PURIFICATION AND CHARACTERIZATION OF PIG LIVER L - α - GLYCEROL PHOSPHATE DEHYDROGENASE

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This is to certify that the

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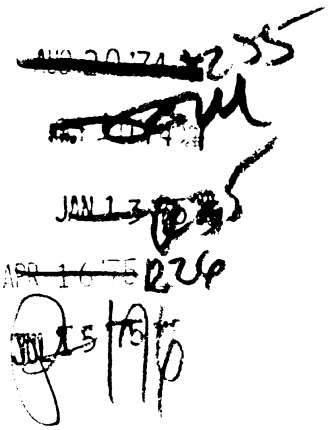

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF PIG LIVER
L-α-GLYCEROL PHOSPHATE DEHYDROGENASE

By

James Nelson Behnke

The enzyme L-α-glycerophosphate dehydrogenase (L-Glycerol-3-phosphate:NAD oxidoreductase, E.C. 1.1.1.8) from pig liver was purified 400-fold to homogeneity (specific activity = 100 μmoles/min/mg) using a six-step procedure that involved calcium phosphate gel adsorption, DEAE cellulose chromatography, phosphocellulose chromatography, preparative sucrose gradient centrifugation and preparative disc gel electrophoresis. The enzyme was judged to be pure on the basis of analytical disc gel electrophoresis, SDS gel electrophoresis, sedimentation velocity and equilibrium experiments in the analytical ultracentrifuge and assays for the enzymes phosphoglycerate kinase, enolase, trisephosphate isomerase, glyceraldehyde phosphate dehydrogenase, aldolase and phosphofructokinase. The native enzyme has a molecular weight of approximately 70,000, a sedimentation coefficient of $s^{0.5\%}_{20,w}$ = 4.89 S and is composed of two subunits. The isoelectric point is 6.8 and the absorption spectrum has a broad maximum near 273 nm and a shoulder near 259 nm. The K_m values for the substrates are: NADH, 8 μM; dihydroxyacetone

phosphate, 0.27 mM; L-α-glycerol phosphate, 0.20 mM; NAD, 57 μM. The enzyme was inhibited by ATP (K_i = 6 mM), ADP (K_i = 17 mM) and 5'-AMP (K_i = 18 mM). Activity stains of disc gels of crude homogenates of pig liver showed one main band and several minor bands of α-glycerophosphate dehydrogenase activity; our highly purified enzyme corresponded to the enzyme in the main band of the crude homogenates. Several important effects of NAD and NADH on the enzyme were observed. In the range of 0.01 mM to 0.5 mM, the pyridine nucleotides: (1) protect the enzyme against heat inactivation at 49°, (2) prevent dissociation of the enzyme at low protein concentration, (3) maintain the activity of the enzyme during disc gel electrophoresis and for pure enzyme yield a single band instead of the diffuse smear observed in its absence, (4) maintain the enzyme activity during ion-exchange chromatography on DEAE and phosphocellulose and modify the binding of the enzyme to the ion exchangers and (5) prevent the inhibition of the enzyme by ATP and other adenine nucleotides. These are the first reports of effects of NADH and NAD on column chromatography and disc gel electrophoresis of the enzyme.

PURIFICATION AND CHARACTERIZATION OF PIG LIVER L-α-GLYCEROL PHOSPHATE DEHYDROGENASE

By

James Nelson Behnke

A THESIS

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DEDICATION

To Helen, Marty and Stevie

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The author wishes to express his appreciation to Dr. W. C. Deal for his guidance and suggestions throughout this research. The author also wishes to thank Drs. Massey and Stancel for their many times of discussion and argument and many suggestions.

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VITA

James N. Behnke was born on May 1, 1943, in Alpena, Michigan. After spending his childhood in several mid-western and western states, he returned to Michigan (Hart) in 1955 and graduated from Hart High School in 1961. He participated in Forensics, attended Michigan Boys State, was elected president of the Hart Chapter of the National Honor Society and graduated second in his class.

After one year at the University of Michigan, he moved to Flint, Michigan, where he worked for the Chevrolet Division of General Motors and attended Flint Junior College. He then transferred to Central Michigan University where he received his B.S. in Chemistry, with a Biology minor, in 1967. While studying at Central Michigan, he was involved in research on the synthesis and decomposition of two furoyl peroxides, under the direction of Dr. Fred Kabbe. He was also involved in teaching Freshman, Analytical and Organic chemistry laboratories and helped organize the CMU Chapter of Student Affiliates of the American Chemical Society. Mr. Behnke received the 1967 Outstanding Chemistry Senior Award, presented by the Midland, Michigan, Section of the American Chemical Society.

Mr. Behnke then began his graduate program in the Department of Biochemistry at Michigan State University.

Under the guidance of Dr. William C. Deal, Mr. Behnke was able to purify and study the enzyme L-α-Glycerol Phosphate Dehydrogenase from pig liver.

Mr. Behnke intends to continue his research career in the Department of Microbiology and Public Health at Michigan State University, in the laboratory of Dr. Leland Velicer. Dr. Velicer has been studying the proteins of the Feline Leukemia Virus grown in cell culture. Mr. Behnke will be involved in characterizing these proteins and studying their arrangement within the virus particle.

Mr. Behnke was married on August 25, 1967, to Helen, and they have two sons, Martin (November 6, 1969) and Stephen (September 4, 1972).

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ABBREVIATIONS

ALD	aldolase
Bis	N,N'-methylenebisacrylamide
BSA	bovine serum albumin
DHAP	dihydroxyacetone phosphate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ENOL	enolase
FDP	fructose-1,6-diphosphate
GAPD	glyceraldehyde phosphate dehydrogenase
α-GDH	α-glycerol phosphate dehydrogenase
Glu-6-P	glucose-6-phosphate
α-GP	L-α-glycerol phosphate
GuHCl	guanidine HCl
LDH	lactate dehydrogenase
MES	N-(N-morpholino)ethane sulfonic acid
mS	milli-Siemens
NBT	nitroblue tetrazolium
NEM	N-ethyl maleimide
PCMB	p-chloromercuribenzoate
PGI	phosphoglucoisomerase
PGK	phosphoglycerate kinase
PGM	phosphoglycerate mutase
Pipes	piperazine-N,N'-bis(2-ethane sulfonic acid)
PK	pyruvate kinase

PMS	phenazine methosulfate
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
TPI	triose phosphate isomerase

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INTRODUCTION

The enzyme α-glycerol phosphate dehydrogenase (L-Glycerol-3-Phosphate:NAD oxidoreductase, EC 1.1.1.8) has been purified and studied from a variety of sources, but most of the detailed physical and chemical studies have been with the rabbit muscle enzyme. For various reasons, it is important to characterize the enzyme from liver sources. For example, the enzyme is positioned at a branch point in metabolism between: (1) glycolysis, (2) lipid synthesis through α-glycerol phosphate and (3) energy transport into the mitochondria via the glycerophosphate shuttle. The limited studies conducted thus far have not shown any evidence of significant regulation of the enzyme, but if such control does exist, it should be detectable with enzyme from liver. In contrast to muscle, which has virtually no fatty acid synthesis, liver is a major tissue for fatty acid synthesis. In order to study any possible control of a liver α-glycerol phosphate dehydrogenase, it was necessary to purify the enzyme.

The pig was chosen as the source of liver for several reasons. Primarily, the pig is quite similar to the human in many respects, especially in the tendency toward obesity. For this reason, this laboratory is involved in a systematic purification of, and studies on, the glycolytic and lipid

synthesizing enzymes from pig liver. Large amounts of the liver are easily available at low cost from local slaughterhouses. The aims of the present study were then to: (1) purify pig liver α-glycerol phosphate dehydrogenase to homogeneity, (2) to characterize it physically, chemically and kinetically, and (3) to investigate the possible control mechanisms of the enzyme. Since this was the initial project in a much broader study, the first studies involved brief characterization of virtually all the glycolytic enzymes from pig liver.

CHAPTER I

LITERATURE REVIEW

Since the work presented in this thesis concerns the cytoplasmic, NADH linked, glycerol-3-phosphate dehydrogenase, only the literature concerning that class of enzymes will be reviewed in detail. That concerning other α-glycerol phosphate dehydrogenases will be only briefly discussed. A summary of the literature concerning the rabbit muscle enzyme is also available in the previous work from this laboratory (Holleman, 1966).

Purification of α-Glycerol Phosphate Dehydrogenase from Various Sources

The rabbit muscle enzyme has been purified by a variety of procedures. Beizenherz et al. (1955) utilized a series of ammonium sulfate precipitation steps and repeated crystallization from ammonium sulfate solution to give enzyme with a specific activity of 75 units/mg. Modifications of this procedure include a final differential centrifugation step (Telegdi, 1964) and a final DEAE cellulose column (van Eys et al., 1959; Otto et al., 1972).

Fondy et al., (1968) purified the rat skeletal muscle enzyme to a specific activity of 115 using ammonium sulfate precipitation, DEAE cellulose chromatography, gel

filtration and crystallization. They also purified the rat liver enzyme (Fondy et al., 1971) using a similar procedure that included ammonium sulfate fractionation, DEAE chromatography and gel filtration. The purified enzyme had a specific activity of 100.

The honeybee thorax enzyme (Marquardt and Brosemer, 1966) was purified using ammonium sulfate fractionation and crystallization from ammonium sulfate solution; the purified enzyme had a specific activity of 170.

Lehmann and Pfleiderer (1968) purified the human liver enzyme by using calcium phosphate adsorption, ammonium sulfate fractionation, a heat step, DEAE Sephadex chromatography and crystallization from ammonium sulfate. The pure enzyme had a specific activity of 288.

The chicken liver and muscle enzymes were purified by White and Kaplan (1969). The muscle enzyme was purified using ammonium sulfate fractionation, a heat step, DEAE and phosphocellulose chromatography and crystallization from ammonium sulfate solution. The liver enzyme was purified by homogenizing in ammonium sulfate, chromatography on DEAE and phosphocellulose and gel filtration. The muscle enzyme had a specific activity of 145 and the liver enzyme 90.

The rabbit liver enzyme was purified by Otto et al., (1972) to a specific activity of 142 using a series of heat and ammonium sulfate fractionation steps, gel chromatography and crystallization from ammonium sulfate solution.

Properties of Various α-Glycerol Phosphate Dehydrogenases

Kinetic Properties

The kinetic properties of some of the α-glycerol phosphate dehydrogenases are given in Table I.

Mechanism

Some work has been done on the mechanism of the rabbit muscle enzyme. Black (1966) did extensive initial velocity and product inhibition studies and concluded that the enzyme had an Ordered Bi Bi mechanism with NADH (or NAD) binding first and NAD (or NADH) leaving last, with the possibility of either an inactive dead-end NADH-enzyme complex or an isomerized NADH-enzyme complex. From further studies Telegdi and Keleti (1968) concluded that abortive glycerophosphate-enzyme complexes were formed and concurred with Black that NAD or NADH is bound first to the enzyme. Apitz-Castro and Suarez (1969) reported that the observed substrate inhibition by NADH, α-glycerol phosphate, and dihydroxacetone phosphate could be overcome by incubating the enzyme with N-ethylmaleimide, giving Michaelis-Menten Kinetics. Presumably the maleimide reacts with a sulfhydryl group which is essential for binding of these compounds. In another interesting study of the rabbit muscle enzyme, Telegdi (1968) reported that the product of α-glycerol phosphate oxidation is either dihydroxyacetone phosphate or glyceraldehyde phosphate, depending on the pH. The pH

TABLE I

SUMMARY OF KINETIC PROPERTIES OF VARIOUS

α-GLYCEROL PHOSPHATE DEHYDROGENASES

	pH Optima		K_m, μM					
	Forward[a]	Reverse[b]	NAD	NADH	DHAP	α-GP	Activator* or Inhibitors	Reference
Rabbit Muscle	7.4	10.0	160	6.3	80	260		Young and Pace, 1968
								Black, 1966
							P_i, PCMB	Fondy et al., 1968
							Palmityl Co A	Werner and Schwandt, 1969
							NADP, 2'AMP, 3'AMP Glu-1-P, FDP	Apitz et al., 1965
							L-α-GP	Blanchaer, 1965
Chicken Muscle			500	6	230	2200-14,000		White and Kaplan, 1969
Honeybee	6.5				330		PCMB, NEM	Marquardt and Brosemer, 1966b
Rat Muscle				10	190	1600		Fondy et al., 1968
Rabbit Liver			265	9	720	1400	Iodoacetate, DTNB, EDTA*	Otto et al., 1972
			190	4	18	680		Lee and Craine, 1971
Chicken Liver			20	1.4	43	80-120		White and Kaplan, 1969
Human Liver	7.8				33		PCMB, DTNB	Lehmann and Pfleiderer, 1968
Rat Liver						100-200		Ross et al., 1971

[a]DHAP and NADH as reactants and αGP and NAD as products

[b]αGP and NAD as reactants and DHAP and NADH as products

optimum for glyceraldehyde phosphate production was 8.6 and that for dihydroxyacetone phosphate production was 9.4.

Apitz-Castro and Suarez (1970) studied the kinetic parameters of the enzyme as a function of pH and concluded from the pK values of 6.61 for the enzyme and 6.72 for the enzyme-substrate complex that one or more histidine residues were involved in the active center. These conclusions were supported by findings that the enzyme was completely inhibited by irradiation in the presence of rose bengal. It was also completely inhibited by modification of 2.14 histidines per molecule with dizao-1-H-tetrazole and inhibited 50 percent upon modification of 1.07 histidine residues per molecule, indicating that two histidines were required at the active site for activity. In a further study on the rose bengal-oxidized enzyme, Kamp and Mauk (1971) found that the rose bengal binding was competitive with NADH binding and that NADH protected the enzyme from photoinactivation.

Kim and Anderson studied the inhibition and inactivation of the enzyme with: (1) a number of N-alkylamaleimides (Anderson, Kim and Wang, 1970), (2) a series of aliphatic carboxylic acids (Kim and Anderson, 1969a), (3) a series of N-alkyl-ammonium chlorides (Kim and Anderson, 1968a), (4) a series of N'-alkylinicotinamide chlorides (Kim and Anderson, 1968b), and (5) the compounds adenylic acid, adenosine diphosphate and adenosine diphosphate ribose (Kim and Anderson, 1969b). They concluded that the binding of the NAD molecule involves the adenosine portion, the

pyrophosphate group and the nicotinamide ring and that a hydrophobic region was located near the binding site.

The enzyme also catalyzes the hydrolysis of p-nitrophenyl acetate (Alfonzo and Apitz-Castro, 1961), but the esterase site is apparently distinct from the dehydrogenase site (Suarez and Apitz-Castro, 1972).

Physical Properties

The physical properties of some of the α-glycerol phosphate dehydrogenases are given in Table II.

Chemical Properties

Several workers have studied the prosthetic group(s) bound to the rabbit muscle enzyme. It can be easily removed by charcoal treatment or precipitation of the enzyme by trichloroacetic acid, perchloric acid, heat or acid-alcohol. The apoenzyme had a normal absorption spectrum, instead of the maximum absorption at 277 nm and high absorption at 260 nm previously observed. Ankel et al., (1960) reported that the group was ADP-ribose or a compound closely related. Later, van Eys (1960) reported that the group was 3-(4-methyl-5[β-hydroxyethyl]-thiazolyl) succinic acid, but Celliers et al. (1963) disagreed, concluding that two unknown compounds of formulae $C_5H_9NO_7$ and $C_4H_7NO_4$ were bound to the enzyme.

The function of the prosthetic group is also unknown; van Eys (1959) reported that the enzyme free of this prosthetic group has the same catalytic behavior as the control enzyme. It is possible that the rabbit liver enzyme also

TABLE II

SUMMARY OF PHYSICAL PROPERTIES OF VARIOUS

αGLYCEROL PHOSPHATE DEHYDROGENASES

	Molecular Weight	Subunits	Amino Acid Composition	Reference
Rabbit Muscle	76,000	2	Yes	van Eys et al., 1964
	173,000			Young and Pace, 1958
	77,000	2		Brosemer, 1969
	68,000			White, 1971
	65,000	2	Yes	Fondy et al., 1969
	74,400	2	Yes	Holleman, 1966
Chicken Muscle	68,000		Yes	White, 1969, 1971
Honeybee	76,000			White, 1971
	77,000	2		Brosemer, 1969
Rat Muscle	63,000	2	Yes	Fondy et al., 1968, 1971b
Rabbit Liver	77,000		Yes	Otto et al., 1972
Chicken Liver	68,000		Yes	White, 1969, 1971
Human Liver	70,000		Yes	Lehmann and Pfleiderer, 1968
Rat Liver	63,000	2	Yes	Ross et al., 1971

contains a similar prosthetic group, since Lehmann and Pfleiderer (1968) report that the enzyme has an absorption maximum near 277 nm, but they did not study it further.

Pfleiderer and Auricchio (1964),using the Sephadex column equilibrium technique, found that the rabbit muscle enzyme binds 2 moles of NADH per mole of enzyme, indicating the presence of two active sites per molecule.

No work has been done on the amino acid sequence of the enzyme, but two studies have found 2 moles of C-terminal methionine per molecule of enzyme (Holleman, 1966; Brosemer and Kuhn, 1969).

Holleman (1966) also did extensive studies on the reversal of the denatured enzyme from guanidine-HCl solutions and found, under the optimal conditions of 0.1 M Tris-HCl, at pH 7.42,0.001 M EDTA, 0.2 M β-mercaptoethanol, and a final enzyme concentration of 0.025 mg/ml, that 90 percent of the enzymatic activity could be recovered.

Liver α-Glycerol Phosphate Dehydrogenases

The liver α-glycerol phosphate dehydrogenases previously isolated are generally similar to the muscle α-glycerol phosphate dehydrogenases. They all have molecular weights of 65,000 to 75,000 and are composed of two subunits, and their kinetic parameters are fairly similar. The liver enzymes are less stable than the muscle enzymes, however. They usually require the presence of reducing agent during purification and the chicken (White and Kaplan, 1969) and

human (Lehmann and Pfleiderer, 1968) liver enzymes require the presence of NADH and NAD, respectively, for stabilizing the enzyme during purification, whereas the muscle enzymes generally do not require either during purification. It has also been reported that the rabbit liver enzyme is allosteric (Lee and Craine, 1971) and that there are multiple forms of the rat liver enzyme (Ross et al., 1971).

The α-Glycerol Phosphate Shuttle and Other Roles of the Enzyme

The enzyme is thought to play at least two roles in the cell: (1) reoxidation of NADH to NAD for continued glycolysis by participation along with the mitochondrial glycerophosphate oxidase in the glycerophosphate shuttle, and (2) synthesis of α-glycerol phosphate for lipid synthesis. The operation of the shuttle was discussed in detail by Boxer and Devlin (1961) and it has been shown to be operative in insect flight muscle (Marquardt and Brosemer, 1966), in human adipose tissue (Galton and Bray, 1967) and in Ehrlich ascites tumor cells (Dionisi et al., 1970). However, two recent studies have concluded that the shuttle is only minimally operative in perfused rat liver (Williamson et al., 1971; Canicero et al., 1972). White and Kaplan (1969) proposed that the chicken liver and muscle enzymes were primarily involved in lipid synthesis and regeneration of NAD, respectively.

Since much of an animal's lipid is made in the liver and transported to other parts of the body (O'hea and Leveille, 1969), it is quite important that there be a controlled supply of α-glycerol phosphate for lipid synthesis. There are other mechanisms for synthesis of glycerol phosphate, such as through glycerol kinase, or synthesis of lipid via the acyl dihydroxacetone phosphate pathway (Hajra and Agranoff, 1968), but most likely, synthesis of α-glycerol phosphate through α-glycerol phosphate dehydrogenase is the most important source for lipid synthesis.

Interestingly, the enzyme is generally absent in tumor cells, leaving lactate dehydrogenase as the chief, if not the only, method of regenerating NAD to allow continuation of the abnormally high rate of glycolysis. This has led to attempts to inhibit tumor growth by use of inhibitors of lactate dehydrogenase, but these attempts have been generally unsuccessful (e.g., see Chasin, 1967).

Other α-Glycerol Phosphate Dehydrogenase

A second type of α-glycerol phosphate dehydrogenase has been partially purified and studied from *E. coli* B and and *E. coli* K-10 strain 8 (Kito and Pizer, 1968). The enzyme is apparently biosynthetic in nature since it utilizes NADPH instead of NADH and it is strongly inhibited by palmitoyl Co-A and stearoly Co-A, the Ki's being approximately 25 μM.

A third type of α-glycerol phosphate dehydrogenase is the mitodiondrial glycerophosphate oxidase which is involved in the glycerophophosphate shuttle. The enzyme contains a flavin prosthetic group and is linked to the electron transport system. Since the enzyme is part of the mitochondrial membrane, its purification has been difficult, but two procedures for partial purification of the enzyme from pig brain mitochondria have been published (Ringler, 1961; Dawson and Thorne, 1969).

Other pyridine nucleotide independent α-glycerol phosphate dehydrogenases have also been found in E. coli K 12 and Streptococcus faecuim F 24 (Kistler et al., 1969; Koditschek and Umbreit, 1969), but have not been studied in detail.

CHAPTER II

MATERIALS AND METHODS

Materials

Fresh hog livers were obtained from the Michigan State University Meats Department slaughterhouse and frozen within 2 hours of killing or from the Peet Packing Co., Chesaning, Michigan, and frozen within 15 minutes of killing. NAD and NADH were obtained from P-L Biochemicals (Milwaukee). DL-α-glycerol phosphate, NBT, PMS, ATP, ADP, 5'-AMP, DHAP dimethyl ketal and BSA were obtained from Sigma (St. Louis, Mo.). The DHAP was converted to the H^+ salt with Dovex 50 and hydrolyzed at 40° for 4 hours and stored frozen at -20°. Phosphocellulose and DEAE cellulose were obtained from Schleicher and Schuell (Keene, N.H.). Ampholine brand ampholyte was obtained from LKB (LKB Produkter AB, Fack, s-161 25, Bromma 1, Sweden). Thioglycerol was obtained from Aldrich (Milwaukee) and MES from Calbiochem (La Jolla, Cal.). The coupling enzymes glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, enolase, aldolase, triose phosphate isomerase, glycerophosphate dehydrogenase and glucose-6-phosphate dehydrogenase were obtained from Boehringer (N.Y.). All other chemicals were of reagent grade, and deionized distilled water was used throughout.

Enzyme Assays

αGlycerol phosphate dehydrogenase was routinely assayed by following the decrease in absorbance at 340 nm due to oxidation of NADH with a Beckman DU monochrometer equipped with a Gilford 2000 multiple sample absorbance recorder at 25°. A stock assay mix containing 0.7 m M DHAP, 0.25 m M NADH, 0.1 M Tris-Cl, 0.001 M EDTA, pH 7.6, was prepared daily; 1-2 µg of enzyme in 5 µl was added to 0.395 ml of assay mix to start the reaction. For kinetic studies, the buffer also contained 0.1 M KCl to maintain essentially constant ionic strength. One unit of enzyme will convert 1 µmole of NADH to NAD per minute at 25°; specific activity is expressed as units per mg protein.

The reverse assay involves following the increase in absorbance at 340 nm due to reduction of NAD. For kinetic studies of the enzyme in the reverse direction, 0.1 M glycine, 0.001 M EDTA, 0.1 M KCl, pH 10.0 or 0.1 M Tris-Cl, 0.001 M EDTA, 0.1 M KCl, pH 7.6 was used. For conditions where activity was low, fluorometric assays were performed with an Aminco-Bowman spectrofluorometer equipped with a Sargent recorder, exciting at 340 nm and observing the change in the fluorescence at 465 nm.

Enzyme Assays--Other Glycolytic Enzymes

The assay conditions of all the other glycolytic enzymes were based on those given by Boxer and Shonk (1960),

assaying either directly or coupling to a NAD linked dehydrogenase and observing the reaction at 340 nm. The buffer used for all assays was 0.1 M Tris-Cl, 0.005 M EDTA, pH 7.6. The stock assay mixes used are as follows:

Glyceraldehyde Phosphate Dehydrogenase

0.375 ml 2 M KCl
0.30 ml 0.2 M sodium arsenate
0.25 ml 0.06 M cysteine
3.28 ml buffer
*0.80 ml 0.01 M NAD

Phosphoglycerate Kinase, Phosphoglycerate Mutase

0.375 ml 2 M KCl
0.25 ml 0.06 M cysteine
0.062 ml 0.2 M ATP
0.045 ml 2 M $MgCl_2$
4.15 ml buffer
*0.13 ml 0.01 M NADH

Phosphofructokinase

0.13 ml 0.01 M NADH
0.045 ml 2 M $MgCl_2$
0.062 ml 0.2 M ATP
4.78 ml buffer

Hexokinase

0.20 ml 0.01 M NADP
0.045 ml 2 M $MgCl_2$
0.062 ml 0.2 M ATP
4.71 ml buffer

Pyruvate Kinase, Enolase

0.13 ml 0.01 M NADH
0.03 ml 2 M $MgCl_2$
0.13 ml 2 M KCl
0.062 ml 0.2 M ADP
4.65 ml buffer

α-Glycerol Phosphate Dehydrogenase, Triose Phosphate Isomerase, Aldolase, Lactate Dehydrogenase

0.13 ml 0.01 M NADH
4.87 ml buffer

The stock assay mixes were prepared fresh daily. The starred components were added immediately before a mix was to be used. The assays were performed at 25° in the Gilford spectrophotometer by the sequential addition of the following:

Glyceraldehyde Phosphate Dehydrogenase 0.385 ml assay mix, 5 μl of enzyme solution, 50 μl of 0.02 M glyceraldehyde-3-phosphate.

Phosphoglycerate Kinase 0.385 ml of assay mix, 5 µl of glyceraldehyde phosphate dehydrogenase (10 µl of mg/ml in 0.04 ml buffer), 5 µl of enzyme solution and 5 µl of 0.2 M 3-phosphoglyceric acid.

Phosphoglycerate Mutase 0.38 ml of assay mix, 10 µl of coupling enzymes (10 µl of 50 mg/ml lactate dehydrogenase, 20 µl of 10 mg/ml pyruvate kinase and 10 µl of 10 mg/ml enolase in 0.46 ml buffer), 5 µl of enzyme solution and 5 µl of 0.2 M 3-phosphoglyceric acid.

Phosphofructokinase 0.37 ml of assay mix, 10 µl of aldolase (10 mg/ml stock centrifuged and the pellet dissolved in buffer equal to the initial volume), 10 µl of α-glycerol phosphate dehydrogenase-triose phosphate isomerase (20 µl of 10 mg/ml in 0.18 ml buffer), 5 µl of enzyme solution and 5 µl of 0.2 M fructose-6-phosphate.

Hexokinase 0.38 ml of assay mix, 10 µl of glucose-6-phosphate dehydrogenase (10 µl of mg/ml in 0.24 ml buffer), 5 µl of enzyme solution and 5 µl of 0.2 M glucose.

Pyruvate Kinase 0.375 ml of assay mix, 10 µl of lactate dehydrogenase (10 µl of 50 mg/ml in 0.49 ml buffer), 5 µl of enzyme solution and 10 µl of 0.2 M phosphoenol pyruvate.

Enolase 0.365 ml of assay mix, 10 µl of coupling enzymes (10 µl of 50 mg/ml lactate dehydrogenase and 20 µl of 10 mg/ml pyruvate kinase in 0.47 ml buffer), 5 µl of enzyme solution and 20 µl of 0.2 M 2-phosphoglyceric acid.

α-Glycerol Phosphate Dehydrogenase 0.385 ml of assay mix, 5 µl of enzyme solution and 10 µl of 0.03 M DHAP.

Triose Phosphate Isomerase 0.365 ml of assay mix, 10 µl of α-glycerol phosphate dehydrogenase (0.1 ml of 10 mg/ml in 0.9 ml buffer), 1 µl of enzyme solutions and 25 µl of 0.02 M glyceraldehyde-3-phosphate.

Aldolase 0.38 ml of assay mix, 10 µl of coupling enzymes (0.1 ml of 10 mg/ml α-glycerol phosphate dehydrogenase-triose phosphate isomerase in 0.9 ml buffer), 5 µl of enzyme solution and 5 µl of fructose-1,6-diphosphate.

Lactate Dehydrogenase 0.385 ml of assay mix, 5 µl of enzyme solution and 10 µl of 0.2 M sodium pyruvate.

Protein Determination

Protein concentration was determined by the modified tannic acid-turbidimetric method of Majbaum-Katzenellenbogen and Dobryszycka (1959).

Tannic Acid Solution	Gum Arabic Solution
98 ml 1 N HCl	0.100 g gum arabic
2 ml phenol	100 ml water
10 g tannic acid	

It was usually necessary to filter the tannic acid solution before it could be used. Both solutions were stored in the cold and warmed to room temperature before use. To 0.50 ml of protein solution containing 5 to 50 μg of protein was added 0.50 ml of tannic acid solution. This was thoroughly mixed and allowed to stand at room temperature for 20.0 minutes, when 1.00 ml of gum arabic solution was added and thoroughly mixed in. After 5 to 10 minutes, the absorbance at 650 nm was read. A standard curve using BSA was constructed for each set of protein determinations.

Conductivity and pH

The conductivities of solutions were measured with a Radiometer CDM 2e conductivity meter and type 114 cell (cell constant 0.56). The pH's were measured with a Radiometer PM-4 pH meter equipped with a GK2321C electrode at the desired temperature.

Electrophoresis

Disc gel electrophoresis was performed by the general method of Ornstein (1964) and Davis (1964). The standard Tris-Cl buffered gel solutions for the running (pH 8.9) and stacking (pH 6.7) gels were used, along with the Tris-glycine (pH 8.3) running buffer.

