Rate with GAPD[a]	Rate with TPI[b]	Rate with GAPD + TPI	Rate (1) + Rate (2)
0.1 M Tris, pH 8.0	0.052	0.060	0.104	0.112
0.1 M Tris, + 1.25 mM GAP[c]	0.032	0.036	0.068	0.068
0.1 M Tris. + 1.25 mM GAP + 1.25 mM NAD	0.032	0.036	0.068	0.068
Complete GAPD Assay Mix, - cysteine[d]	0.032	0.044	0.075	0.076

*All rates expressed as change in absorbance at 450 mμ per minute, after correction for spontaneous hydrolysis of the p-nitrophenyl acetate.

[a]Glyceraldehyde-3-phosphate dehydrogenase.

[b]Triosephosphate isomerase.

[c]Glyceraldehyde-3-phosphate.

[d]See Methods for exact composition of GAPD assay mix.

See Methods for further details.

corrected for spontaneous hydrolysis of the p-nitrophenyl acetate, indicates no effect of triosephosphate isomerase on GAPD under these conditions as judged by measuring the esterase activity.

Surprisingly, these experiments revealed that the triosephosphate isomerase alone catalyzed the hydrolysis of the ester model substrate, p-nitrophenyl acetate. This seems to be an active site catalysis since the triosephosphate isomerase substrate, glyceraldehyde-3-phosphate, inhibited the hydrolysis. Also, the catalysis by triosephosphate isomerase is comparable to the catalysis by GAPD, which is considered to be quire active as an esterase (Tayler et al., 1963). For example, yeast GAPD catalyzes the hydrolysis of p-nitrophenyl acetate at a rate 1.5 times greater than chymotrypsin when the rates of hydrolysis are expressed as μmoles substrate $min.^{-1}$ mg. $protein^{-1}$ (Taylor et al., 1963).

As noted in Table IX one set of conditions used to measure the esterase activity was the complete GAPD assay mix (see Methods) minus cysteine (normally present at a concentration of 0.05 M in the GAPD assay mix). Cysteine had to be omitted from these experiments because this amino acid alone catalyzed the hydrolysis of the p-nitrophenyl acetate at a rate several times that of either of the proteins.

EFFECT OF GLYCERALDEHYDE-3-PHOSPHATE CONCENTRATION ON THE INHIBITION OF GAPD BY TRIOSEPHOSPHATE ISOMERASE. After

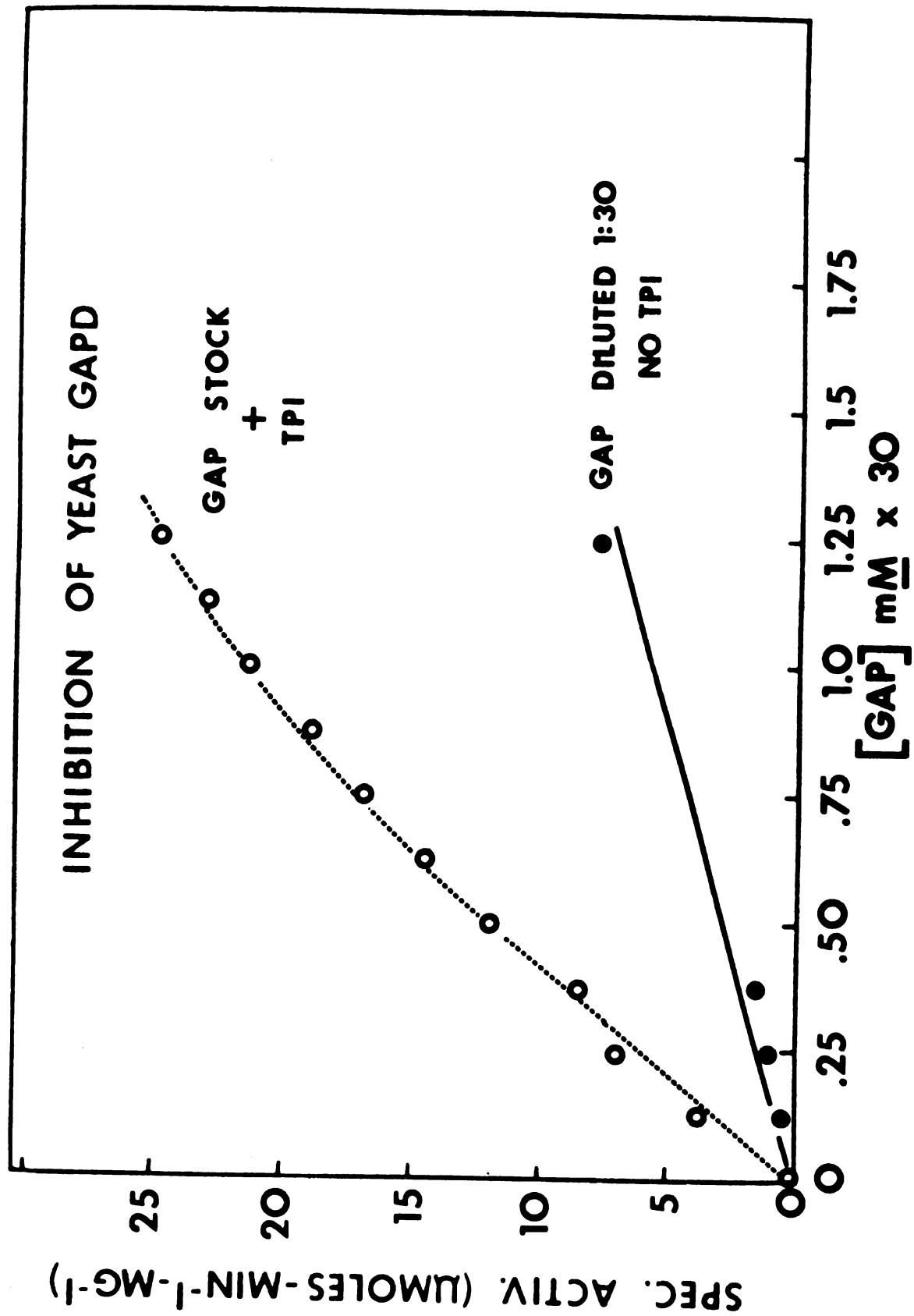
the inhibitor protein was identified as triosephosphate isomerase, it became apparent that some of the previous tests for the effects of triosephosphate isomerase on GAPD could not be properly interpreted since the effective concentration of the substrate glyceraldehyde-3-phosphate in the presence and absence of the triosephosphate isomerase was drastically altered. It was thus not possible to strictly interpret some earlier experiments to decide if the triosephosphate isomerase had any effect per se on the GAPD. In order to do this it is necessary to assay the GAPD, with and without triosephosphate isomerase present, in such a way that the concentration of glyceraldehyde-3-phosphate remains constant in both cases.

