



Escherichia coli B glucose-6-phosphate dehydrogenase : purification and characterization of the enzyme

by Kenneth Geoffrey Dennison Allen

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Biochemistry

Montana State University

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Abstract:

Escherichia coli B glucose-6-phosphate dehydrogenase has been purified by a seven stage procedure involving column chromatography and fractionations. The enzyme was purified 1157-fold to a final specific activity of 82 units per milligram measured at pH 7.4.

The purified dehydrogenase was found to consist of five electrophoretic species, all of which exhibited enzymic activity. Denaturation in 8 M urea or one percent sodium dodecyl sulphate and one percent mercaptoethanol resulted in a single protein band upon electrophoresis. The purified enzyme was found to have a subunit molecular weight of 53×10^3 daltons by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and 26.50×10^3 daltons by ultracentrifugation in the presence of 6 M guanidine-hydrochloride. Equilibrium between these two values was observed in 6 M guanidine-hydrochloride. The enzyme exhibited an activity optimum at pH 8.5 with enzymic activity 2.27-fold of the value at pH 7.4. Maximal stability of the enzyme was observed below pH 8.0. Treatment with the sulphhydryl reagents, p-chloro-mercuribenzoate and N-ethyl maleimide implicated a cysteine residue involved at the active site of the enzyme. The N-terminal amino acid of the dehydrogenase was found to be methionine by chromatography of dansyl derivatives. Kinetic investigations of the purified enzyme gave values for the Michaelis-Menten constants of NADP⁺ and G6P as 1.80×10^{-5} moles and 2.23×10^{-4} moles respectively. Competitive inhibition of the enzyme activity was observed with ATP and NADPH. NAD⁺ appeared to be a noncompetitive inhibitor for both the substrates, and analysis of the kinetic data by Hill plots further implicated an allosteric role for NAD⁺ in controlling glucose-6-phosphate dehydrogenase activity. ''

ESCHERICHIA COLI B GLUCOSE-6-PHOSPHATE DEHYDROGENASE:
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KENNETH GEOFFREY DENNISON ALLEN

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Biochemistry

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ABSTRACT

Escherichia coli B glucose-6-phosphate dehydrogenase has been purified by a seven stage procedure involving column chromatography and fractionations. The enzyme was purified 1157-fold to a final specific activity of 82 units per milligram measured at pH 7.4. The purified dehydrogenase was found to consist of five electrophoretic species, all of which exhibited enzymic activity. Denaturation in 8 M urea or one percent sodium dodecyl sulphate and one percent mercaptoethanol resulted in a single protein band upon electrophoresis. The purified enzyme was found to have a subunit molecular weight of 53×10^3 daltons by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and 26.50×10^3 daltons by ultracentrifugation in the presence of 6 M guanidine-hydrochloride. Equilibrium between these two values was observed in 6 M guanidine-hydrochloride. The enzyme exhibited an activity optimum at pH 8.5 with enzymic activity 2.27-fold of the value at pH 7.4. Maximal stability of the enzyme was observed below pH 8.0. Treatment with the sulphhydryl reagents, p-chloro-mercuribenzoate and N-ethyl maleimide implicated a cysteine residue involved at the active site of the enzyme. The N-terminal amino acid of the dehydrogenase was found to be methionine by chromatography of dansyl derivatives. Kinetic investigations of the purified enzyme gave values for the Michaelis-Menten constants of NADP^+ and G6P as 1.80×10^{-5} moles and 2.23×10^{-4} moles respectively. Competitive inhibition of the enzyme activity was observed with ATP and NADPH. NAD^+ appeared to be a noncompetitive inhibitor for both the substrates, and analysis of the kinetic data by Hill plots further implicated an allosteric role for NAD^+ in controlling glucose-6-phosphate dehydrogenase activity.

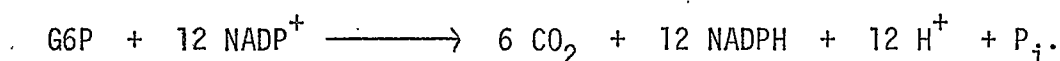
INTRODUCTION

All organisms require energy to sustain the complex array of chemical reactions that constitute a living cell. Heterotrophic organisms, such as animals, fungi and most bacteria, require a supply of organic compounds from their environment for utilization as sources of energy. Ultimately this energy is obtained by oxidation-reduction reactions in the cell. The most commonly employed organic compounds for use as energy sources are the six-carbon sugars, and in particular D-glucose. The initial stage of energy production from glucose common to all heterotrophic organisms is the sequence of anaerobic reactions known as glycolysis. The end product of the glycolytic pathway, pyruvic acid, can be further oxidized under aerobic conditions by the series of enzymic conversions known as the Kreb's tricarboxylic acid cycle (TCA cycle). This TCA cycle is the final common oxidative catabolic pathway of all carbohydrate molecules under aerobic conditions, and coupled to oxidative phosphorylation provides for the production of ATP, the main cellular high energy compound utilized in biochemical processes.

Another pathway of glucose catabolism is possible via the hexose monophosphate shunt (HMP shunt), also known as the pentose phosphate or phosphogluconate pathway. This pathway of glucose metabolism branches off from glycolysis at the common intermediate glucose-6-phosphate (G6P) and provides for the metabolism of glucose independently of the TCA cycle. The branch point at G6P and the possibility of

utilization of this compound by phosphoglucoseisomerase, via glycolysis, or by glucose-6-phosphate dehydrogenase, through the HMP shunt, provides the cell with the first "choice" of pathways in catabolic carbohydrate metabolism. The mechanism of control of the direction of glucose utilization must be linked to the particular metabolic needs of the cell.

The HMP shunt provides for the complete oxidation of glucose-6-phosphate to carbon dioxide in which six molecules of G6P are oxidized to yield six molecules each of ribulose-5- P_4 and carbon dioxide; the net reaction can be considered as

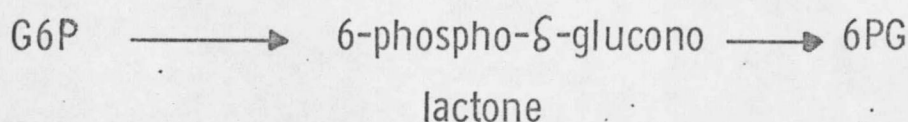
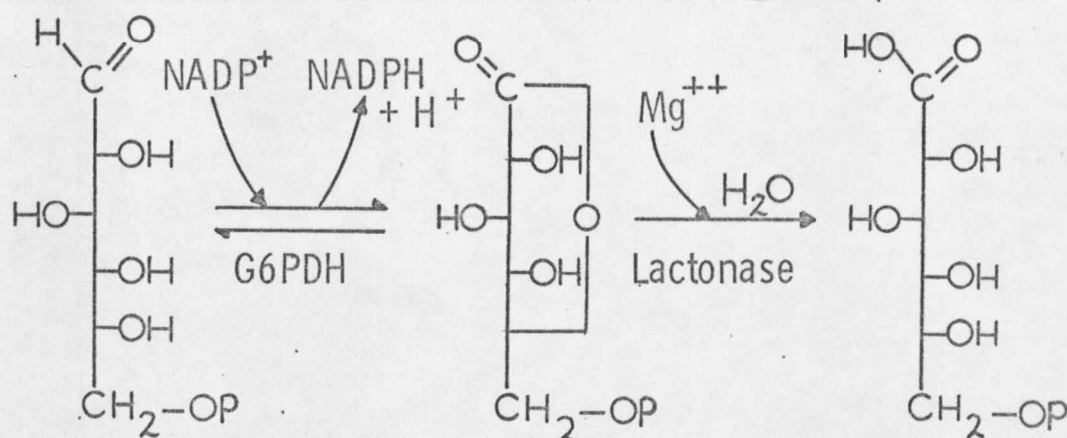


The reduced dinucleotide phosphate when coupled to oxidative phosphorylation would result in the formation of ATP. It is doubtful, however, that this complete oxidative degradation of G6P occurs in the cell. An important consequence of the HMP shunt enzymes is that they facilitate, in concert with the glycolytic sequence, the reversible interconversion of three-, four-, five-, six- and seven-carbon sugars via transaldolase and transketolase reactions.

The study reported here is concerned with the purification and properties of the first enzyme of the HMP shunt, namely glucose-6-phosphate dehydrogenase (G6PDH) [EC. 1.1.1.49]. This enzyme is of particular interest and importance since it catalyzes the dehydrogenation of

glucose-6-phosphate, the branch point intermediary common to both glycolysis and the HMP shunt and is a presumptive point for the control and regulation of cellular metabolism.

The dehydrogenation of glucose-6-phosphate results in the production of 6-phospho- δ -gluconolactone. This enzymic oxidation requires the presence of the coenzyme nicotinamide adenine dinucleotide phosphate (NADP^+) as the hydrogen acceptor. The δ -lactone of 6-phosphogluconic acid is hydrolyzed in vivo by gluconolactonase to produce the free acid.



$$\Delta G^{\circ'} = -0.1 \text{ Kcal/mole}$$

$$\Delta G^{\circ'} = -5.0 \text{ Kcal/mole}$$

The glucose-6-phosphate dehydrogenase catalyzed oxidation of glucose-6-phosphate is readily reversible with $\Delta G^{\circ} = -.1 \text{ kcal mole}^{-1}$ for the reaction. Reversibility has been demonstrated with the yeast enzyme utilizing 6-phospho- δ -gluconolactone and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrates.¹ The hydrolysis of the 6-phospho- δ -gluconolactone acid is highly exergonic, $\Delta G^{\circ} = -5 \text{ kcal mole}^{-1}$, and the equilibrium strongly favors the free acid. The reaction is rapid at physiological pH, and the half-time for the hydrolysis of the lactone ring is 1.5 minutes at pH 7.4.¹ Nevertheless, specific gluconolactonases, which catalyze this lactone hydrolysis, have been identified from numerous bacterial sources. Recently Kupor and Fraenkel² have shown that an Escherichia coli mutant lacking the gluconolactonase enzyme, has only slightly impaired growth rate, and glucose-6-phosphate is still metabolized via the HMP shunt, since the δ -lactone of phosphogluconic acid hydrolyzes spontaneously.

Originally it was assumed that the HMP shunt functioned primarily to provide ribose for the synthesis of nucleotides,⁷ but recently this rationale has come under question. In Escherichia coli the formation of pentoses from glucose occurs by both an oxidative and non-oxidative pathway. The oxidative formation of pentoses is brought about by the HMP shunt, and the non-oxidative synthesis of pentoses arises by transaldolase and transketolase mediated hexose and pentose interconversions. Recently quantitative studies have been undertaken to eluci-

date the relative contribution of these two pathways to the pentose pool. Numerous studies involving C^{14} labeled glucose³⁻⁷ have shown that the HMP shunt accounts for only 10-40% of the glucose metabolized by E. coli. Caprioli and Rittenburg⁷ have shown in studies using $1-0^{18}$ -glucose as the sole carbon source that the labeling pattern in the nucleosides indicated that 70% of the ribose resulted from non-oxidative transketolase catalyzed condensation. The oxidative pathway, namely the HMP shunt, accounted for only 30% of the ribose found in both PNA and DNA. These studies on the origin of cellular ribose throw doubt on the original assumptions for the function of the HMP shunt. Thus it appears that the majority of the ribose in DNA and RNA arises from a non-oxidative pathway.^{6,7} If this is indeed the case, then it is necessary to question the major role of the HMP shunt in the catabolism of glucose. Studies with E. coli, yeast (Candida utilis) and Neurospora crassa have recently shed light on this role. Model and Rittenburg,⁸ in labeling studies with E. coli have shown that a decrease in amino acid biosynthesis resulting from nitrogen starvation causes a concomitant decrease in the utilization of the HMP shunt, and this effect was reversible upon resupplying NH_4^+ to the media. In addition, an E. coli mutant capable of using ribose as the sole carbon source was cultured, and the utilization of the hexose MP shunt was not diminished by the presence of ribose in the culture media. These findings support the postulate that the HMP shunt exists to provide reduced nicotinic adenine

dinucleotide phosphate (NADPH) for biosynthetic purposes, such as the synthesis of amino acids from ammonia, and lipid biosynthesis.

Fuscaldo⁹ et al. have investigated the role of the HMP shunt in Neurospora crassa by the study of HMP mutants on colonial morphology.

Two single site mutants were isolated; one produced a defective G6PDH and the other produced a defective 6-phosphogluconate dehydrogenase (6PGDH), the second enzyme in the HMP shunt. Both of these mutations resulted in reduced participation of the HMP shunt in glucose metabolism.

The morphology of either of the mutants reverted to the wild type by growth on a media supplemented with a carbon source, such as glutamate or acetate, that result in production of NADPH by an alternate pathway.

No phenotypic reversion to wild type morphology was exhibited when either of these mutants was supplemented with ribose as a carbon source. Similar results have been obtained by Osmond and Rees¹⁰ on the HMP shunt of Candida utilis. These studies strongly indicate that

the major role of the HMP shunt is the production of NADPH. Hanson and Leveille¹¹ have suggested that as much as 50% of the cellular NADPH requirement may be provided by the first two dehydrogenases of the HMP shunt. This rationale on the role of the HMP shunt is supported in mammalian systems by its localization in tissues associated with lipid biosynthesis.

Since G6P is the first carbohydrate intermediary common to two metabolic pathways, it can be degraded by phosphoglucose isomerase and

other enzymes of the glycolytic sequence, or by G6PDH through the HMP shunt. The optional utilization of G6P by these two pathways leads to the question of the control of the route of G6P catabolism, and the factors governing the pathway of metabolism of this intermediary. The genetics of E. coli G6PDH and 6PGDH, the first two dehydrogenases of the HMP shunt, have been investigated by Fraenkel and coworkers.¹² These two enzymes are constitutive and their synthesis is at a constant level, unaffected by growth conditions or substrate levels.¹² This does not imply that the utilization of the HMP shunt is unaffected by variations in cellular substrate concentrations, or by the particular metabolic requirement of the cell. Peren and Fraenkel¹² have mapped the loci for the structural genes of G6PDH and 6PGDH by conjugation and transduction experiments in both E. coli and Salmonella typhimurium. In both organisms the loci for the dehydrogenases appeared in the same region of the chromosome but they were not closely linked. In addition, the structural loci for both G6PDH and 6PGDH did not exhibit any linkage to enzymes of glycolysis such as phosphoglucoseisomerase or phosphofructokinase. These results clearly showed that the metabolic linkage of the first two dehydrogenases of the HMP shunt is not reflected in any genetic linkage. These findings in conjunction with the constitutive nature of the activities, precludes any operon control in the classical manner proposed by Jacob and Monod.¹³ Fraenkel and Banjerjee¹⁴ have reported increased levels of G6PDH synthesis in a mutant of E. coli.

This mutant mapped very near to the normal E. coli B wild type locus and the enzyme produced was indistinguishable from the normal protein. Several authors^{8,9,10,15} have attempted to explain control of the HMP shunt in terms of increased or decreased variations of activities of the shunt enzymes acting in response to cellular requirements for NADPH. Both in vivo studies and experiments with cellular extracts have been attempted in order to account for the control of catabolism in the demonstrated absence of genetic control.

G6PDH, or Zwischernferment as it is called, was first identified by Warburg and Christian in 1931.¹⁶ Their investigations led to the discovery of nicotinamide adenine dinucleotide phosphate as an obligatory coenzyme. An active preparation was originally obtained from erythrocytes, and also from Brewer's Yeast (Saccharomyces carlsbergensis).¹⁷ Since these initial studies, the enzyme has been isolated from a wide variety of sources, and several purifications have been accomplished from sources as varied as mammalian mammary glands²³ and molds such as Neurospora crassa.^{18,19,20,21,22,24,25,26} These purifications involved fractionation of crude cellular extracts and chromatography on a wide range of adsorbants.

Escherichia coli B. G6PDH has been partially purified by Scott²⁷ and Sanwall.¹⁵ The initial work by Scott and Cohen²⁷ resulted in a crude extract of the E. coli enzyme, and Sanwall¹⁵ has reported a 170-fold purification from the same source.

MATERIALS AND METHODS

Biochemical Reagents and Supplies

Bacto nutrient broth	Difco Laboratories
Levigated alumina	Norton Company
β -Mercaptoethanol (β ME)	Eastman Kodak Company
Ethylene diamine tetracetic acid (EDTA) disodium salt	Eastman Kodak Company
Tris (hydroxymethyl) amino methane (TRIS)	Sigma Chemical Company
Nicotinamide adenine dinucleotide (NAD^+), sodium salt	Calbiochem
Nicotinamide adenine dinucleotide phosphate (NADP^+) sodium salt	Calbiochem
Nicotinamide adenine dinucleotide, reduced (NADH)	Calbiochem
Nicotinamide adenine dinucleotide phosphate, reduced (NADPH) tetrasodium salt	Calbiochem
Glucose-6-phosphate, disodium salt (G6P)	Calbiochem
6-Phosphogluconate, sodium salt (6PG)	Calbiochem
Adenosine 5' triphosphate, dipotassium salt	Calbiochem
Adenosine 5' monophosphate, sodium salt	Calbiochem
Folin Ciocalteu phenol reagent (2N)	Fischer Scientific Company
Guanidine hydrochloride	Schwartz Mann
5-Dimethyl amino naphthalene sulphonyl chloride (Dansyl-chloride)	Sigma Chemical Company

Dansyl amino acids	Sigma Chemical Company
Deoxyribonuclease I (DNase I)	Worthington Biochemical Corporation
Bovine serum albumin	Sigma Chemical Company
Yeast alcohol dehydrogenase	Sigma Chemical Company
Ribonuclease A (RNase A), bovine pancreas	Mann Research Laboratories
Chymotrypsin, beef pancreas	Mann Research Laboratories
Sodium lauryl sulphate, sodium dodecylsulphate (SDS)	Mann Research Laboratories
Acrylamide	Eastman Kodak Company
N,N'-methylene-bis-acrylamide (Bis)	Eastman Kodak Company
Tetraethylene methylene diamine (TEMED)	Eastman Kodak Company
Riboflavin	Canalco
Phenazine methosulphate	Calbiochem
2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride (nitro Blue tetrazolium)	Sigma Chemical Company
Coomassie Brilliant Blue	Mann Research Laboratories
Amido Schwartz, Naphthol Blue Black	Allied Chemical
Bromophenol Blue	Allied Chemical
Diethyl aminoethyl cellulose, (DEAE-cellulose), Cellex D	BioRad Laboratories
Ammonium sulphate, ultra pure	Mann Research Laboratories

Spectroscopic plates, type 11-G

Eastman Kodak Company

Silica thin-layer chromatograms
type 6060

Eastman Kodak Company

Bacterial Strain

The bacterial strain used was Escherichia coli B, wild type (ATCC 1303). The cells were obtained from General Biochemical Corporation; they were grown in Kornberg media and harvested in late-log phase.

E. coli B, wild type, cells were also grown in 40 liter batches in a chemostat. A slant of E. coli B, wild type, cells was obtained from Dr. P. D. Skaar, Montana State University, Bozeman, Montana, and cultured in a liter of sterile nutrient solution containing 8 gms of Bacto nutrient broth and 5 gms NaCl. The resulting starter culture was grown in a shaker for 12 hours at 37° C. The starter culture was added to 40 liters of sterile nutrient broth in a 60 liter culturing vessel and grown for 12 hours at 37° C with aeration and stirring. The cells were then cooled by passage through a stainless steel coil immersed in ice and collected with a Sharples Centrifuge, type T.1., at an effluent rate of 200 mls per min. The cells were stored at -10° C until used.

Buffer Recipes

0.1 M Tris - 12.11 grams of Tris (0.1 moles) was dissolved in a liter of doubly distilled water, and the pH adjusted to 7.4 at 4° C by the addition of glass distilled hydrochloric acid (HCl).

33 mM Tris buffer - The above buffer was diluted 1:3 with the addition of doubly distilled water.

Phosphate Buffers - Potassium phosphate buffers were prepared by the addition of equimolar solutions of potassium dihydrogen phosphate (KH_2PO_4) and potassium dihydrogen phosphate (K_2HPO_4) until the pH of the resulting solutions was 6.8 at 4° C. The following potassium phosphate buffers were made in this manner: 500 mM; 120 mM; 90 mM; 60 mM and 1.0 mM, all at pH 6.8. 7.46 grams of potassium chloride (KCl) were added to each liter of 1.0 mM potassium phosphate buffers in order to raise the ionic strength. All buffers employed contained 0.1 mls of β -mercaptoethanol per liter of solution (1.3 mM unless otherwise indicated.) The β -mercaptoethanol was added to the buffers both as a sacrificial reductant, and to complex with trace heavy metal contaminants present in the solutions.

Glass Distilled Constant Boiling Hydrochloric Acid

Seven hundred and fifty milliliters of reagent grade hydrochloric acid was diluted 1:2 with glass distilled water and distilled in a 3-necked flask. The first 500 milliliters of distillate was used for general laboratory purposes, the second 500 ml fraction was used for buffer adjustments, and the residue was discarded.