Solution A	Solution B
30 g Tris	5.98 g Tris
0.23 ml TEMED	0.46 ml TEMED
approx. 40 ml 1 N HCl (pH 8.9 - 9.0)	approx. 48 ml 1 N HCl (pH 6.6 - 6.8)
water to 100 ml	water to 100 ml

Solution C	Solution D
28 g acrylamide	10 g acrylamide
0.735 g Bis	2.5 g Bis
water to 100 ml	water to 100 ml

Solution E	Catalyst
4.0 ml riboflavin	14 mg ammonium persulfate
water to 100 ml	water to 10 ml

The 8.0 cm long x 0.7 cm o.d. gel tubes were rinsed with Photoflow (Eastman), dried, marked at 5.5 cm with a fine point marking pen and supported vertically in an apparatus made from Vacutainer tops and a wooden block. They were filled to the mark with the lower gel solution (1 part A: 2 parts C:1 part H_2O:4 parts catalyst), overlayed with water and allowed to stand. After polymerization, the water overlay was removed and 0.2 ml of the upper gel solution added

(1 part B:2 parts D:1 part E:2 parts H_2O), overlayed with water and photopolymerized by placing near a fluorescent lamp. The gels were supported in the electrophoresis apparatus and the reservoirs filled with running buffer (14.4 g glycine, 3.0 g Tris in 1 liter of water with a small amount of bromphenol blue added). Five to 50 μg of protein in 5 to 10 percent sucrose and dilute buffer was layered on each gel and electrophoresed at 4 mA per tube at 4°. If the gels were to be stained for protein, they were removed from the tubes by injecting water between the gel and the tube wall, fixed in 10 percent TCA for 1/2 hour, stained with Coomassie Brilliant Blue R for 1 hour at room temperature (1.25 g Coomassie Blue, 454 ml 50 percent methanol, 46 ml acetic acid) and destained in 7 1/2 percent acetic acid at 40° to 50°. If the gels were to be stained for activity, they were stained with an activity staining solution (Dietz and Lubrano, 1967), which contained, per gel, 150 mg DL-α-GP, 0.1 mg PMS, 0.3 ml of 0.01 M NAD, 0.9 ml of 2 mM NBT and 2.0 ml of 0.3 M Tris-Cl, pH 8.0, for 1 hour in the dark at room temperature. The staining was stopped by placing the gels in 7 1/2 percent acetic acid at 4°; they were also "destained" and stored in 7 1/2 percent acetic acid in the dark at 4°. When protein was electrophoresed in the presence of NADH, 5 ml of 0.25 mM NADH, 2 percent sucrose in the Tris-glycine buffer was placed in the subreservoir above each gel (Massey and Deal, 1973). Before staining for activity, it

was necessary to soak the gels in 0.02 M MES, 0.001 M EDTA, pH 5.9, for 1/2 hour to remove excess NADH which would react with the staining reagents. Control gels were stained in the absence of αGP and showed no activity bands, although the NADH band, which moved with the tracking dye, stained very heavily.

SDS gel electrophoresis was performed with a modification of the method of Weber and Osborn (1969). The following stock solutions were used:

Solution A	Solution B
0.8 g SDS	20 g acrylamide
11.0 g $NaH_2PO_4 \cdot H_2O$	0.735 g Bis
0.35 ml TEMED	water to 100 ml
adjust to pH 7.1	
water to 100 ml	

Catalyst

14 mg ammonium persulfate
water to 10 ml

The 10.0 x 0.7 cm tubes were supported vertically, filled to a mark at 8.5 cm with the gel solution (1 part A:2 parts B: 1 part 20 percent glycerol:4 parts catalyst), overlayed with water and allowed to polymerize. The protein was prepared for electrophoresis by dissolving in 1 percent SDS, 1 percent β-mercaptoethanol, 10 percent glycerol and 0.1 M sodium phosphate, pH 7.1 and incubating at 100° for 15 minutes. Five to 20 μg of this protein in 5 to 20 μl was applied to each gel. The electrophoresis buffer was 0.1 M sodium phosphate, 0.1 percent SDS, pH 7.1 and the tracking dye, bromphenol blue, was added to the protein solutions. The

gels were electrophoresed at 8 mA per tube at room temperature, removed from the tubes and fixed in 10 percent TCA for 1/2 hour. They were stained with Coomassie Brilliant Blue R for a minimum of 2 hours at room temperature and destained in 7 1/2 percent acetic acid at 40° to 50°. The molecular weight of the subunit of the enzyme was estimated by the method of Shapiro et al., (1967) using the molecular weights for the standards given by Weber and Osborn (1969).

Analytical Ultracentrifugation

The molecular weight of the native enzyme was determined by the Yphantis high speed equilibrium method in a Model E analytical ultracentrifuge equipped with interference optics. Five samples were run simultaneously in interference double sector cells with sapphire windows and 12 mm aluminum-filled Epon centerpieces in an AnG rotor, using only the Evapatrol for temperature control (15°). The sectors of the cells were filled using Hamilton syringes; 0.13 ml of buffer was placed in the left sector (screw ring in front) and 0.01 ml of FC-43 Fluorocarbon oil (3-M, St. Paul, Minn.) plus 0.12 ml of protein solution (approximately 0.5 mg/ml) in the right sector. The protein samples had been dialyzed overnight at 4° against 0.02 M MES, 0.1 M KCl, 0.001 M EDTA, pH 5.9, before use. After 24 hours at speed, (23,150 RPM) photographs were taken using Spectrographic II-G plates. The plates were developed in D-19 developer

and after fixing and drying were read with a Bausch and Lomb comparator (van Holde, 1967). The calculations were done manually using standard techniques based on the equation

$$M = \frac{2\ RT}{(1-\bar{v}\rho)\omega^2} \quad \frac{2.303\ d(\log C)}{d\ r^2}.$$

where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solvent, ω is the angular velocity of the rotor, $d(\log C)/d\ r^2$ is the slope of the plot of 3 + log fringe displacement versus r^2 and r is the radial position; ω^2 is equal to 1.097×10^{-2} RPM^2.

The sedimentation velocity experiments were performed using standard single sector sedimentation velocity cells with plane and wedge quartz windows and 12 mm aluminum-filled Epon centerpieces in an AnD rotor and Schlieren optics. The cells were filled with 0.5 ml of a 5 mg/ml solution which had been desalted into 0.02 M MES, 0.001 M EDTA, 0.01 M ammonium sulfate, pH 5.9. Also, NADH had been added to one of the samples to make it 0.5 mM in NADH. The samples were centrifuged at 56,100 RPM at 8°. Photographs were taken at 8 minute intervals using Metallographic plates; they were developed as previously described. The peak positions were read using a Bausch and Lomb comparator. The calculations were performed manually using standard techniques based on the equation

$$s = \frac{1}{(t-t_o)\omega^2} \quad 2.303 \log \frac{r_p\ (t)}{r_p\ (r_o)}$$

where ω is the angular velocity in radians per second and where r_p (t_o) is the radial position of the peak at the time, t_o, of the first picture, and r_p (t) is the radial position of the peak at a later time t. Time is expressed in seconds.

Amino Acid Composition

Three mg of enzyme was reduced in 6 ml of 7.5 M Guanidine HCl in 1.4 M Tris-Cl 0.01 M EDTA, pH 8.6, and 0.1 M dithiothreitol under nitrogen for 4 hours at room temperature and carboxymethylated by addition of 213 mg of iodoacetate dissolved in 1 ml of 1 N NaOH under nitrogen in the dark according to the method of Crestfield et al. (1963), followed by dialysis against 0.02 M ammonium bicarbonate and lyophilization. The dried, reduced, carboxymethylated protein was taken up in 2.0 ml of 6 M GuHCl for determination of tryptophan by the spectrophotometric method of Edelhoch (1967) and again exhaustively dialyzed against 0.02 M ammonium bicarbonate, lyophilized and dried to constant weight at 105°. Samples of 0.5 mg were hydrolyzed in constant boiling HCl at 110° for 24, 48, and 72 hours and aliquots equivalent to 25 µg were chromatographed on a noncommercial, ultrasensitive analyzer (Robertson et al., 1971). The amount of each amino acid present was determined by comparing to the added internal standard, norleucine.

The partial specific volume of the enzyme was calculated from the amino acid composition by the method of Cohn and Edsall (1943).

Isoelectric Focusing

The isoelectric point of the enzyme was determined using an apparatus first described by Massey and Deal (1973), but in a slightly modified form. The column was prepared from 10 mm glass tubing. A slight bulb was blown in one end of the tubing. A 5 percent acrylamide plug was formed in the enlarged end using standard polyacrylamide gel solutions, and soaked in 3 percent sulfuric acid overnight. After the tube was rinsed, a sucrose gradient containing 1 percent ampholyte of the appropriate range and 100 to 200 µg of protein was formed in the column. The column was then supported in an electrophoresis apparatus containing 3 percent sulfuric acid in the lower reservoir (+) and 3 percent ethylene diamine in the upper reservoir (-); this was focused at 200 V for up to 8 hours in the cold. Fractions of 0.2 ml were collected by pumping 50 percent sucrose under the gradient, forcing the gradient out of the top through capillary polyethylene tubing. The pH of each fraction was measured at 0° and the appropriate fractions assayed for enzyme activity.

Analytical Sucrose Gradient Centrifugation

Frozen liver was homogenized in one volume of 0.25 M sucrose, 0.02 M Tris-Cl, pH 7.6. This was then centrifuged

in a SW 50.1 swinging bucket rotor for 1 hour at 30,000 RPM to remove the particulate material and the supernatant was diluted 1:1 with 0.02 M Tris-Cl, pH 7.6, before applying to the gradients. Gradients were prepared from 2.3 ml each of 5 percent and 20 percent (w/v) sucrose in 0.02 M Tris-Cl, pH 7.6, in 5 ml cellulose nitrate tubes using a small plexiglass gradient maker. The gradients were allowed to stand at 4° for several hours and 0.2 ml of the diluted homogenate supernatant was layered on top of each gradient. The gradients were then centrifuged in the SW 50.1 rotor for 17 hours at 40,000 RPM at 0° to 4° in a Spinco Model L ultracentrifuge (Martin and Ames, 1961). The centrifuge was then allowed to coast to a stop and the gradients were fractionated using a device that punctured the bottom of the tube, allowing the gradient to flow out by gravity. Usually 30 fractions of 10 drops each were collected and the appropriate fractions assayed for each of the glycolytic enzymes, except phosphofructokinase and hexokinase. These enzymes could not be analyzed in the homogenate supernatant, presumably because of technical problems with the assay (interfering enzymatic reactions).

Cellular Fractionation

Frozen liver was homogenized in four volumes of 0.25 M sucrose, 0.02 M Tris-Cl, pH 7.6. This was centrifuged at 800 x g (2500 RPM in the Sorvall SS 34 rotor) for 15

minutes. The pellet was then washed twice in the Tris-sucrose buffer by repeatedly drawing into a pasteur pipette and centrifugation, finally suspending in the Tris-sucrose buffer, giving the "nuclear" fraction. The supernatant from the 800 x g centrifugation was centrifuged at 8,000 x g (10,000 RPM) for 15 minutes. The pellet was again washed twice in and resuspended in the Tris-sucrose buffer, giving the "mitochondrial" fraction. The supernatant from this centrifugation was then centrifuged at 24,000 x g (17,000 RPM) for 1 hour. The supernatant from this step was called the "supernatant" fraction and the washed pellet called the "microsomal" fraction. The fractions were then assayed for each of the enzymes.

Ammonium Sulfate Fractionation

Frozen liver was homogenized in 4 volumes of 0.25 M sucrose, 0.02 M Tris-Cl, pH 7.6, and centrifuged at 40,000 RPM in a Spinco 50 fixed angle rotor for 1 hour. Solid ammonium sulfate was added to the supernatant to bring it to 20 percent saturation (10.6 g per 100 ml); it was allowed to stand in ice for 10 to 15 minutes and centrifuged at 15,000 RPM in the SS 34 rotor for 10 minutes. The pellet was resuspended in sufficient buffer to give a volume equal to the initial volume of homogenate supernatant used and this was called the "20 percent pellet." Additional solid ammonium sulfate was added to the supernatant to bring it to 40

percent saturation (11.3 g per 100 ml), followed by standing in ice and centrifugation. The pellet was resuspended in buffer, giving the "40 percent pellet" and the supernatant brought to 60 percent saturation (12.0 g per 100 ml), again followed by standing in ice and centrifugation. The pellet was dissolved in buffer, giving the "60 percent pellet," and the supernatant was brought to 80 percent saturation (12.9 g per 100 ml) and centrifuged, giving the "supernatant" and the "80 percent pellet."

Heat Stability Studies

Liver was homogenized manually in 2 volumes of 0.05 M potassium phosphate, pH 7.8, and centrifuged at 13,000 RPM in the SS 34 rotor for 15 minutes. Saturated ammonium sulfate was then added to bring the solution to 35 percent saturation and the suspension recentrifuged at 13,000 RPM for 15 minutes. The supernatant, which contained the enzyme, was stored in the cold until use. The ammonium sulfate fractionation removed the particulate material which interfered with desalting and the enzyme was relatively stable when stored in this manner. Just before a sample was to be used, it was desalted on a Sephadex G-25 column into 0.05 M potassium phosphate, pH 7.8. Aliquots of 0.5 ml were used for the kinetic studies. The appropriate amount of 10 mM NAD or NADH was added before use and the solution was allowed to stand in the ice for one or two minutes; the sample

was then assayed for the zero time activity. The sample was then placed in a 49° water bath and the clock started. The samples for assay were withdrawn 10 seconds before the indicated times and immediately assayed.

Ion Exchange Celluloses

The ion exchange celluloses (phosphocellulose and DEAE cellulose) were prepared for use by washing once in ethanol, three times (or until the filtrate was colorless) in 0.2 M NaOH, twice in water, three times in 0.2 M HCl, twice in water and fined in water. The cellulose was then suspended in the appropriate buffer, the pH adjusted to the desired pH with 6 N NaOH, and the cellulose washed twice more with buffer. It was then stored in the cold (4°) over chloroform until use. Following use, the cellulose was regenerated by the same method, except the initial ethanol wash was omitted.

Calcium Phosphate Preparation

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938). 141 g of $CaCl_2 \cdot 2H_2O$ was dissolved in 19 liters of water in a 38 liter chromatography jar equipped with a heavy duty mechanical stirrer. 240 g of $Na_3PO_4 \cdot 12\ H_2O$ was dissolved in 750 ml of boiling water and this was mixed with 12 liters of water. The warm sodium phosphate solution was added to the calcium chloride

solution over a period of 2 to 3 hours using a 2 liter separatory funnel and rapid stirring. After the mixing was complete, the gel was allowed to settle overnight and the supernatant was siphoned off. Additional water was added to bring it back to the original volume, the gel was stirred for a brief time (5 to 15 minutes) and allowed to resettle. This washing was repeated 7 times over a period of 10 days; the final 2 or 3 washes contained no Cl^- as determined by addition of Ag^+ to a small aliquot. The gel was harvested by centrifugation at 1500 RPM in the Sorvall GSA rotor for 10 minutes, resuspended in a minimum volume of water and additional water was added to bring the total weight to 3850 g. The suspension thus prepared contained approximately 25 mg of calcium phosphate (dry weight) per ml of mixture and was stored at 4°.

Enzyme Purification

Our purification procedure has been shown to reproduceibly yield pure enzyme with a specific activity of 100 with a 5 percent yield (3 to 5 mg of enzyme from 400 g of liver). The procedure consists of six steps: homogenization, calcium phosphate gel adsorption, DEAE cellulose chromatography, phosphocellulose chromatography, preparative sucrose gradient centrifugation, and preparative disc gel electrophoresis. All centrifugation steps were performed in a Sorvall RC-2B centrifuge at 0° to 4°. All purification

steps were performed at 0° to 4°, except where indicated otherwise.

Homogenization

The frozen liver was broken apart with a hammer and screwdriver and 400 g was homogenized in a Waring blender in two 200 g batches in 800 ml of buffer. The homogenization buffer (buffer A) was 0.02 M MES, 0.001 M EDTA, 0.005 M KCl, pH 5.9; to ensure reproducibility in later steps using this buffer, it was adjusted to a specific conductivity of 1.34 mS at room temperature with 4 M KCl. The homogenate was then centrifuged in the Sorvall GSA rotor at 13,000 RPM for 30 minutes and the supernatant taken off with an aspirator. About 750 to 800 ml of supernatant was usually obtained.

Calcium Phosphate Gel Adsorption

To the homogenate supernatant, 1.5 ml of cold calcium phosphate gel suspension was added per ml of homogenate supernatant. The pH of this mixture was measured directly and adjusted to 6.8 to 6.9, with 1 N NaOH if necessary. The mixture was then centrifuged at 3000 RPM in the Sorvall GS3 rotor for 10 minutes. The supernatant was decanted and saved, and the gel pellet was washed with 1 liter of 0.01 M potassium phosphate buffer, pH 6.8. The gel was suspended in the buffer and then "plumped" (homogenized) in a 1 liter polyethylene graduated cylinder with a loosely fitted Lucite pestle. This suspension was again centrifuged for 10 minutes

at 3000 RPM and the supernatant was decanted and combined with the first supernatant. The pH of the solution was adjusted to 7.8 with 1 N NaOH, NAD was added to 0.1 mM (10 ml of 0.01 M per liter), thioglycerol was added to 10 mM (0.84 ml per liter). The protein was then stored in the cold until the next step.

DEAE Cellulose Pad

A 4.0 cm x 18.5 cm pad of DEAE cellulose was formed in an 18.5 cm Buchner funnel and washed by suction twice with 350 ml aliquots of 0.01 M potassium phosphate, 0.1 mM NAD, 10 mM thioglycerol, pH 7.8. The protein solution from the calcium phosphate step was then filtered through the pad followed by three 0.01 M phosphate washes. The enzyme was then eluted with eight washes (1-8) of a solution of 0.05 M potassium phosphate, 0.1 mM NAD, 10 mM thioglycerol, pH 7.8. The wash fractions were assayed and those with the highest activity were combined, usually fractions 2 through 8. The enzyme was precipitated from solution by adding solid ammonium to 80 percent saturation (516 g per liter) and centrifuged at 9,000 RPM for 30 minutes in the GS3 rotor. The supernatant was discarded and the pellet dissolved in 70 ml of buffer A, giving about 110 ml of protein solution. This was centrifuged at 14,000 RPM for 10 minutes in the SS 34 rotor to remove insoluble protein and desalted in two aliquots at room temperature by passing through a 3.5 cm x 80 cm column of Sephadex G-25 (coarse) equilibrated with

buffer A. The conductivity of each fraction collected was checked to ensure complete desalting. NAD was added to give 0.3 mM (3 ml of 0.01 M per 100 ml) and thioglycerol was added to 10 mM (1 ml of 1 M per 100 ml) and the solution was stored in the cold overnight.

Phosphocellulose Chromatography

A 2.7 cm x 22 cm column of phosphocellulose was poured and equilibrated with buffer A containing 0.3 mM NAD and 10 mM thioglycerol (buffer B). The protein solution was centrifuged at 14,000 RPM in the SS 34 rotor for 10 minutes to remove the precipitate which had formed and the clear, green solution (approximately 200 ml) was applied to the column at room temperature at a rate of 2 to 3 ml per minute. This was followed by an additional 75 ml wash with the initial buffer, and then with a 225 ml wash with buffer B which had been adjusted to a specific conductivity of 5.35 mS with 4 M KCl. The enzyme was then eluted from the column with a 750 ml KCl gradient formed from equal parts of buffer B to which 4 M KCl had been added to give solutions with conductivities of 5.35 mS and 15.2 mS. The protein elution pattern was followed with a Gilson column photometer and a Texas Instruments recorder. The fractions were assayed for enzymatic activity and those with the highest activity were combined, concentrated to 20 ml by diafiltration and stored overnight in the cold.

Sucrose Density Centrifugation

The preparative sucrose density centrifugation was performed with a zonal modified International B-50 ultracentrifuge and a B-30 titanium zonal rotor. A 5 percent to 20 percent (w/w) gradient was formed in 29 equal 20 ml steps by pumping the lightest fraction (20 ml of 5 percent) in from the rim at 15 to 20 ml per minute while the rotor was spinning at 2500 RPM, followed in order of increasing density by the other fractions (19.28 ml of 5 percent plus 0.71 ml of 20 percent, 18.57 ml of 5 percent plus 1.43 ml of 20 percent, through 20 ml of 20 percent). The protein solution was tested to ensure that it floated on 5 percent sucrose and if it did not, it was diluted appropriately. It was then pumped into the core followed by a 30 ml overlay of buffer only. The buffer used for making the sucrose solutions and the overlay was 0.02 M MES, 0.01 M ammonium sulfate, 0.001 M EDTA, 0.3 mM NAD and 10 mM thioglycerol, pH 5.9. The centrifuge was pumped down and the protein centrifuged for 12 hours at 48,000 RPM at 10°. The centrifuge was then slowed with the brake to 2500 RPM and the gradient unloaded from the core by pumping 30 percent sucrose in the rim. The fractions were then assayed for enzyme and protein and those with the highest specific activity were combined and concentrated to 50 ml by diafiltration.

Preparative Disc Gel Electrophoresis

The preparative disc electrophoresis was performed with a Canalco Prep Disc apparatus equipped with a PD 2/70 upper column and a Model 200 constant current power supply. The temperature of the apparatus was regulated at 20° with a Lauda type K2 thermostat. The upper column, with its bottom covered with Saran Wrap, was supported vertically in the clamp supplied with the apparatus. Sufficient 5.5 percent acrylamide gel solution was added to give a 3.0 cm lower gel and this was overlayed with water. The standard polyacrylamide gel solutions were used, but the relative amounts of solution C and water were changed to give 5.5 percent acrylamide. After the polymerization was complete, the water was drawn off, sufficient stacking gel solution was added to give a 1.0 cm stacking gel and this was overlayed with water. The gel was allowed to stand overnight before use. The unit was completely assembled, the lower reservoir was filled with the standard Tris-glycine running buffer and the upper with standard Tris-glycine buffer containing 0.25 mM NADH. The protein sample for electrophoresis (up to 50 mg) was concentrated to 1 to 2 ml by diafiltration, bromphenol blue was added and the mixture was layered on the stacking gel under the buffer. The cooling water and the elution buffer (Tris-glycine running buffer diluted 1:4 with water) were started and the slit was adjusted to 1 to 2 mm. Finally, the electrodes were connected, the current was

turned on and adjusted to 6 mA until stacking was complete and the protein band had entered the upper gel (approximately 1 hour). The current was then increased to 8 mA. The elution buffer was pumped through at approximately 1 ml per minute; 8 ml fractions were collected until just before the tracking dye came off the gel (approximately fraction 20) when the rate was increased to 3 ml per minute and 3 ml fractions were collected. It was usually necessary to adjust the slit during the run to maintain it at 1 to 2 mm. The appropriate fractions were assayed for enzyme and protein, and those with the highest specific activity were combined and concentrated by suction filtration through a dialysis bag. The specific activity after this step was approximately 100 μmoles per minute per mg protein.

CHAPTER III

RESULTS

PRELIMINARY FRACTIONATION STUDIES ON TEN GLYCOLYTIC ENZYMES FROM PIG LIVER

This research marked the beginning of a broad study by this laboratory of the glycolytic enzymes from pig liver. Accordingly, the project began with some general studies on the fractionation properties of almost all of the glycolytic enzymes. This included: (1) a cellular fractionation study, which showed the fractional activity of each enzyme in each cellular fraction; (2) sucrose density gradient ultracentrifugation, which gave the approximate molecular weight of each of the enzymes; and (3) ammonium sulfate fractionation. These studies provided a starting point for purification of all these glycolytic enzymes from pig liver. In the three following sections, activities are expressed as units per g of liver (wet weight) for the crude homogenate and then as percent of the homogenate level for the various fractions.

Cellular Fractionation

The results of cellular fractionation studies are shown in Table III.

It appears that the glycolytic enzymes are generally

TABLE III

CELLULAR FRACTIONATION

	PGI	Ald	TPI	αGDH	GAPD	PGK	PGM	Enol	PK	LDH
Homogenate (U/g)	69	2.6	650	29	33	42	24	27	23	17
Nuclear Fraction (%)	6	0	3	0	0	7	11	0	4	0
Mitochondrial Fraction (%)	7	-	2	0	0	3	8	0	2	0
Microsomal Fraction (%)	13	20	3	0	0	8	11	5	-	10
Supernatant (%)	98	95	80	97	115	95	127	81	96	92

found in the supernatant fraction, but there are some exceptions. Phosphoglucoisomerase, triosephosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase and pyruvate kinase were found in all of the fractions and significant amounts of aldolase and lactate dehydrogenase were found in the "microsomal fraction." There are at least two possible explanations for these enzymes being in the different fractions: (1) these enzymes may be actually associated with these organelles or structures in the cell and remain with them during fractionation, or (2) these observations may be artifacts of the isolation procedure due to nonspecific association of these enzymes with these structures during fractionation. An example of the first case has been described (Wilson, 1968) and it was suggested that this binding may play an important role in the control of the activity of that enzyme.

Sucrose Density Gradient Ultracentrifugation

The samples were prepared and centrifuged as described in Materials and Methods. It was necessary to dilute the high-speed supernatant fraction with buffer so that its density was lower than that of 5 percent sucrose. The results of two gradients from separate centrifuge runs are shown in Figures 1 and 2. Lactate dehydrogenase was used as a standard in both gradients. Its sedimentation coefficient was assumed to be 7.5 S (Kaplan, 1964).

Figure 1.--Sucrose Gradient Centrifugation of Pig Liver Glycolytic Enzymes Liver was homogenized in 1 volume of 0.25 M sucrose, 0.02 M Tris-Cl, pH 7.6, and centrifuged 1 hour at 30,000 RPM. The supernatant was then diluted 1:1 with 0.02 M Tris-Cl, pH 7.6, and 0.2 ml was layered on a 5 percent to 20 percent sucrose gradient and the gradients centrifuged for 17 hours at 0° to 4° in a SW 50.1 rotor. Ten-drop fractions were collected and assayed for the enzymes indicated.