In order to determine what effect, if any the triosephosphate isomerase had on the GAPD reaction other than lowering the effect glyceraldehyde-3-phosphate concentration, the following experiment was performed. A stock solution of glyceraldehyde-3-phosphate (10^{-2} M) was diluted 1:30 with water, and a 0.04 ml aliquot of this diluted solution was added to a cuvette containing the complete GAPD assay mix. No triosephosphate isomerase was added to this cuvette. The cuvette was then allowed to stand at 23° for several minutes, after which an aliquot of GAPD was added to initiate the reaction. A second cuvette was prepared in a similar manner except that a 0.04 ml aliquot of the stock glyceraldehyde-3-phosphate solution (10^{-2} M) was used instead of an aliquot of glyceraldehyde-3-phosphate diluted 1:30. After the

addition of glyceraldehyde-3-phosphate to the second cuvette a large excess of triosephosphate isomerase (relative to the GAPD) was added and the cuvette allowed to stand at 23° for several minutes before initiating the reaction with GAPD. In the second cuvette an equilibrium was established between glyceraldehyde-3-phosphate and dihydroxyacetone phosphate before the addition of GAPD. The equilibrium of the triosephosphate isomerase reaction is such that the dihydroxyacetone phosphate: glyceraldehyde-3-phosphate ratio is 30:1 at equilibrium (Trentham et al., 1969). Therefore, both cuvettes contained the same concentration of glyceraldehyde-3-phosphate, and the only difference was the presence of triosephosphate isomerase in the second cuvette. Similar experiments were performed with two series of cuvettes; comparable cuvettes in each series contained identical glyceraldehyde-3-phosphate concentrations and differed only in the presence or absence of triosephosphate isomerase.

The completely unexpected result of this experiment was that the triosephosphate isomerase seemed to activate the GAPD rather than inhibit (Figure 48). The magnitude of the activation depended on the glyceraldehyde-3-phosphate concentration and varied from 3 to 7-fold under the conditions of the experiment.

Figure 48. Effect of glyceraldehyde-3-phosphate concentration on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by triosephosphate isomerase. The experiments were performed in such a manner that the glyceraldehyde-3-phosphate concentration was the same with and without added triosephosphate isomerase. See text for further details.


INHIBITION OF YEAST GAPD
GAP STOCK
+
TPI
GAP DILUTED 1:30
NO TPI
SPEC. ACTIV. (UMOLES-MIN-1-MG-1)
0
5
10
15
20
25
0
.25
.50
.75
1.0
1.25
1.5
1.75
[GAP] mM x 30

DISCUSSION

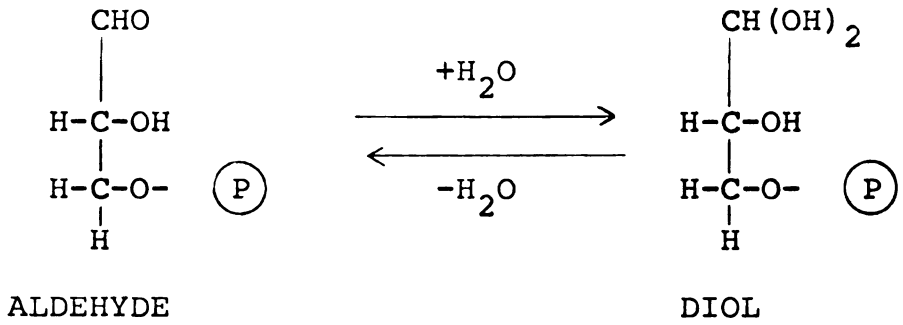
The GAPD inhibitor protein discussed in this chapter of the thesis has clearly been shown to be tirosephosphate isomerase. The evidence for the identification of the inhibitor protein as triosephosphate isomerase is that the values of K_m, specific activity, molecular weight and amino acid composition of the protein isolated in this work (p. 228) are very close to the literature values of these quantities for yeast triosephosphate isomerase (Krietsch et al., 1970).