Glass Distilled Water

House distilled water was redistilled in a Corning Model Ag-2 glass distillation apparatus. The distillate was collected and stored in 10 gallon carboys. This water was used in the preparation of all buffers

and solutions employed in this study.

Saturated Ammonium Sulphate. $[(\text{NH}_4)_2\text{SO}_4]$

A saturated solution of ammonium sulphate (Mann Research Laboratories, ultra pure) was made by adding solid ammonium sulphate to 0.1 molar Tris buffer, pH 7.4, with constant stirring. A small excess of solid was left in the solution to insure saturation. Five mM (1.86gm) of disodium EDTA was added to each liter of saturated solution to chelate residual heavy metals. 0.1 mls of β ME (1.3 mM) was also added to each liter of solution. The pH of the saturated solution was adjusted to neutrality by the addition of a small amount of solid Tris. The solution was stored in a stoppered bottle at 4° C.

Lysis of the E. coli B Cells

E. coli B wild type cells were lysed by the following procedure. 100 grams of frozen E. coli cells were allowed to thaw slowly and 100 mls of 0.1 molar Tris buffer pH 7.4 were added. The resultant slurry was ruptured in 40 ml batches at 20,000 psi by the use of an Aminco French Pressure cell (American Instrument Co., Inc., Silver Springs, Maryland), and the lysate was cooled in ice. 0.6 milligrams of deoxyribonuclease I (DNase I) per 100 gms of cells were added to the lysate and the mixture was left standing at 4° C for 3 hours. By this time the "stringyness" due to DNA in the lysate had disappeared. The cell debris was then centrifuged down at 30,000 xg at 4° C in a Sorvall RCB

centrifuge and discarded. The supernatant was reclarified at 30,000 xg, at 4° C for 30 minutes, and the volume of the crude solution was measured.

Enzyme Activity Measurements and Calculations

The substrates used for enzyme assays were obtained from Calbiochem Co., and were made up in 0.1 M Tris buffer pH 7.4. The solutions were stored at 4° C, and were prepared freshly each week.

Nicotinamide Adenine Dinucleotide Phosphate

Sodium salt, dihydrate MW = 801.4 - A 7.5 mM solution was obtained by dissolving 30 milligrams in 5.0 mls of 0.03 Tris buffer, pH 7.4.

D-Glucose-6-Phosphate

Disodium salt, dihydrate MW = 340.1 - A 12.0 mM solution was prepared by dissolving 0.0408 gms in 10 mls 0.10 M Tris buffer pH 7.4.

6-Phosphogluconic Acid

Sodium salt MW = 342.1 - A 12 mM solution was prepared by dissolving 0.041 gms in 10 mls of 0.1 M Tris buffer pH 7.4.

Magnesium Chloride

MgCl₂·6H₂O MW = 203.3 - A 0.15 molar solution was prepared by dissolving 3.05 gms of crystals in 100 mls of 0.1 M Tris buffer pH 7.4.

Enzyme Assay Solution

Assay solution was prepared freshly each day from the stock solutions. Each milliliter of assay solution contained the following.

0.8 ml	0.1 M Tris buffer pH 7.4
0.05 ml	7.5 mM NADP ⁺
0.05 ml	0.15 M Mg ⁺⁺
0.10 ml	12.0 mM G6P

This solution was used for assaying G6PDH. An identical solution for the assay of 6PGDH activity was prepared except that 0.1 ml of 12 mM 6-phosphogluconate was substituted for the glucose-6-phosphate solution.

Enzyme Assay

The unit of activity of both G6PDH and 6PGDH is defined as the amount of enzyme that reduces one micromole of NADP⁺ per minute. The assay is performed in a Cary Model 14 recording spectrophotometer by measuring the increase in absorbance at 3400 Å. The reduced coenzyme (NADPH) produced by the enzyme dehydrogenation of G6P has an absorption maxima at 3390 Å. The extinction coefficient at this wavelength ($\epsilon_{3390 \text{ Å}}$) has a value of 6.21×10^3 OD units mole⁻¹. Oxidized coenzyme (NADP⁺) does not exhibit an absorption peak at 3390 Å. Calculations of the activity for both dehydrogenases involve the use of the following equation:²⁸

Units of enzyme activity (μ moles of reduced NADPH^+ produced per min) =

$$\frac{\Delta\text{OD}_{340}\text{min}^{-1}}{6.21} \quad (1)$$

Specific activity of the dehydrogenases is defined as enzyme activity units per milligram of protein. The protein concentration determinations are explained later.

Activity determinations for G6PDH and 6PGDH were performed as follows. A measured amount of assay solution was pipetted into a quartz cuvette (1 cm pathlength) in a Cary Model 14 recording spectrophotometer, set at $3390 \text{ \AA}$. The instrument was balanced, and then a known volume of enzyme solution was pipetted into the cuvette to yield a final volume of 1.0 mls. The increase in absorbance at $3390 \text{ \AA}$ was plotted on the recorder.

$$\text{Units ml}^{-1} = \frac{\Delta\text{OD}_{3390}\text{min}^{-1}}{6.21} \times \frac{1}{\text{vol. of enzyme soln. used, ml.}} \quad (2)$$

Since the enzymes G6PDH and 6PGDH are metabolically linked, the enzymic product of G6PDH provides substrate for 6PGDH, and consequently two molecules of NADPH^+ are produced for each molecule of G6P when both enzymes are present. Consequently, in the initial stages of purification when both activities were present in the solution,

it was necessary to determine the activity of 6PGDH independently in order to evaluate that due to G6PDH alone. This was accomplished by spectrophotometric determination of the enzyme solution with G6P containing assay solution, which gave an increase in absorbance due to both G6PDH and 6PGDH activity, and a determination with 6PG containing assay solution which gave an increase in absorbance due to 6PGDH activity alone. The G6PDH activity was calculated as the difference between the two values.

Protein Determinations

Two methods of protein concentration determinations were used. The Lowry method (Folin-Ciocalteu phenol reagent) was used when nucleic acid concentrations were high enough to preclude spectrophotometric measurements of protein. When nucleic acids had been removed, a direct spectrophotometric protein determination was employed.

Lowry Protein Determination²⁹ (Folin-Ciocalteu phenol reagent)

A standard protein concentration versus absorbance curve was made up as follows. Between 0 and 100 μ grams of a standard bovine serum albumin solution, in about 15 μ g increments, was added to a series of six tubes. The volume in each tube was made up to 1 ml by the addition of water, and each tube was shaken thoroughly. An alkaline copper reagent was made by mixing a 2% Na_2CO_3 in 0.1 N NaOH solution with

a 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium citrate in the ratio of 50:1. Five mls of this alkaline copper solution was added to each tube and the mixture was left standing for 10 minutes at room temperature after thorough stirring. After standing, 0.5 mls of 1 N Folin-Ciocalteu phenol reagent was pipetted into each tube and immediately mixed. The optical density of each tube was measured at $7500 \text{ \AA}$ after 30 minutes. The standard curve was obtained by plotting micrograms of protein versus optical density at $7500 \text{ \AA}$. All the reagents employed were stable indefinitely, except for the phenol reagent which was replaced when a greenish hue developed.

The same procedure was employed for determinations of unknown protein concentrations, and several determinations were performed at various dilutions.

Spectrophotometric Protein Determinations

Once the nucleic acids in the protein solutions were removed it was possible to use a direct spectrophotometric protein determination using a Cary 14 recording spectrophotometer. The following equation was employed:

$$\text{mgms protein ml}^{-1} = (\text{Abs}_{2800\text{\AA}}) \times 1.45 - (\text{Abs}_{2600\text{\AA}}) 0.74^{28} \quad (3)$$

Ammonium Sulphate Protein Precipitations

Ammonium sulphate precipitations were accomplished by the drop-wise addition of an appropriate volume of saturated ammonium sulphate to the protein solution with continuous stirring at 4° C. The saturated ammonium sulphate solution was added at the rate of 100-200 mls per hour. Precipitated proteins were left overnight before centrifuging.

Diethyl Amino Ethyl Cellulose (DEAE-cellulose) Anion Exchange Resin

DEAE-cellulose, exchange capacity 0.69 milliequivalents per gram was obtained from BioRad Laboratories. Before use the anion exchanger material was treated to remove any impurities as follows.

1. Approximately 500 gms of DEAE cellulose was soaked in 4 liters of water for one hour.
2. The suspension was stirred and left to settle for 20 minutes and the fine particles poured off. This process was repeated twice.
3. The DEAE was then washed with 3-4 liters of 1/10 N NaOH for 10 minutes and the slurry was vacuum filtered in a Buchner funnel. This procedure was repeated twice.
4. The DEAE was then washed to neutrality with water, 3-4 liters, three times, and again vacuum filtered using a Buchner funnel.
5. 3-4 liters of 95% ethanol was then added and the suspension was stirred and left standing for 10 minutes. The DEAE-cellulose filtered, and the process repeated twice.

6. Ethanol traces were removed by washing with water and filtration.

7. A second washing with 3-4 liters 1/10 N NaOH was performed and repeated twice.

8. The purified DEAE was washed to neutrality with water and filtration.

The purified DEAE was stored in a sealed flask and 1 ml of 0.1 molar sodium azide was added to retard any bacterial growth.

Preparation of Hydroxyl Apatite Anion Exchange Material

Hydroxyl apatite $\text{Ca}_5(\text{PO}_4)_3\cdot\text{OH}$ was prepared using a modification of Levin's³⁰ method.

Two liters of 0.5 M CaCl_2 and 2 liters of 0.5 M Na_2HPO_4 were allowed to flow into a 4 liter glass beaker from two dropping funnels. The flow rates from the two funnels were equal, approximately 15 ml/s per minute. The beaker was fitted with a stirring motor and a glass stirrer whose width was just less than that of the beaker; the stirring rate was 80-100 rpm. When the precipitation of inactive-brushite (calcium phosphate $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$) was completed, the brushite was allowed to settle and the supernatant was aspirated with a water pump. The calcium phosphate was then treated in the following manner to convert it to the active hydroxyl apatite which would be used as an anion exchanger.

1. The brushite was washed with 3-4 liters of water four times; complete settling was allowed each time.

2. Three liters of water was added to the washed brushite and 100 mls of freshly prepared 40% (w/w) NaOH was added. Stirring was commenced at approximately 80 rpm, and the contents of the beaker were heated to boiling within 45 minutes. Very gentle boiling was continued for one hour, with continuous stirring, and the resultant hydroxyl apatite was allowed to sediment for 5 minutes. The supernatant was removed by suction.

3. The hydroxyl apatite was washed with four portions of 3-4 liters of water; the stirrer was operated for 5 minutes and after a sedimentation time of 5 minutes the supernatant was removed by suction.

4. The washed hydroxyl apatite was heated to boiling with 0.01 M Na phosphate buffer, pH 6.8, with stirring once boiling had commenced; the apatite was allowed to settle for 5 minutes and the supernatant was aspirated. This operation was repeated twice with 0.01 M NaPO_4 , and twice with 0.001 M NaPO_4 buffer (pH 6.8). Each time, however, the boiling was continued for 15 minutes.

After the fifth boiling in phosphate buffer the preparation was complete, and the resultant hydroxyl apatite was stored in 0.001 molar sodium phosphate buffer.

Care was taken throughout the preparation to avoid an excessively vigorous stirring rate. The final product consists of minute crystals of hydroxyl apatite either in a free state, or loosely bound to incompletely converted brushite particles. Vigorous mechanical stirring

results in a breakdown such that a column of hydroxyl apatite can only be operated with a pump.

Ammonium Sulphate Precipitation

The crude enzyme preparation (approximate volume 500-600 mls) was precipitated by the addition of saturated ammonium sulphate solution (cf. above) to a final saturation of 55%. The suspension was stored in sealed liter bottles at 4° C until used.

Chromatography on DEAE-Cellulose

Treated DEAE cellulose (cf. above) was packed in a large (5 x 100 cm, bed volume 1800 mls) chromatographic column (Pharmacia). The DEAE was added to the column as a slurry, stirred, and was allowed to settle under gravity. When the adsorbant had settled the stopcock was opened and the DEAE bed was packed down under 10 psi of nitrogen. The column was packed in segments in this manner, and the top of the bed was stirred before each addition of fresh DEAE slurry. When packed the column was washed with 2 liters of 1.0 molar KCl in 0.03 molar Tris buffer, pH 7.4, to remove any residual impurities. The column was then equilibrated with 2-3 liters of 0.03 molar Tris buffer, pH 7.4, and was ready for use.

Approximately 300 mls of the suspension from the ammonium sulphate fractionation was centrifuged at 30,000 xg. The precipitate which contained most of the G6PDH activity (approximately 700 units)

was dissolved in a liter of 0.03 molar Tris buffer, applied to the top of the DEAE column and washed on to equilibrium buffer. The supernatant contained most of the 6PGDH activity and was discarded. The redissolved precipitate was added to the DEAE column at a flow rate of 75-100 mls per hour, and the yellow protein solution could be seen to be absorbed in the top 10% of the column bed. The effluent during the loading procedure was discarded.

A linear KCl gradient was constructed by allowing 1200 mls of 0.35 molar KCl to flow into 1200 mls of 0.15 molar KCl with stirring; both solutions were made in 0.03 molar Tris buffer pH 7.4. The gradient was started and 20 ml fractions were collected. The elution of G6PD was preceded by a yellow fraction, which provided a convenient marker for enzyme elution. 100-120 20 ml fractions were collected before the linear KCl gradient was stopped. Proteins, and nucleic acid, which remained bound to the column, were displaced by washing with 2.0 liters of 1.0 molar KCl in 0.03 molar Tris buffer. The column could be reused after equilibration with 2.3 liters of 0.03 molar Tris buffer. The DEAE column was used repeatedly until irregularities in the loading procedure, such as non-uniform loading and streaking, became evident. The absorbant was then removed, retreated and packed as described above.

Each of the 20 ml fractions was assayed for G6PDH and 6PGDH activity, and the protein concentration was determined by the Lowry

method (cf. above). Tubes containing in excess of 40 units of activity were pooled and precipitated at 60% ammonium sulphate saturation. The protein was stored as a suspension in 60% ammonium sulphate until further use.

Hydroxyl Apatite Column Chromatography

Hydroxyl apatite was introduced as a thin slurry into a Pharmacia column (diam. = 2.5 cm, length = 45 cm, bed vol. = 200 mls) and allowed to pack under gravity at a flow rate of 100 mls per hour. The packed column was washed with 1.0 liters of 500 mM phosphate buffer, pH 6.8, in order to replace the Na^+ ion employed in the preparation with K^+ ion. According to Stoll and Neuman³¹ sodium ions slowly replace Ca in the hydroxyl apatite lattice resulting in breakdown of the absorbance characteristics and a lower flow rate of the hydroxyl apatite exchanger. Potassium ion does not affect this replacement, and sodium ion can be exchanged quantitatively with potassium by shocking with 0.5 mM K^+ . The column was then washed with 1.0 mM K^+ phosphate buffer for 24 hours in order to remove the excess phosphate ions absorbed. The column was not in equilibrium with the regeneration buffer until after about 24 hours of washing.³⁰

The precipitated protein from the linear salt gradient DEAE column chromatography was centrifuged at 30,000 xg and the supernatant discarded. The precipitate was redissolved in 30-40 mls of 1.0 mM PO_4

buffer pH 6.8 containing 0.1 moles/liter of potassium chloride, and assayed for G6PDH and 6PGDH activities. Protein was measured spectrophotometrically, since nucleic acid had been removed. The potassium chloride (0.1 M) was added to the 1.0 mM PO_4 buffer in order to increase the ionic strength and reduce the possibility of enzyme inactivation. The salt does not affect the elution of proteins or the absorbance characteristics of hydroxyl apatite.³⁰ The redissolved protein was applied to the top of the hydroxyl apatite column over a period of 2-4 hours, and could be seen as a tight yellow band at the top. The column was eluted stepwise with the following sequence of K^+ phosphate buffers, pH 6.8: 1.0 mM; 30.0 mM; 60.0 mM; 90.0 mM; 120 mM. One to one and a half column void volumes of each buffer were used; this was sufficient to elute any proteins at the particular buffer concentration. Fractions (4 mls each) were collected throughout the gradient and assayed for G6PDH activity; protein was determined by direct spectrophotometric measurement at 2800 Å and 2600 Å. Tubes with a specific activity in excess of 20.0 units per milligram were pooled and the total volume measured. The pooled fractions were assayed for G6PDH and protein. The activity was precipitated by the addition of saturated ammonium sulphate solution to a final concentration of 60% saturation. The protein was stored as a suspension in 60% ammonium sulphate for further use.

The hydroxyl apatite column was washed free of bound protein by

1.0 liters of 500 mM K^+ phosphate buffer, pH 6.8, and regenerated by washing for 24 hours with 1.0 molar PO_4 buffer, pH 6.8. The column could be used repeatedly and reliably over a period of a year, and the adsorbant was only replaced when it began to disintegrate and exhibit a flow rate of less than a column void volume in five hours.

Exponential Gradient Chromatography on DEAE Cellulose

Treated DEAE cellulose was packed in a Pharmacia column (diameter 1.5 cm, length 45 cm) with a bed volume of 40 mls, as described before. The column was prewashed with 1.0 liters of 0.5 molar KCl in 0.03 M Trizma buffer pH 7.4, and equilibrated with 1.0 liters of 2.0 mM G6P in Trizma buffer. The substrate was added to the equilibration buffer in order to provide substrate protection for the enzyme during the chromatography. The precipitated protein from the hydroxyl apatite column chromatography was centrifuged at 30,000 xg, and the precipitate was dissolved in 10 mls equilibration buffer. The supernatant was discarded. The redissolved protein was applied to the top of the DEAE cellulose column, and washed on with a small amount of buffer.

An exponential gradient was constructed by allowing a solution of 0.33 molar KCl, 2.0 mM G6P, in 0.03 molar Trizma buffer pH 7.4 to flow into a 100 ml (fixed constant volume) of 0.2 molar KCl, 2.0 M G6P in the same buffer with continuous stirring. The gradient was started and applied to the top of the column and 4 ml fractions were collected.

Fractions were assayed for any G6PDH activity present, and the protein concentrations were determined by direct spectrophotometric measurement. The tubes of highest specific activity were pooled, assayed and used as outlined below.

Hydroxyl Apatite Chromatography and Enzyme Storage

A glass column of diameter 1.0 cms and 10.0 cms in length was made and fitted with a feeding device. This column was packed with a 2 ml pad of hydroxyl apatite and treated as described above. The pooled tubes from the exponential DEAE chromatography were applied to the column and washed on with 1.0 mM PO_4 buffer pH 6.8 containing 0.1 moles of KCl per liter. G6PDH was stable when adsorbed on hydroxyl apatite and this technique afforded a convenient method of storage of the activity without the problem of ammonium sulphate precipitation of small volumes of protein solution obtained from the exponential DEAE chromatography. The adsorption of the activity onto a pad of hydroxyl apatite also provided a rapid and simple procedure for concentration of the activity, in addition to a stable storage method. By passing a small volume of 120 mM K^+PO_4 buffer pH 6.8 through the pad the activity was eluted in a small volume, and any decomposition products were removed by a short prewash with 1.0 mM PO_4 buffer.