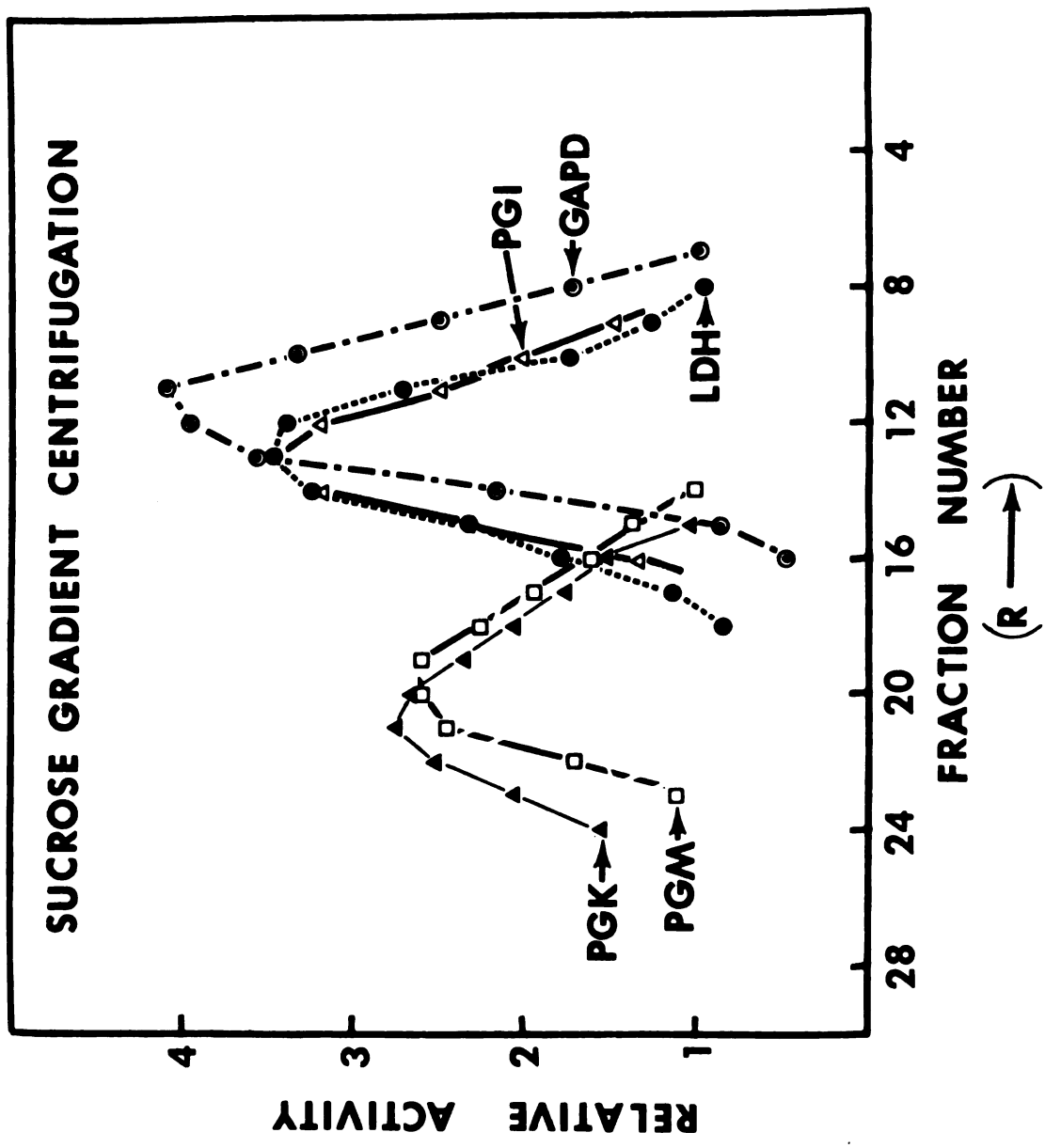
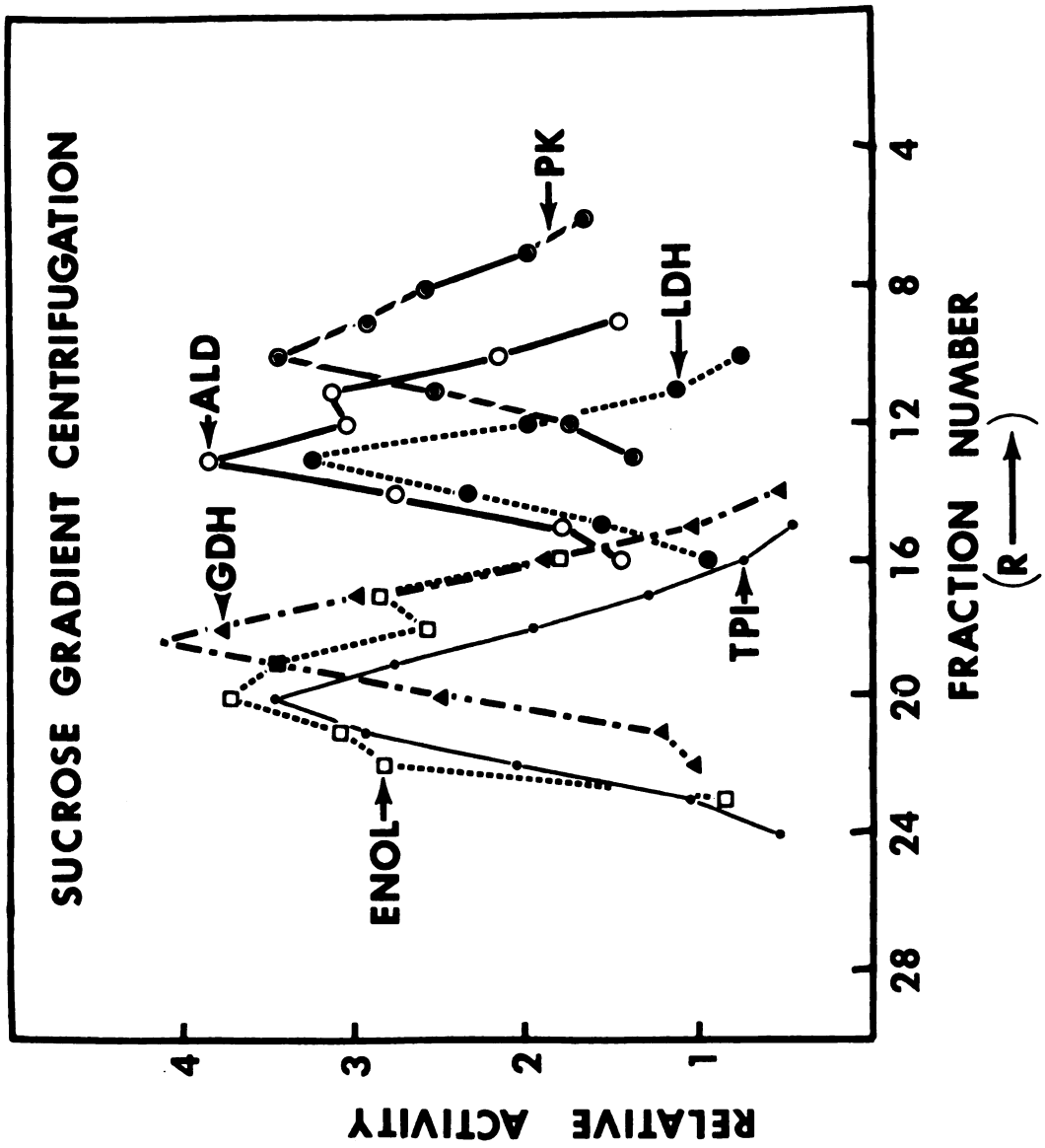

SUCROSE GRADIENT CENTRIFUGATION
PGI
GAPD
LDH
PGK
PGM
RELATIVE ACTIVITY
1
2
3
4
FRACTION NUMBER
(R
4
8
12
16
20
24
28

Figure 2.--Sucrose Gradient Centrifugation of Pig Liver Glycolytic Enzymes. Conditions same as Figure 1.


SUCROSE GRADIENT CENTRIFUGATION
ALD
PK
LDH
GDH
TPI
ENOL
RELATIVE ACTIVITY
4
3
2
1
28
24
20
16
12
8
4
FRACTION NUMBER
(R ⟶)

It appears that there are two general size classes of the glycolytic enzymes assayed. The class of small enzymes consists of enolase, triose phosphate isomerase, phosphate glycerate mutase, α-glycerol phosphate dehydrogenase and phosphoglycerate kinase. They have sedimentation coefficients in the range of 3.5 S to 5.5 S. The other class consists of aldolase, lactate dehydrogenase, phosphoglucoisomerase, glyceraldehyde phosphate dehydrogenase and pyruvate kinase with sedimentation coefficients in the range of 7.0 S to 9.5 S. Those in the first class appear to be monomeric or dimeric enzymes with molecular weights of 50,000 to 80,000 and those in the second class appear to be tetrameric enzymes with molecular weights of 120,000 to 220,000 (Holleman, 1966; Darnall and Klotz, 1972).

Ammonium Sulfate Fractionation

The ammonium sulfate fractionation results are shown in Table IV. It appears that ammonium sulfate fractionation would be a suitable purification step for most of the enzymes, since they are found primarily in one or two fractions. However, the enzymes phosphoglycerate kinase, phosphoglycerate mutase and pyruvate kinase seem to precipitate in several of the fractions, which would make ammonium sulfate fractionation quite inefficient as a purification step.

The activities of several enzymes in the homogenate

TABLE IV

AMMONIUM SULFATE FRACTIONATION

	PGI	Ald	TPI	α-GDH	GAPD	PGK	PGM	Enol	PK	LDH
Homogenate Supernatant (U/g)	48	1.8	670	15	13	12	13	13	20	7
20 percent precipitate (%)	6	a	b	b	12	12	6	b	a	b
40 percent precipitate (%)	7	0	b	b	18	23	9	b	35	b
60 percent precipitate (%)	55	22	5	62	12	51	44	18	33	68
80 percent precipitate (%)	39	52	18	31	135	68	26	64	18	82
80 percent supernatant (%)	4	0	46	0	71	23	7	0	0	0

[a]The 20 percent fraction was not taken; the first pellet was the 40 percent pellet.

[b]The first pellet was the 60 percent pellet.

supernatant of the ammonium sulfate fractionation study were lower than the corresponding activities in the homogenate in the cellular fractionation study. This is probably due to the length of time required to finish the ammonium sulfate fractionation (2 days).

The high recoveries of glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase in the ammonium sulfate study (200 percent) could be due to erroneous results in the original assays of the homogenate supernatant; the enzymes were probably inhibited by competing enzyme reactions. Alternatively, the crude homogenate could contain inhibitors of the enzymes under study and the inhibitors were removed by the fractionation. Also, ammonium sulfate might activate these enzymes, yielding greater activity in the solutions with ammonium sulfate.

CHAPTER IV

PRELIMINARY ENZYME PURIFICATION STUDIES

Multiple Forms of the Enzyme

In beginning the process of working out a purification procedure for the enzyme, it was desirable to determine how many different active forms of α-glycerol phosphate dehydrogenase were present in the homogenate supernatant. To do this, protein samples were electrophoresed on polyacrylamide disc gels as usual and stained for enzymatic activity. A gel of a sample from a typical crude homogenate electrophoresed in the absence of NADH is shown in Figure 3. Bands 1, 2, 3, and 4 are numbered in the Figure.

We normally observed one major band of activity accounting for about 90 percent of the total activity (band 1) and several minor bands of activity. There were always at least three minor bands (bands 2, 3, and 4), and often as many as six or seven. More bands of activity were observed when samples were electrophoresed in the presence of NADH than in the absence of NADH. This effect is discussed in detail in a later section (page106). Most of the activity was in band 1, with small amounts in bands, 2, 3 and 4 (in order of decreasing mobility; band 4 is the more mobile of the two heavy bands at the top of the gel).

Figure 3.--α-Glycerol Phosphate Dehydrogenase Activity in Disc Gels of Pig Liver Homogenate Supernatant. Liver was homogenized in 0.02 M MES, 0.001 M EDTA, 0.005 M KCl, pH 5.9 and centrifuged at 40,000 RPM for 1 hour; 25 µl of the supernatant was applied to the gel. The gel was then electrophoresed and stained as described in the text. Bands 1, 2, 3 and 4 are identified.

To partially characterize these isomers, and see if they were molecular weight or charge isomers, their migration was studied on gels of various acrylamide concentrations. The mobilities were calculated and plotted according to Ferguson (1964). A Ferguson plot of the data is shown in Figure 4. The fact that the lines for bands 2, 3, and 4 are parallel indicates that they are primarily charge isomers, with approximately equal molecular weights. Band 1 is a smaller protein, since its retardation coefficient (the slope of the line) is smaller than the retardation coefficients of bands 2, 3 and 4.

Part A of Figure 5 shows a densitometer tracing of a typical activity pattern obtained with an electrophoresis gel without NADH using the crude homogenate from an adult pig liver; only one major activity band is seen. Generally, the relative intensities of the various activity bands were similar from various livers; livers from a number of young pigs (0-3 weeks) gave patterns similar to the normal adult pattern, with only one major band. However, in two livers from 3-week pigs the intensities of the normally minor bands were much greater (Figure 5B), two of the minor bands were approximately as intense as the major band, band 1.

Figure 4.--Ferguson Plot for α-Glycerol Phosphate Dehydrogenase Activity Bands 1, 2, 3 and 4. Liver was homogenized by hand in 0.05 M potassium phosphate, pH 7.8, and centrifuged for 1 hour at 40,000 RPM; 25 µl of the supernatant was applied to each gel. The gels were prepared by using appropriate amounts of Solution C and water to give the indicated acrylamide concentrations and were electrophoresed and stained as described in Materials and Methods. For band 4, the ordinate is Mobility x 10.

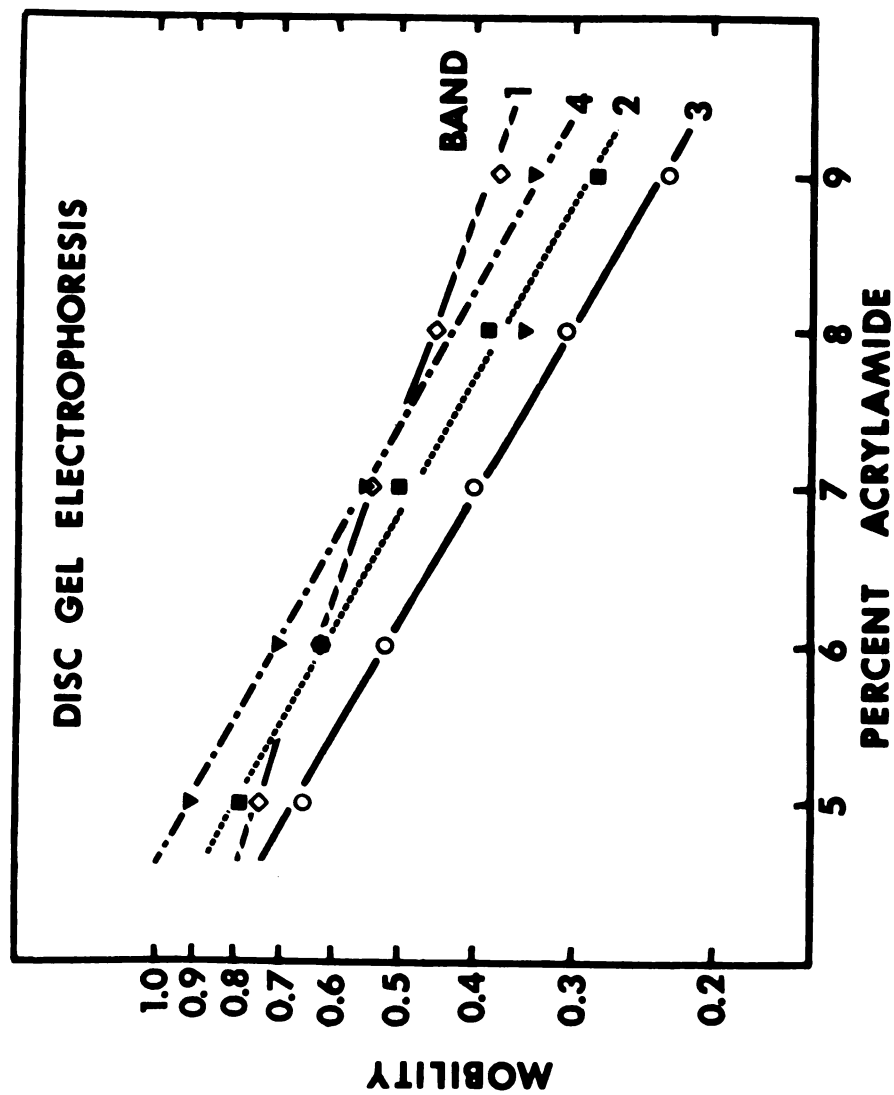
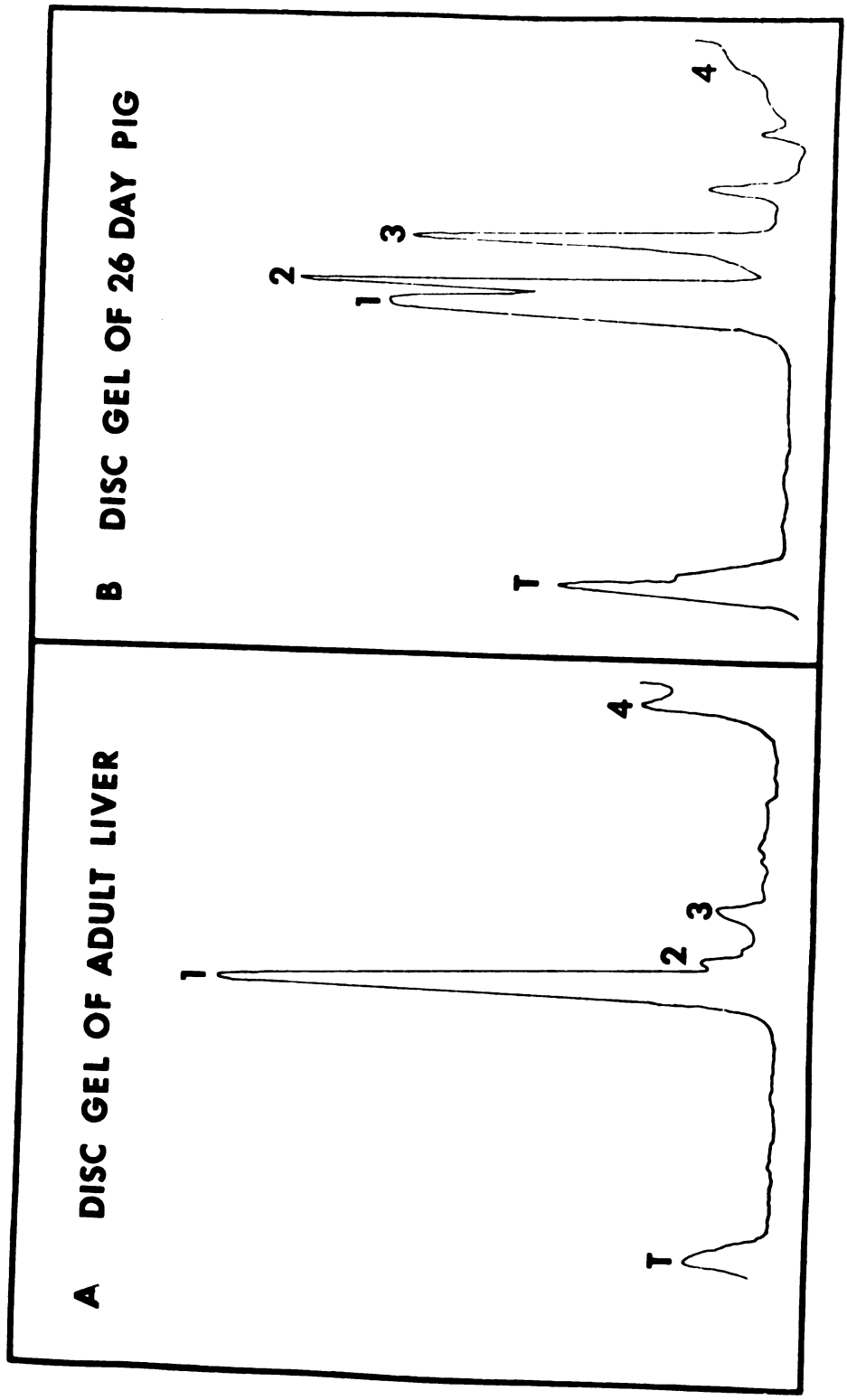

DISC GEL ELECTROPHORESIS
BAND
1
4
2
3
MOBILITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
5
6
7
8
9
PERCENT ACRYLAMIDE

Figure 5.--α-Glycerol Phosphate Dehydrogenase Activity in Disc Gels of Pig Liver Homogenate Supernatant. A. Densitometer tracing of a typical adult electrophoretic pattern. Gels were electrophoresed and the densitometer tracing taken using a Joyce-Loebl Automatic Recording Microdensitometer, model MK111C. B. Densitometer tracing of an atypical electrophoretic pattern from a 26-day old piglet. The liver was homogenized in 0.25 M sucrose, 0.02 M Tris, pH 7.4 and centrifuged at 40,000 RPM for 1 hour. T indicates the position of the tracking dye; migration was to the left. Bands 1, 2, 3 and 4 are identified.


A DISC GEL OF ADULT LIVER
1
2
3
4
T
B DISC GEL OF 26 DAY PIG
1
2
3
4
T

Key Findings Enabling Purification of α-Glycerol Phosphate Dehydrogenase

Protection of α-Glycerol Phosphate Dehydrogenase Against Heat Denaturation by NAD and NADH

Heat denaturation studies were performed to determine whether a heat step could be used in the purification procedure and also to study the stabilizing effects of NAD and NADH on the enzyme. In order to study the binding to and stabilization of the enzyme by the nucleotides, it was necessary to have the enzyme in a controlled environment, removed from all the small molecules present in a normal homogenate supernatant. To accomplish this, simple ammonium sulfate precipitation and desalting steps were used.

The studies were performed in phosphate buffer at 49°. This temperature was quite critical; at a temperature of 1 or 2 degrees lower, the denaturation slowed drastically (the half-time for the reaction increased 5 to 10 fold); at a temperature 1° or 2° higher, the half-time decreased four-fold. This indicates a high activation energy for the denaturation process. The results for NAD and NADH as stabilizers are shown in Figure 6 and Figure 7, respectively. There appears to be an initial activation of the enzyme by the NADH, followed by the heat denaturation. NADH protects the enzyme against heat denaturation somewhat better than NAD does; 0.25 mM NADH given nearly complete protection, but 0.5 mM NAD is required to give equivalent protection. NAD was used for stabilizing the enzyme during three of the

Figure 6.--Protection of α-Glycerol Phosphate Dehydrogenase by NAD Against Heat Denaturation at 49°. Pig liver was homogenized in 0.05 M potassium phosphate, pH 7.8, and ammonium sulfate added to 35 percent saturation. After centrifugation, the supernatant was removed and stored in the cold. Before use, a sample was desalted on Sephadex G-25 into 0.05 M potassium phosphate, pH 7.8, and the appropriate amount of 10 mM NAD was added to give the indicated concentration. The zero time assay was taken from the sample while it was in ice; the other assay aliquots were removed from the sample 10 seconds before the indicated times and assayed immediately.

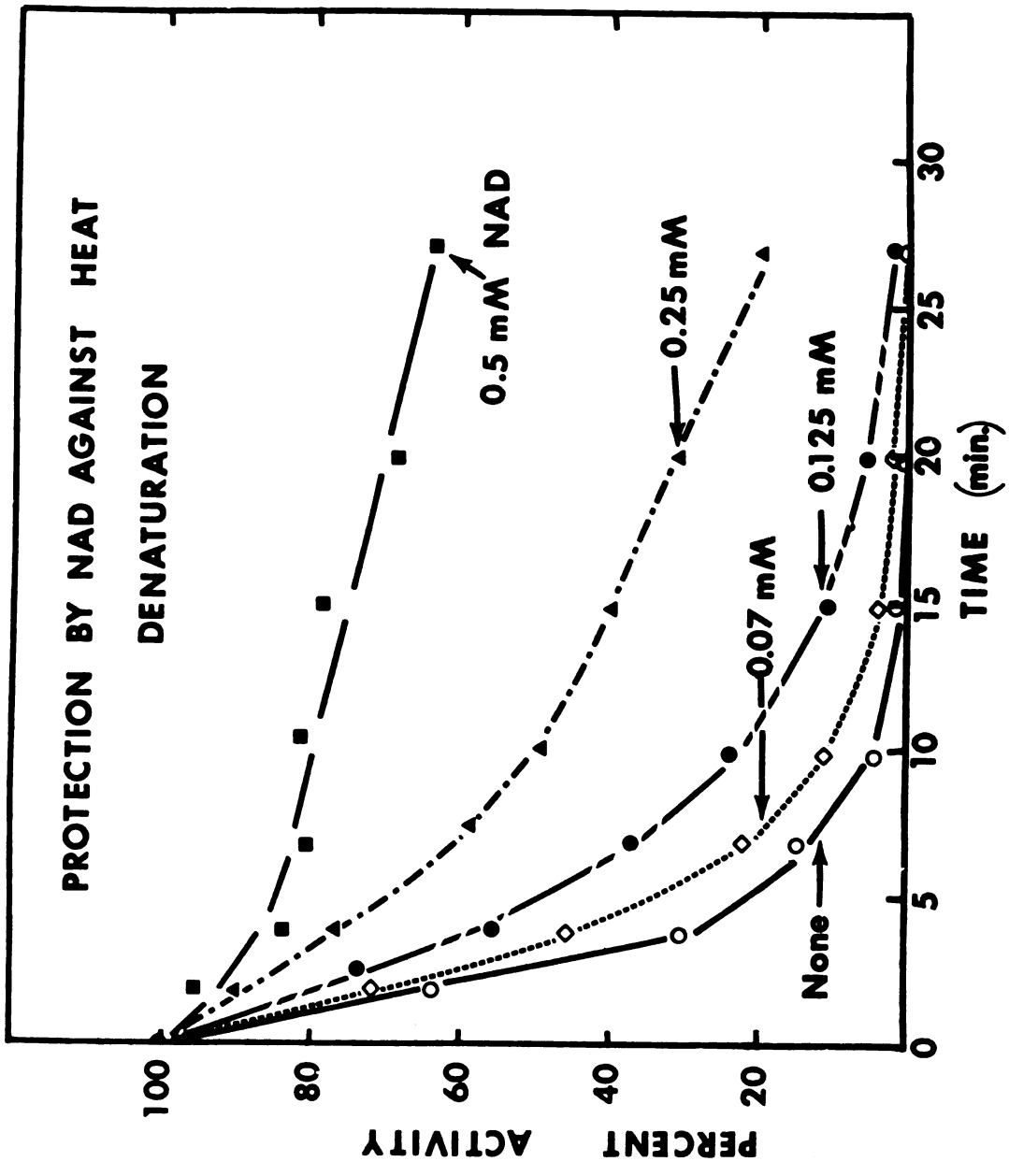
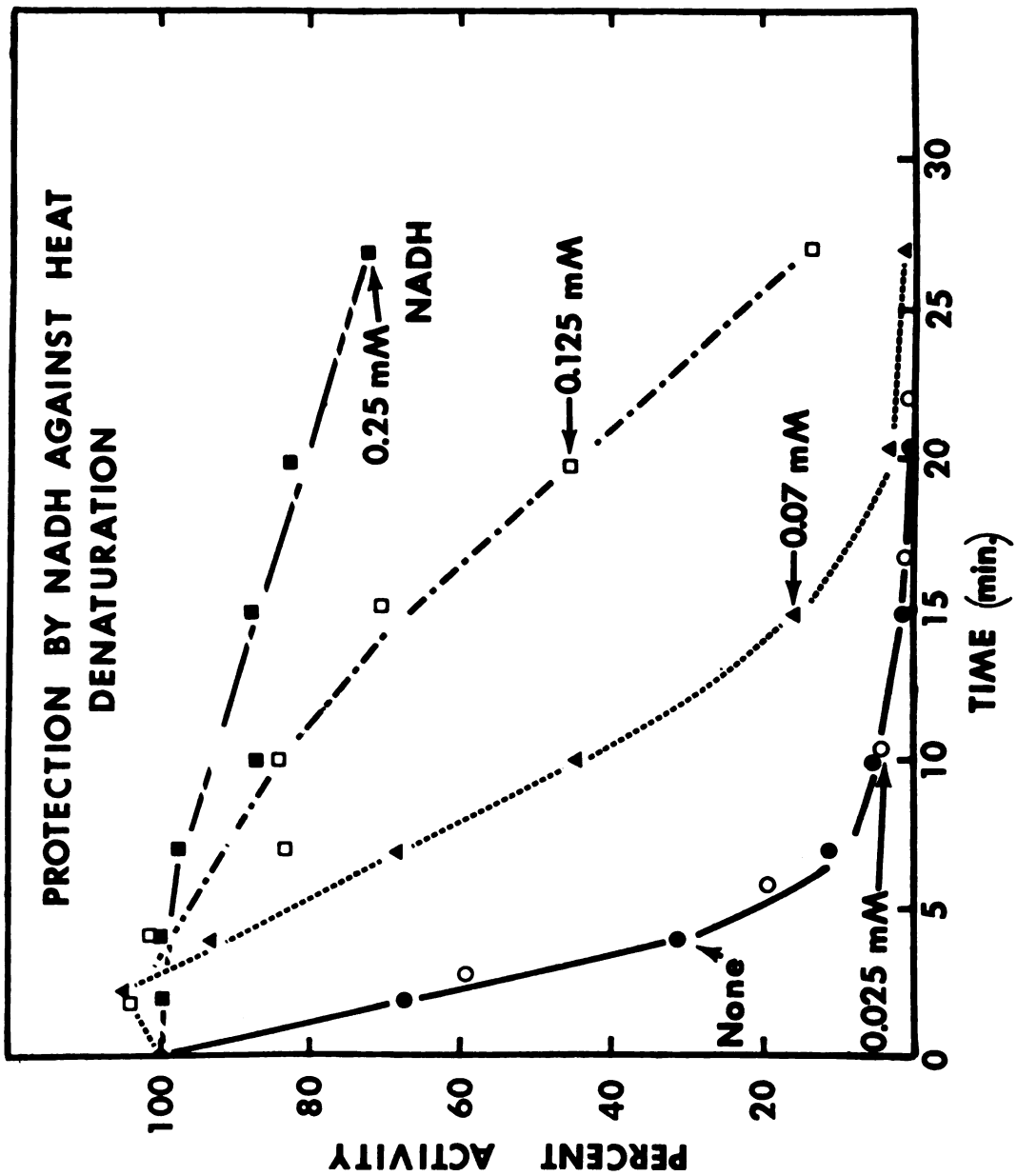

PROTECTION BY NAD AGAINST HEAT DENATURATION
0.5 mM NAD
0.25 mM
0.125 mM
0.07 mM
None
PERCENT ACTIVITY
100
80
60
40
20
0
5
10
15
20
25
30
TIME (min.)

Figure 7.--Protection of α-Glycerol Phosphate Dehydrogenase by NADH Against Heat Denaturation at 49°. Conditions same as Figure 5, except appropriate amounts of 10 m$\underline{M}$ NADH were added instead of NAD.


PROTECTION BY NADH AGAINST HEAT DENATURATION
NADH
0.25 mM
0.125 mM
0.07 mM
0.025 mM
None
PERCENT ACTIVITY
100
80
60
40
20
0
5
10
15
20
25
30
TIME (min.)

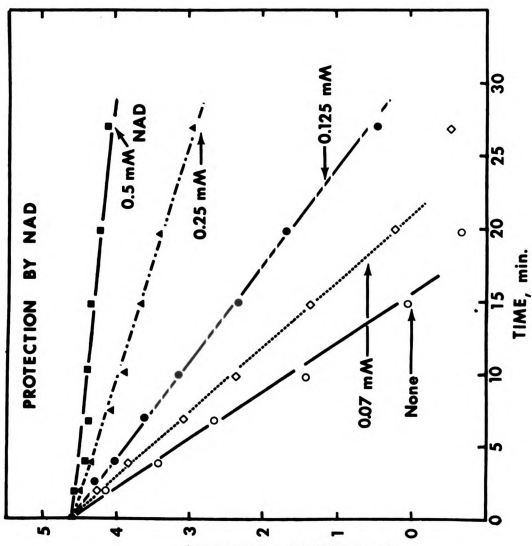
purification steps primarily because it is less expensive than NADH, because it is more stable at pH 5.9 than NADH (Lowry et al., 1961) and because it adequately protects the enzyme. For the DEAE cellulose pad, 0.1 mM NAD was sufficient to protect the enzyme, but the phosphocellulose column and the preparative sucrose gradient required the presence of 0.3 mM NAD to protect the enzyme. NADH was required in the preparative disc gel electrophoresis step, because NAD gave no protection during the procedure. The NAD may not migrate into the gel, or it may migrate with the enzyme, but not protect it.