It has also been clearly shown that the inhibition of yeast GAPD is due to the trisoephosphate isomerase per se, since the ratio of the specific activity of the triosephosphate isomerase to the specific activity of the inhibitor protein remains constant during purification of the inhibitor protein from yeast extracts (p. 231).

The only question which remains is "What is the precise mechanism of the inhibition of GAPD by triosephosphate isomerase?" In order to answer this question it is first necessary to consider in detail the rather complex effect of triosephosphate isomerase on the glyceraldehyde-3-phosphate concentration in the GAPD assay.

EFFECT OF GLYCERALDEHYDE-3-PHOSPHATE CONCENTRATION ON THE INHIBITION OF GAPD BY TRIOSEPHOSPHATE ISOMERASE. The problem of the inhibition of GAPD by triosephosphate isomerase is complicated by the chemical state of the

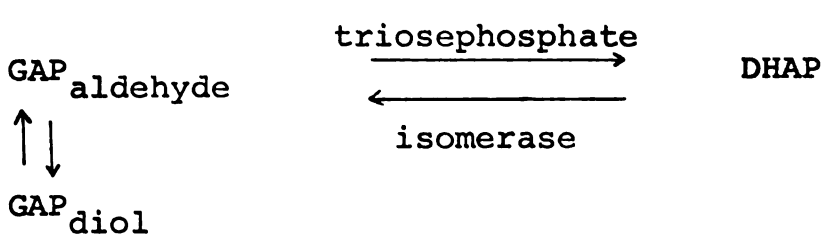
substrate glyceraldehyde-3-phosphate, which exists in solution in two forms, the aldehyde and the hydrated diol (Trentham et al., 1969). This is illustrated below.



In aqueous solutions the interconversion of the two forms is very rapid; the first order rate constants for diol formation and for diol breakdown are 2.5 $sec.^{-1}$ and 8.7 x 10^{-2} $sec.^{-1}$ respectively (Trenthan et al., 1969).

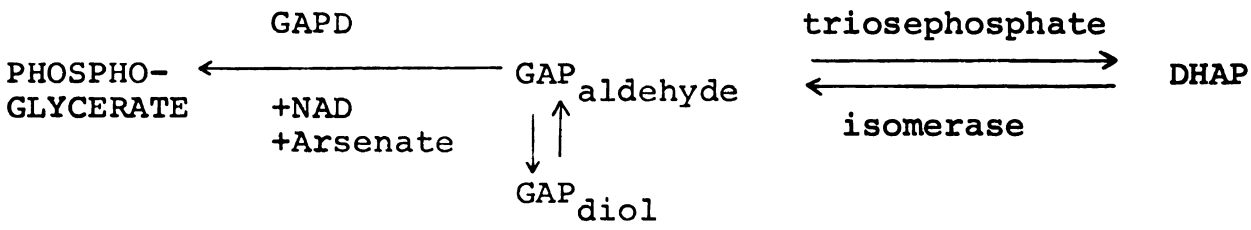
In aqueous solution (pH 7.3-8.6, 20°) the equilibrium ratio of DIOL:ALDEHYDE is 29:1 (Trentham et al., 1969). Furthermore, the aldehyde is the only form utilized by GAPD, triosephosphate isomerase and aldolase; also, the product of the aldolase and triosephosphate isomerase reactions is the aldehyde (Trentham et al., 1969).

In the presence of triosephosphate isomerase alone, there are thus three forms of triosephosphate in solution:



In the presence of triosephosphate isomerase, the equilibrium ratios of $GAP_{aldehyde}$:GAP_{diol}:DHAP are 1:29:660.

Finally, in the presence of both GAPD and triosephosphate isomerase there are multiple processes occurring, and these are illustrated below:



Thus, one of the effects of adding triosephosphate isomerase to the GAPD assay system must be to lower the concentration of $GAP_{aldehyde}$, which is the substrate for GAPD. Along with this effect, the concentration of $GAPD_{diol}$ must also be lowered.

MECHANISM OF THE INHIBITION OF GAPD BY TRIOSEPHOSPHATE ISOMERASE. The lowering of the glyceraldehyde-3-phosphate concentration should certainly cause an apparent inhibition of GAPD. Thus, this accounts for the bulk of the inhibition of GAPD by triosephosphate isomerase. Furthermore, a number of physical tests (p. 208 to p. 225) failed to demonstrate any physical interaction between GAPD and triosephosphate isomerase.