Polyacrylamide Gel Electrophoresis

The purified E. coli B G6PDH was electrophoresed on polyacrylamide gels using a modification of the procedure of Ornstein³² and Davis.³³ Prior to electrophoresis the enzyme solution was dialysed for 3 hours, 4° C, against 33 mM Trizma buffer pH 7.4, this procedure was employed to remove the potassium salts present in the sample and thus reduce the time required for electrophoresis. The polyacrylamide gels were prepared in glass tubes, 10 cms long and 5 mm in diameter, held upright in rubber caps which fitted snugly enough to prevent leakage during the preparation of the gels. The procedures of Davis³³ were followed during the preparation of the polyacrylamide gels with the following modifications. The small pore, or separatory, gel, was prepared first to yield 1.8 mls of a 5% polyacrylamide gel with 0.8% cross linkage due to NN'-methylene bis acrylamide. The polymerization of this gel was accomplished with ammonium persulfate catalysis, and was complete in about 20-30 minutes. After the preparation of this gel, the gel tubes were rinsed with large pore, or stacking, gel solution to remove the water exuded during the polymerization of the small pore gel. The tubes were then dried with lint free paper and the top of the small pore gel was touched to remove traces of the rinsing solution. Large pore gel solution (0.6 mls) was then added on top of the small pore gel and photopolymerized under a fluorescent light with riboflavin catalysis. Polymerization was complete in about 45 minutes and yielded

a 3% polyacrylamide gel, 8% cross linkage due to NN'-methylene bis acrylamide. The gel tubes were removed from the rubber caps and placed in the electrophoresis chamber and Tris/glycine electrophoresis buffer added. The sample gel procedure of Davis³³ was omitted, and instead a few crystals of sucrose were dissolved in the protein sample solution to increase the solution density, and measured aliquots layered on top of large pore gel. Electrophoresis was performed at 1.5 mA per tube and the direction of electrophoresis was from the top to the bottom of the chamber. In some cases a drop of Bromophenol Blue was added to the upper chamber and its passage through the gel served as a convenient marker of the electrophoretic front. The electrophoresis was run at 4° C and was complete in 2-4 hours. Removal of the gels from the tubes and the protein staining and destaining procedure was performed according to Davis.³³ In most cases staining of the protein bands by Amido Schwartz (Naphthol Blue Black) was allowed to occur overnight, before the destaining procedure was employed. After destaining, the gels could be stored in 7% acetic acid for several weeks. Gels were also stained for the localization of enzyme bands on the basis of the catalytic activity performed by the protein. A modification of the procedure developed by Rattazzi et al.³⁴ was used. The activity staining procedure is based on the hydrogen ion released by the enzymic dehydrogenation of glucose-6-phosphate. The activity staining solution was made as follows:

Tris-HCl	1.0 M, pH 8.6, 4mM EDTA	12.5 mls
NADP	10 mg ml ⁻¹	1.0 mls
G6P	25 mg ml ⁻¹	1.5 mls
Phenazine methosulphate	2 mg ml ⁻¹	2.5 mls
Nitro Blue Tetrazolium	2 mg ml ⁻¹	2.5 mls
Cobaltous chloride	0.5 M	<u>1.0 mls</u>
		21.0 mls

This volume of staining solution was sufficient for the staining of five gels; the purple activity bands developed within 30 minutes when incubated at 37° C. The activity stain diffused very rapidly, and the gels became coated with a purple precipitate very shortly after development. Storage in 10% formalin solution diminished the diffusion of the bands, but resulted in fading of the stain.

6 M Urea Polyacrylamide Gel Electrophoresis

Basic 6 M urea polyacrylamide gels were prepared in glass tubes 10 cms long and 5 mm in diameter. The gels were prepared as described above with the following modifications. Urea was added to all the gel solutions to yield a 6 M solution, and consequently the preparative solutions had to be warmed in a water bath (60° C) to dissolve the urea prior to preparation of the gels. A 3% separatory gel (0.8% cross linked) and a 3% stacking gel (8% cross linked) were prepared, both 6 M with respect to urea. The polymerization of those gels required longer

periods than necessary for the urea free polyacrylamide gels. When prepared, the gel tubes were placed in the electrophoresis chamber and Tris/glycine electrophoresis buffer, pH 8.4, containing 6 M urea, was added.

Purified E. coli B G6PDH was incubated for 24 hours at room temperature in 8 M urea, and layered on top of the urea gels with the aid of a Hamilton microliter syringe. In most cases 100-150 micrograms of urea incubated protein was employed. Gels were run at 5 mA per tube for a period of 4 hours. The gels were then removed from the tubes, stained, and destained as described previously.

Sodium Dodecyl Sulphate Gel Electrophoresis

The molecular weight of the purified E. coli B G6PDH protein was estimated by sodium dodecyl sulphate (SDS) gel electrophoresis of a protein sample dissociated in the presence of SDS and β -mercaptoethanol. The procedure employed was based on the technique developed by Weber and Osborn,³⁵ and their analysis of the reliability of this method of molecular weight determination.

The SDS gels were prepared in glass tubes 10 cms long with an internal diameter of 5 mm. For a typical run, 15 mls of 10% acrylamide solution (0.3% bis acrylamide) was mixed with 13.5 mls of gel buffer containing 56 mM of NaH_2PO_4 , 144 mM of Na_2HPO_4 , 0.2% SDS, 0.045 ml of N,N,N',N'-tetramethylene diamine and 1.5 ml of freshly prepared

ammonium persulphate (15 gms per ml) and polymerization was allowed to occur for about 30 minutes. E. coli B G6PDH and standard protein samples were dissociated with SDS and β ME in the following manner. Incubations were run at 55° C for one hour in 10 mM sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% β ME. When the incubations were complete the solutions were allowed to cool and 50 μ grams of the SDS-dissociated protein was mixed with 3 μ liters of 0.05% bromophenol blue, 1 drop of glycerol, 5 μ liter of β ME and 50 μ l of 10 mM Na phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% β ME. The polymerized SDS polyacrylamide gels were placed in the electrophoresis chamber and the electrophoresis buffer, 28 mM NaH_2PO_4 , 72 mM NaHPO_4 , containing 0.1% SDS, was added. The protein samples were loaded on top of the gels and the electrophoresis was performed at 8 mA per tube with the anode in the lower chamber. The electrophoresis was run for 2-4 hours, by which time the marker dye, bromophenol blue, had moved three quarters through the gel. After electrophoresis the gels were removed from the tubes with the aid of a long syringe needle. The length of the gel and the distance moved by the marker dye were measured and recorded.

The removed gels were stained with Amido Schwartz, and in some cases with Coomassie Brilliant Blue. The Amido Schwartz stain was prepared by dissolving 1 gm of solid in 100 mls of 7% acetic acid. Coomassie Brilliant Blue fixative stain was prepared by dissolving 1.25 gms in a mixture of 454 mls 5% methanol and 46 mls of glacial acetic acid.

The gels were placed in the fixative stain and left overnight. When the Amido Schwartz stain was employed the gels were destained electrophoretically in 7% acetic acid. Coomassie Blue stained gels were destained electrophoretically in a solution containing 75 ml of glacial acetic acid, 50 ml of methanol and 875 ml of glass distilled water. After destaining was complete the length of the gels and the positions of the protein bands were measured and recorded.

High Speed Sedimentation Equilibrium Ultracentrifugation

The method of Yphantis³⁶ was used to determine the subunit molecular weight. A small length of dialysis tubing was boiled in 100 mls of 100 mM EDTA in glass distilled water. The tubing was boiled for one hour and allowed to cool. Ninety-five percent ethanol was then added to the solution to yield a final concentration of 70%; the treated tubing was stored in this solution for future use.

Prior to sedimentation analysis it was necessary to denature the protein by dialysis against the appropriate denaturant.

A 6 molar solution of guanidine-hydrochloride, containing 0.2 moles of β -mercapto ethanol, was prepared in 0.1 M Trizma hydrochloride buffer and the pH was adjusted to 6.0 by the addition of glass distilled hydrochloric acid. Two ml of a purified sample of E. coli B G6PDH, containing approximately 300 μ grams per ml of protein, was denatured by the addition of solid guanidine-hydrochloride sufficient to yield a

6 M solution. β -Mercapto ethanol was added to this solution to yield a final concentration of 0.2 moles. The treated dialysis tubing was presoaked in 100 ml of 6 M guanidine-hydrochloride, pH 6.0, for several hours, and the denatured G6PDH solution was then placed in the tubing. The protein sample was dialysed against 250 ml of the stock guanidine-hydrochloride solution for 72 hours with five changes of dialysate. When the dialysis was complete the protein solution was removed from the dialysis bag and placed in a tightly capped vial; a 5 ml sample of the final dialysate was also removed and stored in a similar vial for future use.

A double sector equilibrium sedimentation cell with sapphire windows, obtained from Beckman Instruments, Palo Alto, California, was cleaned and assembled. Fifty microliters of fluorocarbon #43 (Beckman Instruments) was introduced into each sector of the cell with a Hamilton microliter syringe. This quantity of fluorocarbon served to raise the cell contents above the bottom of the cell. One hundred and ten microliters of the dissociated and dialysed G6PDH solution was placed in the right hand sector of the cell, and 110 microliters of the final dialysate was placed in the left hand sector. The two small loading apertures of the cell were sealed with plastic gaskets secured by screws.

The sealed double sector cell was placed in a An-D aluminum rotor (Beckman Instruments), and balanced with a counterweight. The rotor

was placed in the chamber of a Spinco Model E analytical ultracentrifuge, equipped with Rayleigh optics, and the chamber was evacuated and cooled. The rotor speed was raised to 40,000 rpm and maintained at this value for 24 hours. Interferograms of the spinning cell contents were taken at this time and again after an additional 12 hours. The exposures were taken on a Kodak Spectroscopic plate which was developed immediately. The rotor speed was then lowered and the procedure repeated. Pictures were obtained at 24 and 36 hour intervals for three different centrifugation speeds at each protein concentration. Three different protein concentrations, 280, 140 and 85 μg per ml, were employed in the ultracentrifuge estimation of the molecular weight. In each case the rotor was spun at 40,000 rpm for 24 hours prior to lowering the speed to the value at which pictures were first obtained.

The spectroscopic plates were allowed to dry thoroughly after development, and were then analyzed using a Nikon Profile Projector (Nippon Kogaku KK., Tokyo, Japan). Each exposure on the plate was aligned on the rotating table of the projector, and the distance from the reference hole to the solution meniscus was measured and recorded. A convenient Rayleigh fringe, on the solution, was selected and the fringe height at 0.1 mm increments was recorded from the solution meniscus to the bottom of the solution column. The fluorocarbon #43 meniscus marked the bottom of the solution column, and served to raise the solution column above the bottom of the cell. Each exposure on each plate

was read and recorded in this manner.

N-Terminal Amino Acid Determinations

Two milliliters of a purified sample of E. coli B G6PDH, containing 0.3 mg/ml protein, was made alkaline by the addition of sufficient solid sodium carbonate to yield a 0.1 M solution. The solution was then boiled in a water bath for 5 mins, and allowed to cool to room temperature. An equal volume of an acetone sample containing 2.0 mg/ml of 5-dimethyl amino naphthalene-sulphonyl chloride (Dansyl-chloride) was added to the heat denatured protein solution. The mixture was allowed to react until the yellow color due to the Dansyl-chloride had disappeared; this was complete within 5 minutes. The solution was then evaporated to dryness in a vacuum dessicator, and the small amount of solid was dissolved in 1 ml of 5.7 M hydrochloric acid prepared by diluting reagent grade hydrochloride 1:1 with glass distilled water. The solution was then transferred to a Carius tube which was cooled in an acetone-dry ice bath and evacuated with a vacuum pump. The Carius tube was sealed under vacuum with a flame. One mg samples of standard Dansyl-amino acid derivatives, obtained from Sigma Chemical Co., St. Louis, Missouri, were also dissolved in 1.0 ml of 5.7 N hydrochloric acid and sealed in evacuated Carrius tubes as described above. These acid solutions were then heated in an oven at 110° C for 8 hours, cooled in an acetone-dry ice bath, and broken open. The solutions were again evaporated to

dryness in a vacuum dessicator, usually within 24 hours, and the solid taken up in 1.0 ml of acetone. The samples were transferred to tightly stoppered vials and stored at 4° C.

Thin layer chromatography was performed on Eastman Chromagram Sheets, Eastman Kodak Co., Rochester, New York, silica gel thin layer plates prepoured on inert polyethylene terephthalate. The plates were dried in a 40° C oven for several hours prior to use. Twenty microliter samples, containing 20 μ g of the acetone solution of the standard Dansyl amino acid derivatives were spotted on the lower end of the thin layer plate about 2 cms apart. Between 100-200 microliters of the G6PDH N-terminal Dansyl derivative was similarly spotted on the thin layer plate. During this spotting procedure a small stream of nitrogen was directed on the thin layer plate; this facilitated rapid evaporation of the acetone and insured small spot sizes.

The spotted plates were placed upright in glass chromatography tanks and developed until the solvent system reached the top of the plate. Care was taken to insure that the sample spots on the plate were positioned above the solvent meniscus when the plate was placed in the chromatography tank. The solvent reached the top of the thin layer plate within 2-3 hours, and the plates were removed from the tank and allowed to air dry for 20 minutes. The developed plates were observed under an ultraviolet light, and the Dansyl amino acid derivative spots fluoresced yellow. Unreacted Dansyl-chloride, and the Dansyl-

amino acid derivative spots fluoresced yellow. Unreacted Dansyl-chloride, and the Dansyl-hydroxide and amine formed during the hydrolysis appeared as blue spots.

The solvent systems used were prepared freshly for each development, and discarded after use. The following solvent systems were employed.

1.	Chloroform	38 parts
	Ethanol	4 parts
	Acetic Acid	3 parts
2.	Chloroform	70 parts
	Benzyl Alcohol	30 parts
	Acetic Acid	3 parts
3.	Benzene	80 parts
	Pyridine	20 parts
	Acetic Acid	5 parts
4.	N-Butanol	30 parts
	Pyridine	20 parts
	Acetic Acid	6 parts
	Water	24 parts

Thin layer plates were also developed in which the G6PDH N-terminal Dansyl derivative was co-spotted with a standard Dansyl amino acid derivative. These plates were treated and developed in the same manner as described previously.

Stability and Activity Studies

All activity and stability studies were performed using a Cary 14 recording spectrophotometer obtained from Varian Associates, Palo Alto, California. Activity measurements were made at 3390 Å. Studies were performed using purified E. coli B G6PDH which had been previously dialysed for 6 hours against 0.1 M Tris-hydrochloride buffer, pH 7.4, without the addition of β -mercapto ethanol.

(a) pH Activity Studies

A purified sample of G6PDH, specific activity 20, 148 μ grams per milliliter, was diluted 1:40 and assayed for activity as described previously. In each case, however, the assay was performed using 0.1 Tris-hydrochloride buffers ranging in pH from 10.0 to 5.5 by 0.5 increments. Enzyme activities were measured and reported as increments in adsorption, at 3390 Å, per minute, per ml of enzyme aliquot.

(b) pH Stability Studies

A series of 0.1 M Tris-hydrochloride buffers, in glass distilled water, was prepared ranging in pH from 10.0 to 6.0 by 0.5 increments. β -Mercaptoethanol was omitted from these buffers. 0.9 ml of each of the Tris buffer was pipetted into a series of nine vials, and 100 microliters of the G6PDH solution specific activity 67, was then added to each vial. Each of these incubation solutions was immediately assayed for activity, at pH 7.4, as described previously. The pH incubation solutions were stored at 4° C and assayed every 24 hours, over a period of five days; these assays were again performed at pH 7.4. Enzyme

activities were reported as increments in adsorption at 3390 Å per minute, per ml of enzyme aliquot.

Investigation of Sulphydryl Groups Involved in Enzyme Activity

A sample of purified E. coli B G6PDH (specific activity 85 units/mg) in 0.1 M Tris-hydrochloride, pH 7.4, was used for this investigation. A series of six vials was prepared and the purified G6PDH was incubated in solution containing buffer (0.1 M Tris pH 7.1), 5 mM NEM, and 5 mM NEM solutions with 3.75 mM NADP⁺, 6 mM ATP, 6 mM G6P, 3 mM ATP/3mM G6P mixture, and finally a 3 mM ATP/1.9 mM NADP⁺ mixture. In all incubations the NEM solution was added last. Incubations were performed at 4° C and room temperature for a period of 30 hours.

A similar series of incubation vials was prepared using p-chloromercuribenzoic (PCMB) in place of the NEM. PCMB incubations were performed at 4° C for 1.5 hours. The PCMB solution was added to the incubation vial last.

The incubated samples were assayed at pH 7.4 using a Cary Model 14 recording spectrophotometer, as described previously.

Kinetic Studies

In preparation for these studies, a sample of purified E. coli B G6PDH was dialysed against 1 liter of 0.1 M Tris-hydrochloride buffer, pH 7.4, for six hours. Kinetic studies were performed using a Cary Model 14 recording spectrophotometer set at a wavelength of 3390 Å.

In a typical experiment the initial reaction rate of the enzymic dehydrogenation was measured under conditions where the concentration of one of the substrates, NADP^+ or G6P, was varied from a saturating concentration to a value 100-fold less.

A series of six vials was prepared containing concentrations of the variable substrates ranging from a saturating concentration to a value of approximately 100-fold less. These solutions were prepared in 0.1 M Tris hydrochloride pH 7.4, and stored at 4° C. A solution containing a saturating concentration of the other, non-variable, substrate was also prepared in the same buffer. The variable substrate, 150 microliters, was pipetted into a 3 ml quartz cuvette, and 100 microliters of the dialysed enzyme solution was also added. The cuvette was then placed in the spectrophotometer, and 2.85 ml of the non-variable substrate solution was pipetted in rapidly enough to insure mixing of the cuvette contents. The recorder was employed to measure the initial rate of the enzymic reaction, by measuring the initial slope produced. These rates were recorded as changes in absorbance, measured at 3390 Å, per minute at 25° C. This procedure was repeated for each concentration of the variable substrate. In each case a fixed enzyme aliquot (100 µl) and a fixed aliquot of non-variable substrate solution (2.85 ml) was employed. It was important to insure rapid and thorough mixing of the cuvette contents in order to be able to measure the initial reaction rates. For this reason 3 ml cuvettes were chosen

in favor of the 1 ml cuvettes employed in previous assays.

Kinetic experiments were performed allowing each substrate (NADP or G6P) to be the variable, while the other was maintained at a fixed saturating concentration. The procedure described above was employed. In addition, kinetic analyses in the presence of certain nucleotides were investigated. In these experiments the nucleotide, at a fixed concentration, was added to the solution containing the non-variable substrate and pipetted into the cuvette last. In all kinetic experiments the initial rates of the enzymic reaction were recorded as a function of the variable substrate concentration. Kinetic investigations in the presence of the following nucleotides were made.

1. Adenosine 5' monophosphate (AMP)
2. Adenosine 5' triphosphate (ATP)
3. Nicotinamide adenine dinucleotide (NAD^+)
4. Nicotinamide adenine dinucleotide, reduced (NADH)
5. Nicotinamide adenine dinucleotide phosphate, reduced (NADPH)

RESULTS AND DISCUSSION

Enzyme Yield from Lysis of E. coli B Cells

E. coli B cells, harvested in the late log stage of growth, were ruptured in a French Pressure Cell as described in the Materials and Methods section. Table I shows typical yields of enzyme units obtained from the rupture of the E. coli cells.

Table I

Units of Escherichia coli B Glucose-6-phosphate dehydrogenase Obtained by Cell Rupture with a French Pressure Cell

Gms E. coli B Lysed	Tot. Units G6PDH	Tot. Units 6PGDH	Tot. mg Protein	Units G6PDH/ mg Protein	Units 6PGDH/ mg Protein
1000	4,400	1,409	55,880	.078	.031
1000	4,352	2,082	57,408	.975	.035

Rupture with the aid of a French pressure cell provided much better yields than cell lysis by grinding with levigated alumina in a mortar and pestle. Table II compares the yields obtained from these two methods of rupture.