Analysis of the Binding of NAD to α-Glycerol Phosphate Dehydrogenase

Because of the importance of the protection of the enzyme by NAD and NADH in the purification of the enzyme, it was of great interest to analyze further the binding of the nucleotides to the enzyme. The initial activation of the enzyme by NADH makes it difficult to analyze further the NADH protection data. No such activation is observed with NAD and the analysis is straightforward. The data were replotted in Figure 8 as ln percent activity remaining (0 time = 100) versus time to test whether they fit first-order kinetics. As can be seen by inspecting Figure 8 virtually all the data fit nicely on a straight line.

If the rate constant k for the inactivation of the enzyme is defined as the slope of the first order plot of the data then the plot of 1/k versus $(NAD)^2$ shown in Figure

Figure 8.--Kinetic Analysis of Heat Denaturation Data. The data from Figure 6 were analyzed and plotted as ln percent activity versus time to test for first order kinetics.


PROTECTION BY NAD
0.5 mM NAD
0.25 mM
0.125 mM
0.07 mM
None
ln PERCENT ACTIVITY
TIME, min.
0
1
2
3
4
5
0
5
10
15
20
25
30

9A is obtained. This plot shows that: (1) the rate constant for inactivation is inversely proportional to the square of the NAD concentration, and (2) the rate constant approaches zero as the NAD concentration increases. This first observation suggests that the binding of two molecules of NAD to each enzyme molecule is involved in the protection of the enzyme. To test this hypothesis, the data was further treated as follows (Kuczenski and Suelter, 1970, 1971). For each concentration of NAD used, a corresponding value of α was calculated (α is the fraction of binding sites occupied) using the following equation

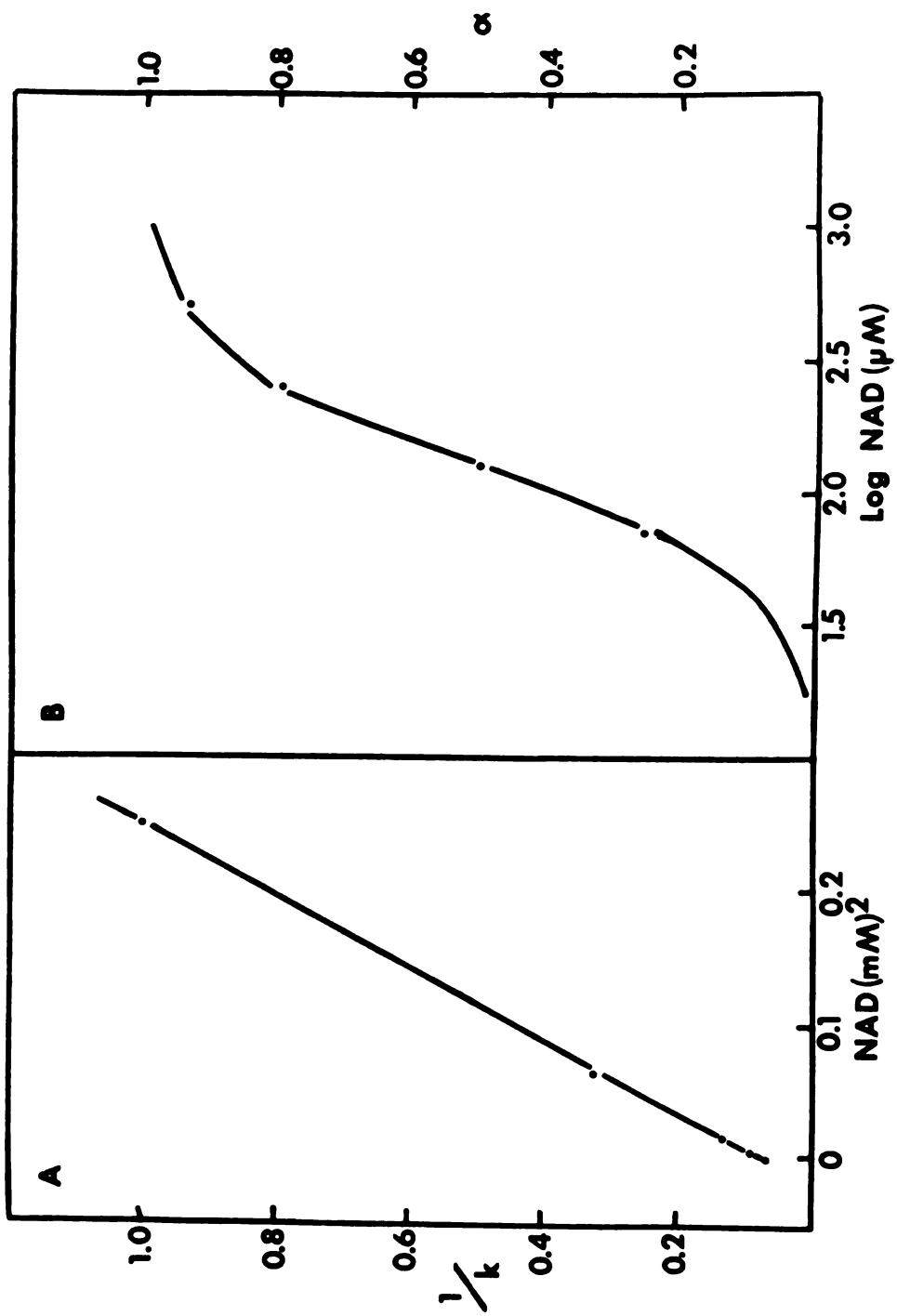
$$\frac{k_o - k_x}{k_o - k_\infty}$$

In this equation, k_x is the denaturation rate constant (slope of the semilog plot) for the NAD concentration of interest, k_o is the rate constant in the absence of NAD and k_∞ is the rate at high NAD concentration, assumed to be zero for this purpose. The values of α versus log (NAD) are shown in Figure 8B. The line drawn between the points is the theoretical line calculated for the case of n = 2 and an average binding constant (K_{av}) of 126 μM, using the equation

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

where p (NAD) is the negative logarithm of the NAD concentration and pK_{av} is 4.100 (see appendix for the derivation of this equation). As can be seen, the experimental points fit the theoretical curve quite well. This is additional evidence that two NAD molecules per enzyme molecule are

Figure 9.--Theoretical Treatment of Heat Denaturation Data. A. The slope of each line in Figure 5 was taken as k for that NAD concentration. This was then plotted in the form 1/k versus $(NAD)^2$. B. For each NAD concentration α was calculated as described in the text, and plotted versus log (NAD), giving the points shown. The line is the theoretical line for the case of n = 2 and Kav = 126 $\mu\underline{M}$.


A
B
1.0
0.8
0.6
0.4
0.2
1/k
0
0.1
0.2
NAD(mM)2
α
1.5
2.0
2.5
3.0
Log NAD(μM)

involved in protecting the enzyme, with an average binding constant of approximately 125 μM.

Protection of α-Glycerol Phosphate Dehydrogenase by MES, Sucrose and Ammonium Sulfate

If the crude enzyme is stored in MES buffer at pH 6, it retains essentially all of the activity after 24 hours at 4°. In phosphate buffer it retains 90 percent of the zero time activity, but in cacodylate buffer it loses almost all the activity. In the heat denaturation studies, if MES buffer was used instead of phosphate buffer, an increase of 6° in the temperature was necessary to obtain the same denaturation rate. In preliminary studies on stabilizing the enzyme in crude solution, 10 mM ammonium sulfate was found to maintain the enzyme activity for over a week at 4° (MES buffer, pH 5.9), while the sample without ammonium sulfate lost 40 percent of its activity. In a separate study using Tris (pH 7.8) as the buffer, the enzyme stored in 10 percent sucrose had approximately twice the activity of the control sample without sucrose after 18 hours.

CHAPTER V

FINAL PURIFICATION PROCEDURE

A summary of the major steps in the purification procedure in shown in Table V. Enzyme with a specific activity of between 95 and 100 μmoles/min/mg protein was obtained each time the purification procedure was run. The preparation takes three days, with the homogenization, calcium phosphate gel absorption, DEAE cellulose pad and ammonium sulfate concentration steps on the first day, the phosphocellulose column on the second, the sucrose density gradient during the night of the second and the preparative disc gel electrophoresis on the final day. This preparation uses only 400 g of liver instead of a whole liver (1200 g) primarily because of logistical problems associated with the centrifugation and desalting steps. Even so, one can obtain approximately 5 mg of pure enzyme quite easily from 400 g of liver.

Homogenization

Generally, the liver was still partially frozen when it was homogenized. This did not interfere with the homogenization and helped ensure against overheating during homogenization. Initially, homogenization was in 0.05 M potassium phosphate, pH 7.8, but use of the MES buffer gave

TABLE V

SUMMARY OF PURIFICATION PROCEDURE[a]

	Volume, ml	Total Protein, mg	Total Units	Specific Activity
Homogenate Supernatant	730	33,000	8,500	0.26
Calcium Phosphate Gel	2,515	22,000	7,500	0.34
DEAE Cellulose Pad	2,760	7,700	6,400	0.83
Phosphocellulose Column	340	240	2,700	11
Sucrose Density	50	55	1,650	30
Disc Electrophoresis	12	5.6	555	100

[a]The results of the most recent purification. It should be noted that the following specific activity values are more typical: homogenate supernatant, 0.15; phosphocellulose column, 20-30; sucrose density, 50.

the proper homogenate conductivity and pH for the calcium phosphate gel step which followed, without any dialysis or adjustment of the pH. The pellet obtained after centrifugation was not tightly packed, so some of it was usually removed during the aspiration of the supernatant.

Calcium Phosphate Adsorption

This step usually gave a two-fold purification, and removed most of the hemoglobin and some of the contaminating particulate material which was not removed by the initial centrifugation. The step is easy to perform and takes less than an hour. Only one-third of the enzyme binds to the calcium phosphate gel, and most of that which does bind is washed off with the 0.01 M phosphate wash. It is quite important that the pH after the addition of the gel be 6.8 to 6.9, since if it is too low, the enzyme binds too tightly to the gel and cannot be washed off.

DEAE Cellulose Pad

The pad step was devised after it was found that the enzyme was unstable when bound to DEAE cellulose without NAD but was stable for about 45 minutes when bound in the presence of NAD. Using the DEAE cellulose in the form of a pad allows the enzyme to be adsorbed and then eluted in less than 30 minutes. Also, the pad removes the remaining particulate material present, giving a clear, greenish-colored

solution. Usually, a two-to four-fold purification is obtained with this step.

Because the protein solution was so dilute, it was necessary that the solution be brought to 80 percent ammonium sulfate saturation to precipitate the enzyme. Under the usual conditions, where the protein is at higher concentrations, only 55 to 60 percent saturation was required to precipitate the enzyme. It was critical that the enzyme be completely desalted following the ammonium sulfate precipitation step, because any residual ammonium sulfate interferes with the binding of the enzyme to the phosphocellulose column. Consequently, any desalted enzyme fractions which had a conductivity greater than 1.8 mS were not used.

Phosphocellulose Chromatography

This step usually took from four to five hours to run and gave a 20- to 30-fold purification, with a greater than 50 percent yield in enzyme. Very little contaminating protein binds to the column under the conditions used, and if there is no ammonium sulfate present, all of the α-glycerophosphate dehydrogenase binds to the column. Generally, there is no problem with column capacity. The column can be checked visually for overloading, since α-glycerol phosphate dehydrogenase is bound to the column in a region between the very tightly bound hemoglobin (or another red protein) right

at the top of the column, and a less tightly bound green protein which, because of competition with stronger binding proteins, finds adsorption sites only near, or below, the middle of the column. If the green protein washes off the column initially, the column is overloaded. An initial step elution with 5.35 mS KCl is necessary to elute the green protein ahead of the α-glycerol phosphate dehydrogenase; if only a linear gradient was used, they were not separated well, giving a lower purification. The results of a typical run are shown in Figure 10.

Sucrose Density Gradient Centrifugation

This purification step was used instead of a gel filtration step. It is faster and requires less buffer than gel chromatography, and the zonal rotor is superior to a standard swinging bucket rotor, since larger samples can be used and much higher forces are obtained, significantly shortening the centrifugation time. This step is the only purification step in the procedure which is based on molecular size. It is relatively simple to run, and usually yields enzyme with a specific activity of 50, but has given a sample with a specific activity of 90. It removes a yellow-colored protein which is a major contaminant, giving a clear, colorless enzyme solution. The results of a typical run are shown in Figure 11. The only problem generally encountered was loss of a portion of the gradient, due to leakage around the plug in the rotor. This decreases the

Figure 10.--Purification of α-Glycerol Phosphate Dehydrogenase by Phosphocellulose Chromatography. The concentrated enzyme from the DEAE cellulose pad was applied to the column at room temperature in buffer consisting of 0.02 M MES, 0.001 M EDTA, 0.005 M KCl, 0.3 mM NAD, 10 mM thioglycerol, pH 5.9, specific conductivity 1.34 mS. The enzyme was eluted from the column with an initial 5.35 mS wash, followed by a 5.35 mS to 15.2 mS KCl gradient. Fractions 92 through 132 were combined and concentrated. Enzyme activity is in units of ΔOD/min.

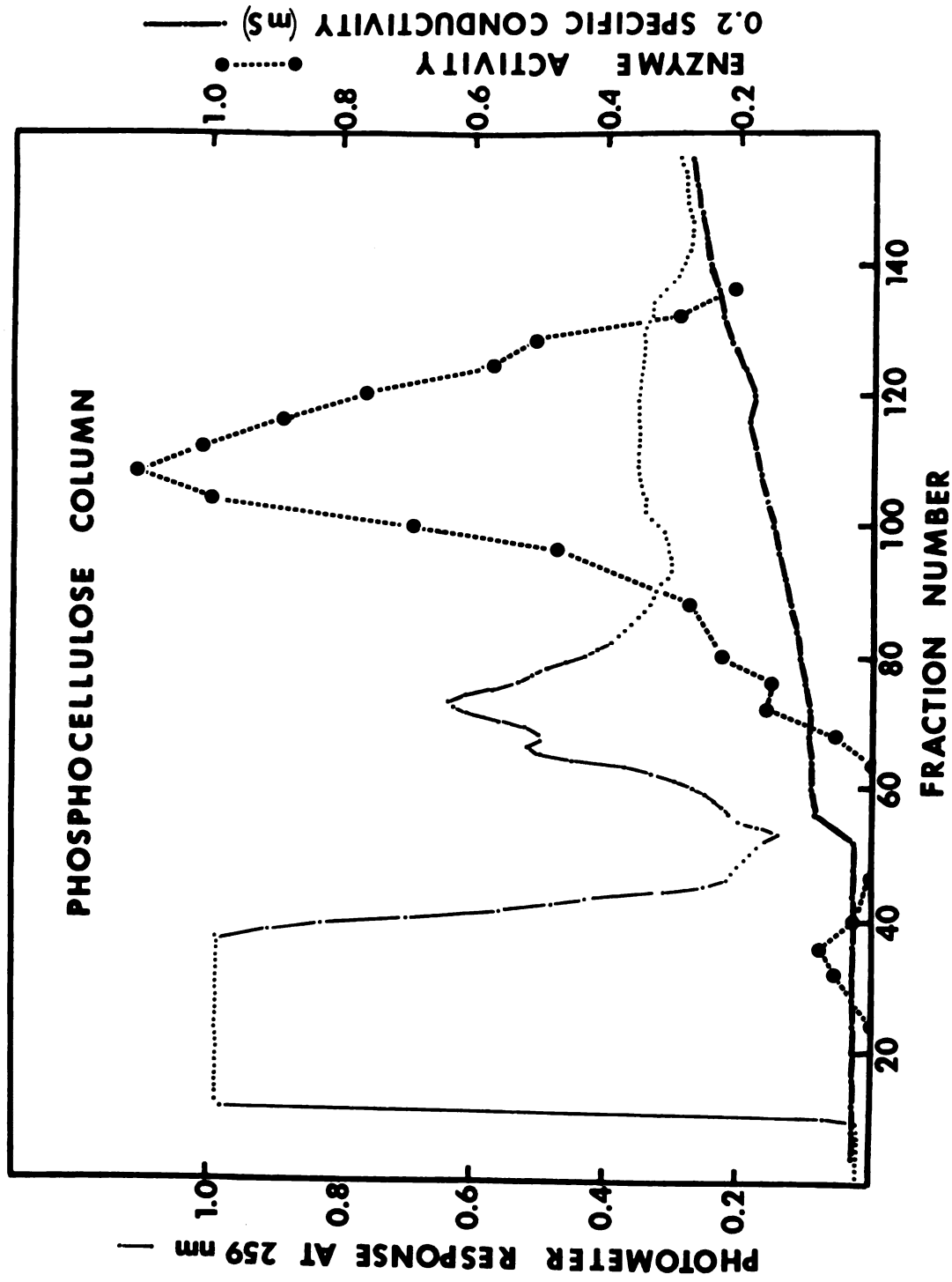
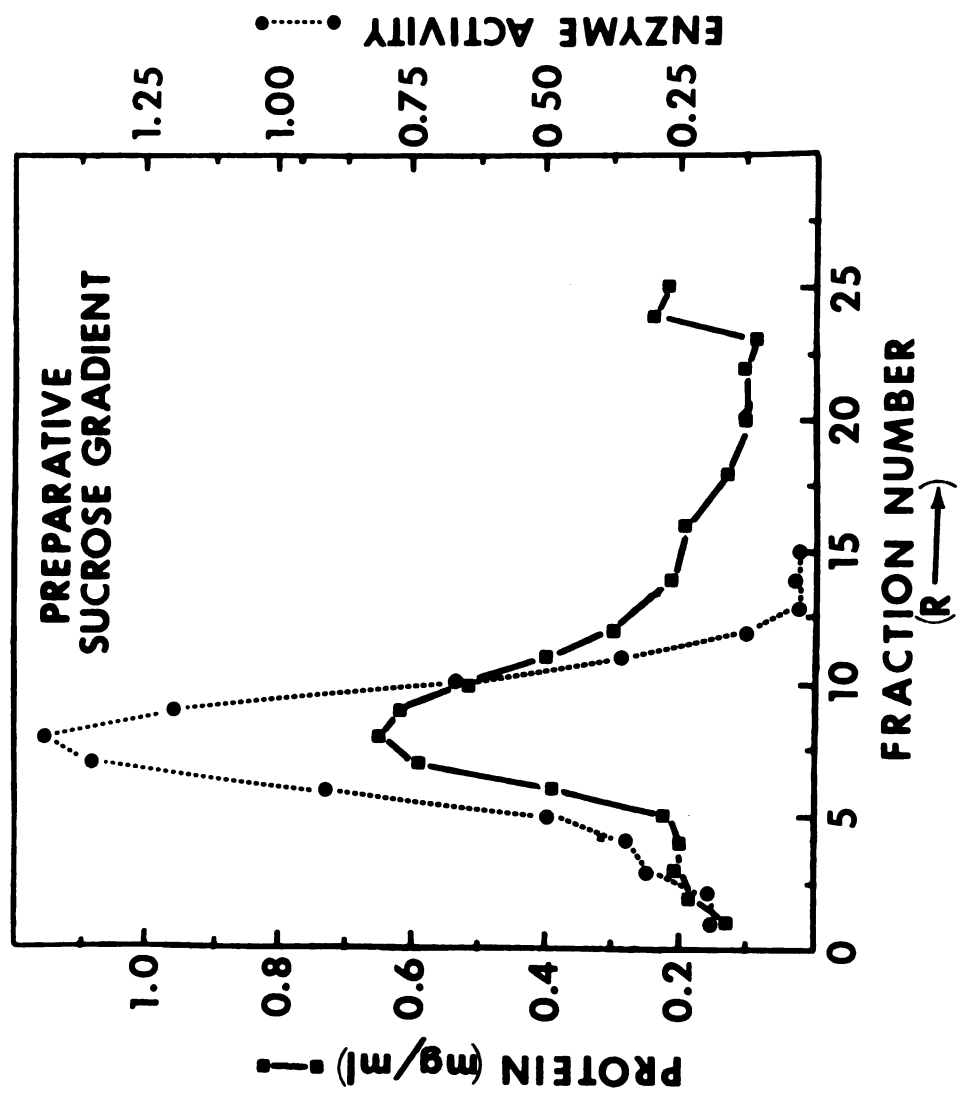

PHOSPHOCELLULOSE COLUMN
PHOTOMETER RESPONSE AT 259 nm
ENZYME ACTIVITY
0.2 SPECIFIC CONDUCTIVITY (mS)
FRACTION NUMBER
0.2
0.4
0.6
0.8
1.0
20
40
60
80
100
120
140

Figure 11.--Preparative Sucrose Density Ultracentrifugation. The concentrated α-glycerol phosphate dehydrogenase from the phosphocellulose column step was applied to a 5 percent to 20 percent sucrose gradient in 0.02 M MES, 0.001 M EDTA, 0.01 M ammonium sulfate, 0.3 mM NAD, 10 mM thioglycerol, pH 5.9 and centrifuged at 48,000 RPM for 12 hours at 10° in a zonal modified International B-60 ultracentrifuge with a B-14 titanium rotor. Fractions 1 through 10 were combined and concentrated. Enzyme activity in units of ΔOD/min.


PREPARATIVE
SUCROSE GRADIENT
PROTEIN (mg/ml)
ENZYME ACTIVITY
FRACTION NUMBER
(R)
1.0
0.8
0.6
0.4
0.2
1.25
1.00
0.75
0.50
0.25
0
5
10
15
20
25

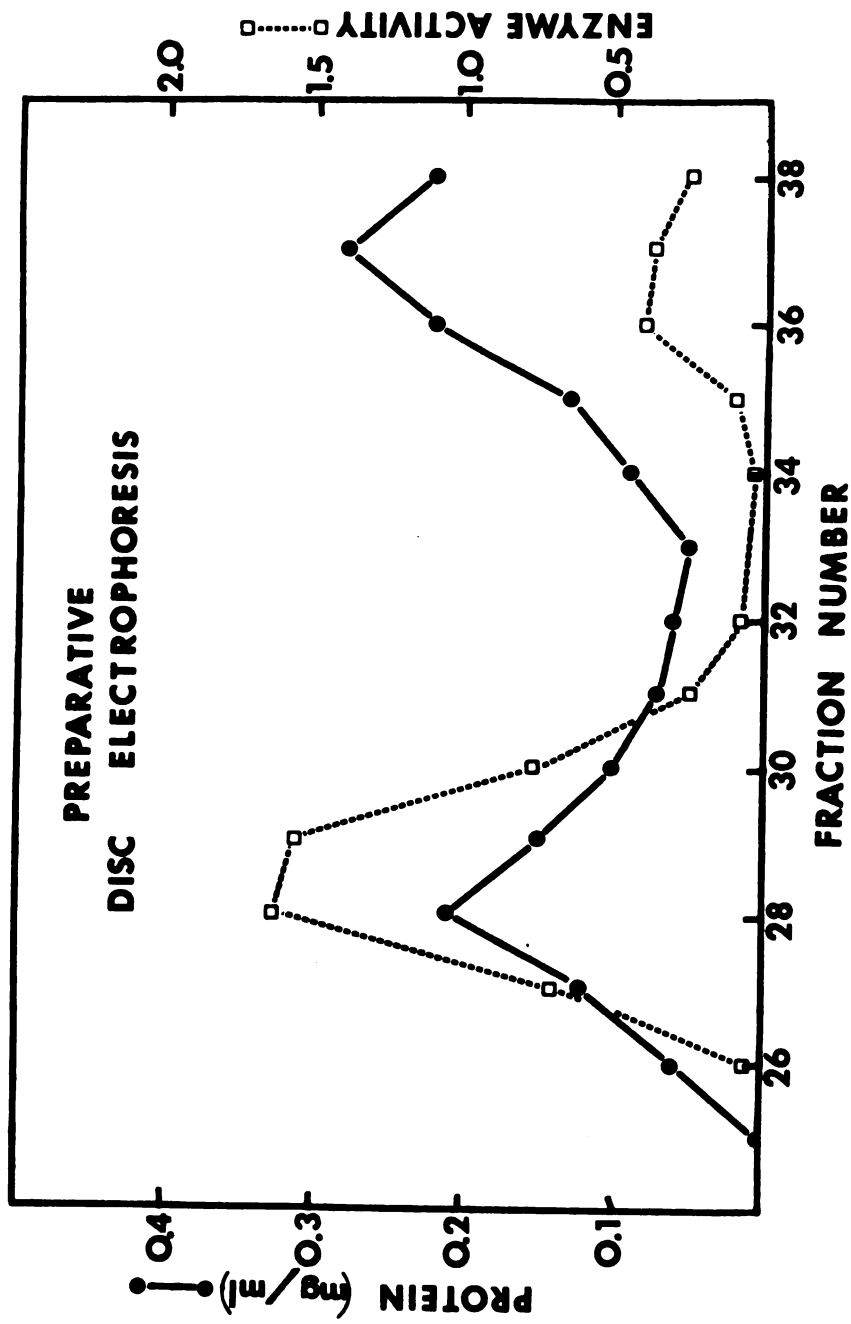
resolution at the top of the gradient and since the enzyme generally sediments only about 1/3 of the way through the gradient, this can interfere with the results. On one of five runs, 100 ml was lost, making it necessary to repeat the centrifugation.

Preparative Disc Gel Electrophoresis

The results from a preparative disc gel electrophoresis run are shown in Figure 12. Two things are important for the success of this step: the use of NADH in the running buffer and the use of a 5.5 percent acrylamide gel. When purified enzyme is electrophoresed in the absence of NADH, a smear of protein is obtained, with few or no distinct bands. However, if NADH is present, distinct bands are obtained, with much greater enzymatic activity than in the absence of NADH. The NADH also protects the enzyme after it elutes from the column, since under normal conditions, the enzyme is relatively unstable in Tris buffer at high pH. If a higher gel concentration is used, the enzyme migrates too slowly and loses activity. If a lower gel concentration is used, the enzyme migrates with the tracking dye. After the preparative sucrose gradient step, most of the enzymatic activity present electrophoreses as a single band, with perhaps 25 percent of the total activity in the minor bands (Figure 12, also see page 47).

This major band of activity runs right behind the

Figure 12.--Preparative Disc Gel Electrophoresis. α-Glycerol phosphate dehydrogenase from the sucrose gradient step was concentrated to 1 to 2 ml and applied to a 5.5 percent acrylamide gel column and electrophoresed in a Canalco Prep Disc apparatus with a PD 2/70 upper column. The running buffer was the analytical Tris-glycine buffer (page 19) diluted 1:4 with water. Fractions 27 through 30 were combined and concentrated. Enzyme activity is in units of ΔOD/min.


PREPARATIVE
DISC ELECTROPHORESIS
PROTEIN (mg/ml)
ENZYME ACTIVITY
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
1.0
1.5
2.0
26
28
30
32
34
36
38

tracking dye on a 5.5 percent gel. Therefore, it is on the gel for only a short time and runs as a sharp band, eliminating much of the dilution problem. The running and elution buffers are not standard Canalco buffer systems, but they have given reproducibly good results. Sufficiently high voltages were obtained so that it took only two hours to run the protein sample through the gel. Since specially purified reagents were not used, there was a small problem with the gel swelling, narrowing the slit (Canalco instruction booklet). If this becomes too great a problem in future runs, the use of recrystallized acrylamide and bis acrylamide should minimize the swelling.

It was found that the purified enzyme was quite sensitive toward mechanical denaturation. This prevented concentration of the enzyme by diafiltration. The enzyme must be handled gently during pipetting to prevent foaming, also. To concentrate the enzyme, a simple device made from a suction flask, a rubber stopper, a glass funnel and a 6" to 10" length of dialysis tubing was used (D. D. Randall, 1970, Ph.D. Thesis, Michigan State University). The dialysis tubing was tied at one end and the other end was put through an appropriate size hole in the rubber stopper. The funnel was then put inside the open end of the dialysis tubing and the two were then fitted tightly in the hole in the stopper. This stopper-funnel-dialysis bag apparatus was then put in the suction flask, the enzyme solution was poured into the

bag and the flask was evacuated with an aspirator. Using this device, a 12 ml sample was concentrated to 2 to 3 ml in approximately 2 hours, with no loss of protein due to denaturation.

Other Steps Tested But Not Adopted

Tests were made of several other standard purification steps, but these were not used for various reasons. Ammonium sulfate fractionation had been used as the initial purification step, but was discarded because it gave inconsistent yields and purification; there was also a great deal of residual ammonium sulfate which was inconvenient for subsequent steps and difficult to remove because of the large volumes of solution involved. An ammonium sulfate precipitation step is still used after the DEAE cellulose pad, however, to concentrate the protein. At this point in the purification, the volume is small enough that the residual salt can be removed by desalting on Sephadex G-25.

A 49° heat step in the presence of NADH and a pH 4.5 acid denaturation step were also tested but abandoned; it was impossible to obtain active enzyme from the calcium phosphate gel it these two steps preceded the calcium phosphate step. Gel filtration chromatography on Sephadex G-200 was also tried, but it required a large amount of rather expensive buffer (0.02 M MES plus 0.3 mM NAD) and took two days to run, whereas the preparative sucrose gradient requires only 12 hours and much smaller amounts of buffer.