These observations suggested that the only effect of triosephosphate isomerase was to lower the glyceraldehyde-3-phosphate concentration. On the other hand there

are a number of observations that could not be explained by the triosephosphate isomerase catalyzed conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate. These observations are as follows:

(1) Increasing the glyceraldehyde-3-phosphate concentration did not completely overcome the inhibition; above 1.5 mM glyceraldehyde-3-phosphate the GAPD activity showed a plateau with increasing substrate (p. 185).

(2) The inhibition of GAPD in the presence of triosephosphate isomerase was reversible by dilution (p. 173); e.g., samples diluted from a stock solution mixture of GAPD and triosephosphate isomerase showed a decreasing % inhibition as the total concentration of the mixture was decreased.

(3) At a definite molar stoichiometry of 1:1 (GAPD:triosephosphate isomerase, see p. 204) a maximal inhibition was observed; this suggests an interaction between enzymes rather than a competition for substrate, since a coincidental ratio of 1:1 seems highly unlikely in view of the fact that the specific activity of the triosephosphate isomerase is 10 to 20 times that of GAPD.

(4) When GAPD is assayed, with and without triosephosphate isomerase present, in such a manner that the concentration of glyceraldehyde-3-phosphate is the same in both cases (p. 239), the triosephosphate isomerase seems to activate the GAPD.

These observations suggest that the triosephosphate isomerase physically interacts with the GAPD.

Since there is no single satisfactory explanation for all the data, further experiments are needed to conclusively prove or disprove that the triosephosphate isomerase physically interacts with the GAPD.

HYDROLYSIS OF p-NITROPHENYL ACETATE BY TRIOSEPHOSPHATE ISOMERASE. The observation in this work that triosephosphate isomerase has considerable esterase activity with the model substrate p-nitrophenyl acetate (p. 235) is itself interesting, and was rather unexpected. The specific esterase activity (μmoles $min.^{-1}$ mg. $protein^{-1}$) of the triosephosphate isomerase is the same as that of GAPD, which is considered to be quite active as an esterase with p-nitrophenyl acetate as a substrate (Taylor et al., 1963). For example, yeast GAPD is 1.5 times as active as chymotrypsin (Taylor et al., 1963).

The esterase activity of the triosephosphate isomerase also seems to be a specific effect, since the substrate glyceraldehyde-3-phosphate inhibits the hydrolysis of the p-nitrophenyl acetate. Glyceraldehyde-3-phosphate

also inhibits the esterase activity of GAPD (Taylor et al., 1963). It thus seems likely that a study of the esterase activity of the triosephosphate isomerase might provide valuable information about the active site of this enzyme, just as studies of the esterase activity of GAPD have provided valuable information (Taylor et al., 1963).

At present no biochemical significance can be assigned to this unusual catalytic function of triose-phosphate isomerase.

METHODS

ASSAY FOR INHIBITION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. Inhibition was measured by assaying an aliquot of a solution of GAPD (usually 0.2-0.4 mg/ml in a volume of 0.5-1.0 ml) to determine the 100% activity value, adding a small amount of inhibitor protein (25-125 μg) to the GAPD solution, and then assaying an aliquot of the mixture for GAPD activity. Assays were performed as previously described (p. 95). The solvent in which the two proteins were mixed was 0.1 M Tris, pH 8.0, containing 0.1 M β-mercaptoethanol. The inhibitor protein was added and mixed gently at room temperature. Identical values for the % inhibition were obtained if the GAPD and inhibitor protein were added separately to the GAPD assay cuvette, instead of pre-mixing. Since pre-mixing of the two proteins was more convenient, this technique was routinely used to measure the inhibition.

REAGENTS. The p-nitrophenyl acetate used for the esterase assays was obtained from Sigma (St. Louis, Mo.), and the ampholytes used for the isoelectric focusing were obtained from LKB (LKB Produkter AB, Fack, s-161 25, Bromma 1, Sweden). The reagents used in the GAPD assays have previously been described (p. 95). All other reagents were the best commercial grade available.

ISOELECTRIC FOCUSING. Isoelectric focusing was performed with either an LKB 110 ml apparatus or an LKB 440 ml apparatus and a Savant high voltage power supply unit.

The anode solution was prepared by mixing 0.8 ml of sulfuric acid, 56.0 ml of water and 48 g. of sucrose. The cathode solution was 0.8 ml of ethylendiamine and 40.0 ml of water.

For the narrow pH gradient (4-6), the ampholyte-sucrose column (440 ml) was prepared by layering a series of 46 fractions (9 ml each) of decreasing sucrose density on 75 ml of anode solution. Fractions were layered with tubing attached to a 10 ml funnel. The fractions contained various proportions of a dense sucrose-ampholyte solution and a light aqueous ampholyte solution. The dense solution (40% sucrose) contained 7.6 ml of ampholytes, 142 ml of water and 100 g. of sucrose. The light solution contained 2.4 ml of ampholytes and 213 ml of water. Starting with pure dense solution (1st 9 ml fraction) the 9 ml fractions were prepared by successively decreasing the amount of dense solution by 0.2 ml and increasing the

amount of light solution by 0.2 ml. Thus, the second 9 ml fraction contained 8.8 ml of dense solution and 0.2 ml of the light solution. The 46th fraction was pure light solution. Cathode solution was then added to a level 1 cm above the platinum electrode. The procedure was analogous for the broad pH range (3-10), except that 2.25 ml fractions were used with the 110 ml column.