Table II

Yield of Escherichia coli B Glucose-6-phosphate dehydrogenase
by Method of Rupture

Method of Rupture	Units G6PDH per g cells	Units 6PGDH per g cells	G6PDH/ mg Protein	6PGDH/ mg Protein	Units G6PDH Units 6PGDH
French Pressure Cell	4.3	2.1	.075	.035	2.05 (3.0)
Alumina Grinding	1.8	1.51	.030	.017	1.15

Figure 1

Ammonium Sulphate Fractionation of Crude Escherichia coli B

Glucose-6-phosphate dehydrogenase

Enzyme Preparation

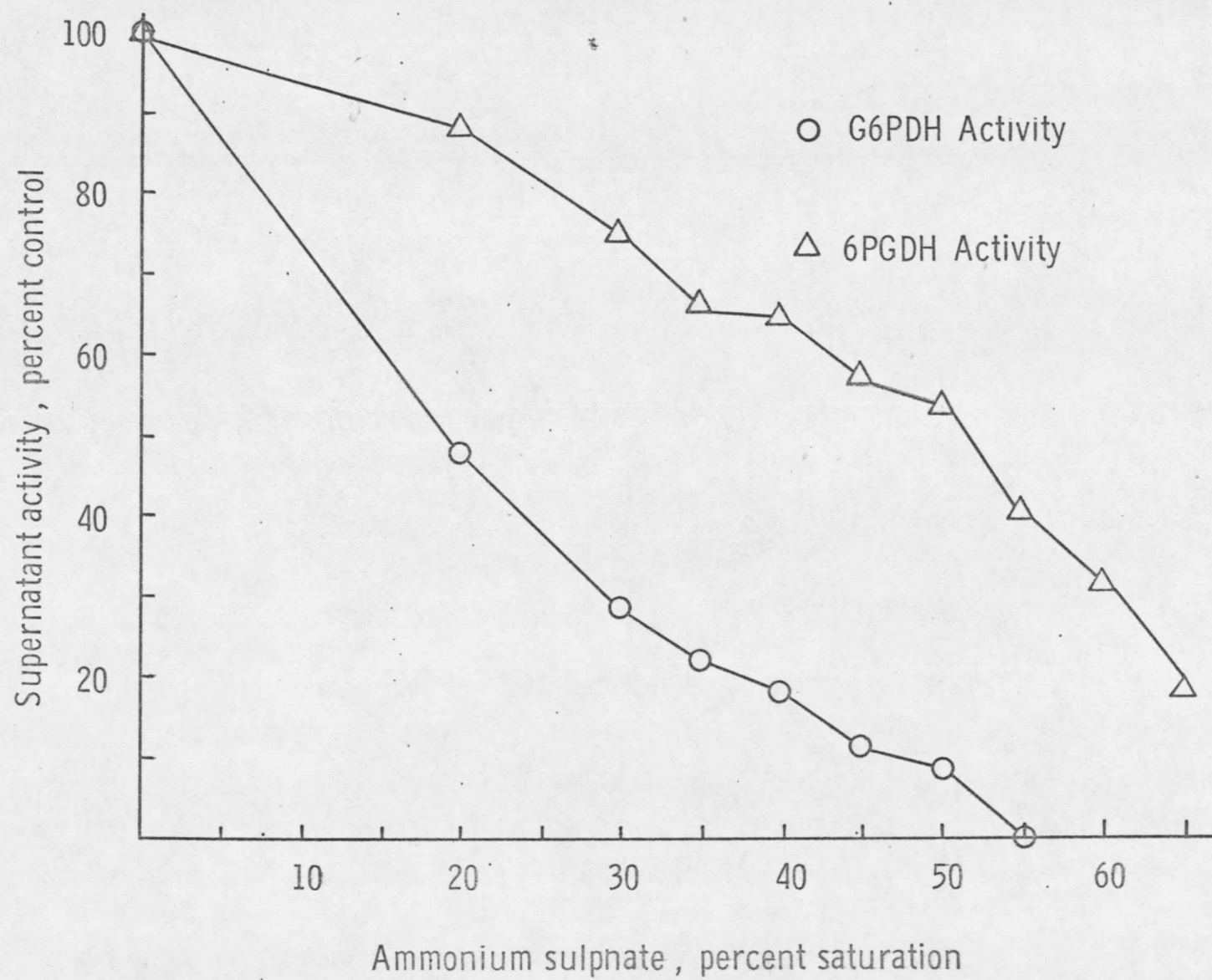


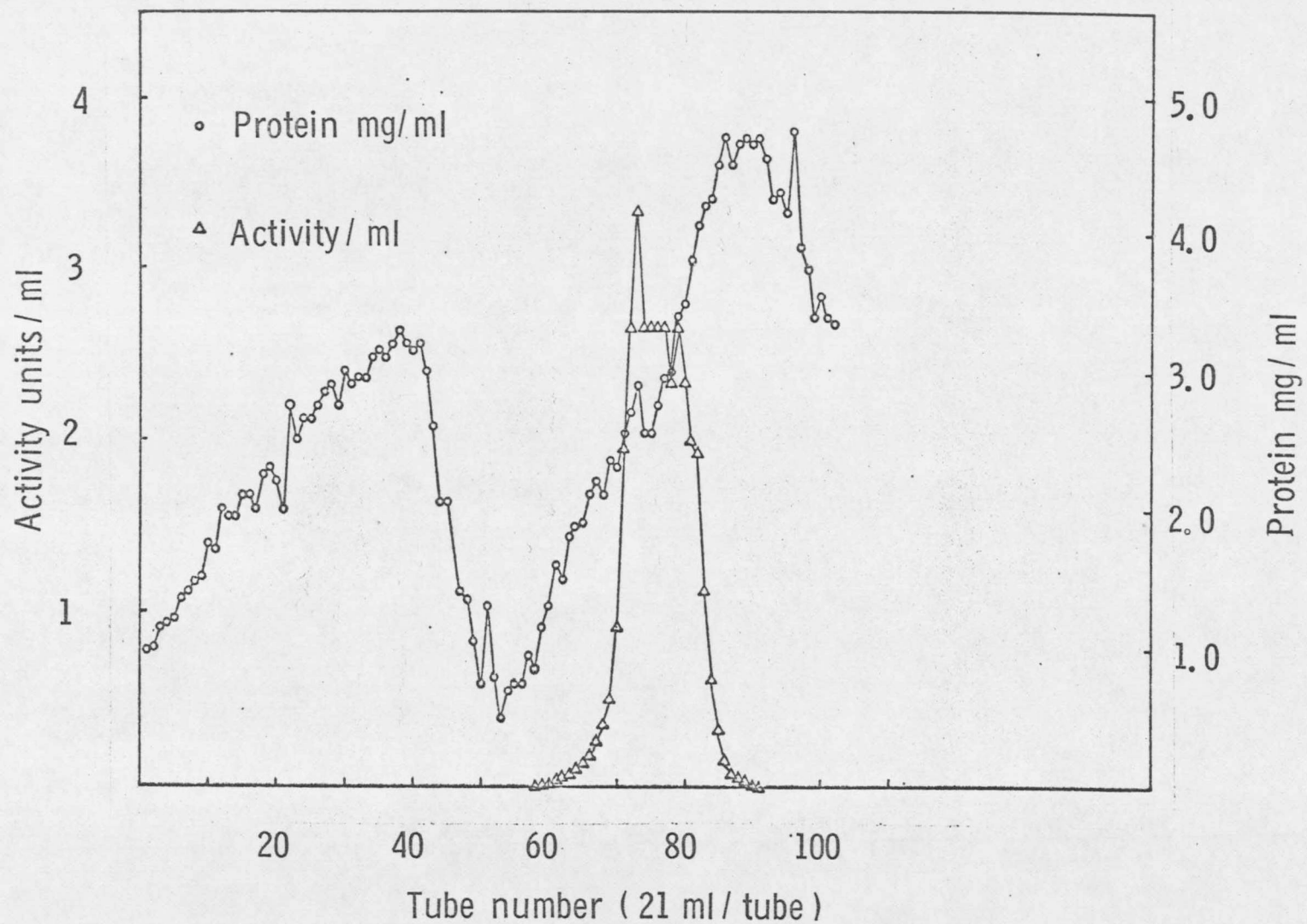
Table II indicates that French pressure cell lysis not only results in larger yields of G6PDH but in addition increases the ratio of G6PDH to 6PGDH activities.

Ammonium Sulphate Fractionations

The crude enzyme preparation was fractionated with a saturated solution of ammonium sulphate as described previously. The fractionation was performed in order to precipitate the G6PDH protein from the solution of the crude preparation and leave the majority of 6PGDH activity in the supernatant. Figure 1 shows the percentage activities of the two dehydrogenases remaining in the supernatant upon fractionation with various saturations with ammonium sulphate. The graph shows that by bringing the crude extract to 50% saturation, by the addition of saturated ammonium sulphate solution, 91% of the G6PDH activity is precipitated while 53% of the 6PGDH activity remains in the supernatant. At 55% saturation all the G6PDH activity was precipitated and 41% 6PGDH remained in the supernatant. Consequently ammonium sulphate precipitations of the crude cell lysate were performed between these two values. It was found that the most convenient method of storage of the activity was precipitation of the crude preparation at 55% ammonium sulphate saturation, and storage of the resultant mixture in stoppered bottles. When used at a later date, the slurry was centrifuged (30,000 xg) and the supernatant discarded. When storage in this

Figure 2

Elution profile of protein and glucose-6-phosphate dehydrogenase from DEAE-cellulose chromatography with linear potassium chloride gradient.



manner was employed, the G6PDH activity was stable indefinitely. Storage of G6PDH activity in this manner for six months resulted in 95% recovery of the activity.

The 55% ammonium sulphate precipitation resulted in a two-fold purification of the G6PDH activity. The values for a typical fractionation are shown in Table III.

DEAE-Cellulose Chromatography with a Linear Potassium Chloride Gradient

The 55% ammonium sulphate precipitate was dissolved and applied to the top of a large (5 x 1000 cm) DEAE-cellulose column as described in the Materials and Methods section. In a typical run, 700 units of G6PDH activity, and approximately 200 units 6PGDH activity, in 1000 mls of buffer were loaded onto the column at a rate of 75-100 ml per hour. The gradient application, and tube collections are described under the Materials and Methods section. A yellow fraction, presumably a flavin-linked protein, preceded the elution of G6PDH protein, and served as a marker for the elution of the activity. Table III shows the data obtained from a typical separation on DEAE-cellulose, and Figure 2 illustrates the elution profile of a typical potassium chloride gradient.

Purification as high as 14-fold has been obtained by this chromatographic procedure; however, 8 to 10-fold values are typical. The yields from the DEAE-cellulose chromatography remain in the 80-85% range despite small variations in the purifications obtained. The

Table III

Chromatography of Escherichia coli B Glucose-6-phosphate dehydrogenase
on DEAE-Cellulose Using a Linear Potassium Chloride Gradient

Applied				Recovered					
Tot. Units G6PDH	Tot. Units 6PGDH	Protein (mg)	Spec. Act. (units/mg)	Tot. Units G6PDH	Tot. Units 6PGDH	Protein (mg)	Spec. Act. (units/mg)	Fold Pur.	% Rec.
666	166	6.25	0.104	585	0	946.1	0.82	8	87.6

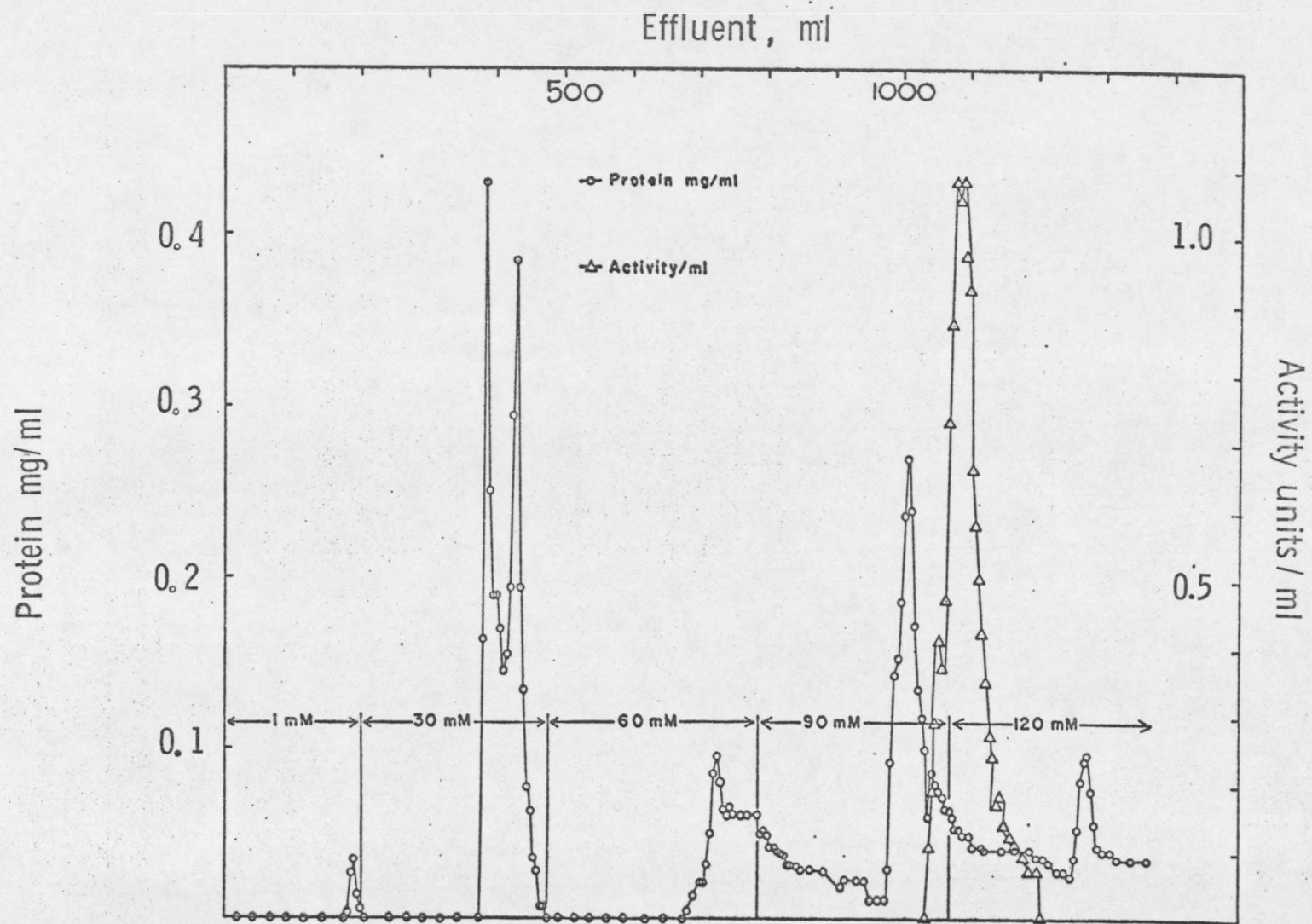
DEAE-cellulose was used repeatedly over periods of six months, and was only treated and repacked when poor separations and irregularities in loading were exhibited. Treating and packing procedures are described under the Materials and Methods section. The chromatographic technique provided G6PDH activity free from 6PGDH activity, and in addition removed 90% of the nucleic acids present in the ammonium sulphate fractionation. Precipitation of the recovered activity at 60% ammonium sulphate saturation provided the best means of storing the protein. This precipitation also resulted in a 2-fold purification (Table VIII).

Hydroxyl Apatite Chromatography

The preparation of the hydroxyl apatite adsorbant yielded 300 ml of the adsorbant. The properties of the adsorbant varied between preparations; however, these variations were in the concentration of the potassium phosphate buffer necessary to displace the activity. Resolution and capacity of the hydroxyl apatite remained constant for several preparations. It was found that replacement of the sodium ion, used in the adsorbant preparation, with potassium resulted in a much longer column life for the hydroxyl apatite. When sodium phosphate buffers were employed in the chromatography, the sodium ion replaced calcium in the hydroxyl apatite lattice and this resulted in smaller crystal size and lower flow rate; consequently, after three or four uses this necessitated operating the column with increased pressure. The use of potassium phosphate buffers eliminated this problem and

Figure 3

Elution profile of protein and glucose-6-phosphate dehydrogenase from stepwise hydroxyl apatite chromatography with potassium phosphate buffers.



hydroxyl apatite columns could be used repeatedly without any appreciable decrease in the flow rate.³¹

The ammonium sulphate precipitated protein from the DEAE-cellulose chromatography was dissolved and chromatographed on hydroxyl apatite as described under the Material and Methods section. Figure 3 illustrates the elution profile obtained with this method of separation. When the protein solution was adsorbed onto the hydroxyl apatite column it appeared as a light yellow band at the top of the column. This yellow component was eluted in the 30 mM phosphate wash. Table IV shows the data obtained from a typical separation on hydroxyl apatite.

Table IV

Hydroxyl Apatite Chromatography of Escherichia coli B Glucose-6-phosphate dehydrogenase by Stepwise Elution Using Potassium Phosphate Buffers, pH 6.8

Applied			Recovered				
Tot. Units G6PDH	Protein (mg)	Spec. Act. (units/mg)	Tot. Units G6PDH	Protein (mg)	Spec. Act. (units/mg)	Fold Pur.	% Rec.
368	310.2	1.2	333	16.8	20.3	17	90

This method of chromatography provided the best separation of components in the partially purified enzyme preparation. Yields were consistently in the 85-95% range, with purifications of from 15 to 20-fold. Hydroxyl apatite column chromatography is based on affinity

chromatography, and most commonly a stepwise gradient is employed (Levin³⁰). At each step the R_f of an eluted protein is increased from zero to one, and consequently one to one and a half column volumes of each phosphate buffer is sufficient to elute a protein. The elution profile (Figure 3) shows that the protein displaced by the 90 mM phosphate wash consists of two components appearing very close together. The second component contains the G6PDH activity and the first, non-active component, is approximately 20% larger. Similarly, a two-component peak is obtained in the 30 mM phosphate wash.

A two component peak such as this is unusual in hydroxyl apatite separations, and the binding constants for the two components must differ only very slightly. Levin³⁰ has stated that a single protein may appear as a split peak due to a too low displacing concentration of phosphate. However, a two component system within a single phosphate wash has not been reported before. The elution profile illustrated in Figure 3 indicates that fine resolution within a particular stepwise concentration of phosphate buffer is feasible.

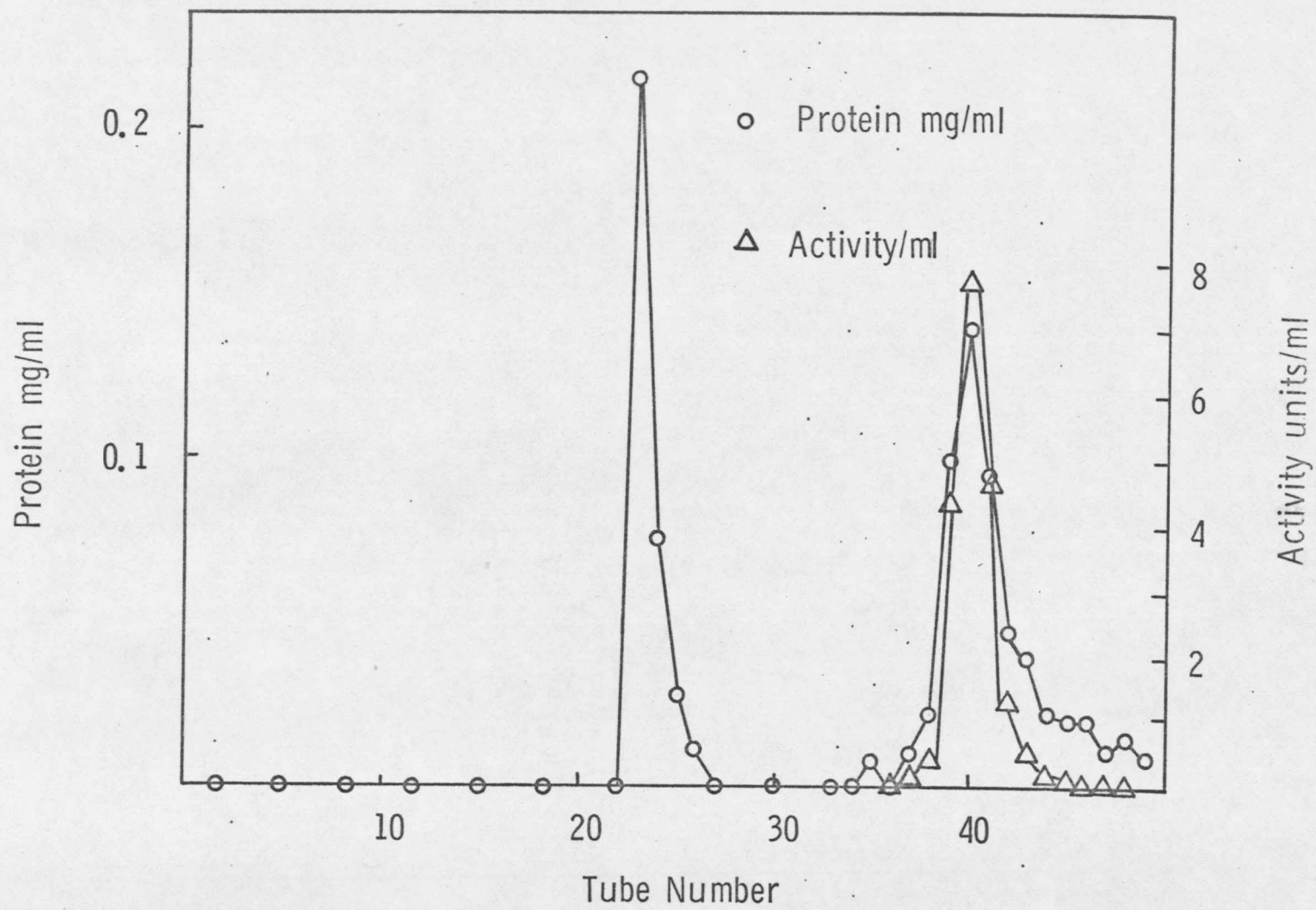
Tubes containing G6PDH activity with a specific activity of 20.0 units/mg or more were pooled and precipitated at 60% ammonium sulphate saturation. The protein was also stored in this manner.

DEAE-Cellulose Chromatography by Exponential Potassium Chloride Gradient

Chromatographic separation of the partially purified E. coli B G6PDH was accomplished using an exponential potassium chloride gradient

Figure 4

Elution profile of protein and glucose-6-phosphate dehydrogenase from
DEAE-cellulose chromatography with exponential potassium chloride gradient.



on DEAE-cellulose. The protein from the hydroxyl apatite chromatography was collected, dissolved and adsorbed onto the DEAE-cellulose column as described in the Materials and Methods section.

Figure 4 illustrates the elution profile obtained with the exponential gradient. The gradient described in the Materials and Methods section was chosen since it provided the best separation of the two peaks diagrammed in Figure 4. A steeper gradient resulted in overlap of the active and non-active peaks. A shallower gradient, while providing separation of the two peaks, produced a wide, dilute enzyme peak. The exponential DEAE-cellulose chromatography provided a 3-fold purification; the amounts of protein in the active, non-active and 500 mM KCl wash peaks were equal.