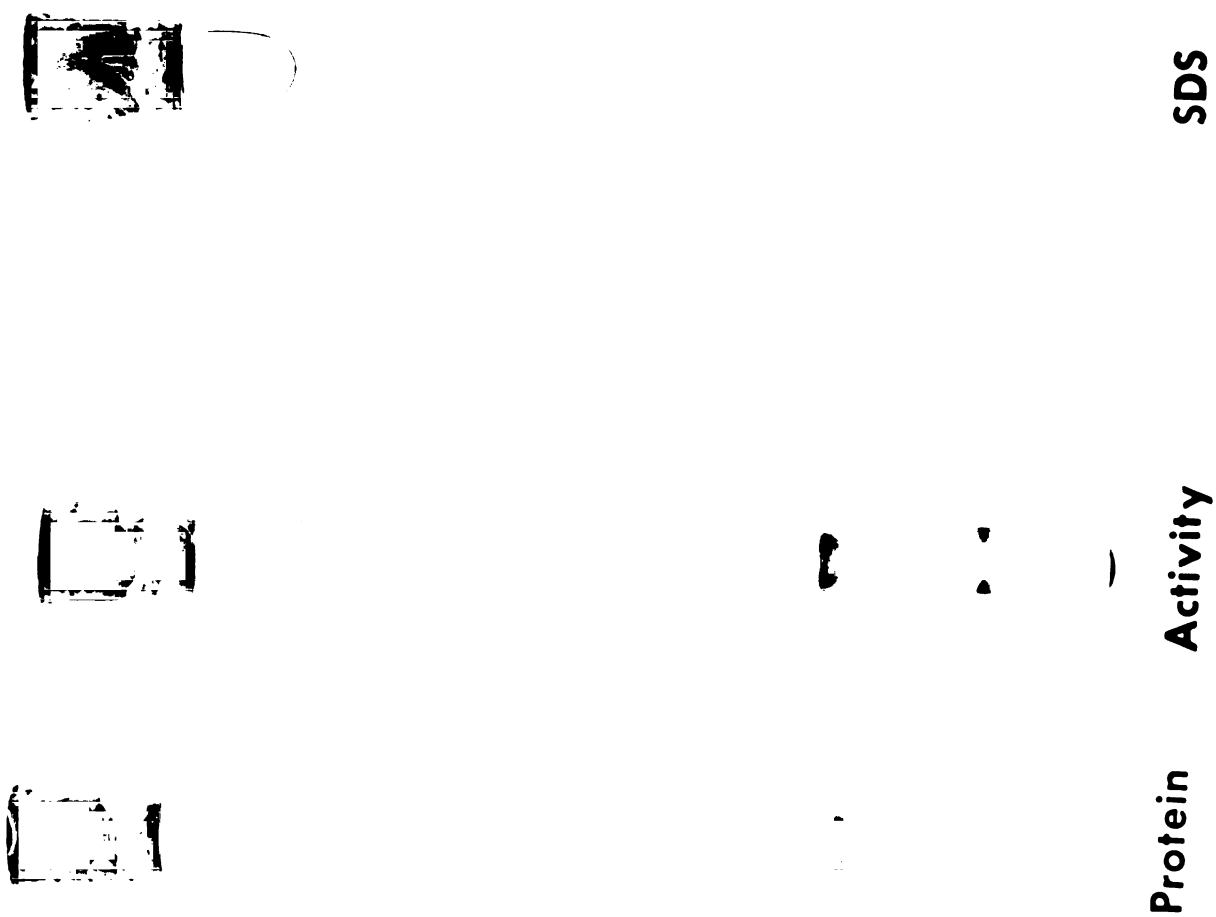
A final DEAE Sephadex A-25 chromatography step was attempted, but conditions which gave reproducibly good yields and purity were not found.

Purity of the Enzyme

The estimate of purity of the enzyme was based on several criteria. The primary estimate was based on the results of standard disc gel electrophoresis and SDS gel electrophoresis. These results are shown in Figure 13. On the standard disc gel stained for protein, the enzyme runs as a broad band which sometimes appears to be partially resolved into two sub-bands. The broad band generally has a somewhat broader blurred background; there are also other faintly staining areas. The activity and protein bands clearly coincide. The relative mobility of the purified enzyme corresponds to that of band 1, the major band, in the crude homogenates. On the SDS gel, there is the single major band of enzyme and two very minor bands that migrate faster than the enzyme. The enzyme appears to be greater than 95 percent pure.

The enzyme showed no signs of impurity in the two ultracentrifuge experiments, the sedimentation velocity and sedimentation equilibrium experiments (see pages 94 and 88). While these methods are not as sensitive to impurities as the electrophoresis experiments, they are consistent with homogeneity of the enzyme. Finally, an enzyme sample with a specific activity of 100 was tested for the presence of

Figure 13.--Analytical Gel Electrophoresis of Purified α-Glycerol Phosphate Dehydrogenase. Analysis of enzyme carried through the preparative disc gel electrophoresis step. Protein and activity gels: Disc gel electrophoresis of approximately 15 µg of the purified enzyme in the presence of NADH. Gels stained as indicated. SDS gel: An SDS gel of approximately 15 µg of the purified enzyme.


SDS
Activity
Protein

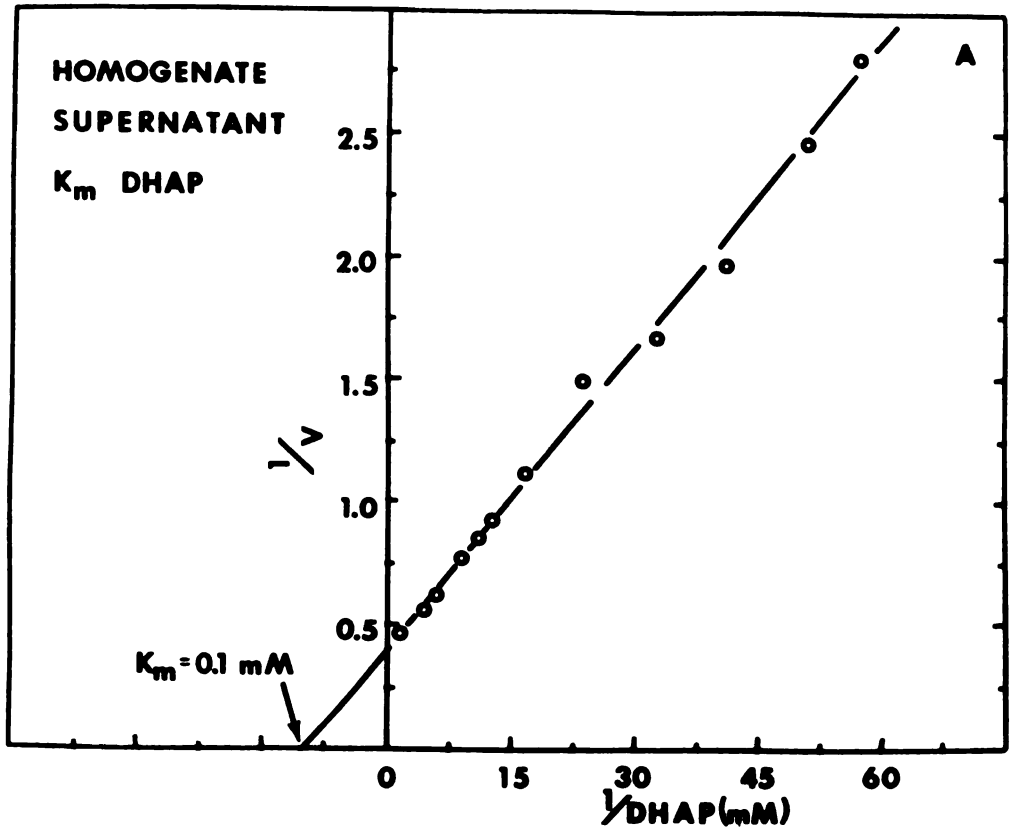
some of the other glycolytic enzymes. Phosphofructokinase, phosphoglycerate kinase, aldolase, trisephosphate isomerase and enolase were all assayed; the activity of each of these enzymes was less than 0.0001 percent of the α-glycerol phosphate dehydrogenase activity present. This is especially satisfying in the case of aldolase, because with one purification procedure the rabbit muscle α-glycerol phosphate dehydrogenase and aldolase partially co-purify (Beizenherz et al., 1955).

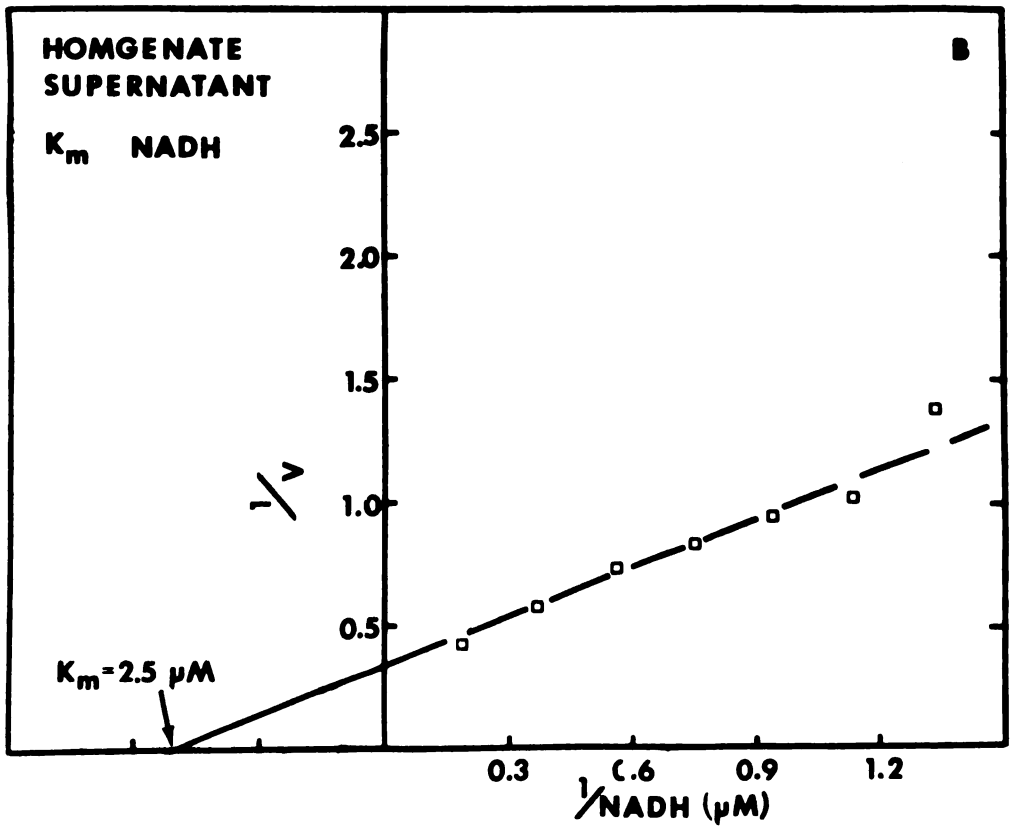
Several tests indicate that no modification of the enzyme occurs during the purification procedure. The enzyme purified by this procedure migrates the same as Band 1 observed on activity stains of gels of the crude homogenate (see page 47). Secondly, the purified enzyme has a sedimentation coefficient determined in the Model E of 4.9 to 5.1 S, while the enzyme activity in crude solutions is accounted for in a peak with a sedimentation coefficient of 5.2 S as determined from the sucrose density experiments (see page 39). Finally, the K_m values for the crude enzyme (Figure 14), 0.1 mM (DHAP) and 2.5 μM (NADH), were the same order of magnitude as the values for purified enzyme, 0.27 mM (DHAP) and 8 μM (NADH).

Storage of the Enzyme

Although extensive studies on the optimal storage conditions for the purified enzyme have not been carried out, two methods were used. To store the enzyme in soluble

Figure 14.--Determination of K_m for Substrates for the Crude Enzyme. A. Liver was homogenized manually in 0.05 M potassium phosphate, pH 7.8 and centrifuged for 1 hour at 45,000 RPM. The assays were performed spectrophotometrically in 0.1 M Tris-Cl, 0.001 M EDTA, pH 7.6 at 25°. The NADH concentration was 0.25 mM. The velocity is in units of ΔOD/min. B. K_m for NADH. Homogenization and assay conditions same as Figure 13, except the DHAP concentration was 0.7 mM. The velocity is in units of relative activity.


HOMOGENATE
SUPERNATANT
K_m DHAP
A
2.5
2.0
1.5
1.0
0.5
1/v
K_m = 0.1 mM
0
15
30
45
60
1/DHAP(mM)


HOMGENATE
SUPERNATANT
K_m NADH
B
2.5
2.0
1.5
1.0
0.5
1/v
K_m = 2.5 μM
0.3
0.6
0.9
1.2
1/NADH (μM)

form, buffer containing 10 percent sucrose, 0.02 M MES, 0.001 M EDTA, 0.01 M ammonium sulfate, 0.3 mM NAD and 10 mM thioglycerol, pH 5.9, was used and the protein concentration was made greater than 1 mg/ml. Otherwise, it was stored as the ammonium sulfate precipitate (70 percent saturated) under the same buffer.

CHAPTER VI

STUDIES ON THE PURIFIED ENZYME

Chemical and Physical Characterization

As indicated earlier, NADH and NAD profoundly affect the activity and heat stability of α-glycerol phosphate dehydrogenase. Because of this, we were interested in investigating the possibility of any effects of NADH and NAD on the structure of the enzyme.

Molecular Weight

The molecular weight of the native enzyme was determined using the Model E analytical ultracentrifuge and the meniscus depletion method (Yphantis, 1964) of high speed sedimentation equilibrium. A protein sample of approximately 1 mg/ml which had been purified through the disc gel electrophoresis step (specific activity = 100) was prepared by dialysis against 0.02 M MES, 0.001 M EDTA, 0.1 M KCl, pH 5.9, overnight at 4°. Samples of 0.36, 0.55 and 0.76 mg/ml were prepared by diluting the stock enzyme with the appropriate amount of dialysis buffer. A 0.55 mg/ml sample which contained 0.5 mM NADH was also prepared by adding the appropriate amount of 10 mM NADH to both the sample and solvent sectors of the ultracentrifuge cell. The results of the experiments for the 0.55 mg/ml samples in the absence and

presence of NADH are shown in Figures 15 and 16 (see Materials and Methods).

Clearly, the sample with no NADH is heterogeneous since the plot is curved. This could be due to either the presence of an impurity or to dissociation or association of the protein. Since the other sample, which was identical except for the presence of 0.5 m$\underline{M}$ NADH, shows no evidence of heterogeneity, the curved line must be due to the presence of either dissociated or associated protein. From the slope of the line in Figure 16, a molecular weight of 70,400 is calculated for the enzyme in the presence of 0.5 m$\underline{M}$ NADH. From the limiting slope at the bottom of the cell in Figure 15, a value of M_w = 68,200 was obtained for the high molecular weight material in the sample without NADH. This value agrees quite well with the molecular weight of the enzyme in the presence of NADH, after correction for the presence of 2 moles of NADH bound per mole of enzyme in the presence of NADH. We conclude that the heterogeneity observed in the absence of NADH is due to dissociation of the enzyme at the low protein concentration near the meniscus and that the native enzyme has a molecular weight of 68,000 to 69,000. A value of M_w = 53,300 was calculated for the dissociated material near the meniscus; hence, the enzyme is only partially dissociated. Assuming that the native enzyme is a dimer (see next section), then the mixture consists of approximately 50 percent monomer and 50

Figure 15.--High Speed Sedimentation Equilibrium of α-Glycerol Phosphate Dehydrogenase. The sample was prepared and the experiment performed as described in Materials and Methods.

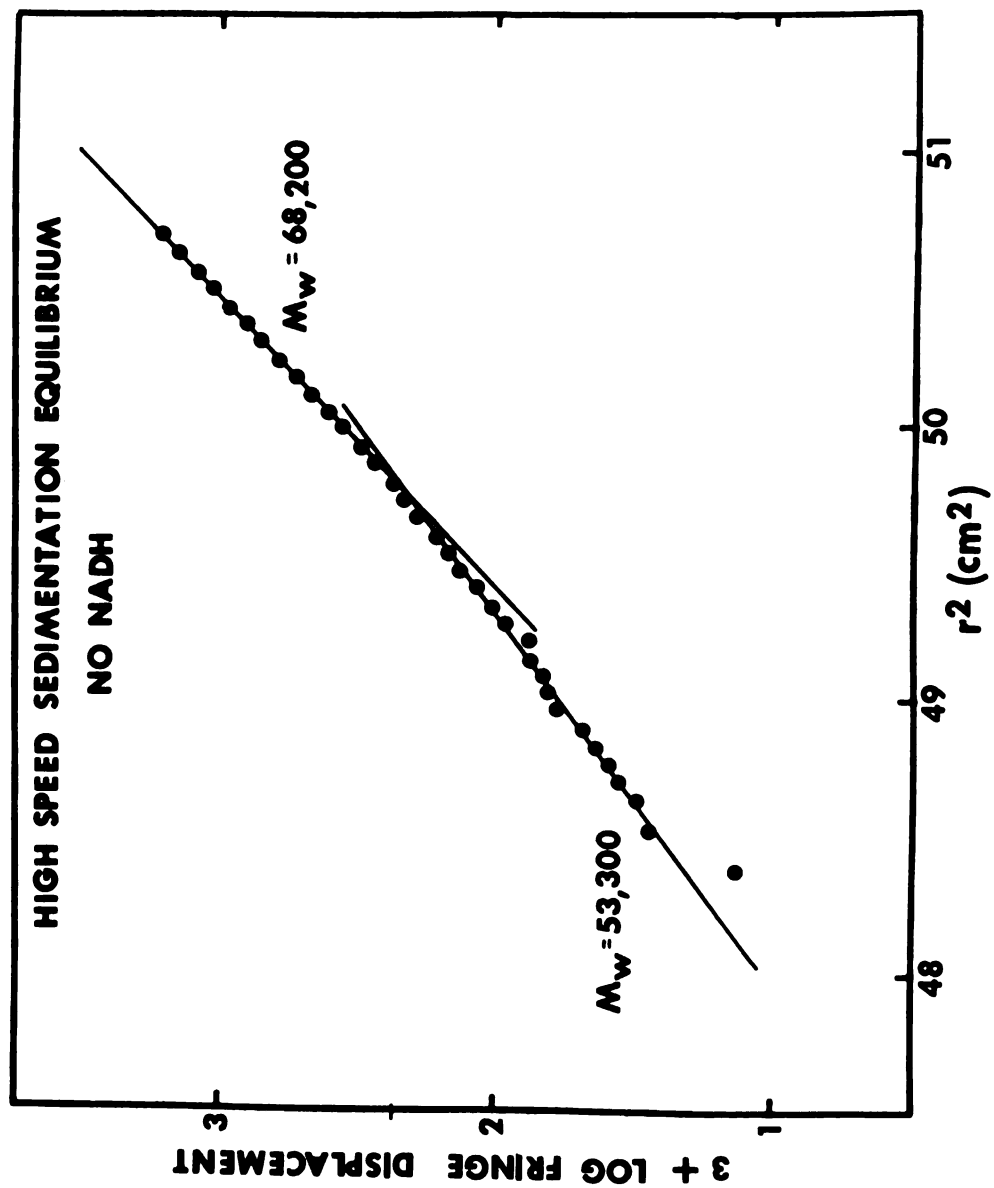
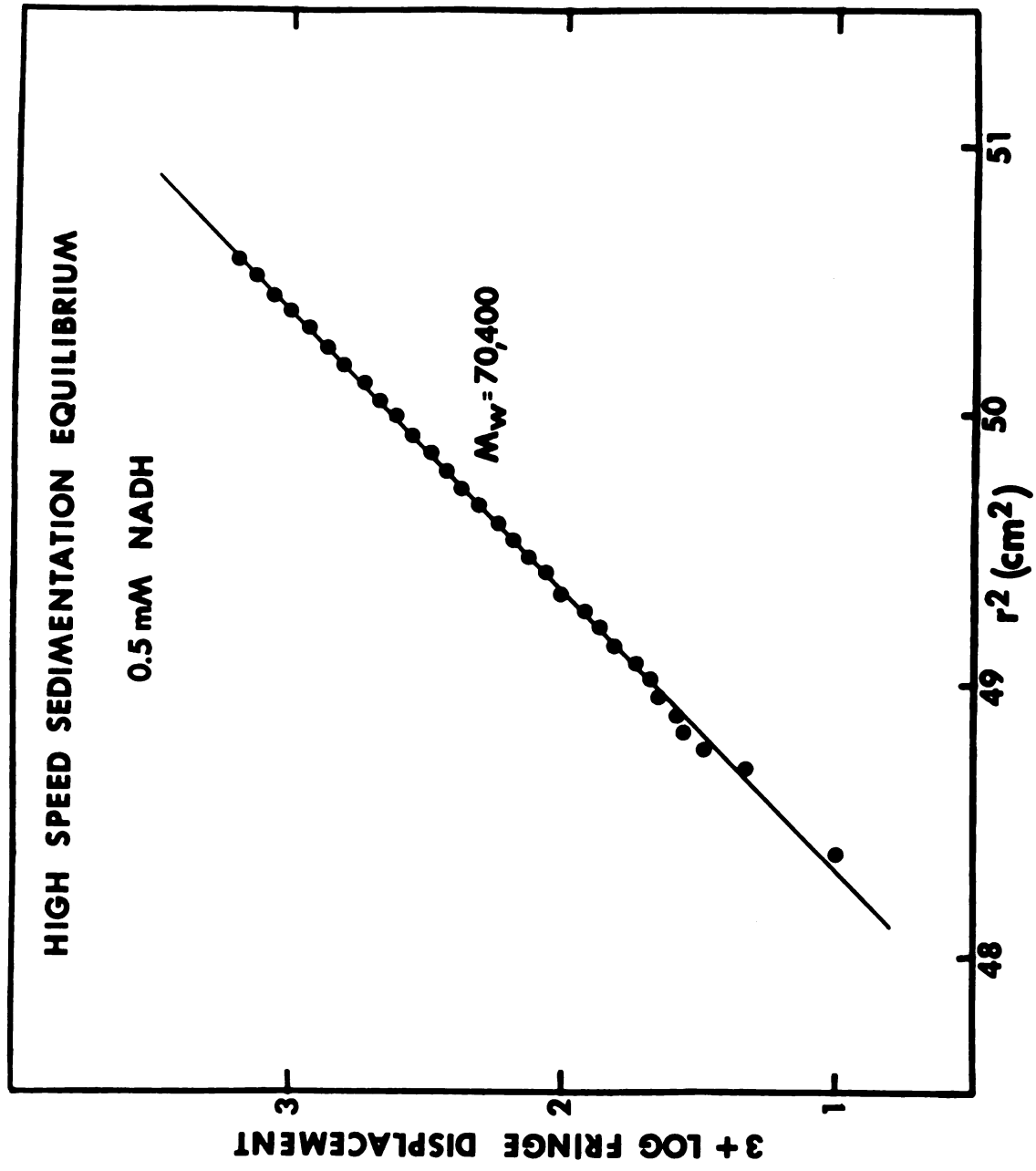

HIGH SPEED SEDIMENTATION EQUILIBRIUM
NO NADH
$M_w = 68,200$
$M_w = 53,300$
3 + LOG FRINGE DISPLACEMENT
r^2 (cm^2)
1
2
3
48
49
50
51

Figure 16.--High Speed Sedimentation Equilibrium of α-Glycerol Phosphate Dehydrogenase. Same as Figure 15, except that 0.5 m<u>M</u> NADH was present.


HIGH SPEED SEDIMENTATION EQUILIBRIUM
0.5 mM NADH
M_w= 70,400
3 + LOG FRINGE DISPLACEMENT
1
2
3
48
49
50
51
r^2 (cm^2)

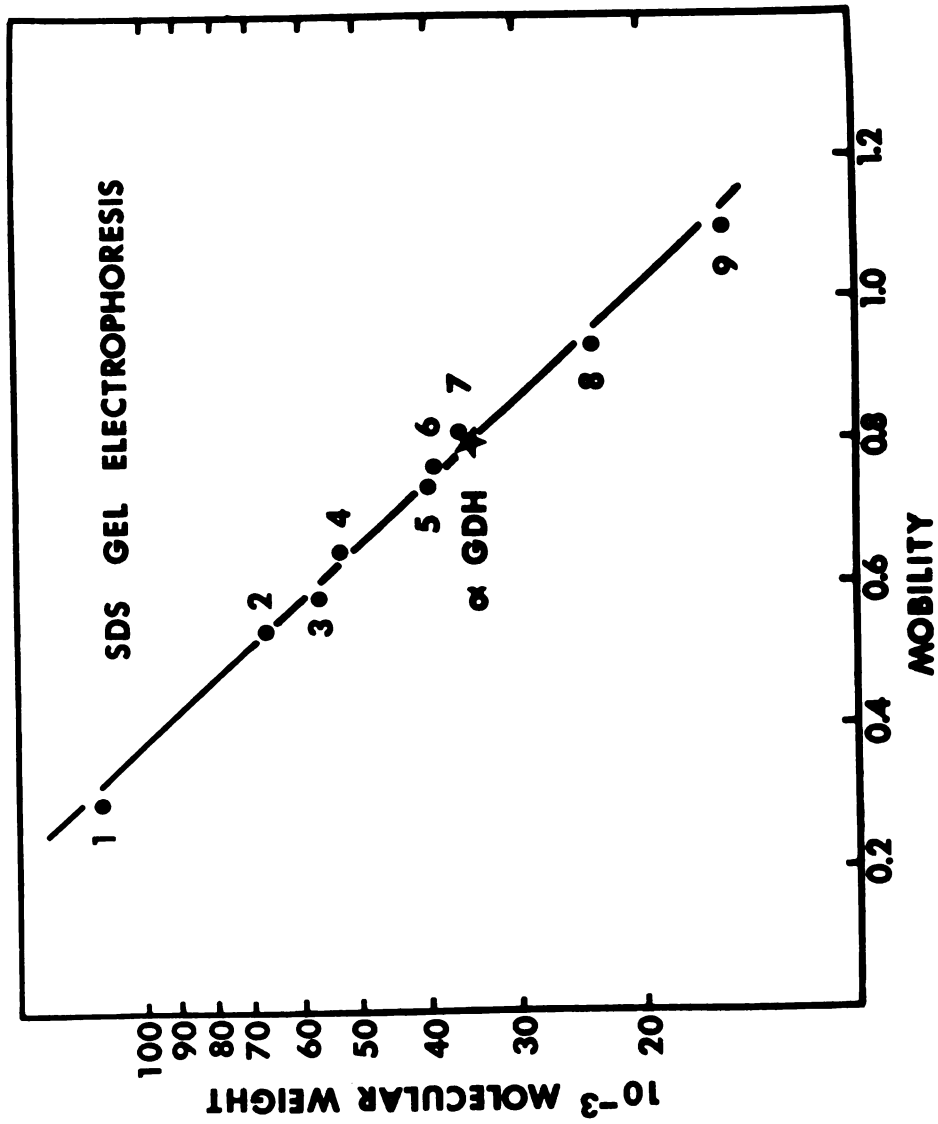
percent dimer at a protein concentration of 0.05 mg/ml (assuming a fringe shift of 4 fringes for 1 mg/ml). This value of protein concentration which yields 50 percent dissociation is approximately numerically equal to the dissociation equilibrium constant, in units of mg/ml.

The molecular weight of the subunit of the enzyme was estimated from SDS gel electrophoresis, using standard techniques, with slight modifications. The results were plotted in the form of log molecular weight versus mobility (relative to the tracking dye) and are shown in Figure 17. The mobility of 0.80 observed for the enzyme corresponds to a molecular weight of approximately 35,000 for the subunit. Since there are occasionally charge-related and shape-related artifacts in SDS gel electrophoresis experiments with various proteins (Segrest et al., 1971; Furthmayer and Timpl, 1971), the molecular weights obtained by this method are only estimations, but usually have an accuracy of 5 to 10 percent. The value of 35,000 for the SDS subunit molecular weight is very near one-half the native enzyme molecular weight (68,000 to 69,000). Hence, we conclude that the native enzyme is a dimer, composed of two similar, if not identical, subunits of molecular weight of about 35,000.