The protein, 25 mg (in 5 ml) for the 110 ml column and 100 mg (in 20 ml) for the 440 ml column, was applied by adding small aliquots (each aliquot was 1 ml) of the protein samples to the 20 ampholyte-sucrose fractions used to form the middle of the column.

A potential difference of 600 v (110 ml column) or 1000 v (440 ml column) was used. The temperature was maintained at 2° with a circulating water bath. After focusing for 72 hrs., the fractions were collected with a Gilson fraction collector.

ANTIBODY PREPARATION AND IMMUNODIFFUSION TESTS. For the primary immunization, GAPD and the inhibitor protein were dialyzed overnite against 0.15 M NaCl and 10^{-4} M EDTA at 23°. Emulsions were prepared by mixing equal volumes of dialyzed protein and Freund's adjuvant. Male-Wistar rabbits were then given 3 ml of the emulsion (containing 7 mg of protein) in six injections (2 foot pads, 2 sides of the neck, and 2 sides of the torso anterior to the hind leg), i.e., 0.5 ml per injection site.

Three weeks after the primary immunization the rabbits were given a booster (IV) in the ear vein. The booster was 2 mg of protein (previously dialyzed as noted above) in a volume of 0.5 ml.

Eight days after the booster, 15-20 ml of blood was collected by cardiac puncture. The blood was left overnite in the cold, the clot was removed by centrifugation and the serum was collected.

For the preparation of the double diffusion plates, one gram of agar was mixed with 25 ml of buffer (see below) and 75 ml of water. The mixture was warmed until the agar completely dissolved, and 1 ml of 1% merthiolate was added. The agar was stored in the refrigerator until used.

The buffer used in the agar solutions consisted of 0.05 M sodium diethylbarbiturate, 0.01 M diethylbarbituric acid and 0.05 M sodium acetate. The pH of this buffer solution was 8.6 and the ionic strength was 0.1.

FLUORESCENCE MEASUREMENTS. Fluorescence measurements were made at 20° with an Aminco-Bowman spectrophotofluorometer equipped with an X-Y Recorder (American Instruments Co., Silver Springs, Md.). Fluorescence was measured at right angles to the illumination. The NADH was excited at a wavelength of 340 mμ and the fluorescence emission was measured at a wavelength of 465 mμ. The temperature was thermostat controlled.

Titrations of fluorescence change were begun with a 1.0 ml sample of NADH in 0.1 M Tris, pH 8.0, which contained 0.1 M β-mercaptoethanol, in a fused quartz cell. Small aliquots of the enzyme titrant (GAPD or the inhibitor protein) in the same solvent as the NADH were added and mixed with a magnetic stirrer. The fluorescence intensity after each addition was then read and corrected for dilution.

The initial concentration of NADH in these experiments was either 1×10^{-5} M or 2.5×10^{-5} M.

AMINO ACID ANALYSIS. The protein samples (0.2 ml of 1.0 mg/ml) were de-salted by passage through a 1.6 x 12 cm column packed with Sephadex G-25, which had been equilibrated with distilled de-ionized water. The samples were lyophillized in hydrolysis tubes (200 µg/tube), 6 N HCl was added and the tubes were placed in an acetone/dry ice bath. The tubes were then evacuated and sealed. After hydrolysis at 110° for 24 or 48 hrs., 25 µg samples of protein were analyzed.

The analysis was performed on an amino acid analyzer constructed by Dr. D. Robertson and Dr. W. A. Wood of this department.

ASSAY FOR TRIOSEPHOSPHATE ISOMERASE ACTIVITY. Triosephosphate isomerase activity was routinely measured in a coupled assay using α-glycerophosphate dehydrogenase. The assay contained the following components (the final

concentration of each component in the assay is given in parenthesis) which are listed in the order of addition to the assay cuvettes: Tris buffer (0.1 M), pH 8.0; rabbit muscle α-glycerophosphate dehydrogenase (25 µg/ml); NADH (1 mM); triosephosphate isomerase (0.1 -1.0 µg/ml); glyceraldehyde-3-phosphate (1 mM). The total volume was 0.4 ml.

The decrease in absorbance at 340 mµ was followed with a Gilford Model 2000 Spectrophotometer equipped with temperature control and multiple sample absorbance recorder. Assays were performed at 25°.

ASSAYS FOR ESTERASE ACTIVITY USING p-NITROPHENYL ACETATE. The esterase activity of GAPD and triosephosphate isomerase was measured with the model substrate p-nitrophenyl acetate as described by Taylor et al., (1963). The assay contained the following components (final concentrations in the assay are given in parenthesis) which are listed in the order of addition to the assay cuvettes: Tris buffer (0.1 M), pH 8.0; GAPD or triosephosphate isomerase (0.1-0.5 mg); p-nitrophenyl acetate (2.17×10^{-3} M). Total volume was 0.4 ml.