The data in Table V illustrate the results obtained in a typical separation on DEAE-cellulose.

Table V

DEAE-Cellulose Chromatography of Escherichia coli B Glucose-6-phosphate dehydrogenase with Exponential Potassium Chloride Gradient

Applied			Recovered				
Total Units G6PDH	Total mg Protein	Specific Activity	Total Units G6PDH	Total mg Protein	Specific Activity	Fold Pur.	% Rec.
116.6	5.0	23.3	75.0	1.1	68.3	3.1	64.3

Yields from this chromatographic separation vary from 60-70%, and the final specific activity ranges from 70-80 units/mg. This purification

was obtained when 5.0 mgm, or less, of protein were applied to the exponential DEAE-cellulose column. Larger loadings were possible, but the purifications obtained were not as high. For example, Table VI shows the values obtained when a sample containing 25.8 mgms of protein was applied to the column.

Table VI

DEAE-Cellulose Chromatography of Escherichia coli B Glucose-6-phosphate dehydrogenase with Exponential Potassium Chloride Gradient

Applied			Recovered				
Total Units G6PDH	Total mg Protein	Specific Activity	Total Units G6PDH	Total mg Protein	Specific Activity	Fold Pur.	% Rec.
466	25.8	18.0	336	8.4	40.3	2.24	66

Larger protein loading, as illustrated in Table VI results in similar enzyme recoveries, but at a 30% lower purification than obtained with 5 mg or less applications.

Final Hydroxyl Apatite Chromatography and Storage of G6PDH

A small hydroxyl apatite pad was used for the final chromatography and storage of the G6PDH activity as described under Materials and Methods. G6PDH samples of specific activity 65-80 units/mg, obtained from small loading on the column, could be stored on the hydroxyl apatite pad, but no further purification was obtained. G6PDH samples of specific activity 40-50 units/mg, obtained from the larger loadings on the DEAE-cellulose column, could be further purified to a value of

65-80 units/mg in addition to storage on the hydroxyl apatite.

Table VII illustrates this data.

Table VII

Hydroxyl Apatite Chromatography of Escherichia coli B
Glucose-6-phosphate dehydrogenase

Applied			Recovered				
G6PDH units	Protein mgms	Spec. Act. (units/mg)	G6PDH units	Protein mgms	Spec. Act. (units/mg)	Fold Pur.	% Rec.
336	8.4	40.3	240	3.8	65.2	1.6	71.4
75.0	1.1	68.3	80	1.18	70.3	1.06	100

The data in Tables VI and VII indicate that the larger loadings on the exponential DEAE-cellulose column result in a 50% overall recovery of G6PDH activity; 66% in the DEAE-cellulose chromatography and 70% in the final hydroxyl apatite step. The smaller loadings, 5 mg or less, on the DEAE-cellulose column yielded purified enzyme at a yield of 70% and after storage on hydroxyl apatite could be 100% recovered. Thus the smaller DEAE-cellulose separations, although more tedious, provided better overall yield of the purified enzyme. For this reason the smaller loadings were employed in most cases.

When adsorbed onto the hydroxyl apatite pad the purified G6PDH activity was stable. In one experiment 95% of the purified enzyme was recovered after storage for a period of one week. In addition the enzyme could be concentrated two-fold when displaced by 120 mM phosphate buffer.

Table VIII

Purification of Escherichia coli B Glucose-6-phosphate dehydrogenase

Purification Step	Total Units G6PDH	Total Protein (mg)	Specific Activity units/mg	Purif. per Step	Accumulative Purification	% Yield
Crude Cellular Extract	4352	57.41×10^3	0.075	-	-	100
55% $(\text{NH}_4)_2\text{SO}_4$ Fractionation	4004	37.77×10^3	.106	1.4	1.4	93
DEAE-Cellulose Chromatography Linear KCl-Gradient	3507	4.27×10^3	0.82	8	11.2	81
60% $(\text{NH}_4)_2\text{SO}_4$ Fractionation	3366	2.59×10^3	1.3	1.6	17.9	77.7
Hydroxyl Apatite Chromatography	3029	1.49×10^2	20.3	16	286.4	69.9
DEAE-Cellulose Chromatography Exponential KCl-Gradient	2000	48.7	41	2.02	579	46
Hydroxyl Apatite Chromatography	1420	17.3	82	2.0	1157	32.6

Table VIII outlines the results for the total preparation. In preparing the enzyme, the various stages were run stepwise. The total amount of crude cellular extract was not used in one preparative sequence. Instead, fractions of the protein were run at each stage of purification. Consequently the data in Table VIII represent an average result for the complete preparation. The final specific activity of the G6PDH varies from 70-80 units/mg; however, specific activities as high as 92 have been obtained, representing a 1227-fold purification. The purification procedure described results in 1157-fold purification of E. coli B G6PDH to a specific activity of 82 units/mg measured at pH 7.4. Purifications from several other bacterial sources have been reported. Sanwall¹⁵ has obtained a 167-fold purification of the E. coli B enzyme to a specific activity of 5.7 units/mg measured at pH 8.0. Levy²⁶ has reported a 114-fold purification of G6PDH from Leuconostoc mesenteroides; the purified protein had a specific activity of 266 units/mg at pH 7.8. G6PDH from Candida utilis, specific activity 314 units/mg at pH 9.1, has been purified 475-fold by Engel et al.¹⁹ Noltmann, Gubler and Yue²¹ have reported a 2535-fold purification of G6PDH from Saccharomyces carlbergensis in a ten step procedure. The final specific activity obtained was 678 units/mg measured at pH 8.0.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed using a 5% separatory gel and a 3% stacking gel as described under the Materials and

Figure 5

Polyacrylamide gel electrophoresis of purified Escherichia coli B glucose-6-phosphate dehydrogenase. The procedure employed was described under the Materials and Methods section.

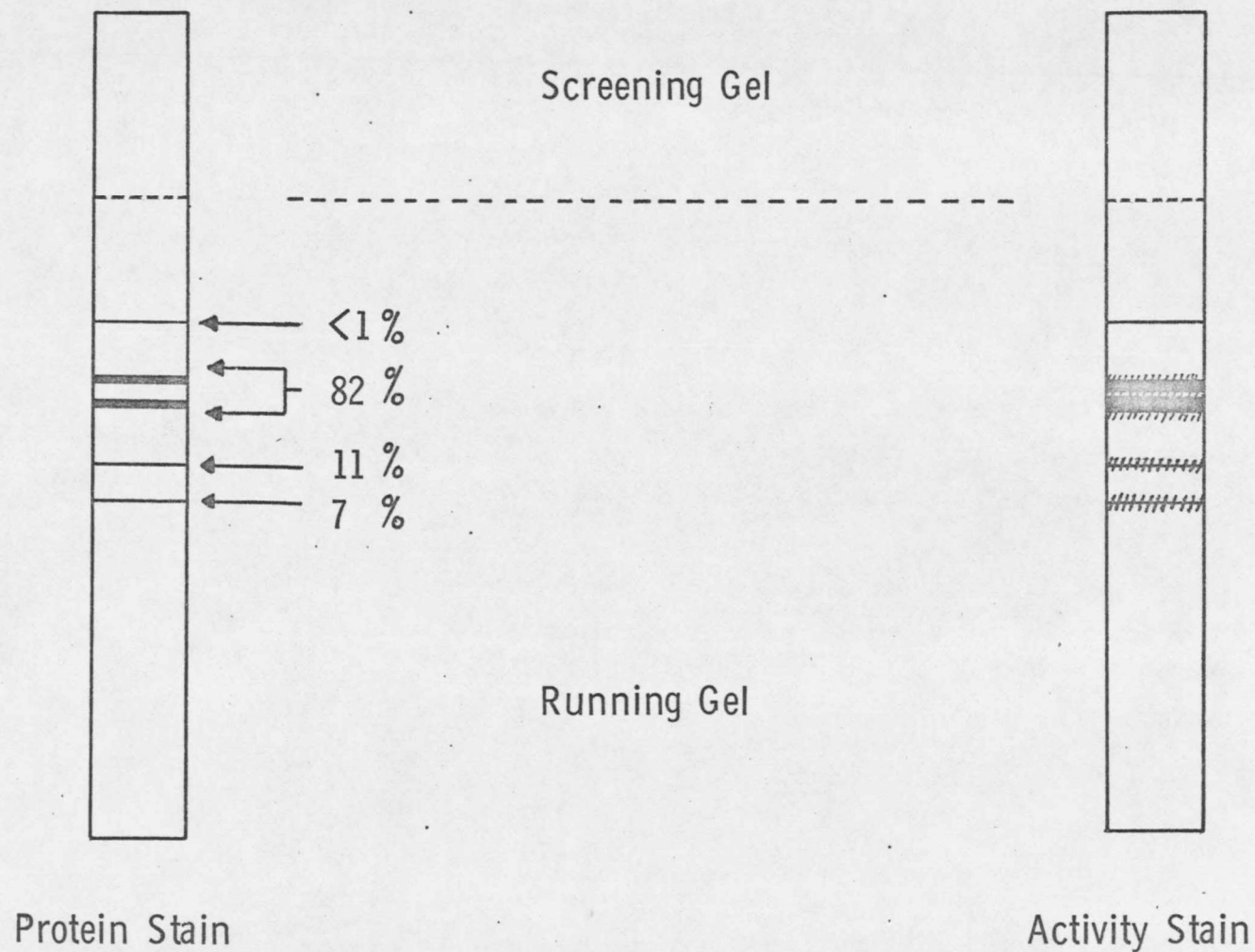
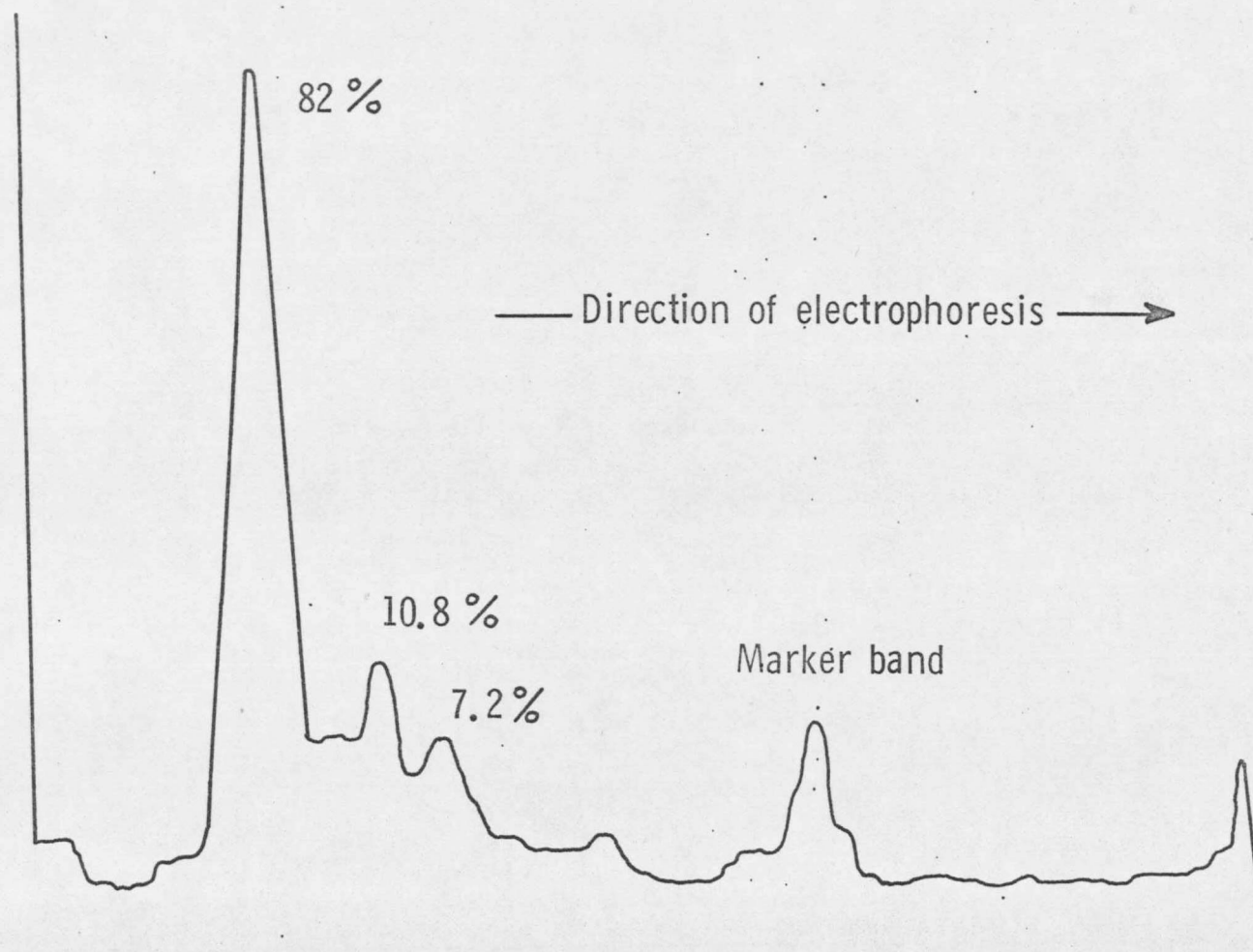


Figure 6

Densitometer scan of Amido Schwartz stained polyacrylamide gel electrophoresis of Escherichia coli B glucose-6-phosphate dehydrogenase. The scan was performed using a Joyce-Loebel densitometer.



Methods section. The purified E. coli B G6PDH protein gave a distribution of five protein bands, each of which was active when stained with the activity solution. This five-banded pattern was reproducible for various preparations of the enzyme. In all cases, each of the five protein bands had enzyme activity. Figure 5 illustrates the band distribution obtained, and shows the coincidence of the protein and activity stains. A densitometer scan of the amido schwartz stained gel is shown in Figure 6. Approximately 82% of the protein occurs in two very closely spaced bands; 10.8 and 7.2% occur in the other two faster moving bands (Figure 6). The slowest moving band, which is also the narrowest, contains very little protein but does stain with activity solution. The interesting factor about the protein distributions in the acrylamide gels is the reproducibility of the pattern between enzyme preparations. The distribution of protein within the five active protein bands also remains constant between preparations. Several attempts were made to change, or to reduce, the band complexity obtained. Gels were normally run at pH 8.4, although pH 7.4 gels produced the same pattern and distribution. In many cases it has been suggested that the ammonium persulphate present in the gels can cause multiple bands due to oxidation of the protein. Consequently gels were pre-electrophoresed in order to remove the ammonium persulphate present in the gels. Subsequent loading and development of the gels gave the same five band distribution. This indicates that the band distribution obtained is not a result of oxidation of the enzyme by the persulphate. Attempts to reduce the complexity of

the protein bands by incubation of the enzyme with β ME and also coenzyme (NADP^+) and subsequent electrophoresis with β ME or NADP^+ in the gel buffer did not change the band distribution. Incubation with 0.2 M β ME at pH 9.0 for 24 hours and running the gels with gel buffer (pH 8.4) containing 0.2 M β ME resulted in the same band distribution; however, the pattern obtained was rather more diffuse. It is interesting to note that the G6PDH is still active in 0.2 M β ME, which indicates that the maintenance of accessible disulphide bonds is not necessary for activity.

Despite this distribution of five protein bands produced in polyacrylamide gel electrophoresis, the G6PDH activity exhibited by each protein band indicates that the prepared enzyme contains no contaminating proteins. Three explanations could account for the five band distribution obtained with the gels. One possibility is that the five protein bands represent various aggregates of the enzyme; however, the irregular spacing of the gel bands would tend to discount this possibility. The electrophoretic displacements of the five protein bands, calculated with the top of the running gel as the origin, were calculated. The electrophoretic displacements of the five bands, in order of increasing displacement (Figure 5) were 0.196, 0.286, 0.329, 0.421 and 0.475, respectively. These values expressed as a ratio of electrophoretic displacements, with the least displaced band as unity are 1.0, 1.46, 1.68, 2.15 and 2.42 respectively. If the five bands represented various aggregates of a single protomer then a more

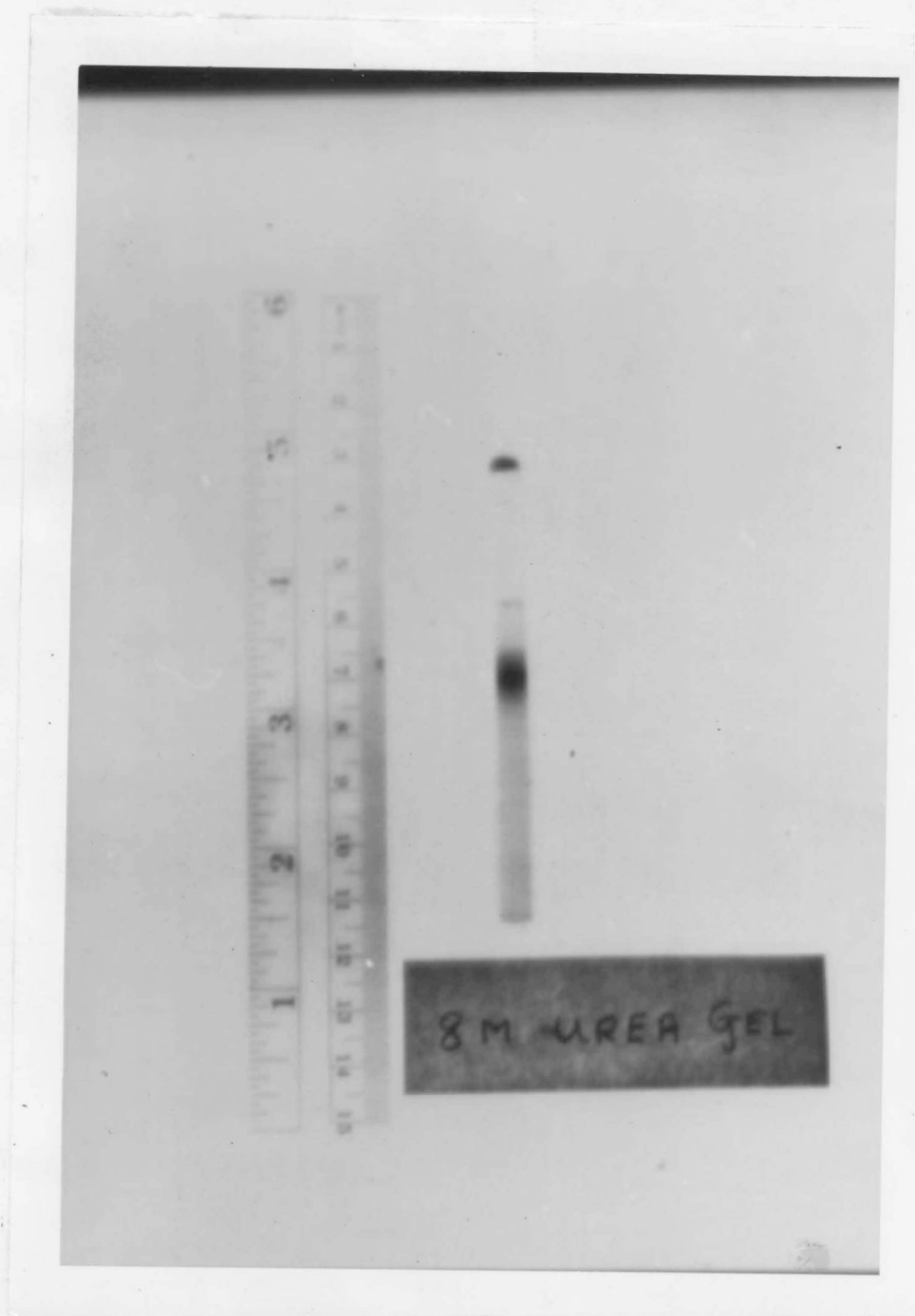
regular spacing of the bands would be expected. However, it is possible that aggregation of a single protomer species could result in changes in the charge to mass ratio of the aggregated species as compared to the protomer. In this case the five aggregates of the protomer would result in a non-regular spacing of the bands. In any case, if the presence of "aggregers" is responsible for the five band distribution, disulphide bonds cannot be the principal bonds involved since β ME did not change the band pattern. Conformational states of a single enzyme species could also account for the five banded distribution. In addition, if the G6PDH enzyme consisted of two, non-identical subunits, A and B, and only tetramers of these two subunits were enzymically active, then a five band enzyme distribution would result. In this case the active tetramers would be A_4 , A_3B , A_2B_2 , AB_3 , and B_4 .