Sedimentation Coefficient

The sedimentation coefficient of the enzyme was determined in the Model E analytical ultracentrifuge as described earlier. The protein sample used had been

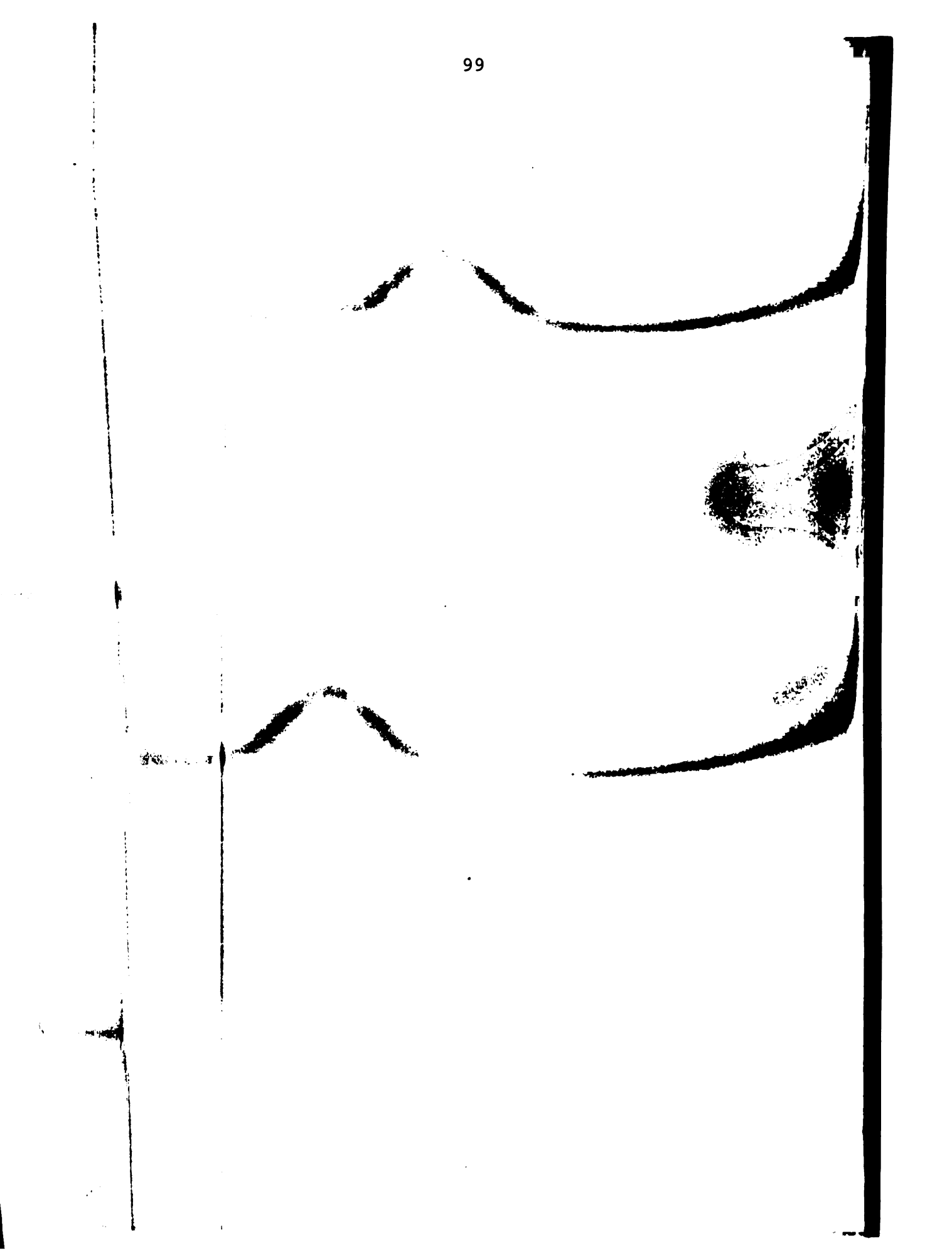
Figure 17.--α-Glycerol Phosphate Dehydrogenase Subunit Molecular Weight by SDS Gel Electrophoresis. The samples were prepared and the gels run as described in Materials and Methods. Each gel contained 10 to 20 μg protein. The standards used were: (1) β-galactosidase, (2) bovine serum albumin. (3) pyruvate kinase, (4) glutamate dehydrogenase, (5) aldolase, (6) rabbit muscle α-glycerol phosphate dehydrogenase, (7) lactate dehydrogenase, (8) trypsin, and (9) hemoglobin.


SDS GEL ELECTROPHORESIS
1
2
3
4
5
6
7
8
9
α GDH
10⁻³ MOLECULAR WEIGHT
100
90
80
70
60
50
40
30
20
0.2
0.4
0.6
0.8
1.0
1.2
MOBILITY

purified to a specific activity of 90 by purification through the preparative sucrose density gradient step. The protein (5 mg/ml) was prepared for centrifugation by equilibrating with the buffer on a small Sephadex G-25 column equilibrated with the centrifugation buffer (see Methods). The sample which contained 0.5 m$\underline{M}$ NADH (upper pattern) was prepared by addition of the appropriate amount of 10 m$\underline{M}$ NADH to an aliquot of the equilibrated protein. The photograph taken 56 minutes after reaching speed (56,100 RPM) is shown in Figure 18. The average sedimentation coefficient for the enzyme in the absence of NADH is $s^{0.5\%}_{20,w}$ = 4.89 S and in the presence of 0.5 m$\underline{M}$ NADH, the value is 5.09 S. These values are quite reasonable for an enzyme with a molecular weight of 69,000 as estimated from a plot of log S versus log molecular weight for several proteins of known molecular weight (Holleman, 1966). From this plot a sedimentation coefficient of 5.0 S is equivalent to a molecular weight of approximately 76,000.

From the appearance of both of the patterns in Figure 18, the enzyme seems to be quite pure, even though the enzyme was not purified through the final step. Both peaks are symmetrical and there is no indication of higher and lower molecular weight material. (The rise in the baseline near the bottom of the cell was much the same in the zero time photograph, so it would seem to be an artifact in the optical system.) Hence, these schlieren patterns are consistent with, although not proof of, purity.

Figure 18.--Sedimentation Velocity Patterns for α-Glycerol Phosphate Dehydrogenase. The sample was prepared and the experiment performed as described in Materials and Methods. The photograph was taken 56 minutes after reaching speed. The upper sample contained 0.5 mM NADH. The protein concentration was 5 mg/ml and the phase plate angle was 55°.



Isoelectric Point

The isoelectric point of partially purified enzyme (purified through the preparative sucrose gradient, specific activity = 55) was determined by isoelectric focusing using a small, mini-column technique developed in this laboratory (Massey et al., manuscript in preparation). The enzyme was focused for 7 hours at 4° in a pH 5 to 8 gradient and the various fractions collected and assayed for enzymatic activity. The results shown in Figure 19 indicate an isoelectric point of 6.8. This agrees quite well with the value of 6.7 we obtained earlier by isoelectric focusing in a large, 110 ml LKB isoelectric apparatus.

Absorption Spectrum

The absorption spectrum of the purified enzyme (specific activity = 100) was determined manually with a Beckman DU monochrometer equipped with a Gilford absorbance indicator, using a sample which had been dialyzed against 0.02 M MES, 0.001 M EDTA, 0.1 M KCl, pH 5.9. The results are shown in Figure 20. There is a broad absorption maximum centered around 273 nm, and perhaps a small shoulder at 259 nm, indicating the possibility of a bound nucleotide; the 280/260 ratio is 1.1.

Amino Acid Composition

The amino acid composition of the purified, carboxymethylated enzyme (specific activity = 100) is shown in

Figure 19.--Isoelectric Focusing of α-Glycerol Phosphate Dehydrogenase. The enzyme was focused in a small, noncommercial isoelectric focusing column as described in Materials and Methods for 7 hours using a pH 5-8 ampholyte gradient. The fractions collected were assayed for activity and the pH measured at 0°. The enzyme activity is in units of units/ml.

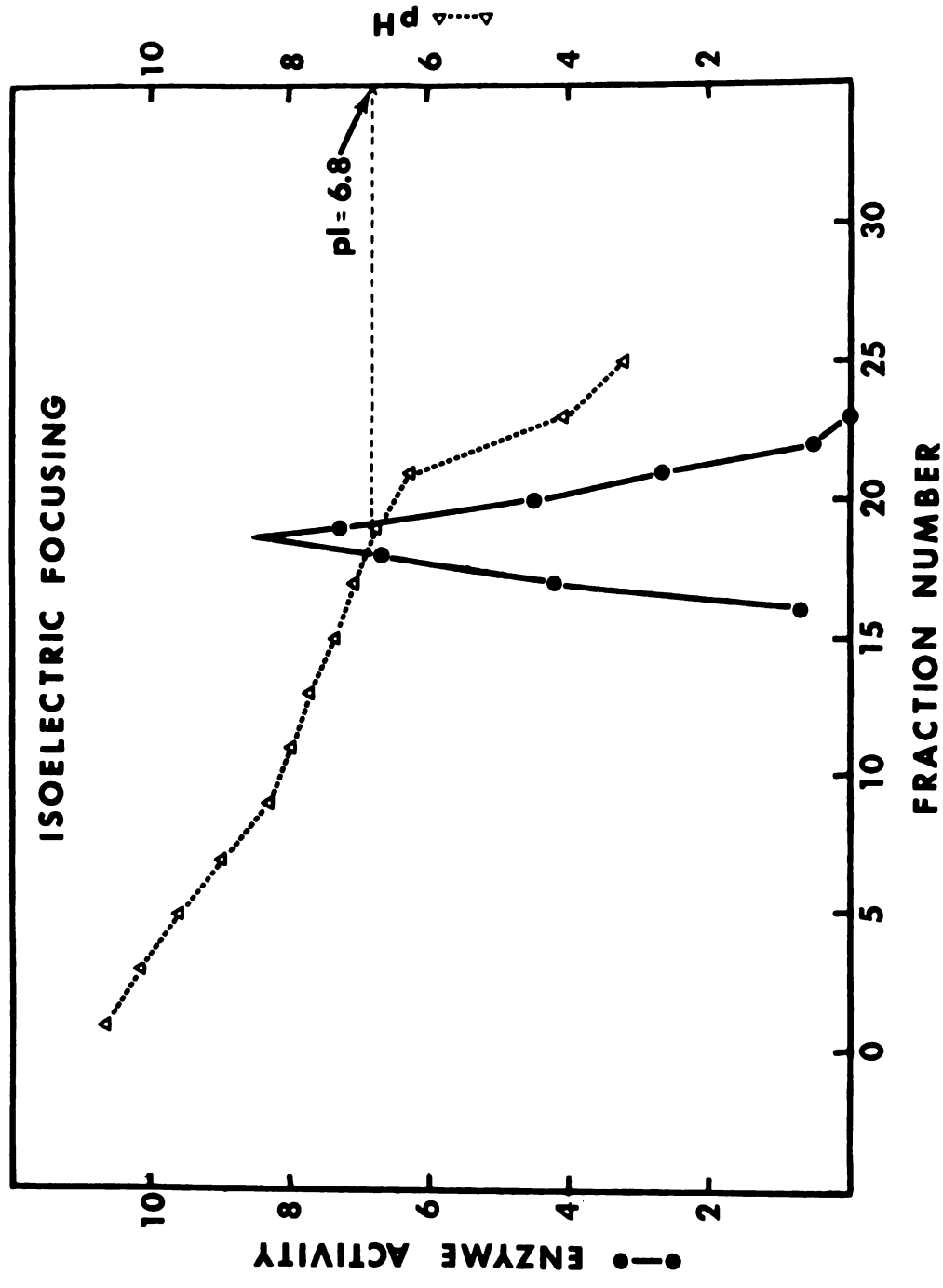

ISOELECTRIC FOCUSING
pH
pI = 6.8
ENZYME ACTIVITY
FRACTION NUMBER
0
5
10
15
20
25
30
2
4
6
8
10

Figure 20.--Absorption Spectrum of α-Glycerol Phosphate Dehydrogenase. The absorption spectrum of a 0.93 mg/ml sample was determined manually using a Beckman DU monochrometer equipped with a Gilford absorbance meter. The protein sample has been dialyzed against 0.02 M MES, 0.001 M EDTA, 0.1 M KCl, pH 5.9, and the buffer was used as the blank.

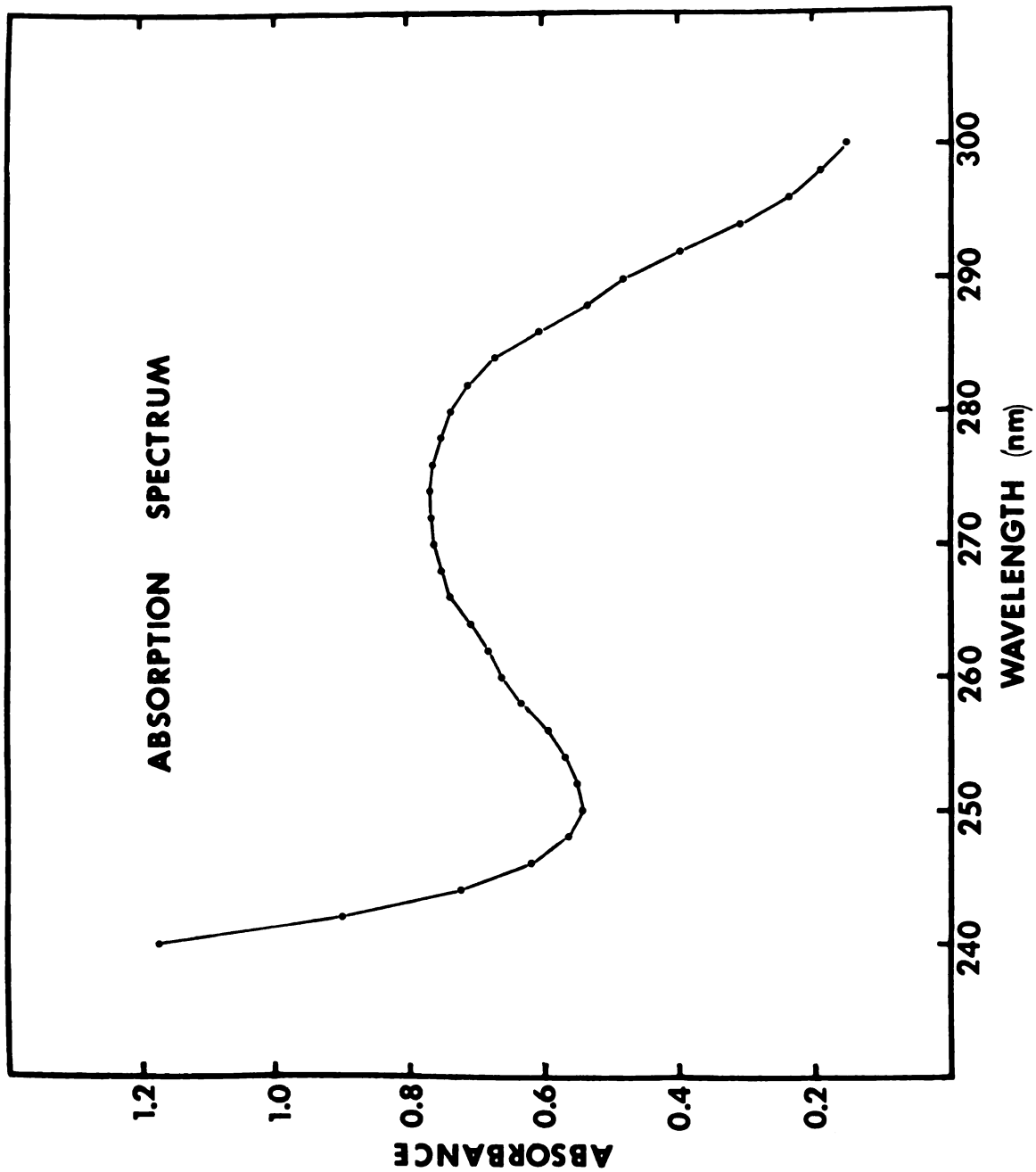

ABSORPTION SPECTRUM
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
240
250
260
270
280
290
300
WAVELENGTH (nm)

Table VI, along with the results for the rabbit muscle enzyme as reported by Holleman (1966). The compositions of the two proteins are quite similar. However, the rabbit muscle enzyme contains significantly more isoleucine, phenylalanine and lysine and less serine, proline and arginine.

TABLE VI

AMINO ACID COMPOSITION

	Pig Liver	Rabbit Muscle	
	Residues Per 69,000	Residues Per 76,000[a]	Residues Per 69,000[a]
Lysine	32	55	50
Histidine	16	18	16
Arginine	18	16	15
Aspartate	50	56	51
Threonine	24	28	25
Serine	28	21	19
Glutamate	64	82	74
Proline	36	31	28
Glycine	60	77	70
Alanine	50	57	52
Valine	48	62	56
Methionine	12	15	14
Isoleucine	36	57	52
Leucine	50	58	53
Tyrosine	10	10	9
Half-Cystine	18	21	19
Tryptophan	5	4	4
Phenylalanine	24	32	29

[a]Values taken from Holleman (1966)

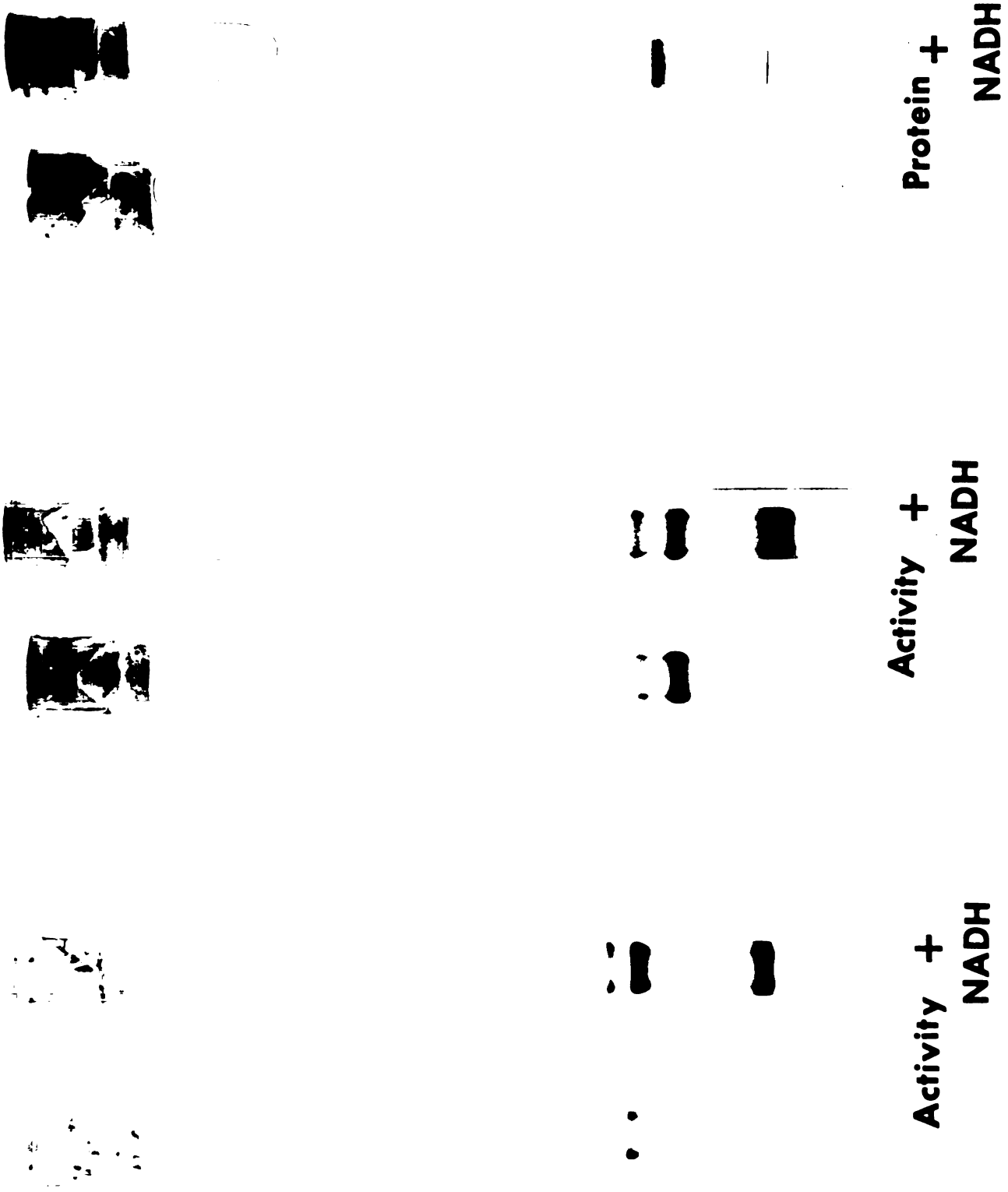
Using this amino acid composition for the pig liver α-glycerol phosphate dehydrogenase, a partial specific volume of 0.737 cc/g was calculated.

Effects of NADH on Disc Gel Electrophoresis of Pig Liver α-Glycerol Phosphate Dehydrogenase

The effects of NADH on the electrophoretic behavior of the enzyme are shown in Figure 21. The two major effects are: (1) Nearly pure or pure enzyme electrophoreses as distinct bands in the presence of NADH, but as smears of indistinct bands in the absence of NADH; (2) With crude homogenate, which contains multiple forms of α-glycerol phosphate dehydrogenase, some bands which do not possess enzymatic activity when electrophoresed in the absence of NADH do exhibit activity when run in the presence of NADH. To do the electrophoresis in the presence of NADH, small subreservoirs filled with the Tris-glycine buffer containing sucrose and 0.25 m<u>M</u> NADH were used. Since the NADH is anionic at pH 8.3, it migrates through the gel ahead of the protein; much of it is concentrated at the buffer discontinuity interface, giving rise to the heavily stained band at the tracking dye position on gels stained for activity.

The first effect is most clearly seen with pure enzyme (specific activity = 100, right set). On the gel that was electrophoresed in the absence of NADH, there was only a smear of protein. In contrast, the gel run in the presence of NADH had one major band of enzyme, with several minor areas of possibly contaminating protein. This effect

Figure 21.--Effects of NADH on Gel Electrophoresis of α-Glycerol Phosphate Dehydrogenase. Left Set. Electrophoresis of adult liver homogenate supernatant stained for activity. Center Set. Electrophoresis of nearly pure enzyme (specific activity = 90) stained for activity. Right Set. Electrophoresis of pure enzyme (specific activity = 100) stained for protein. Gels labeled "+NADH" were electrophoresed in the presence of 0.25 mM NADH.


Protein + NADH
Activity + NADH
Activity + NADH

is probably due to stabilization of the native dimer form of the enzyme during electrophoresis by NADH, giving a sharp band, while in the absence of NADH, an equilibrium between the monomer and dimer is established, giving rise to the observed smear of protein.

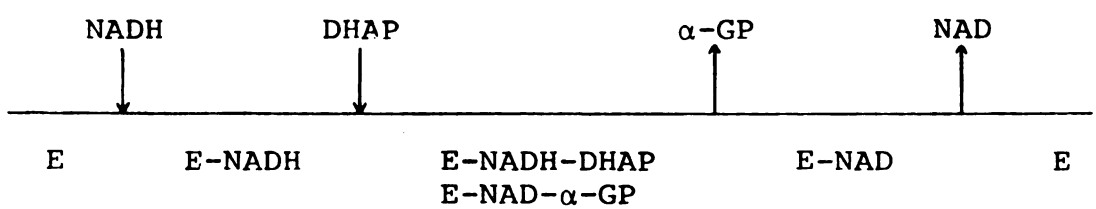
The other effect of NADH on electrophoresis of the enzyme is seen on the left and center sets. Electrophoresis of the crude homogenate (left set) and partially purified enzyme (specific activity = 90, center set) in the absence of NADH gives one and two major activity bands, respectively. But if these same samples are electrophoresed in the presence of NADH, one additional major and one additional minor band appear on the gel of the crude homogenate and four additional minor bands of activity appear on the gel of the partially purified enzyme.

CHAPTER VII

KINETIC CHARACTERIZATION

Kinetic Mechanism

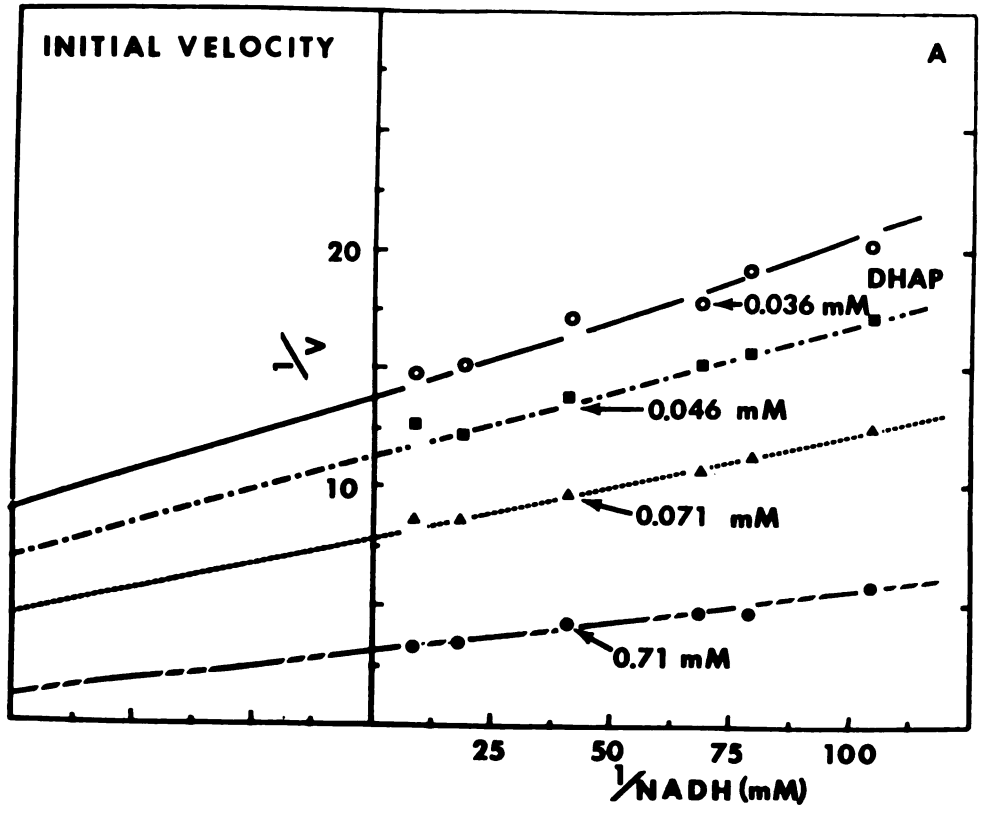
From his extensive studies on the rabbit muscle enzyme, Black (1966) concluded that its mechanism was Ordered Bi Bi, with NADH adding first and NAD leaving last, as shown below:

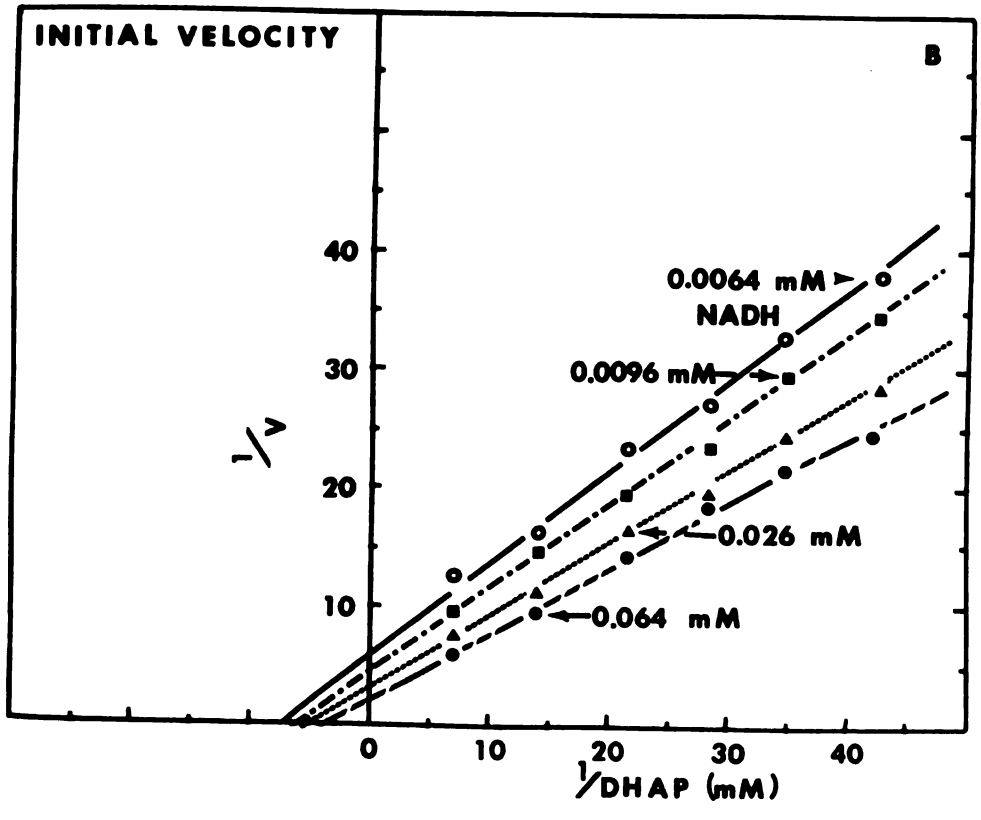


The mechanistic studies reported here were limited in scope, but detailed enough to confirm that the pig liver enzyme has the same mechanism.

Initial velocity studies were performed spectrophotometrically with both NADH and DHAP as the variable substrates (Figure 22). The data obtained were plotted in the form 1/v versus 1/s. The enzyme used had been purified to a specific activity of 65. Each point shown on the graph is the average of two assays. Although the lines on the graphs have not been extended to show the points of intersection, in both cases they intersect below the abscissa, in the third quadrant. These results are consistent with a

Figure 22.--Effect of Substrate Concentration on Initial Velocity of α-Glycerol Phosphate Dehydrogenase. A. Effect of NADH Concentration. Assays were performed in duplicate spectrophotometrically at 25°, with the indicated DHAP and NADH concentrations. The buffer was 0.1 M Tris-Cl, 0.001 M EDTA, 0.1 M KCl, pH 7.6. The velocity is in units of $\overline{\Delta}$OD/minute. B. Effect of DHAP concentration. Conditions same as Part A.


INITIAL VELOCITY
A
20
10
1/v
DHAP
0.036 mM
0.046 mM
0.071 mM
0.71 mM
25
50
75
100
1/NADH (mM)


INITIAL VELOCITY
B
40
30
20
10
1/v
0.0064 mM
NADH
0.0096 mM
0.026 mM
0.064 mM
0
10
20
30
40
1/DHAP (mM)

sequential mechanism, but give no indication as to whether the reaction is ordered or random, and if ordered, which substrate binds first.