Since the p-nitrophenyl acetate is unstable in aqueous solutions, a stock solution (6.3 mg/ml, 3.5×10^{-2} M) was prepared in absolute methanol and stored at 0° until used. Stock solutions were never used more than 3 days after they were prepared.

The reaction was initiated with p-nitrophenyl acetate and the increase in absorbance at 400 mμ (due to the release of p-nitrophenol) was followed with a Gilford Model 2000 Spectrophotometer equipped with temperature control and a multiple sample absorbance recorder. Assays were performed at 25°. Blank rates, obtained from cuvettes minus protein samples, were used to correct all reported rates for spontaneous hydrolysis of the p-nitrophenyl acetate.

Assays could not be performed in the presence of cysteine, because of the high blank rates observed (without the addition of either protein) when cysteine was present in the assay mixture. This high blank rate apparently arose from the catalysis of the hydrolysis of the p-nitrophenyl acetate by the cysteine itself.

TURBIDIMETRIC MEASUREMENT OF PROTEIN CONCENTRATION. The procedure used to locate the protein peaks on the sucrose density gradients (Figures 43 and 44) was exactly as described by Mejbaum-Katzenellenbogen and Dobryszycka (1959).

CHAPTER SIX

KEY FINDINGS OF THE RESEARCH AND SUGGESTIONS FOR FUTURE WORK

DISSOCIATION OF YEAST GAPD IN ATP

(1) Tetrameric yeast GAPD (7.5 S) dissociates into subunits at 0° in the presence of ATP. Ultracentrifugal analysis reveals that the first observable product of the dissociation is a 3.0 S subunit (incubation time less than 12 hrs). This is the expected sedimentation coefficient for a globular or folded subunit of molecular weight 36,000 (Holleman, 1966). After longer incubation times (24 hrs) a 1.6 S subunit is observed. This is the expected sedimentation coefficient for an extensively unfolded subunit, since GAPD subunits in 8 M urea have a sedimentation coefficient of 1.5 S (Deal and Holleman, 1964). After a very long incubation time (3-7 days) at 0° in ATP, a 0.9 S particle is observed. A sedimentation coefficient of 0.9 S corresponds to a particle approximately one-half the molecular weight of the GAPD subunit of 36,000.

The yeast GAPD-ATP dissociation is rather unique since selective treatment can be used to obtain a folded or unfolded subunit. This is in contrast to most dissociation systems which also produce extensive unfolding of the individual polypeptide chains. This system is thus ideally suited to answer a number of questions concerning subunit interactions in polymeric enzymes.

Several questions raised by the observation of the 0.9 S species are "What is the exact nature of the 0.9 S species?", "Does the production of the 0.9 S species

involve the rupture of peptide bonds?" and "Is the production of the 0.9 S species a spontaneous process?"

The first question, pertaining to the nature of the 0.9 S species, can be answered by careful molecular weight measurements. The technique best suited for this measurement is probably electrophoresis in sodium dodecyl sulfate (Weber and Osborn, 1969). Conventional sedimentation equilibrium studies would check the molecular weight of the 0.9 S species obtained by this technique.

The second question, whether or not peptide bonds are ruptured in the formation of the 0.9 S species, can also be answered by available experimental techniques. This can be done by incubating GAPD at 0° in ATP solutions prepared with O^{18}-enriched water, and then determining whether O^{18} is incorporated into the terminal carboxyl groups of the 0.9 S species.

Finally, experiments should be performed to determine whether the production of the 0.9 S species is a spontaneous process requiring only the incubation of GAPD with ATP at 0°, or whether any other protein, such as a protease contaminant in the GAPD preparation, is required. This question can be answered by observing the rate of appearance of the 0.9 S species from native GAPD samples pre-treated in several ways. The first sample would be GAPD prepared as described in Methods, Chapter Three. The second sample would be GAPD prepared as above and then passed over a

Sephadex G-200 column. The third sample would be GAPD prepared as above and then "further purified" by isoelectric focusing. Production of the 0.9 S species at the same rate from all the GAPD samples so pre-treated would be strong evidence that the 0.9 S species arose solely from the incubation of native GAPS with ATP at 0°.

(2) A systematic study of the variables affecting the dissociation of GAPD at 0° in ATP reveals that electrostatic and hydrophobic interactions are both important in the dissociation process. Thus dissociation involves: (a) conformational changes in the individual subunit polypeptide chains produced as a result of electrostatic repulsions between the negatively charged phosphate portion of ATP and the negatively charged groups on the protein when ATP is bound. (b) This conformational change alters the subunit contact sites so that hydrophobic interactions assume a more critical role in maintaining the native tetrameric structure. (c) Lowering the temperature to 0° then weakens hydrophobic interactions between subunits enough to cause dissociation.

This interpretation is also consistent with the results of studies of the assembly of yeast GAPD subunits produced in 8 M urea (Deal, 1969), with the results of studies of the binding of adenine nucleotides (Yang and Deal, 1969a;b) and with the results of the kinetic analysis of dissociation of yeast GAPD at 0° in ATP. Therefore, the

nature of the forces involved in the dissociation process seems well established.