Urea Polyacrylamide Gel Electrophoresis

Polyacrylamide gels containing 6 M urea were prepared as described under the Materials and Methods section. Incubation of the purified G6PDH in 8 M urea for 24 hours and subsequent gel electrophoresis with a 3% separatory and 3% stacking gel resulted in only a single band (Figure 7). Dissociation of the G6PDH protein reduced the five band structure to a single protein band. This strongly suggests that the prepared enzyme consisted of a mixture of five aggregates, since only one protein band was obtained. In addition, this result indicates that the bonding responsible for the appearance of aggregates is due

Figure 7

6 M urea polyacrylamide gel electrophoresis of Escherichia coli B glucose-6-phosphate dehydrogenase. A 3% polyacrylamide gel was employed.



to salt bridging, hydrogen bonding or hydrophobic associations, or some combination of the three. Disulphide bonding of the aggregates is not indicated since incubation of the proteins in 0.2 M β ME and subsequent electrophoresis did not reduce the band complexity. The single band obtained with the 6 M urea gels also strongly suggests that the enzyme subunits are identical. However, it is still possible that the G6PDH consists of two similar, but non-identical, subunits which appear as one band in the urea gels.

Incubation of the G6PDH in 6 M urea was insufficient to reduce the gel pattern to a single band. It was found that 8 M urea incubation over a period of 24 hours was necessary for complete dissociation. In addition, 3% separatory gels allowed for much more rapid development than the 5% gels. In gel electrophoresis the denatured protein would migrate extremely slowly in a 5% separatory gel.

Sodium Dodecyl Sulphate Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gels were prepared and run as described under the Materials and Methods section. Purified E. coli B G6PDH protein and standard proteins, bovine serum albumin, yeast alcohol dehydrogenase, chymotrypsin and ribonuclease A, were dissociated with SDS and β ME as described previously.

The purified G6PDH protein gave only one band after SDS incubation and gel electrophoresis. This result again indicates that the G6PDH consists of very similar or identical subunits.

Table IX

Sodium dodecyl sulphate electrophoresis of sodium dodecyl sulphate-dissociated *Escherichia coli* B glucose-6-phosphate dehydrogenase and standard proteins. Molecular weights of standards obtained from Weber and Osborn.³⁵

Protein Sample	Length of Gel Before Staining (cm)	Length of Gel After Destaining (cm)	Migration of Marker Dye Before Destaining (cm)	Migration of Protein Band (cm)	Mobility	Molecular Weight (Daltons)
<i>E. coli</i> B G6PDH	6.9	7.6	5.6	3.1 (5)	.517	---
Chymo- trypsin	7.0	7.5	5.1	4.7	.86	22.5×10^3
Yeast Alcohol Dehydro- genase	6.9	7.5	5.1	3.7	.665	37×10^3
RNAse A	6.9	7.5	5.0	5.3	.97	13.7×10^3
Bovine Serum Albumin	6.9	7.5	5.2	1.7	.332	67×10^3

Electrophoresis of G6PDH protein and other globular protein samples as standards provided a convenient method of molecular weight determination. The gels were developed, measured and treated as described previously. The data in Table IX were obtained for a typical electrophoretic experiment. The protein mobility was calculated according to the following equation:

$$\text{Mobility} = \frac{\text{Distance of Protein Migration}}{\text{Dye Migration}} \times \frac{\text{Length before staining}}{\text{Length after destaining}}$$

The mobilities of the protein standards were plotted versus their molecular weights (log scale) to give a standard molecular weight distribution curve. The molecular weight of the purified E. coli B G6PDH was determined from this curve by interpolation of the mobility value. This data is illustrated graphically in Figure 8. In three separate experiments the subunit molecular weight values obtained for the G6PDH protein were 53, 53, and 49×10^3 daltons.

The presence of only one band from the dissociation of the purified enzyme also provided a purity check for the enzyme preparation (Figure 9). It was found that staining the developed SDS gels with the Amido Schwartz stain instead of the Coomassie Brilliant Blue resulted in clearer and more easily detected protein bands. In addition the use of the Amido Schwartz protein stain facilitated the destaining of the gels.

Figure 8

SDS polyacrylamide gel electrophoresis. Mobilities of the protein standards were plotted as a function of their molecular weight (log scale). The molecular weight of Escherichia coli B glucose-6-phosphate dehydrogenase was determined by interpolation of the mobility value.

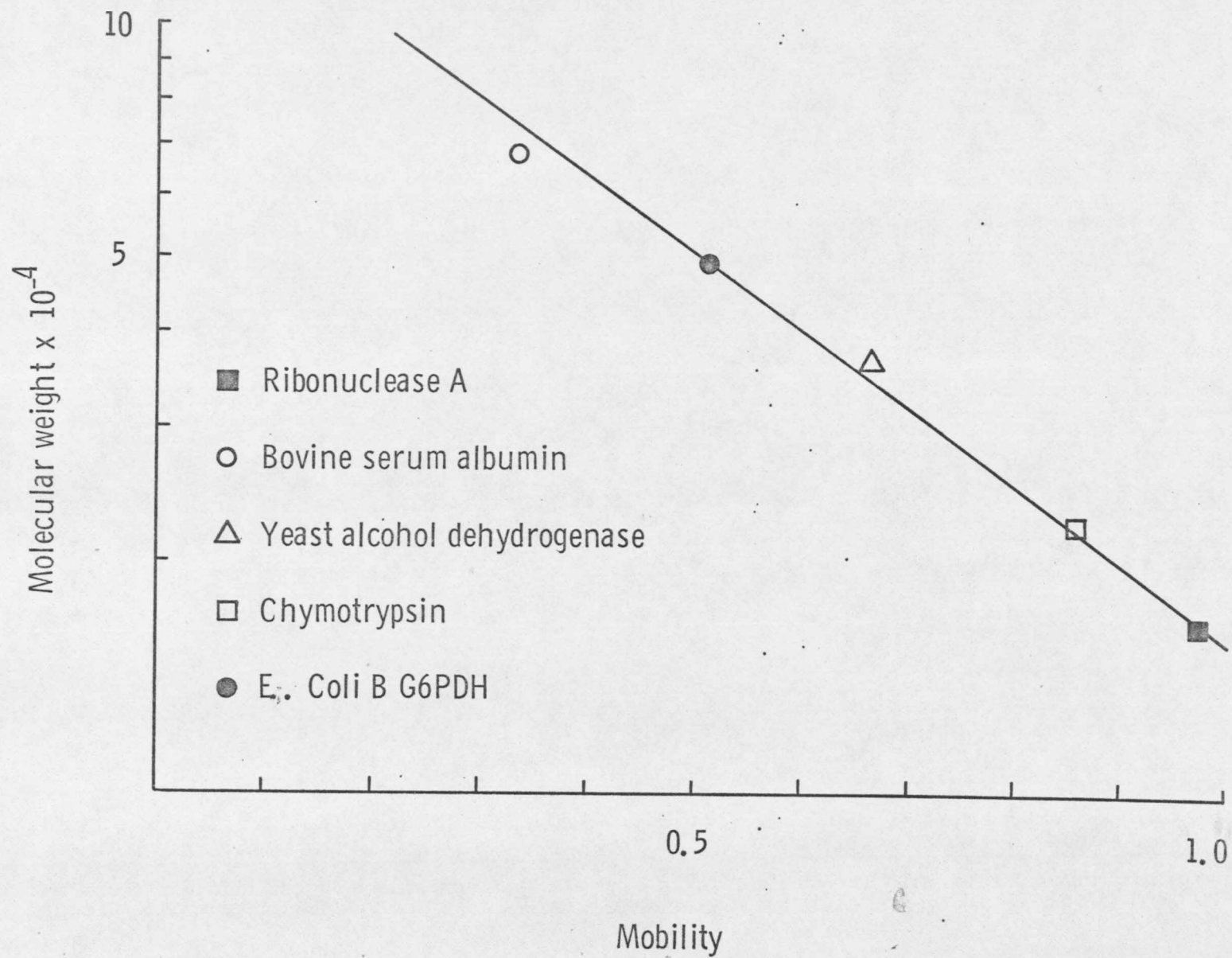


Figure 9

SDS Polyacrylamide Gel Electrophoresis of Escherichia coli B

Glucose-6-phosphate dehydrogenase.

Procedure as Described under Materials and Methods Section.

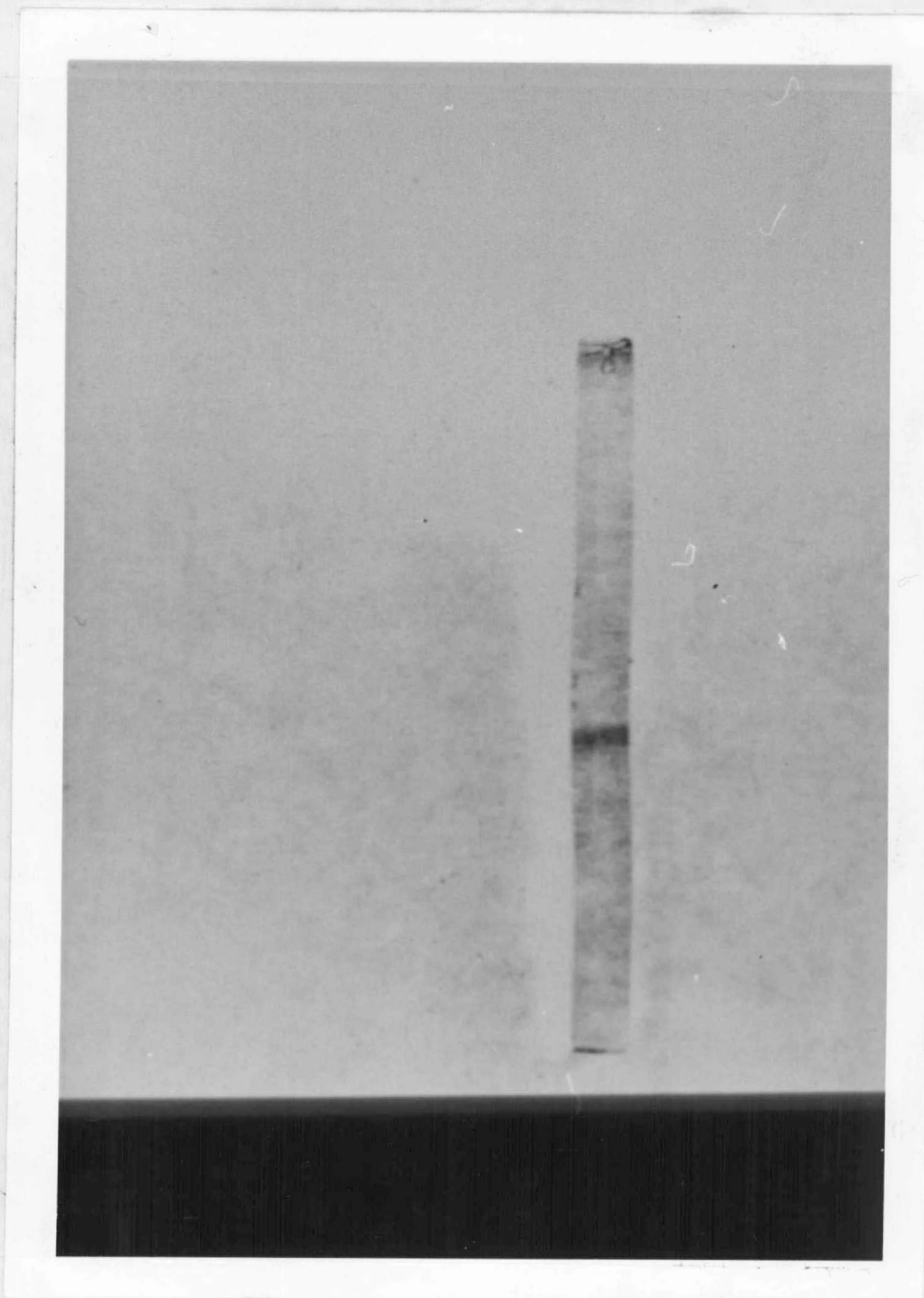


Figure 10

High speed sedimentation ultracentrifugation of Escherichia coli B glucose-6-phosphate dehydrogenase. Interferogram obtained from 28 micrograms per milliliter Escherichia coli B glucose-6-phosphate dehydrogenase, centrifuged at 32,000 rpm for 70 hours.

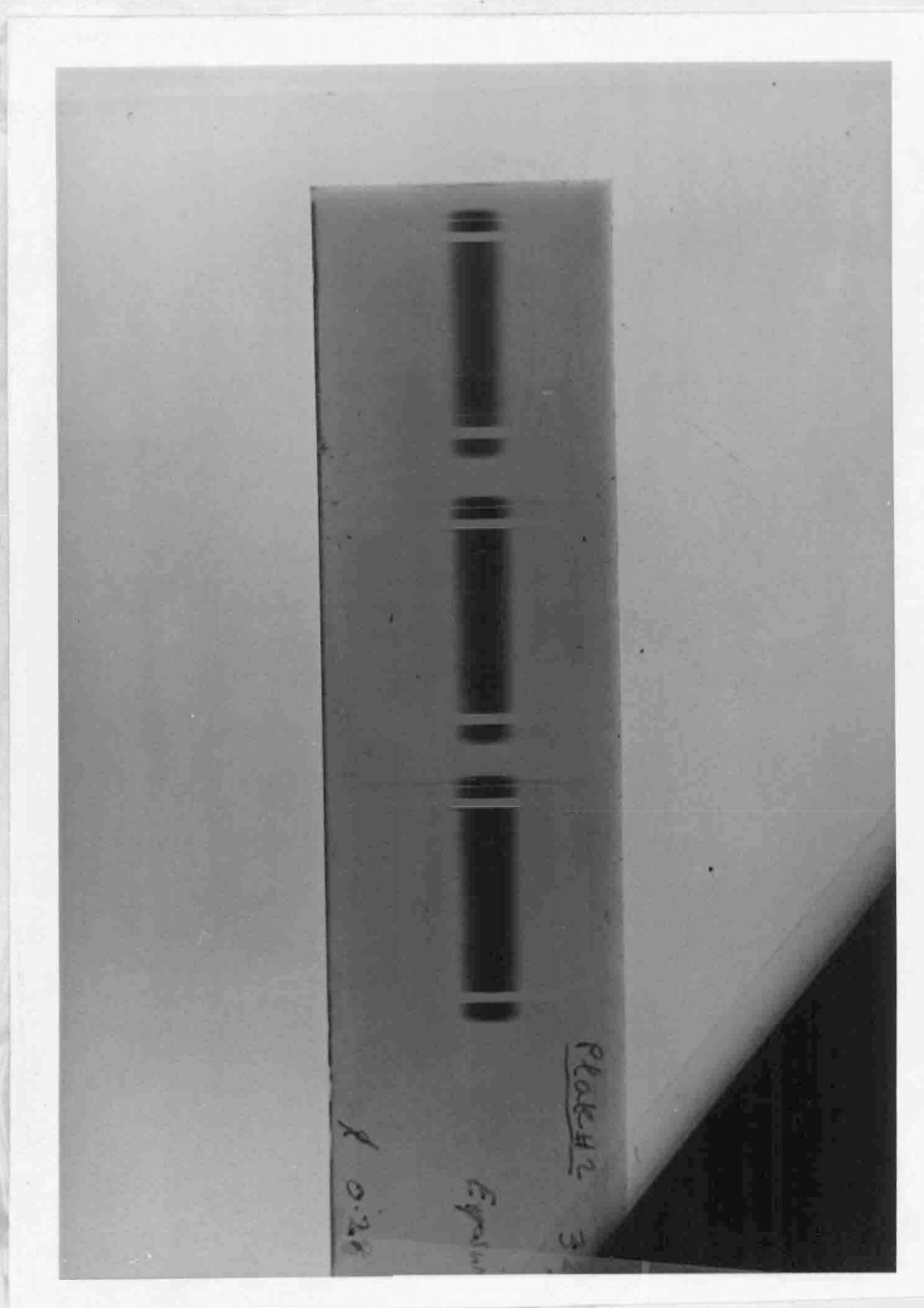


Figure 11

Graph of $\log_{10} (\Delta y \times 10^3)$ versus r^2 . The solution column contained 280 micrograms per milliliter Escherichia coli B glucose-6-phosphate dehydrogenase in 6 M guanidine-hydrochloride and was centrifuged at 34,000 rpm for 28 hours.

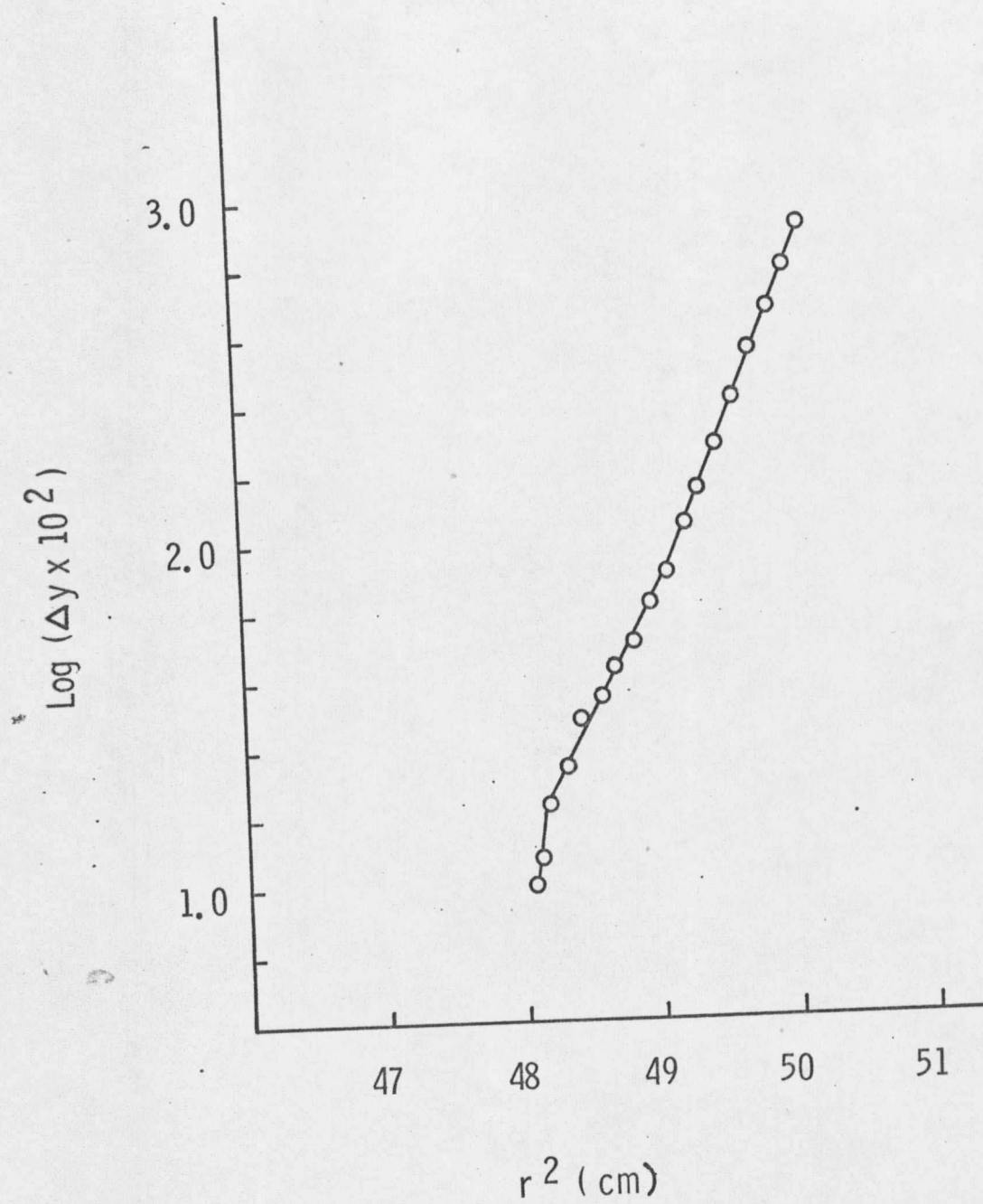


Figure 12

Graph of $\log_{10} (\Delta y \times 10^3)$ versus r^2 . The solution column contained 140 micrograms per milliliter Escherichia coli B glucose-6-phosphate dehydrogenase in 6 M guanidine-hydrochloride and was centrifuged at 26,000 rpm for 26 hours.