To answer these questions, product inhibition studies were performed. Using the Ordered Bi Bi mechanism of the rabbit muscle enzyme as a model, the following product inhibition pattern would be predicted (Plowman, (1972):

Inhibitory Product	Type of Inhibition	
	NADH Variable	DHAP Variable
α-glycerol phosphate	non-competitive	non-competitive
NAD	competitive	non-competitive

From this pattern, it was decided to use NADH as the variable substrate for our studies, since the results would probably be less ambiguous than those with DHAP. Since product inhibition is always non-competitive for the Random Bi Bi mechanism (Plowman, 1972), this case should be ruled out by the NADH-variable studies. These assays were performed fluorometrically because of the low NADH concentrations required. Because of large fluctuations in the intensity of the xenon lamp of the fluorometer, it was necessary to correct the assay slopes for these changes. A graph of fluorometer response as a function of NADH concentration using repeated measurements was constructed. A line fitting all the points was then taken as the average fluorometer response. The correction for an assay was obtained

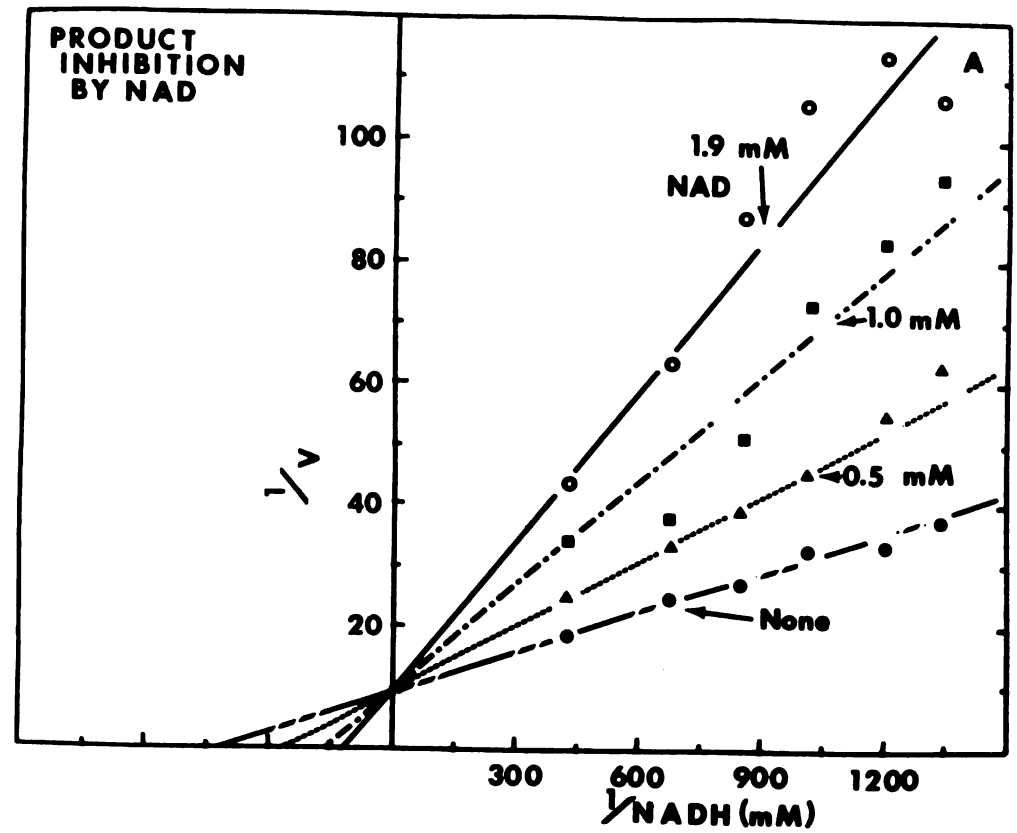
by determining the ratio of the fluorometer response before addition of enzyme to the standard response for that NADH concentration and dividing the assay slope by that ratio. The appropriate response data were not available for the studies using NAD as inhibitor, so this correction could not be made. The results of these studies are shown in Figure 23. In spite of the scatter in the data of the inhibition by NAD, the lines clearly intersect on the ordinate (competitive inhibition). It would be very difficult or impossible to draw the lines such that they intersect on the abscissa or were parallel.

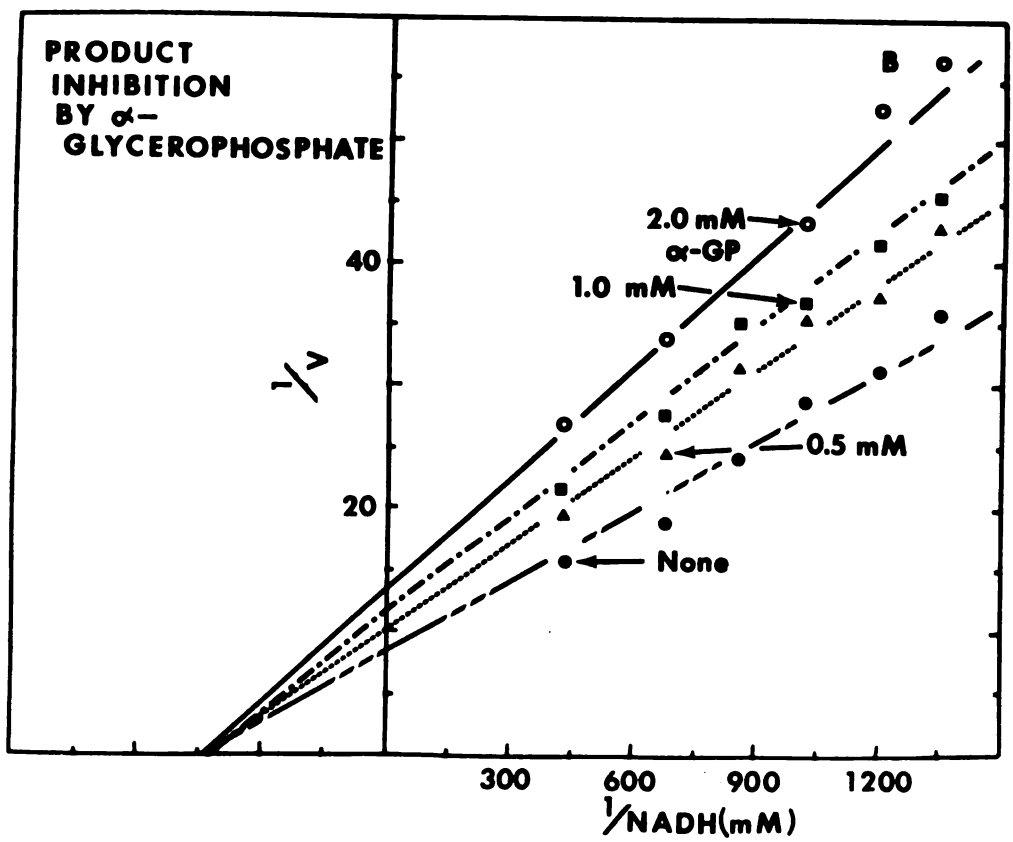
However, the data for the α-glycerol phosphate inhibition is more ambiguous. The lines are drawn as intersecting on the abscissa (non-competitive inhibition), as one would expect for an Ordered Bi Bi mechanism. They could be drawn as parallel, however (uncompetitive inhibition). If one assumes that this ambiguity arises from the DHAP concentration being too high, then either type of inhibition fits the Ordered Bi Bi mechanism.

Michaelis Constants

Using the data from the previous section (Figure 22) the apparent Michaelis constants for NADH and DHAP for the reaction in the forward direction were calculated. To obtain data for calculation of Michaelis constants for NAD and L-α-glycerol phosphate in the reverse direction,

Figure 23.--Inhibition of α-Glycerol Phosphate Dehydrogenase by Products. A. Inhibition by NAD. The assays were performed in duplicate fluorometrically at 25°. The assays contained the indicated amounts of NAD and NADH; the DHAP concentration was 0.071 mM. The buffer was 0.1 M Tris-Cl, 0.001 M EDTA, 0.1 M KCl, pH 7.6. The velocity is in units of relative activity. B. Inhibition by L-α-Glycerol Phosphate. Conditions same as Part A, except the data was treated to remove scatter due to fluctuation of the light source (see text).


PRODUCT
INHIBITION
BY NAD
A
1.9 mM
NAD
1.0 mM
0.5 mM
None
100
80
60
40
20
1/v
300
600
900
1200
1/NADH (mM)

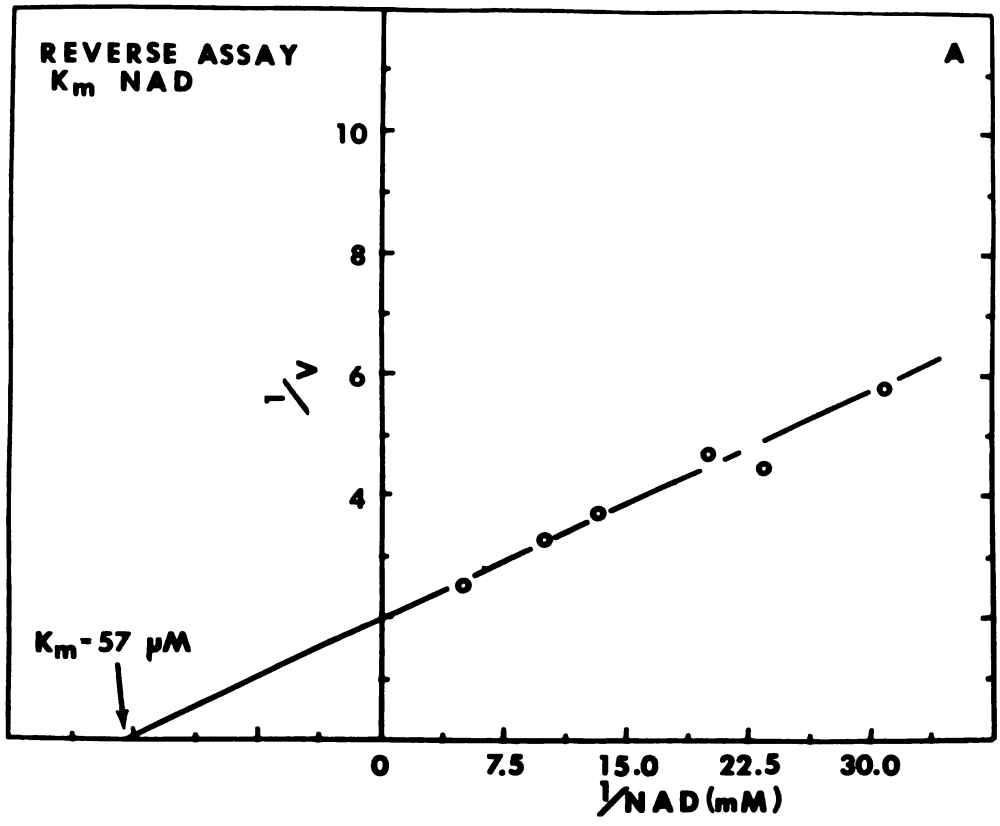

PRODUCT
INHIBITION
BY α-
GLYCEROPHOSPHATE
B
2.0 mM
α-GP
1.0 mM
0.5 mM
None
40
20
1/v
300
600
900
1200
1/NADH(mM)

experiments were performed spectrophotometrically at pH 10.0. The results obtained are shown in Figure 24. The apparent K_m values obtained for each of the substrates are: NADH, 8 μM; DHAP, 0.27 mM; L-α-glycerol phosphate, 0.20 mM; NAD, 57 μM. The apparent K_m for NAD at pH 7.6 of 80 μM was estimated from the data on the inhibition of the enzyme by ATP, ADP and 5'AMP in the reverse direction (see next section).

Inhibition by Adenine Nucleotides

The inhibition of the enzyme by adenine nucleotides was studied using an enzyme sample which had been purified through the sucrose gradient step (specific activity = 55). Spectrophotometric assays were performed at pH 7.6 in both the forward and reverse directions. The data were plotted in the form 1/v versus inhibitor concentration (Dixon plot). The results for the various nucleotides (ATP, ADP and AMP) are shown in Figures 25 through 27. From the intersection points of the lines, the inhibition constant (K_i) for each nucleotide can be calculated. The values obtained for each nucleotide are: 5'-AMP, 18 mM (forward) and 18 mM (reverse); ADP, 17 mM (f) and 16 mM (r); ATP, 6 mM (f) and 8 mM (r). The inhibition in each case is competitive with respect to the pyridine nucleotide, as might be expected, since a portion of the pyridine nucleotide molecule is identical to an ADP molecule.

Figure 24.--Determination of K_m for Substrates in the Reverse Assay of α-Glycerol Phosphate Dehydrogenase. A. K_m for NAD. Assays were performed spectrophotometrically in duplicate at 25° in 0.1 M glycine, 0.001 M EDTA, 0.1 M KCl, pH 10.0. The L-α-GP concentration was 8 mM. The velocity is in units of ΔOD/minute. B. K_m for L-α-Glycerol Phosphate. Conditions same as Part A, except 5 mM NAD was used.


REVERSE ASSAY
Km NAD
A
10
8
6
4
1/v
Km-57 μM
0
7.5
15.0
22.5
30.0
1/NAD(mM)

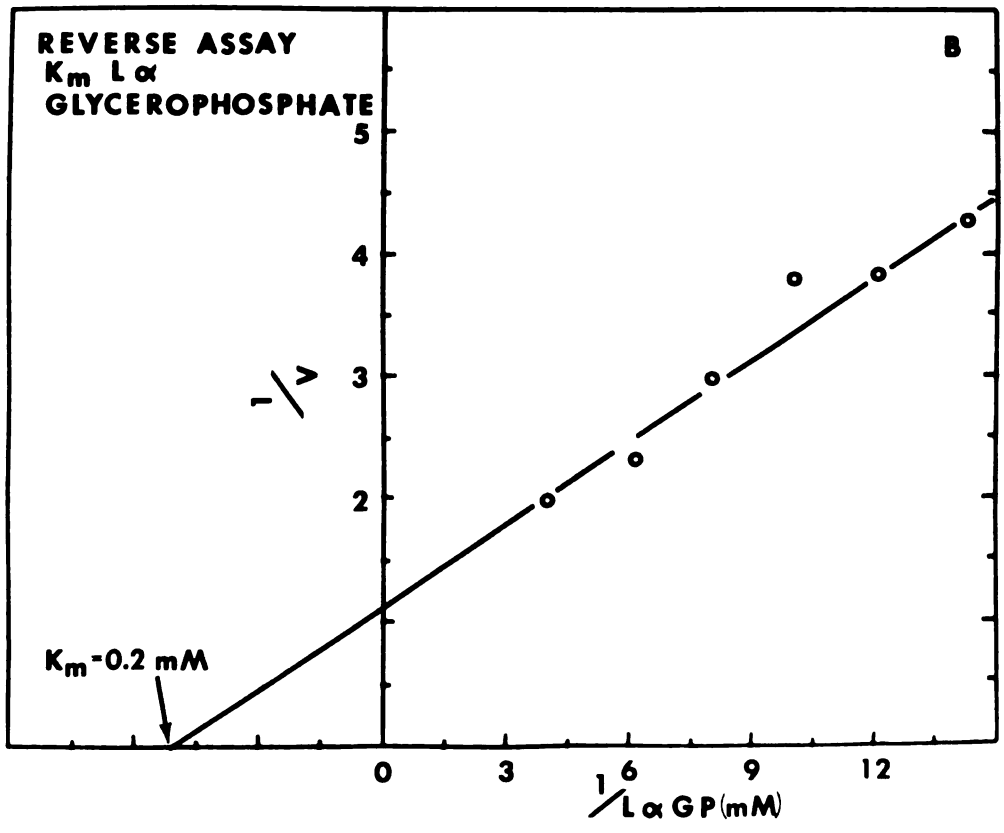
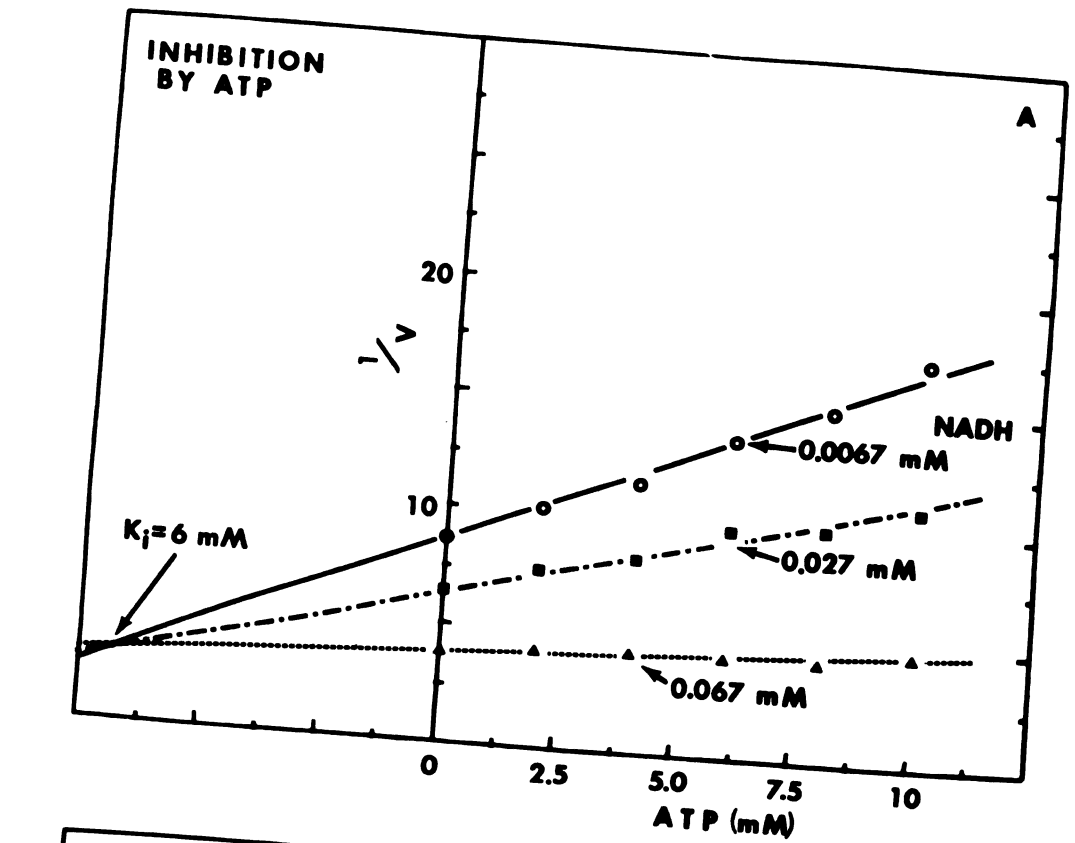

REVERSE ASSAY
Km L α
GLYCEROPHOSPHATE
B
5
4
3
2
1/v
Km-0.2 mM
0
3
6
9
12
1/L α GP(mM)

Figure 25.--Inhibition of α-Glycerol Phosphate Dehydrogenase by ATP. A. Forward Assay. Assays were performed spectrophotometrically in duplicate in 0.1 M Tris-Cl, 0.001 M EDTA, 0.1 M KCl, pH 7.6. The indicated amounts of ATP and NADH were added; the DHAP concentration was 0.71 mM. The data were plotted in the form of the Dixon plot, 1/v versus i. The velocity is in units of ΔOD/minute. B. Reverse Assay. Conditions same as Part A, except the indicated amounts of NAD were added; the L-α-glycerol phosphate concentration was 0.5 mM.


INHIBITION
BY ATP
A
20
1/v
10
NADH
0.0067 mM
K_i=6 mM
0.027 mM
0.067 mM
0
2.5
5.0
7.5
10
ATP (mM)

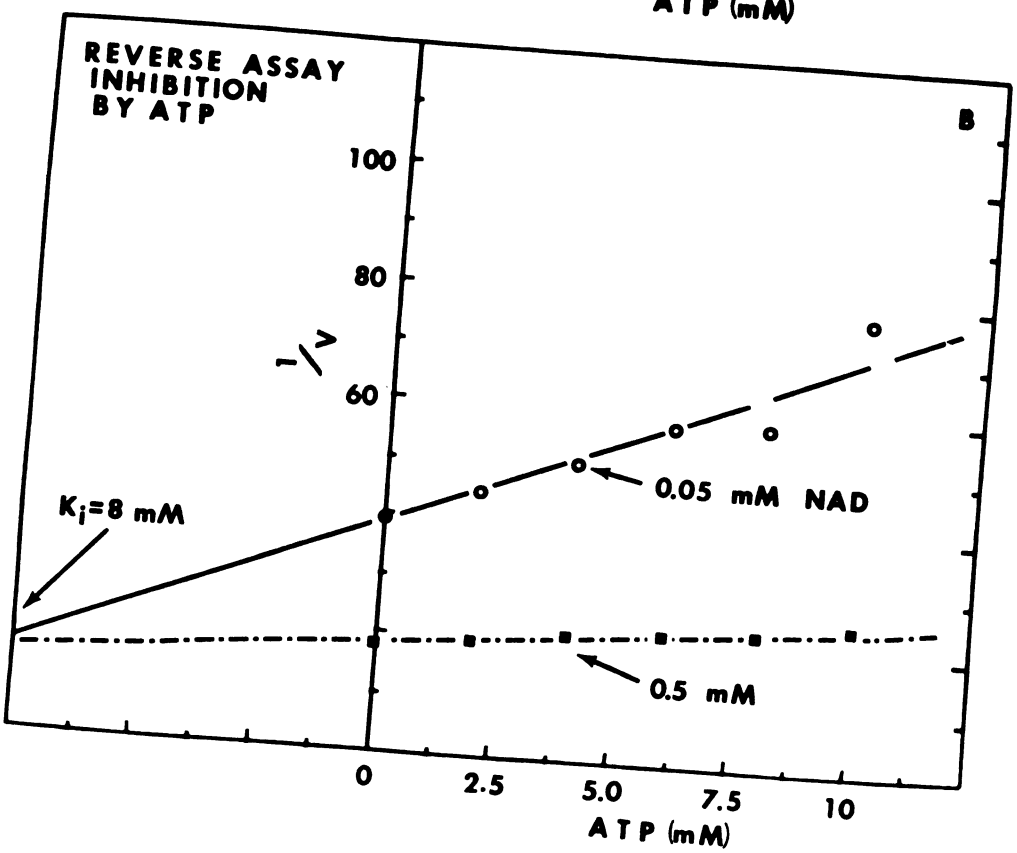
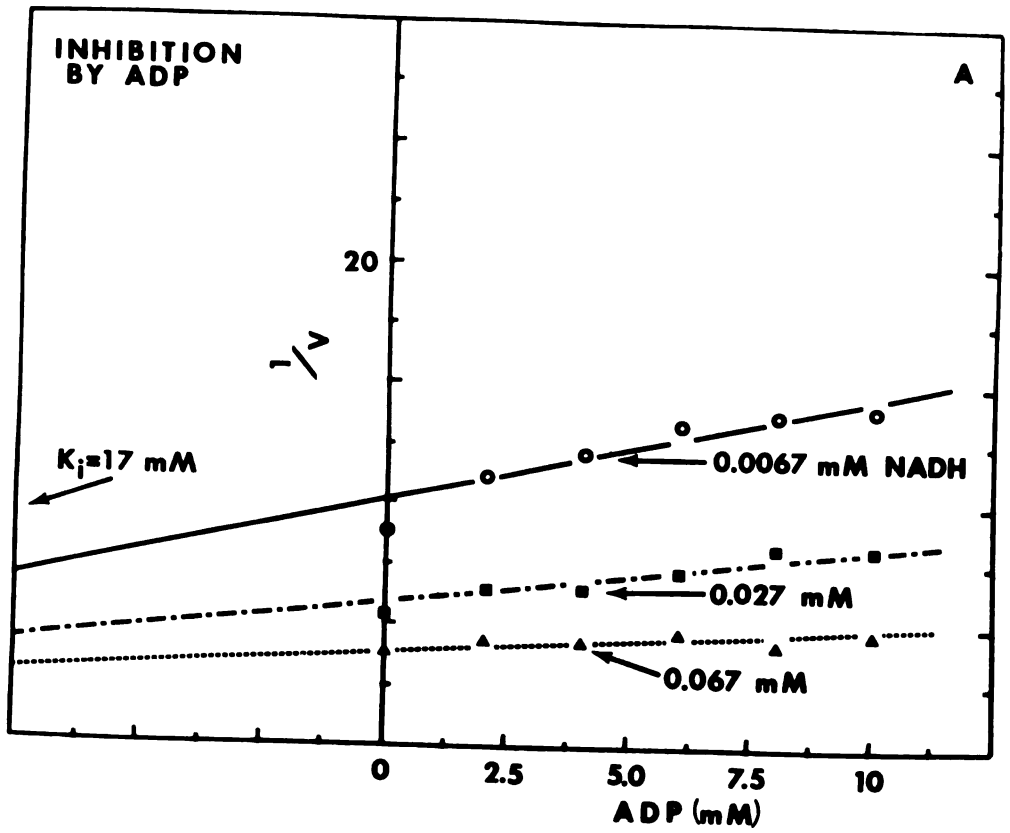

REVERSE ASSAY
INHIBITION
BY ATP
B
100
80
1/v
60
0.05 mM NAD
K_i=8 mM
0.5 mM
0
2.5
5.0
7.5
10
ATP (mM)

Figure 26.--Inhibition of α-Glycerol Phosphate Dehydrogenase by ADP. A. Forward Assay. Conditions same as Figure 25, except ADP was used. B. Reverse Assay. Conditions same as Part A.