(3) A kinetic analysis of the dissociation reveals that the overall process is bi-phasic, involving a fast initial step followed by a slow second step.

The fast step can be described by a rapid reversible equilibrium between tetramers and dimers. The kinetic data can be treated to obtain an equilibrium constant for this initial process. The calculated equilibrium constant (K_{eq} = 10.2 x 10^{-8} M) for this initial dissociation yields a value of 8.9 kcal/mole for ΔG° at 0°.

The slow step can be described by the dissociation of dimers to monomers, which is essentially irreversible at 0°. The average constant for binding of ATP to the dimer is 0.75 mM, and there is no measureable interaction between ATP binding sites.

The methods devised in this thesis for obtaining K_{eq} (at 0°) in the presence of ATP for reversible dissociation of tetramers to dimers can now be used to obtain a complete thermodynamic description of this initial dissociation as follows: (a) K_{eq} can be measured at different temperatures between 0° and 23°, and ΔG° as a function of temperature may then be calculated from

$$\Delta G^{\circ} = -RT \ln K_{eq}$$

(b) The slope of a plot of ln K_{eq} vs 1/T at any temperature will then yield - $\Delta H^\circ/R$ at that temperature, since

$$\frac{d \ln (K_{eq})}{d (1/T)} = \frac{-\Delta H^\circ}{R}$$

(c) The values of ΔH° and ΔG° at any temperature can then be used to obtain ΔS° from

$$- \Delta S^\circ = \frac{(\Delta G^\circ - \Delta H^\circ)}{T}$$

If a method can be devised to measure the constant for binding of ATP to the <u>tetramer</u> of GAPD, the first Law of Thermodynamics can be used to obtain values for the thermodynamic parameters of the tetramer-dimer dissociation in the <u>absence</u> of ATP. This possibility is discussed in Chapter Four of this thesis.

A kinetic analysis of the dissociation process at different temperatures can also be used to obtain a rate constant, k, for dimer dissociation at different temperatures. The activation energy, E_a, for this process can then be calculated from the Arrhenius equation

$$\ln k = \frac{-E_a}{RT} + \ln A$$

where A is the constant obtained upon integration of the differential form of the equation.

REVERSAL OF DISSOCIATION

(4) Globular subunits (3.0 S), produced by incubation of native GAPD with ATP at 0°, can be reassembled to yield native 7.5 S tetramers by raising the temperature (to 15° or above) in the presence of ATP, 10% sucrose and reducing agent. This discovery is especially exciting since the reassembly of folded monomers (this work) may now be compared to reassembly of unfolded subunits produced in 8 M urea (Deal, 1969).

The most striking similarity between these two reassembly systems is the nucleotide requirement for in vitro reassembly. Reassembly of folded monomers requires ATP and reassembly of unfolded monomers requires NAD. Contrary to widespread views, these results clearly show that the amino acid sequence alone is not adequate to determine either the folding or association of yeast GAPD subunits, thus raising the possibility that either folding or association steps, or both, are subject to in vivo control by metabolite levels (see Deal, 1969).

There are many other similarities between the two reassembly systems: (a) the pH profiles of reassembly show no significant dependence upon pH in the range of pH 6.8. (b) Both systems require reducing agent for complete activity recovery upon reassembly. (c) The protein concentration which yields maximum activity recovery for both reassembly systems is 0.04 mg/ml, suggesting a

concentration dependent equilibrium step in the reassembly process. (d) Both reassembly systems exhibit a very similar dependence on temperature for activity recovery. (e) The half-time for activity recovery of unfolded subunits is 7.0 min (95 µg/ml, pH 6.9, 0.3 M KCl, 16°), and 7.5 min for folded subunits (100 µg/ml, pH 7.0, 0.3 M KCl, 23°, 10% sucrose), (f) The half-times of activity recovery for both systems increase with decreasing protein concentration. These results suggest that the mechanism of assembly is very similar for both reassembly systems.

The only two major differences between the different reassembly processes seem to be the previously noted nucleotide specificity and the effect of salt on reassembly. KCl (0.3 M) yields a 5-fold increase in activity recovery upon reassembly of unfolded subunits (Deal, 1969), but does not affect the total activity recovery upon reassembly of folded subunits. Thus it is clear that the effect of KCl on the reassembly of unfolded subunits is on a folding step and not on an association step (Deal, 1969). Furthermore, since KCl substitutes for NAD in the reassembly of unfolded subunits, it is also clear that the effect of NAD upon reassembly of unfolded subunits is an effect on the folding of the polypeptide chains (Deal, 1969).

Taken together, the studies reported in this thesis and the studies of Deal (1969) on the assembly of yeast GAPD subunits to native enzyme provided one of the most

informative and comprehensive analyses available of the assembly of a polymeric enzyme from its subpolymers.

(5) A kinetic study of the reassociation of globular subunits (Chapter Four) reveals that the over-all order of the reassociation process changes from second order at low protein concentrations to first order at higher protein concentrations. This is a significant observation because it indicates that association of folded subunits is not simply a sequence of bimolecular associations, but rather the process may involve a first order isomerization reaction as the final step in reassembly of the native enzyme. This study should serve as a valuable model for the association steps in a complete in vitro synthesis of polymeric enzymes, a goal which biochemists are rapidly approaching.