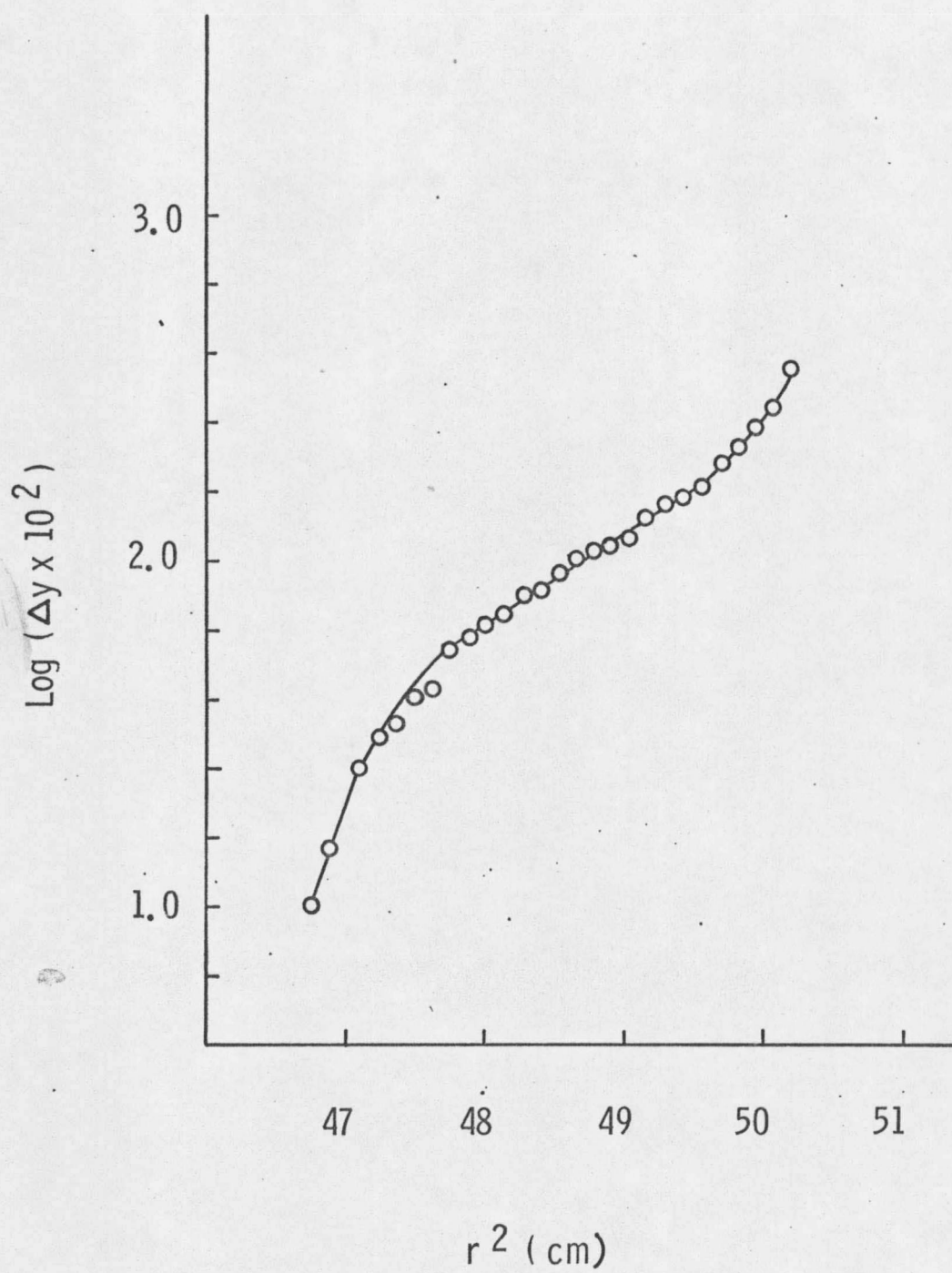
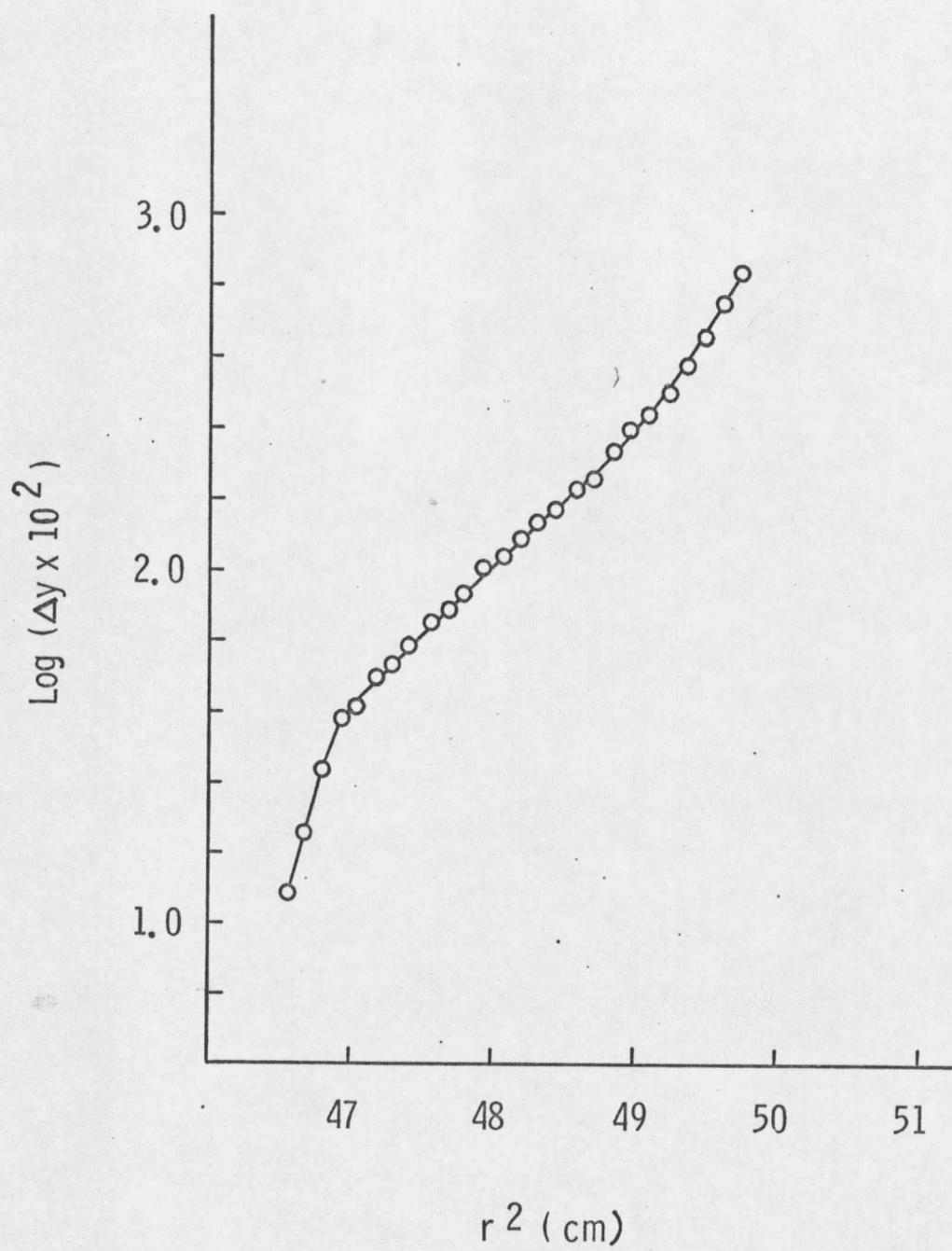


Figure 13

Graph of $\log_{10} (\Delta y \times 10^3)$ versus r^2 . The solution column contained 85 micrograms per milliliter Escherichia coli B glucose-6-phosphate dehydrogenase and was centrifuged at 30,000 rpm for 30.25 hours.



High Speed Sedimentation Equilibrium Ultracentrifuge Determination of G6PDH Subunit Molecular Weight

The spectroscopic interferograms obtained in the high speed sedimentation equilibrium ultracentrifugation were analyzed as described in the Materials and Methods section. Figure 10 shows a typical interferogram obtained. The fringe height at 0.1 mm increments was measured across a single fringe from the solution meniscus to the bottom of the solution column. The results were plotted as $\log_{10} (\Delta y)$ versus r^2 , where Δy (the fringe height) is a function of the enzyme concentration and r^2 the square of the distance from the reference hole to a point on a particular fringe in the solution column. Figures 11, 12 and 13 illustrate this plot for three G6PDH concentrations at three different speeds; 280 $\mu\text{g/ml}$ centrifuged at 34,000 rpm; 140 $\mu\text{g/ml}$ centrifuged at 26,000 rpm and an 85 $\mu\text{g/ml}$ solution centrifuged at 30,000 rpm. For each graph of $\log \Delta y$ versus r^2 , slopes were measured at six points along the plot. Tables X, XI and XII show the values of $\frac{d \log (\Delta y)}{dr^2}$ as a function of r^2 , and the corresponding weight-average molecular weights at these various positions in the solution column.

Molecular weights were calculated according to Yphantis³⁶ using the following equation:

$$\bar{M}_w, \text{ app.} = \frac{2 RT \times 2.303 \frac{d \log c}{dr^2}}{(1 - v_p) \omega^2}$$

$\bar{M}_w$, app. = apparent weight-average molecular weight

R , gas constant = 8.3143×10^7 ergs $^{\circ}\text{K}^{-1}\text{mole}^{-1}$

T = Temperature in degrees Kelvin

$\bar{v}$ = partial specific volume of the solute (G6PDH) =
0.72 cc/gm

ρ = density of 6 M Guanidine Hydrochloride

ρ at 6.90°C = 1.1503

6.95°C = 1.1503

8.25°C = 1.1498

ω = rotational speed = $\left(\frac{2\pi}{60} \times \text{rpm}\right)^2$

$\frac{d \log c}{dr^2}$ = slope of the $\log \Delta y$ versus r^2 graph

* The value of the partial specific volume ($\bar{v}$) of the solute was assumed to be 0.72 cc g^{-1} , a value obtained by Yue, Noltman and Kuby³⁷ for yeast G6PDH. The density of 6 M guanidine-HCl solution at the temperature of the centrifugation was determined from data obtained by Reithel, Robbins and Gorin.³⁸

Table X

Apparent Weight-average Molecular Weight of Glucose-6-phosphate dehydrogenase as a Function of Position in the Solution Column. Eighty-five Micrograms per Milliliter of Glucose-6-phosphate dehydrogenase in 6 M Guanidine-HCl, 0.2 M BME Centrifuged at 30,000 rpm for 30.25 hrs at 6.95° C.

Slope = $\frac{d \log_{10} (\Delta y \cdot 10^3)}{dr^2}$	$r^2 (\text{cm}^2)$	$\bar{M}_w$, app. (Daltons)
0.700	49.6	44.26×10^3
0.575	49.2	36.36×10^3
0.457	48.8	28.90×10^3
0.405	48.4	25.61×10^3
0.400	48.0	25.29×10^3
0.393	47.6	24.85×10^3

Table XI

Apparent Weight-average Molecular Weight of Glucose-6-phosphate dehydrogenase as a Function of Position in the Solution Column. One hundred and forty Micrograms per Milliliter of Glucose-6-phosphate dehydrogenase in 6 M Guanidine-HCl, 0.2 M β ME Centrifuged at 26,000 rpm for 26 hrs at 8.25° C.

Slope = $\frac{d \log_{10}(\Delta y \times 10^3)}{dr^2}$	$r^2(\text{cm}^2)$	$\bar{M}_w$, app. (Daltons).
0.575	50.0	48.53×10^3
0.375	49.6	31.65×10^3
0.325	49.2	27.43×10^3
0.263	48.8	22.20×10^3
0.263	48.9	22.20×10^3
0.250	48.0	21.94×10^3

Table XII

Apparent Weight-average Molecular Weight of Glucose-6-phosphate dehydrogenase as a Function of Position in the Solution Column. Two hundred and eighty Microliters per Milliliter of Glucose-6-phosphate dehydrogenase in 6 M Guanidine-HCl, 0.2 M β ME, Centrifuged at 34,000 rpm for 28 hrs.

Slope = $\frac{d \log_{10}(\Delta y \times 10^3)}{dr^2}$	$r^2(\text{cm}^2)$	$\bar{M}_w$, app. (Daltons)
1.100	50.0	54.14×10^3
1.000	49.6	49.22×10^3
0.840	49.2	41.35×10^3
0.650	48.9	31.99×10^3
0.475	48.7	23.38×10^3
0.450	48.5	22.15×10^3

Figure 14

Apparent Weight-average Molecular Weight Plotted as a Function of the Position in the Solution Column. In this Experiment a Solution Containing 280 Micrograms per Milliliter of Glucose-6-phosphate dehydrogenase, in 6 M Guanidine-Hydrochloride, Centrifuged at 34,000 rpm for 28 Hours.

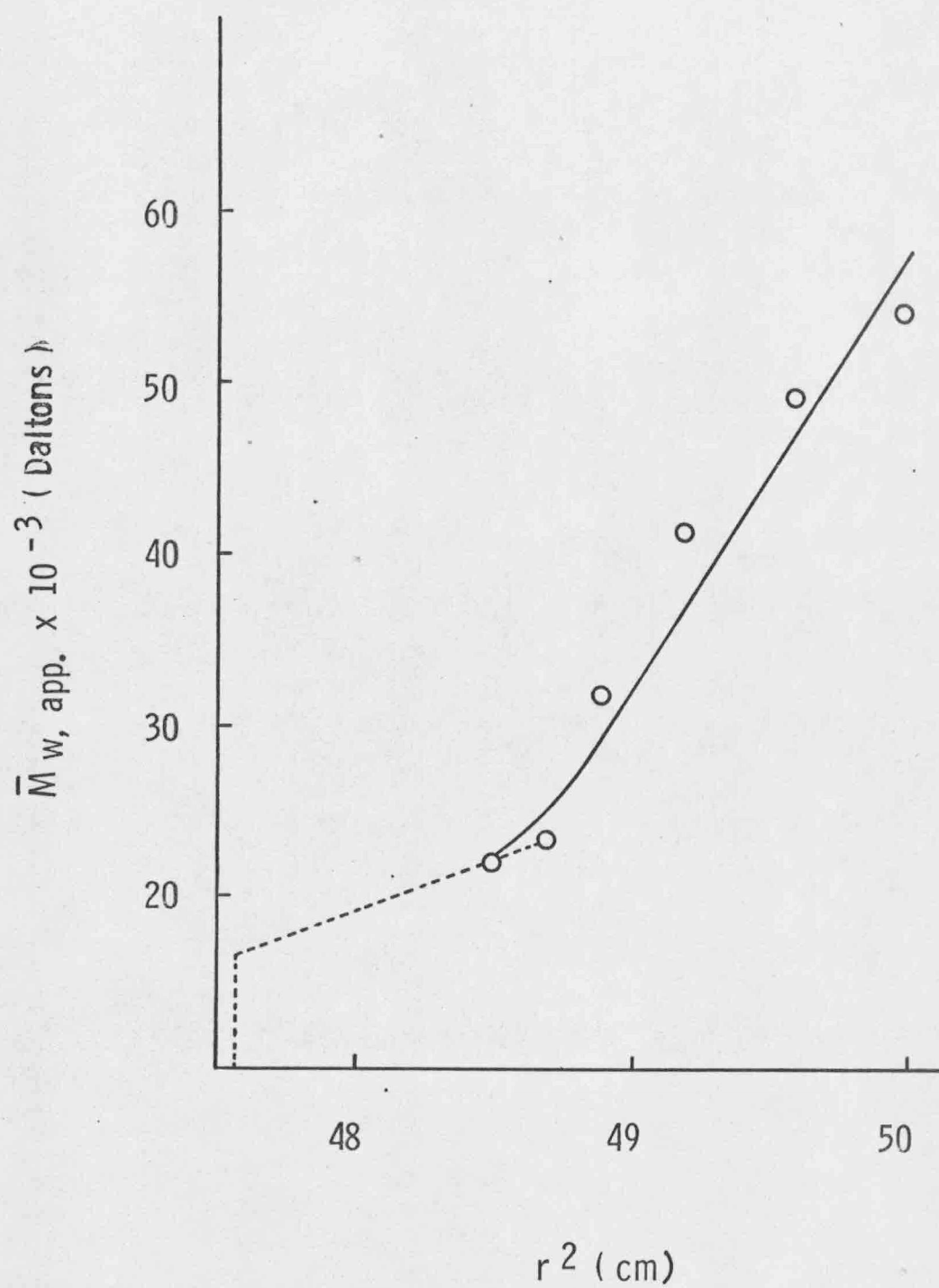


Figure 15

Apparent weight-average molecular weight plotted as a function of the position in the solution column. In this experiment a solution containing 140 micrograms per milliliter glucose-6-phosphate dehydrogenase in 6 M guanidine-hydrochloride, centrifuged at 36,000 rpm for 26 hours.

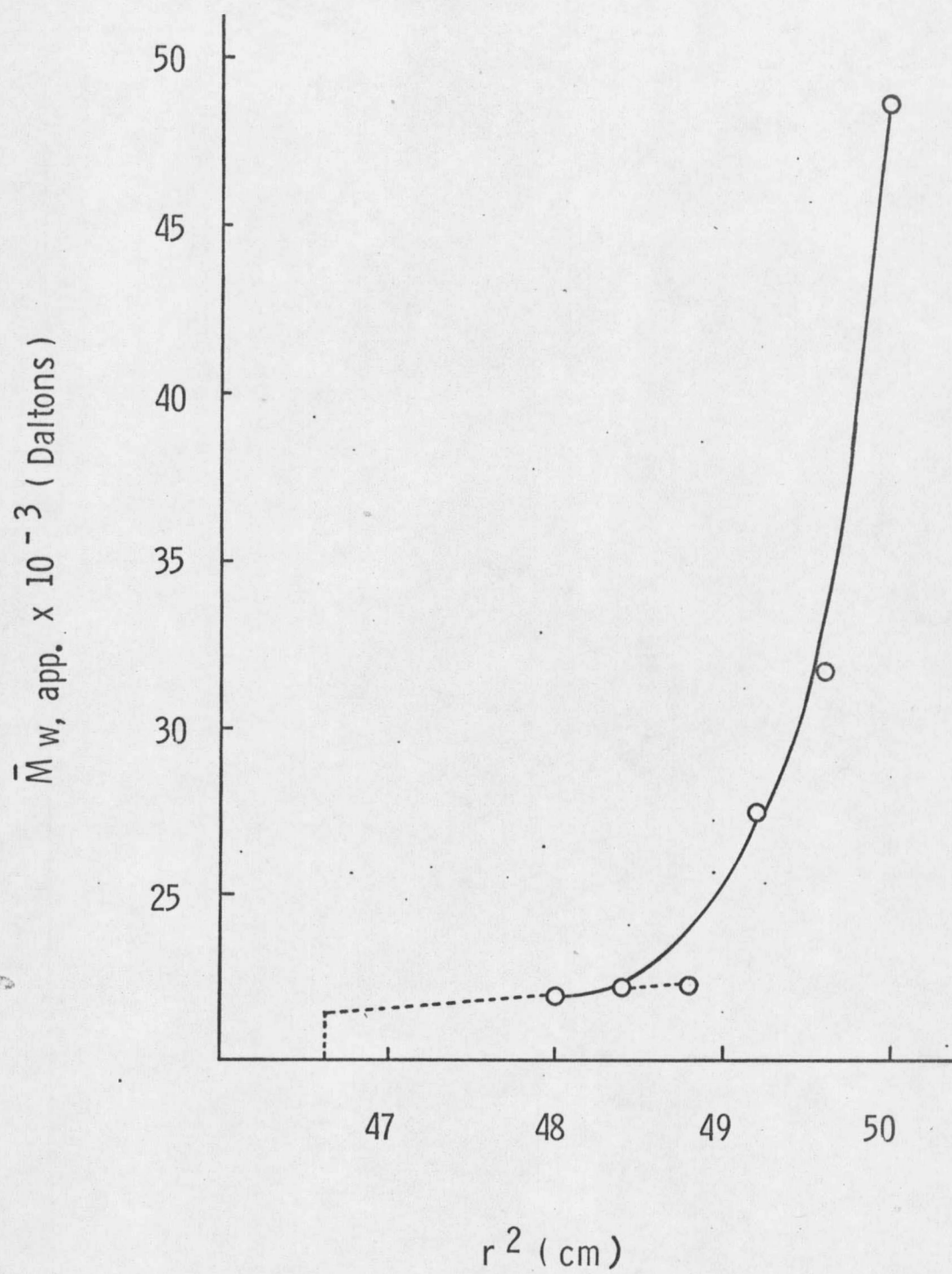
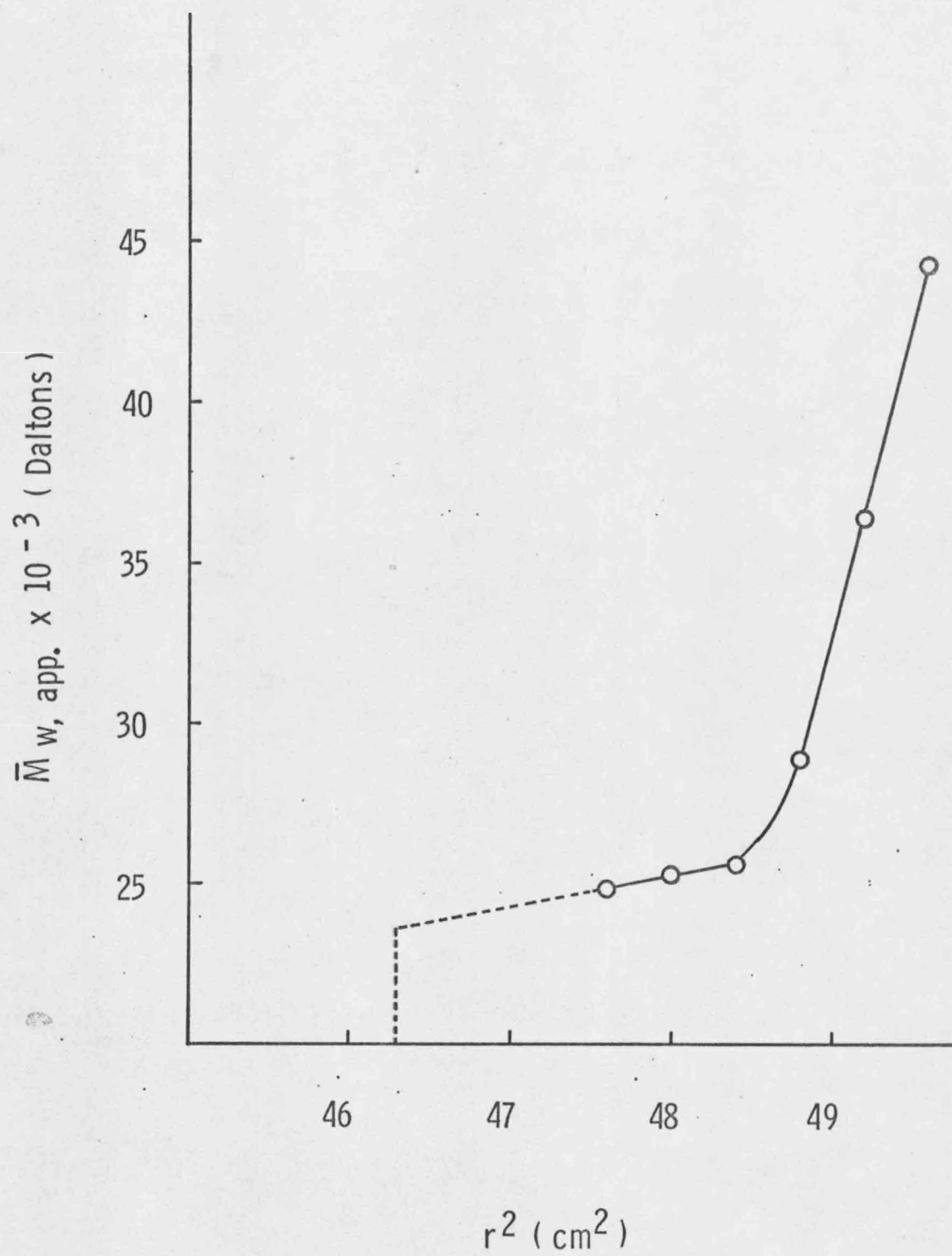


Figure 16

Apparent weight-average molecular weight plotted as a function of the position in the solution column. In this experiment a solution containing 85 micrograms per milliliter glucose-6-phosphate dehydrogenase in 6 M guanidine-hydrochloride, centrifuged at 30,000 rpm for 30.25 hours.