INHIBITION
BY ADP
A
20
1/v
K_i=17 mM
0.0067 mM NADH
0.027 mM
0.067 mM
0
2.5
5.0
7.5
10
ADP (mM)

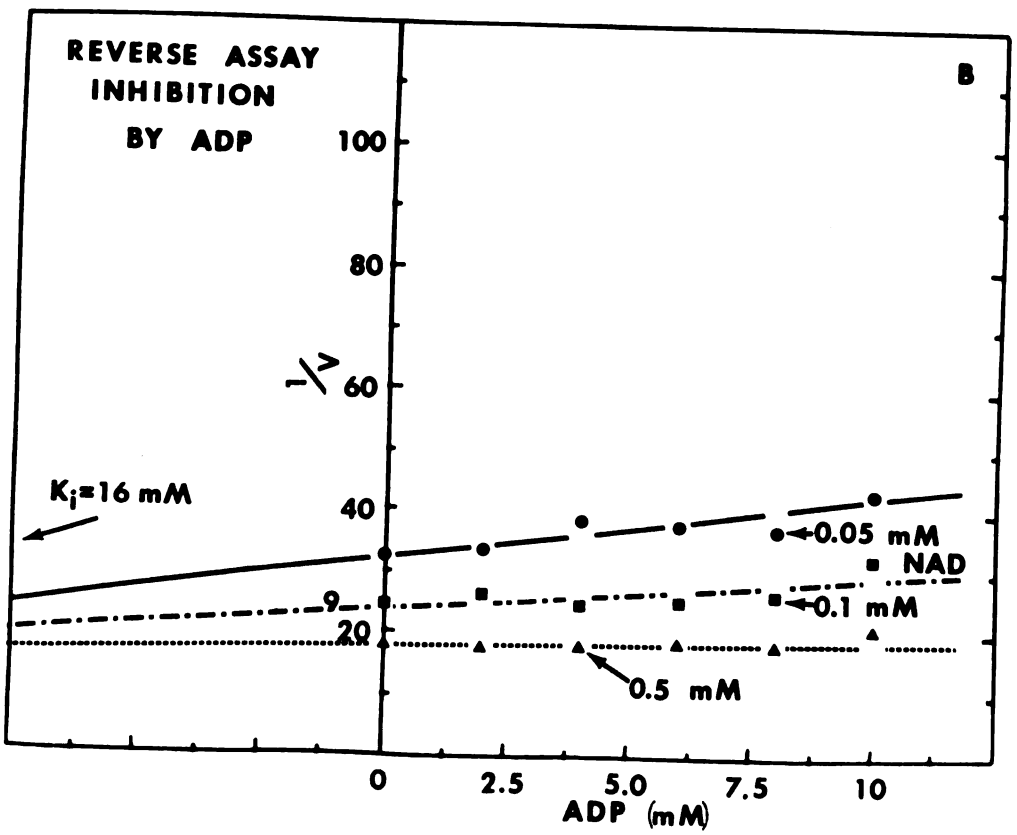
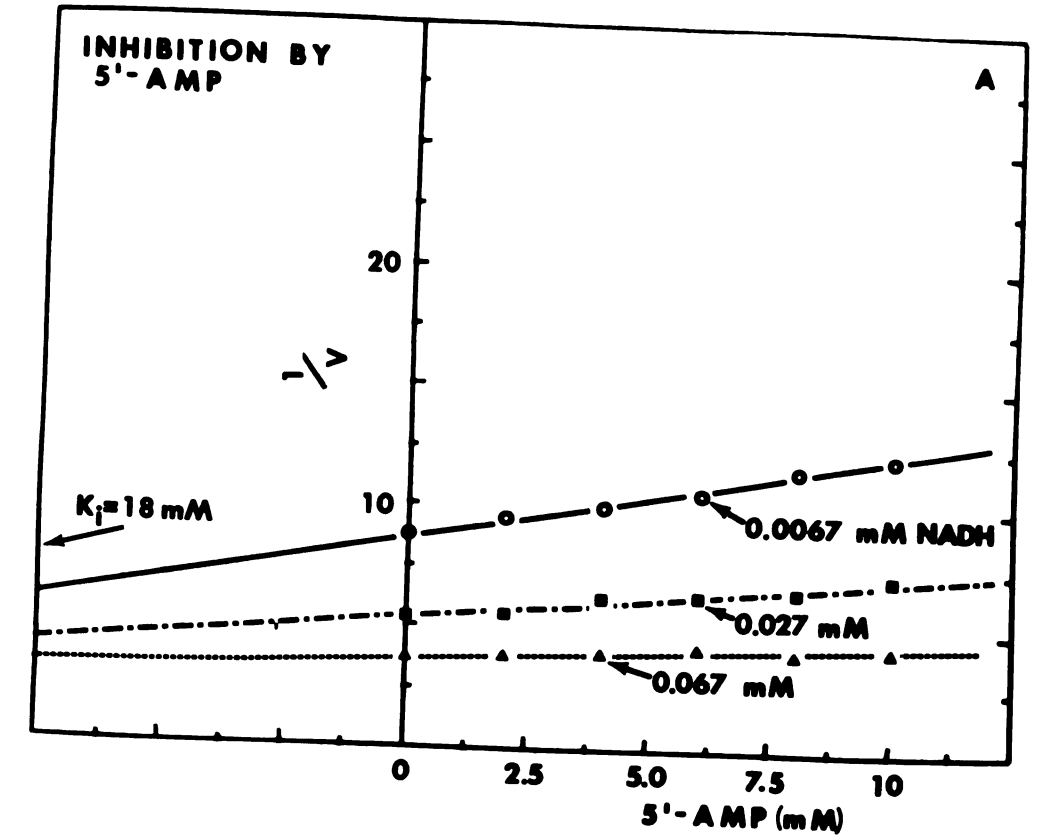
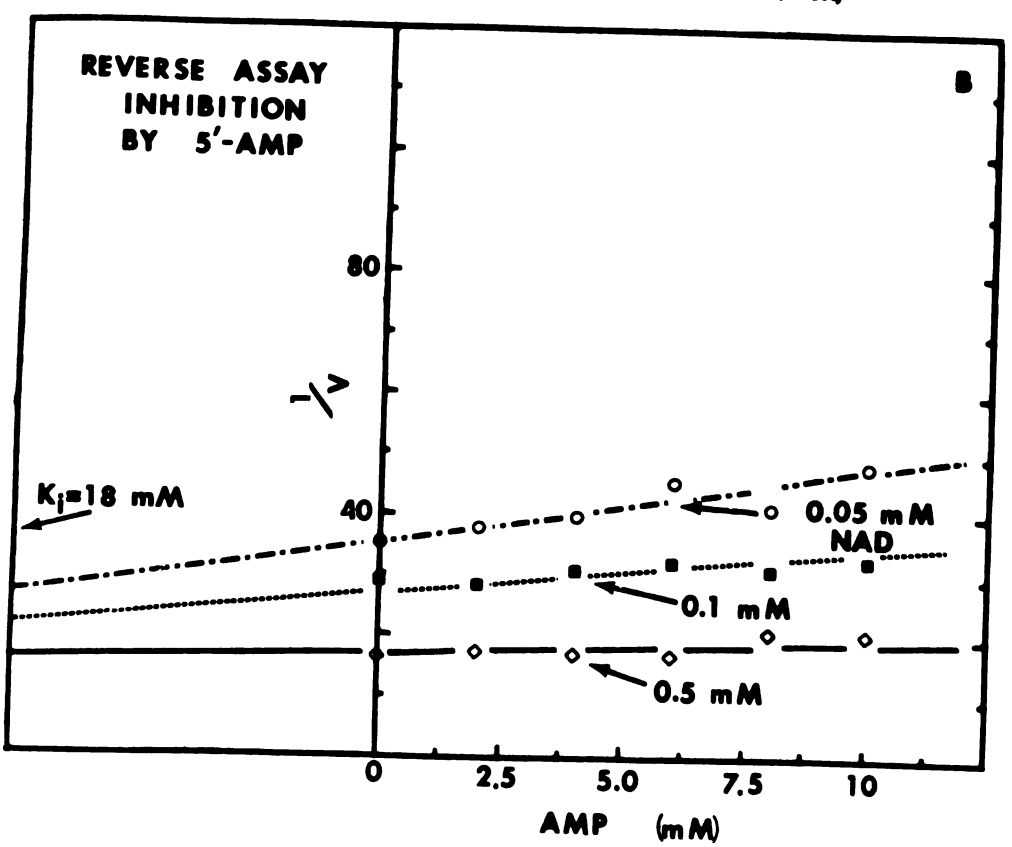

REVERSE ASSAY
INHIBITION
BY ADP
B
100
80
1/v
60
K_i=16 mM
40
0.05 mM
NAD
9
0.1 mM
20
0.5 mM
0
2.5
5.0
7.5
10
ADP (mM)

Figure 27.--Inhibition of α-Glycerol Phosphate Dehydrogenase by 5'-AMP. A. Forward Assay. Conditions same as Figure 25, except 5'-AMP was used. B. Reverse Assay. Conditions same as Part A.


INHIBITION BY
5'-AMP
A
20
1/v
10
K_i=18 mM
0.0067 mM NADH
0.027 mM
0.067 mM
0
2.5
5.0
7.5
10
5'-AMP (mM)


REVERSE ASSAY
INHIBITION
BY 5'-AMP
B
80
1/v
K_i=18 mM
40
0.05 mM
NAD
0.1 mM
0.5 mM
0
2.5
5.0
7.5
10
AMP (mM)

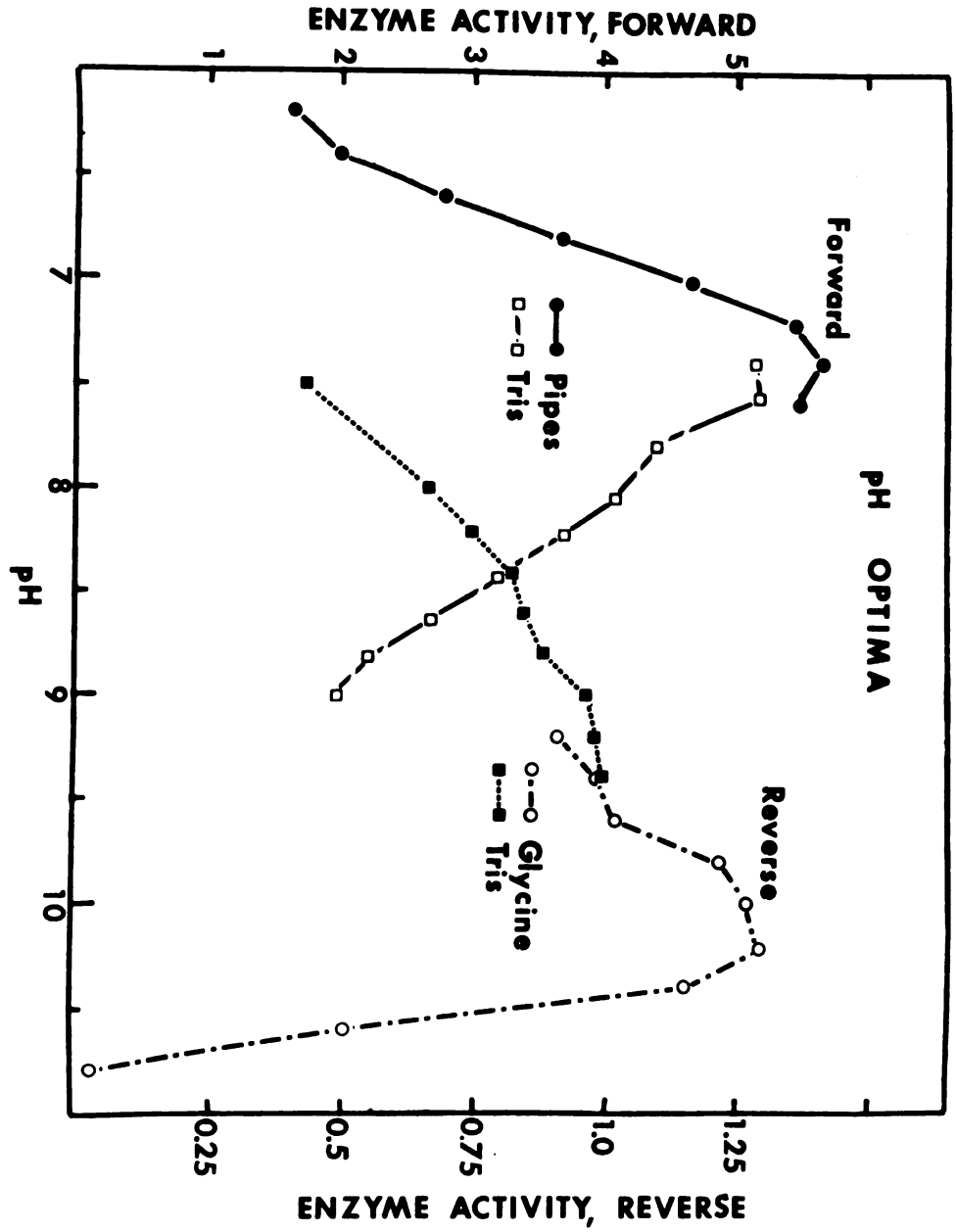
We also tested the following compounds for inhibition in the forward direction at low levels of substrate (0.01 mM NADH and 0.36 mM DHAP): 3',5'-cylic AMP (5 mM), citrate (20 mM), fructose-1,6-diphosphate (10 mM), lactate (10 mM) and acetyl Co A (0.31 mM). No appreciable inhibition (less than 15 percent) was observed under these conditions with these compounds. ATP (10 mM) and ADP (10 mM) inhibited approximately 60 percent and 40 percent, respectively, under these conditions. Several of these compounds were also tested for inhibition in the reverse direction under low substrate conditions (0.05 mM NAD and 0.5 mM L-α-glycerol phosphate). No inhibition was observed with 3',5'-cyclic AMP (5 mM) and lactate (10 mM), but an inhibition was observed with citrate (20 mM) and with FDP (10 mM) both of which seemed to affect the extent of the assay reaction along with the velocity of the reaction.

pH Optima

The pH optima for the reactions in the forward and reverse directions were determined with an enzyme sample which had been purified through the phosphocellulose column step (specific activity = 15). A stock assay mix containing the appropriate buffer (0.05 M), 0.1 M KCl, and substrates (0.7 mM DHAP and 0.25 mM NADH or 8 mM L-α-glycerol phosphate and 5 mM NAD) was prepared and adjusted to the desired pH values by addition of 1 N NaOH or 1 N HCl with a

microliter syringe. For each pH, an aliquot of the assay mix was removed and assays were performed in duplicate immediately. The results obtained are shown in Figure 28. The pH optimum for the reaction in the forward direction is approximately pH 7.5 and that for the reverse direction is approximately pH 10.0. The sharp decrease in enzymatic activity above pH 10.2 was due to inactivation of the enzyme during the assay due to the high pH.

Figure 28.--pH Optima for α-Glycerol Phosphate Dehydrogenase. A stock assay mix containing the appropriate buffer (0.05 M), 0.1 M KCl, and substrates (0.7 mM DHAP and 0.25 mM NADH or 8 mM L-α-glycerol phosphate and 5 mM NAD) was prepared and adjusted to the desired pH's by addition of 1N NaOH or 1N HCl. The assays were performed immediately in duplicate. The enzyme activity is in units of ΔOD/minute.


ENZYME ACTIVITY, FORWARD
1
2
3
4
5
pH
7
8
9
10
Forward
Pipes
Tris
pH OPTIMA
Reverse
Glycine
Tris
0.25
0.5
0.75
1.0
1.25
ENZYME ACTIVITY, REVERSE

CHAPTER VIII

DISCUSSION

Physical and Chemical Characterization

The molecular weight value of approximately 69,000 of the native enzyme is similar to the molecular weights reported for the α-glycerol phosphate dehydrogenases from other sources, which range from 63,000 for the rat muscle and liver enzymes (Fondy et al., 1968, 1971a) to 77,000 for the rabbit muscle (Brosemer, 1969), rabbit liver (Otto et al., 1972) and honeybee (Brosemer, 1969) enzymes. All of the α-glycerol phosphate dehydrogenases for which a subunit structure has been reported are dimers, so the pig liver enzyme is typical in this respect, also.

The pig liver enzyme sediments as a single symmetrical peak (Figure 18) and yields a sedimentation coefficient of $s^{0.5\%}_{20,w}$ = 4.89 S, which agrees quite well with the value of $s^{o}_{20,w}$ = 4.86 S reported for the rabbit muscle enzyme (Holleman, 1966). However, it is higher than the $s_{20,w}$ values of 3.0 S and 3.6 S reported for the rat liver enzyme (Ross et al., 1971) and the $s_{20,w}$ value of 2.8 S value for the rat muscle enzyme (Fondy et al., 1968).

The absorption spectrum observed with the pig liver α-glycerol phosphate dehydrogenase is similar to that of

α-glycerol phosphate dehydrogenases isolated from various sources. The rabbit muscle enzyme has an absorption maximum near 270 nm (van Eys, et al., 1959) and a 280/260 ratio of 1.1 and the rabbit liver enzyme has an absorption maximum near 273 nm and 280/260 ratio of 1.2 (Otto et al., 1972). The rabbit muscle enzyme is known to have a bound nucleotide, but its structure is presently unknown.

The partial specific volume of 0.737 cc/g calculated for the pig liver α-glycerol phosphate dehydrogenase is within the range of 0.70 to 0.76 cc/g normally observed for proteins. However, it is lower than the value of 0.747 cc/g reported for the rabbit and rat muscle enzymes (Holleman, 1966; Fondy et al., 1968). Ross et al. (1971) reported values of 0.744 and 0.737 cc/g for the two forms of the rat liver enzyme.

Kinetic Characterization

While our studies seem to indicate that the enzyme has an Ordered Bi Bi mechanism, they have not ruled out the Theorell-Chance mechanism. It would be necessary to do product inhibition studies with DHAP as the variable substrate to definitely prove this. However, since this mechanism was ruled out for the rabbit muscle enzyme, we can probably rule it out for the liver enzyme, also.

Kim and Anderson (1968) studied the inhibition of the rabbit muscle enzyme by several adenine nucleotides.

They studied the inhibition of NAD binding at pH 7.85 and found the following inhibition constants: adenosine, 4.3 m$\underline{M}$; 5'-AMP, 2.1 m$\underline{M}$; ADP, 0.7 m$\underline{M}$ and ADP-ribose, 0.2 m$\underline{M}$. They did not mention inhibition by ATP and did not discuss any possible in vivo significance of their findings, since they were primarily interested in studying the binding of NAD to the enzyme molecule. The inhibition of pig liver α-glycerol phosphate dehydrogenase by ADP and 5'-AMP is much different. The inhibition constants for ADP and 5'-AMP are 16 m$\underline{M}$ and 18 m$\underline{M}$, respectively, for the pig liver enzyme.

The finding in our studies that the inhibition constant in the forward reaction for each nucleotide is approximately equal to that in the reverse reaction would seem to indicate a simple, competitive inhibition where the K_i is actually the equilibrium constant for the binding of the nucleotide to the enzyme. Since the adenine nucleotides inhibit competitively with the pyridine nucleotides, it is likely that they are binding to the same site on the enzyme. Hence, the adenosine moiety in the pyridine nucleotide plays an important role in binding the nucleotide to the enzyme. However, the "adenine-phosphate" portion of the binding site is not the major factor, since the pyridine nucleotides are bound 100 to 1000 times as tightly as are the adenine nucleotides. Further, since cyclic AMP does not significantly inhibit the enzyme, it appears that the adenine ring itself plays only a minor role in the binding of the nucleotide to the enzyme.

This inhibition of the enzyme by ATP, ADP and AMP appears to be quite significant in playing a role in the control of the enzyme *in vivo*. Its importance is dependent on the levels of NADH and the adenine nucleotides in the cell, however. There are major problems involved in estimation of the level of free NADH in a cell, but it appears that the concentration is in the range of 1 to 10 μmoles per kg (1 to 10 μM) (Biochemists' Handbook; Krebs, 1967; Willms *et al.*, 1970) for liver from fed animals. The levels of the adenine nucleotides are easier to estimate and are approximately 3 μmoles per g for ATP, 0.6 μmoles per g for ADP and 0.1 μmoles per g for AMP (mM), (Willms *et al.*, 1970; Williamson and Corkey, 1969). The levels of ADP and AMP are not high enough to give significant inhibition and control the enzymatic activity. However, the ATP concentration is high enough to be important if the NADH level is near the low end of the concentration range mentioned (1 to 4 μM). One can calculate the inhibition expected using the equation:

$$v = \frac{V}{1 + K_{m/s}\,(1 + i/K_i)}$$

Assuming K_m = 8 μM, NADH = 2 μM, K_i = 6 mM and ATP = 3 mM, an inhibition of 40 percent would be observed. Any inhibition present could be easily overcome by an increase in the NADH concentration however, as might occur during an increased glycolytic flow.

The pH optima of 7.5 and 10.0 observed are similar

to those reported for α-glycerol phosphate dehydrogenase from other sources. Young and Pace (1958) reported optima of 7.4 and 10.0 for the rabbit muscle enzyme, Lehmann and Pfleiderer (1968) reported an optimum of 7.8 for the human liver enzyme in the forward direction and Marquardt and Brosemer (1966) reported an optimum of 6.5 for the forward reaction of the honeybee enzyme.

Effects of NAD and NADH on the Enzyme

Several important effects of NAD and NADH on the pig liver enzyme have been observed during these studies and some have been discussed earlier in this thesis. They protect the enzyme against heat inactivation, prevent dissociation at low protein concentration, affect both the electrophoretic pattern and activity of the enzyme on disc gel electrophoresis, affect both the stability and chromatographic behavior of the enzyme on ion exchange chromatography and control the inhibition of the enzyme by ATP and other adenine nucleotides. These effects are quite significant and indicate the importance of the pyridine nucleotides in maintaining the structure and activity of the enzyme.

While this report of stabilization of α-glycerol phosphate dehydrogenase is not unique, the effects of NAD on the binding of the enzyme during ion exchange chromatography and the effects of NADH on electrophoresis of the enzyme have not been reported before. During our work on

the purification of pig liver α-glycerol phosphate dehydrogenase, the reports of the use of NAD during the heat step in the human liver enzyme and the use of NADH during the heat step in the purification of the chicken liver enzyme were published. However, none of the published purification procedures utilize NAD during column chromatography steps.

Effect of NAD on Heat Inactivation

Protection against heat denaturation may merely be a laboratory phenomenon, but it is quite possible that this protection is physiologically significant. The heat denaturation studies were performed at 49° instead of 37° primarily because the rate of denaturation of the enzyme at 49° is such that the protection could be studied easily. Even though the rate at 37° would be lower, it should still be significant. From interpolation between the denaturation rate at 4° (half-life = 48 hours) and at 49° (half-life = 2.5 minutes), we estimate that the half-life of denaturation at 37° of α-glycerol phosphate dehydrogenase in the absence of NAD or NADH would be in the range of 20 minutes under our experimental conditions (phosphate buffer, pH 7.8). The levels of NADH *in vivo* are probably too low to protect the enzyme against heat denaturation, but the levels of NAD are high enough that they would protect the enzyme against simple heat denaturation quite well. The NAD concentration *in vivo* in rat and guinea pig liver is approximatey 0.5 mM (Williamson and Corkey, 1969; Willms *et al.*, 1970); under

these conditions the protection would be nearly complete, maintaining a high level of active enzyme. But if the NAD concentration were to drop below 0.2 mM for any reason, the loss of enzyme *in vivo* due to heat inactivation could become quite significant. Although studies on protection against proteolytic degradation by the nucleotides have not been performed, it is also quite possible that high levels of NAD and NADH would protect the enzyme against this type of denaturation. Protection against these two types of denaturation could play a major role in determining the level of active enzyme in the cell.

It is difficult to decide whether the "protective" nucleotide binding sites are identical to the catalytic binding sites or not. The only pertinent evidence available is the data for the binding constants from the kinetic data and the denaturation data. The K_m for NAD was determined at pH 10.0, so it is of little use in this comparison. However, a K_m of 80 mM at pH 7.6 was estimated from the adenine nucleotide inhibition data. Also, one can determine the K_i for NAD at pH 7.6 from the product inhibition studies (page), by replotting the data in the form of 1/v versus NAD at the various levels of NADH (not shown). This is the standard Dixon plot, and it gives a value of approximately 0.5 mM. This is about four fold higher than the binding constant determined from the heat denaturation data, 0.125 mM. These values are somewhat different, but this could be

due to differences in pH (7.6 for kinetics and 7.8 for heat denaturation), buffer species and ionic strength. However, there is a large difference in the binding constant for NADH as determined by the two methods. The binding constant (K_{ia}) for NADH from the kinetic data is approximately 2 μM (Plowman, 1972, page 139). This value is the point at which the lines intersect in the initial velocity studies (Figure 22). Even though the heat denaturation data for NADH could not be analyzed because of complications, we estimate a binding constant of 0.05 to 0.15 mM from inspection of the data shown in Figure 7. This is 100 fold greater than the binding constant from the kinetic data and this difference is much too large to be due to differences in the buffer or pH. A reasonable explanation for this large difference is that the large difference in temperature causes a rather drastic change in the conformation of the enzyme, which in turn changes the affinity of the enzyme for the nucleotide. To test this hypothesis, however, further binding experiments would be necessary.

Effect of NADH on Dissociation at Low Protein Concentration

This effect was observed during the high speed sedimentation equilibrium experiments. In all three samples without NADH, there was clearly low molecular weight material present (Figure 15), but in the presence of 0.5 mM NADH, there was no evidence of low molecular weight material.

Also, an earlier experiment using material purified through the sucrose gradient step (specific activity = 90) indicated that 0.5 mM NAD also prevented the dissociation at low protein concentration. Nucleotides may protect the enzyme from heat inactivation by preventing dissociation. It is quite possible that the monomer is less stable to heat than the native dimer. If so, the binding of the nucleotide to the enzyme would prevent the stable dimer from dissociating to unstable monomer, thereby greatly slowing down the inactivation of the enzyme.

Since the intercellular α-glycerol phosphate dehydrogenase concentration is on the order of 0.1 to 0.3 mg/ml (calculated from the crude homogenate levels observed and an assumed specific activity of 100 units/mg), the dissociation could occur in vivo but would be somewhat limited.

Effects of NADH on Electrophoretic Behavior

The effects of NADH on the enzyme in disc gel electrophoresis experiments were quite important in the purification of α-glycerol phosphate dehydrogenase. The use of NADH in the running buffer was critical to the success of the preparative disc gel electrophoresis step.

The finding that electrophoresis of crude or partially purified enzyme in the presence of NADH increases the number of activity bands was quite interesting. An enzyme sample of high apparent purity (purified through the sucrose density gradient; specific activity = 90 and one

peak in the Model E) electrophoresed in the absence of NADH showed at least 8 protein bands, but only 2 activity bands (Figure 21. When the sample was electrophoresed in the presence of NADH, 6 of the bands had activity. Several electrophoretic forms of the enzyme are not active unless electrophoresed in the presence of NADH. Apparently, the NADH binds to these forms during electrophoresis and prevents their denaturation. NADH also protects those forms of the enzyme which retain activity when electrophoresed in the absence of NADH, since it is necessary to stain gels electrophoresed in the absence of NADH at least twice as long as gels electrophoresed in the presence of NADH to obtain activity bands of equal intensity.

Ross et al. (1971) reported the existence of several forms of α-glycerol phosphate dehydrogenase in rat liver. The forms were separated by isoelectric focusing on polyacrylamide gels, and the enzymatic activity was visualized by using an activity stain similar to the one used in this thesis. They observed five different forms of the enzyme using this technique and were able to purify the two major forms. They found that the enzymes were similar kinetically and had similar molecular weights, but differed in their amino acid compositions.

Effects of NAD on Ion Exchange Chromatography

Early attempts to purify the enzyme using ion exchange chromatography were futile because no NAD was used in the

buffers. DEAE, sulfoethyl, carboxymethyl and phosphate ion exchangers were tried, and in every case, enzymatic activity could be recovered only if the enzyme did not bind to the column. If the enzyme did bind, no activity could be recovered. Later, the protective effect of NAD was found and the two ion exchange steps finally incorporated into the purification procedure were worked out. The simplest explanation for the instability of the enzyme toward ion exchange chromatography is that there is a portion of the native enzyme molecule--either a small molecule, such as a nucleotide, or a small peptide--which is necessary for the enzyme to be active that is removed from the enzyme during chromatography. In the presence of NAD, this removable portion is bound to the enzyme more tightly and the enzyme can be eluted from the column in an active form. A second explanation is that there is a reactive group on the enzyme that is modified while the enzyme is bound to the column; when NAD is bound to the enzyme, this group is masked and protected.

Another effect of NAD on the chromatographic behavior was a change in the binding of the enzyme to the phosphocellulose with a change in the NAD concentration. In chromatography experiments with 0.1 m$\underline{M}$ NAD present, the enzyme bound to the column in the presence of 0.02 $\underline{M}$ KCl (3.7 mS) and eluted from the column at approximately 0.10 $\underline{M}$ KCl (13 mS). However, if 0.3 m$\underline{M}$ NAD was present, then it

was necessary to use 5 mM KCl (1.34 mS) initially and the enzyme eluted near 0.065 M KCl (8.8 mS). This different behavior of the enzyme in the two systems was quite reproducible. Approximately two-fold higher yields were obtained in the presence of 0.3 mM NAD, and this required that the conditions for chromatography be redefined for the new system.

Effect of NAD on Inhibition by Adenine Nucleotides

Since the K_m for NADH is in the range of NADH concentrations observed in liver, a major controlling factor in the inhibition of the enzyme by ATP *in vivo* is the NADH level. Earlier in this thesis, it was calculated that under well-fed conditions (NADH = 2 μM and ATP = 3 mM), the enzyme would be inhibited 40 percent. However, if the NADH level increased to 10 μM, as might occur under fasting or fat-fed conditions (Krebs, 1967; Willms *et al.*, 1970) then the inhibition would be decreased to 20 percent. Accompanying this decrease in inhibition would be a three-fold increase in the overall rate of the enzyme reaction, due only to the increase in the NADH concentration near the K_m. These two effects would combine to play an important role in the modulation of the enzymatic activity *in vivo*.

Implications for Further Research

Multiple Forms of Pig Liver α-Glycerol Phosphate Dehydrogenase

There are several interesting areas of study involving the multiple forms of the enzyme that should be

investigated. The different forms may be produced during different stages of development, but we have not been able to show this in our brief experiments with livers from pigs of different ages. More extensive studies with fetal and newborn pigs should show if they are different developmental forms. Whatever the source of the various forms, it would be interesting to investigate which forms are present in one or more hepatomas. Such a study would show whether the decrease in the α-glycerol phosphate dehydrogenase activity in the tumors is due to a decrease in the levels of several or all the forms or to a decrease in or disappearance of only the major forms.

One of the extra forms is partially purified during the preparative disc gel electrophoresis step (Figure 12, fractions 36 through 38). While this enzyme fraction does not have a high specific activity (fraction 36 has a specific activity of 23) it may be quite pure. Analytical disc gel electrophoresis should show its purity; if it is grossly impure, a second preparative disc gel electrophoresis step using a lower gel concentration should increase the purity. This form of the enzyme could then be studied to see how it differs from the major form isolated and characterized in this thesis. If this second form can be obtained in pure form, experiments should be performed to see if it can be converted to another form; no conversion of the purified major form to any of the other forms was observed in our studies.

Binding of NAD and NADH to Pig Liver α-Glycerol Phosphate Dehydrogenase

Earlier, the question of the identity of the "catalytic" nucleotide binding sites and the "protective" nucleotide binding sites was discussed. This question could be answered using fluorescence titration of the enzyme with NADH. If there are two types of NADH binding sites with binding constants of 2 μM and 75 μM as suggested by the kinetic and heat denaturation data, they should be clearly observable with this technique. However, if there is only one type of site, and the weak binding of NADH to the enzyme observed in the heat denaturation studies is due to the high temperature, only one type of site should be observed with the fluorescence studies. The titrations could best be studied following the fluorescence of bound NADH activated by energy transfer from the protein molecule or the change in the fluorescence of the protein upon binding of the nucleotide (Velick, 1958).

An alternative method of studying the question is performing both the heat denaturation studies and the kinetic studies at the same temperature, such as 37°.

APPENDIX

APPENDIX

Derivation of Equation 1

(Patterned after Kuczenski and Suelter, 1970, 1971)

Assuming the successive equilibria

$$E + NAD \rightleftarrows E\text{-}NAD \qquad K_I = \frac{[E]\ [NAD]}{[E\text{-}NAD]}$$

$$E\text{-}NAD + NAD \rightleftarrows E\text{-}NAD_2 \qquad K_{II} = \frac{[E\text{-}NAD]\ [NAD]}{[E\text{-}NAD_2]}$$

through

$$E\text{-}NAD_{n-1} + NAD \rightleftarrows E\text{-}NAD_n \qquad K_n = \frac{[E\text{-}NAD_{n-1}]\ [NAD]}{[E\text{-}NAD_n]}$$

If $K_I > K_{II} > K_n$, then

$$K_I + K_{II} + - - + K_n = [NAD]\left(\frac{[E]}{[E\text{-}NAD]} + \frac{[E\text{-}NAD]}{[E\text{-}NAD_2]} + - - - + \frac{[E\text{-}NAD_{n-1}]}{[E\text{-}NAD_n]}\right)$$

$$\text{since} \quad \left(\frac{[E]}{[E\text{-}NAD]} + \frac{[E\text{-}NAD]}{[E\text{-}NAD_2]} + - - - + \frac{[E\text{-}NAD_{n-1}]}{[E\text{-}NAD_n]}\right) = \frac{1 - \alpha}{\alpha}$$

then taking logarithms

$$pK_I + pK_{II} + - - - + pK_n = p\ [NAD]\ \log \frac{\alpha}{1 - \alpha}$$

or, rearranging

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

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