CHAPTER SEVEN

APPENDICES AND LIST OF REFERENCES

APPENDIX 1

DETERMINATION OF REACTION ORDER BY THE HALF-LIFE METHOD

For any rate expression of the type

$$dx/dt = k\ (a - x)^n \qquad (1)$$

the half-life may be defined for all values of n, the order of the reaction, as

$$t_{1/2} = f\ (n,k)/a^{n-1} \qquad (2)$$

where f is some function of the order of the reaction, n, and the rate constant, k, and a is defined as the initial concentration of reactant (Frost and Pearson, 1961). Expression (2) may be placed in logarithmic form to yield (3)

$$\log\ (t_{1/2}) = \log\ (f[n,k]) - (n-1)\ \log\ a \qquad (3)$$

Therefore a plot of the half-time of a given process vs the initial concentration of reactant, a, should yield a straight line with a slope of (1-n).

APPENDIX 2

DERIVATION OF THE INTEGRATED RATE EQUATION FOR A TETRAMER-DIMER EQUILIBRIUM

The tetramer-dimer equilibrium is represented by the process given below

$$A \underset{k_{24}}{\overset{k_{42}}{\rightleftharpoons}} 2B \qquad K_{eq} = \frac{[B]^2}{[A]}$$

If we start at time zero with A and no B, and let x equal to the amount of A which is converted to B at any time, the concentrations of A and B at time zero, time t and at equilibrium (t_e) will be those given below

$$A \underset{k_{24}}{\overset{k_{42}}{\rightleftharpoons}} B$$

TIME		
t_o	a_o	0
t	(a_o-x)	$(2x)$
t_e	$a_e=(a_o-x)_e$	$(2x)_e$

We may then write an expression for the rate of change of x as

$$dx/dt = k_{42}\ (a_o-x) - k_{24}\ (2x)^2 \qquad (1)$$

At equilibrium, dx/dt = 0, and from (1) we may therefore write

$$0 = dx/dt = k_{42}\ (a_o - x)_e - k_{24}\ (2x)^2 \qquad (2)$$

This may then be rearranged to give

$$k_{24} = k_{42}\ (a_e / (2x)^2_e) \qquad (3)$$

Substitution of this value of k_{24} into (1), yields

$$dx/dt = k_{42}\ (a_o - x) - (k_{42}\ a_e / 4x^2_e)\ (2x)^2 \qquad (4)$$

We now define a constant b, such that

$$b = a_e / x^2_e \qquad (5)$$

After substitution of b, (4) reduces to

$$dx/dt = k_{42}\ (a_o - x - bx^2) \qquad (6)$$

Separation of variables yields

$$dx/(-bx^2 - x + a_o) = k_{42}\ dt \qquad (7)$$

Integration of this relationship yields

$$D^{1/2}\ k_{42}\ t = \log \left[\frac{|(x-2a_o)| - |xD^{1/2}|}{|(x-2a_o)| + |xD^{1/2}|} \right] \qquad (8)$$

where D is a constant given by

$$D = 1 + 4a_o b \tag{9}$$

Now, x was originally defined as the amount of A (tetramer) which is converted to B (dimer) at any given time. Therefore, since the tetramer is active and the dimer is inactive, a measure of the quantity x at any time is given by the difference in enzymatic activity at time zero and encymatic activity at any time, t.

$$x = A_o - A_t \tag{10}$$

If we now let A_o be the measured enzymatic of a solution of active tetramers (at a fixed protein concentration, c), and A_t the enzymatic activity of a mixture of active tetramers and inactive dimers at a total concentration equal to c, we may re-write (8) in terms of enzymatic activity as

$$D^{1/2} k_{42} t = \log \left[\frac{|(A_o - A_t - 2A_o)| - |(A_o - A_t)| D^{1/2}}{|(A_o - A_t - 2A_o)| + |(A_o - A_t)| D^{1/2}} \right] \tag{11}$$

The term D in (11) must also be expressed in terms of enzymatic activity, and this is given below

$$D = 1 + \frac{4A_o A_e}{(A_o - A_e)^2} \tag{12}$$

where A_o is the activity at time zero and A_e is the activity at equilibrium for the tetramer-dimer equilibrium. This value, A_e, is thus seem to be the value of the activity obtained by extrapolation of the slow process (dimer breakdown) of dissociation in ATP to time zero, since this extrapolation provides a measure of the activity one would observe at a given protein concentration for an equilibrium mixture of tetramers and dimers if the dimer did not breakdown further to yield monomers.

Expression (11) may now be further rearranged to its final usable form

$$(T)\ k_{42}\ t = \log \left[\frac{|(\ -A_o - A_t\ )| \ - \ |(\ A_o - A_t\ )(T)|}{|(\ -A_o - A_t\ )| \ + \ |(\ A_o - A_t\ )(T)|} \right]$$

where $\underline{T}$ is simply the square root of $\underline{D}$ as given in (12).

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