The data in each of Tables X, XI and XII indicated that the values of the apparent weight-average molecular weight ($\bar{M}_w$, app.) varied from 50×10^3 daltons at the bottom of the solution column, to a value of $20\text{-}25 \times 10^3$ daltons at the top of the solution column. These values indicated that an equilibrium between a 50×10^3 and 25×10^3 dalton species of G6PDH was occurring in the 6 M guanidine-hydrochloride denaturant. In each case the 50×10^3 dalton value was obtained in the lower region of the solution where $\frac{d \log c}{dr^2}$ was highest. In this region of the solution column the protein concentration was highest and consequently the 50×10^3 dalton species was favored. In the upper part of the solution column the $\frac{d \log c}{dr^2}$ value was small and corresponded to the region of lowest protein concentration. The equilibrium in this region of the solution column favored the 25×10^3 dalton species of G6PDH.

The data in Tables X, XI and XII were plotted graphically to show the variations in the apparent weight-average molecular weight ($\bar{M}_w$, app.) as a function of various r^2 values in the solution column. Figures 14, 15 and 16 illustrate these plots. In each of these plots, the $\bar{M}_w$, app. value at zero protein concentration was obtained by extrapolation of r^2 at $y = 0$. The values of $\bar{M}_w$, app. at zero protein concentration for the three experiments are shown in Table XIII. It will be noticed that the extrapolated values for the three different protein concentrations decrease as the protein concentration increases. This was due to

Table XIII

Apparent Weight-average Molecular Weight, Extrapolated to Zero Protein Concentration, of 6 M Guanidine-Hydrochloride Dissociated Glucose-6-phosphate dehydrogenase as a Function of the Protein Concentration Used in the Centrifugation Experiment.

Protein Concentration and Centrifuge Speed	$\bar{M}_w$, app. at Zero Protein Concentration (Daltons)
280 $\mu\text{g/ml}$ 34,000 rpm	16.60×10^3
140 $\mu\text{g/ml}$ 26,000 rpm	21.40×10^3
85 $\mu\text{g/ml}$ 30,000 rpm	23.60×10^3

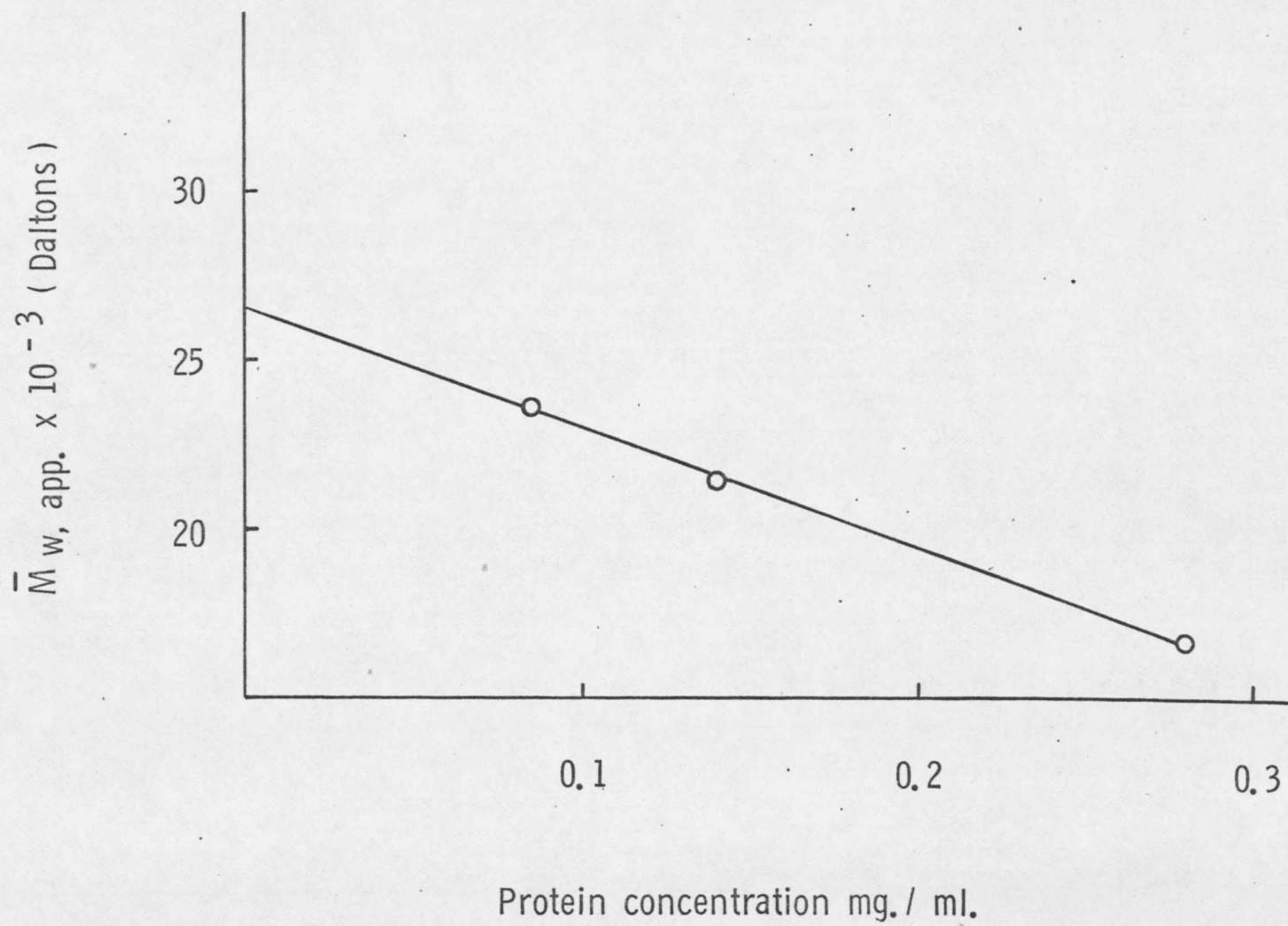
the increase in slope of the $\log \Delta y$ versus r^2 curve. This was especially evident in the 280 $\mu\text{g/ml}$ G6PDH centrifugation in which the high protein concentration and the 34,000 rpm rotor speed combined to yield a steep plot of $\log \Delta y$ versus r^2 . Consequently the graph of $\bar{M}_w$, app. at various r^2 values in the solution column (Figure 16) yielded a steep slope that did not exhibit the linear portion evident

in Figures 14 and 15. This resulted in a value of 16.60×10^3 daltons for the $\bar{M}_w$, app. extrapolated to zero protein concentration. This is 7.0×10^3 daltons lower than the smallest measured value, and is due to the steepness of the $\bar{M}_w$, app. versus r^2 curve. The data obtained for the 85 $\mu\text{g/ml}$ and 140 $\mu\text{g/ml}$ G6PDH centrifugations did not exhibit such a steep $\bar{M}_w$, app. versus r^2 plot (figures 14 and 15) and in both cases the graphs exhibited a region where the $\bar{M}_w$, app. value became linear.

The three values obtained for the apparent weight-average molecular weight ($\bar{M}_w$, app.) at zero protein concentration were plotted as a function of the enzyme concentration used in each determination. This plot is illustrated in Figure 17, and the value of $\bar{M}_w$, app. extrapolated to zero protein concentration yielded a value of 26.5×10^3 daltons as the subunit molecular weight of E. coli B G6PDH.

Figure 17

Apparent weight-average molecular weight values plotted as a function of the protein concentration used in each determination. The apparent weight-average molecular weight value at zero protein concentration was obtained by extrapolation of the data to zero protein concentration.



The high speed Yphantis³⁶ method of molecular weight determination necessitated the measurement of slopes of the plots of protein concentration versus r^2 graphs (Figures 11, 12 and 13). These values were then used to determine $\bar{M}_w$, app. values throughout the solution column as described previously. In the study outlined it was found that an equilibrium between 50×10^3 and 25×10^3 dalton subunit species of G6PDH was occurring in the denaturant solution. The $\bar{M}_w$, app. value of the smaller species was calculated from the upper region of the solution column, where the value of $d \log c/dr^2$ was smallest. Consequently, the error in this region of the data was maximal. The $\bar{M}_w$, app. value in the region of 5% and 10% levels of error in Δy provided a value of $\bar{M}_w$, app. for the smallest subunit species. Estimation of the error in this region gave a value of 22.8%. In addition, the value of the partial specific volume ($\bar{v}$) of the E. coli B G6PDH was assumed to be 0.72, a value obtained for yeast G6PDH. If it is assumed that $\bar{v} = 0.72 \pm 0.01$, then an additional error of 6.8% resulted. Thus the maximum error in $\bar{M}_w$, app. was calculated to be 32%.

Sodium dodecyl sulphate (SDS) gel electrophoresis indicated values of 53×10^3 and 49×10^3 daltons for the subunit molecular weight of E. coli B G6PDH. The Yphantis high speed equilibrium centrifugation indicated an equilibrium between a subunit species of approximately 50×10^3 daltons, and another of 26.5×10^3 daltons. Weber and Osborne³⁵ have reported that SDS polyacrylamide gel electrophoresis molecular weight determinations are in good agreement with the values obtained

by other physical methods, such as ultracentrifugation, x-ray analysis and osmotic pressure measurements. However, in the study of the molecular weights of G6PDH by SDS polyacrylamide gel electrophoresis and high speed ultracentrifugation, conflicting data were obtained for the molecular weight value of the smallest species. The conflict in the values obtained by the two methods arose from the apparent level of denaturation of the G6PDH. SDS denaturation of G6PDH resulted in a single subunit species of molecular weight approximately 50×10^3 daltons. When ultracentrifugation was conducted in the presence of the denaturant, 6 M guanidine-HCl, the data indicated an equilibrium between 50 and 25×10^3 dalton subunit species. Thus, in this instance, the molecular weight values obtained by the two different methods of measurement reflect the level of dissociation effected by the particular denaturant employed. It is interesting to note that the presence of the 25×10^3 dalton subunit was only detected in the region of the solution column where the protein concentration was low. Thus, in order to dissociate G6PDH to a 25×10^3 dalton subunit, it is necessary to have a low protein concentration in addition to the presence of an effective denaturant. This low protein concentration would generally tend to favor the smaller species in the equilibrium, and would not be evidenced in areas of higher protein concentrations. Both SDS and guanidine-hydrochloride rupture similar bonds in the quaternary structure of proteins, i.e., hydrophobic bonds, hydrogen bonds and salt bridges. Thus low protein concentrations are necessary in addition to the presence of a denaturant to show the presence of the

25×10^3 dalton species.

The calculated maximum error in the ultracentrifugation molecular weight determinations was estimated to be 32%. In order to preclude the existence of the 26.5×10^3 dalton species, the maximum error would have to be in the neighborhood of 100%. Thus, it was concluded that the $\bar{M}_w$, app. value of 26.5×10^3 daltons represented a real value and was not due to errors in the method employed.

N-Terminal Amino Acid Determination⁴⁰

The dansyl derivative of the N-terminal amino acid of E. coli B G6PDH was prepared as described under the Materials and Methods section. This derivative was chromatographed with dansyl amino acid standards on Eastman silica gel thin-layer sheets.

Table XIV shows the R_f values for the G6PDH N-terminal dansyl amino acid and several N-dansyl amino acid standards in three different solvent systems. It was found that the benzene (80 parts), pyridine (20 parts) and acetic acid (5 parts) solvent system provided for the best resolution of the dansyl amino acid standards and N-terminal dansyl derivative of G6PDH. Figures 18, 19 and 20 illustrate the results of typical chromatograms performed using the three different solvent systems. Several of the dansyl-amino acid standards produced multiple spots upon development of the chromatogram. In particular, the alanine and tyrosine derivatives consistently produced several fluorescent spots. The N-dansyl-methionine standard was found to

Table XIV

Silica Gel Thin Layer Chromatography
of N-Dansyl Amino Acid Derivatives

Solvent System	R _f Values			
	<u>E. coli</u> B G6PDH N-terminal dansyl derivative	N-dansyl methionine	N-dansyl alanine	N-dansyl tyrosine
Benzene 80 Pyridine 20 Acetic Acid 5	.74 .76 .73	.73 .77 .77	.68	0.74
Chloroform 70 Benzyl alcohol 30 Acetic Acid 3	0.82	0.82	0.74	0.90
Butanol 30 Pyridine 20 Acetic Acid 6	0.65	0.67	0.66	0.74
Chloroform 38 Ethanol 4 Acetic Acid 3	0.78	0.77	0.83	0.97

Figure 18

Silica gel thin layer chromatograph of the N-terminal dansyl derivative of Escherichia coli B glucose-6-phosphate dehydrogenase, and amino acid dansyl standards. The solvent system employed was n-butanol 30 parts, pyridine 20 parts, acetic acid 6 parts and water 24 parts. The dansyl derivatives spotted on the thin layer plate were as follows: 1. Escherichia coli B. glucose-6-phosphate dehydrogenase; 2. valine; 3. isoleucine; 4. proline; 5 phenyl alanine; 6. leucine; 7. tyrosine; 8. hydroxyproline; 9. histidine; 10. tryptophan; 11. alanine; 12. methionine; and 13. serine. Open circles represent yellow fluorescent spots due to the N-terminal derivatives of the amino acids, and broken circles blue fluorescent spots due to the dansyl reagent.

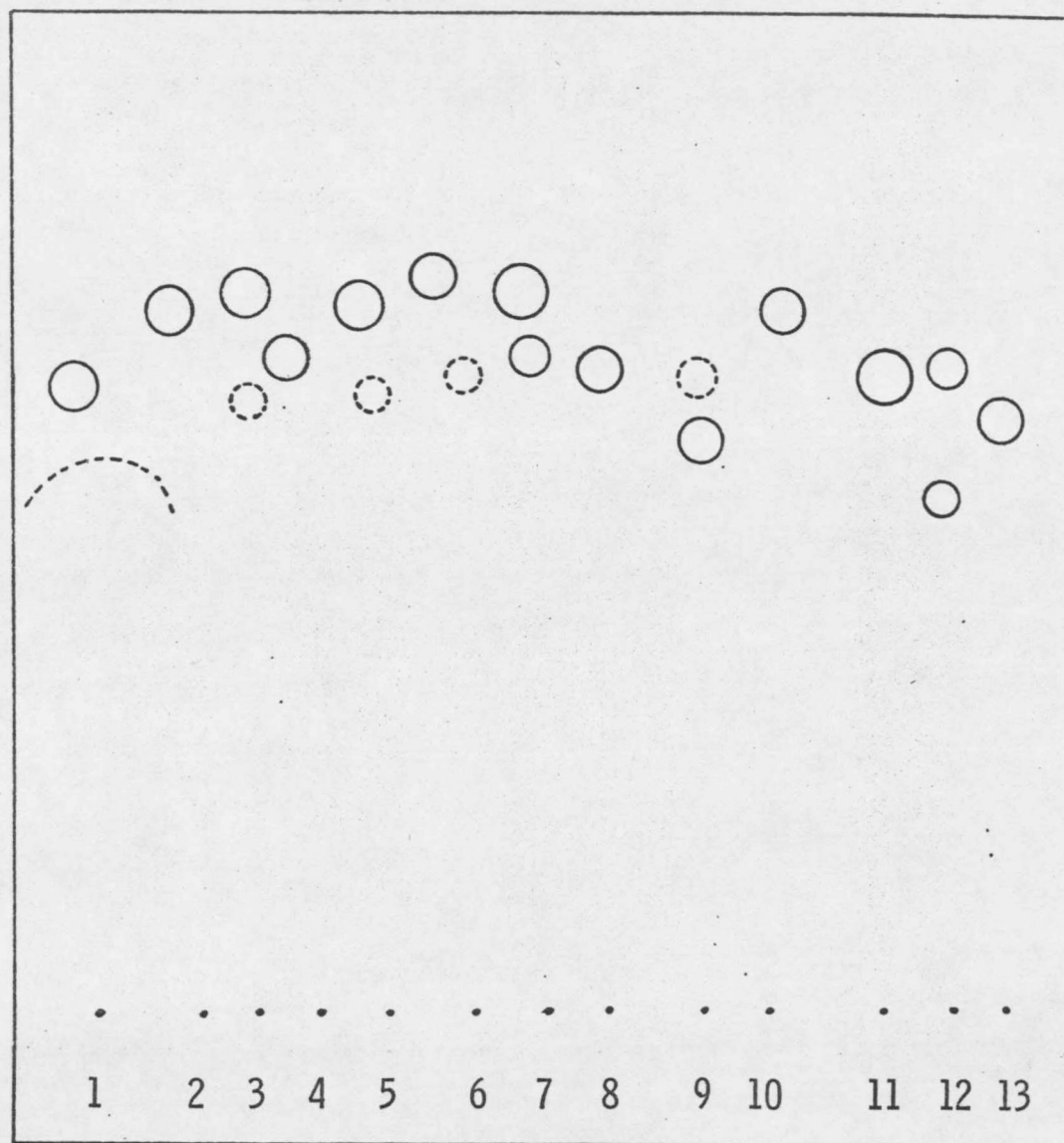


Figure 19

Silica gel thin layer chromatography of N-terminal dansyl derivative of Escherichia coli B glucose-6-phosphate dehydrogenase and amino acid dansyl standards. The solvent system employed was chloroform 70 parts, benzyl alcohol 30 parts and acetic acid 3 parts. The dansyl derivatives spotted on the thin layer plates were as follows:

1. Escherichia coli B glucose-6-phosphate dehydrogenase; 2. valine; 3. isoleucine; 4. proline; 5. phenyl alanine; 6. leucine; 7. tyrosine; 8. hydroxyproline; 9. histidine; 10. tryptophan; 11. alanine; 12. methionine; and 13. serine. Open circles represent yellow fluorescent spots due to the N-terminal derivatives of the amino acids, and broken circles blue fluorescent spots due to the dansyl reagent.

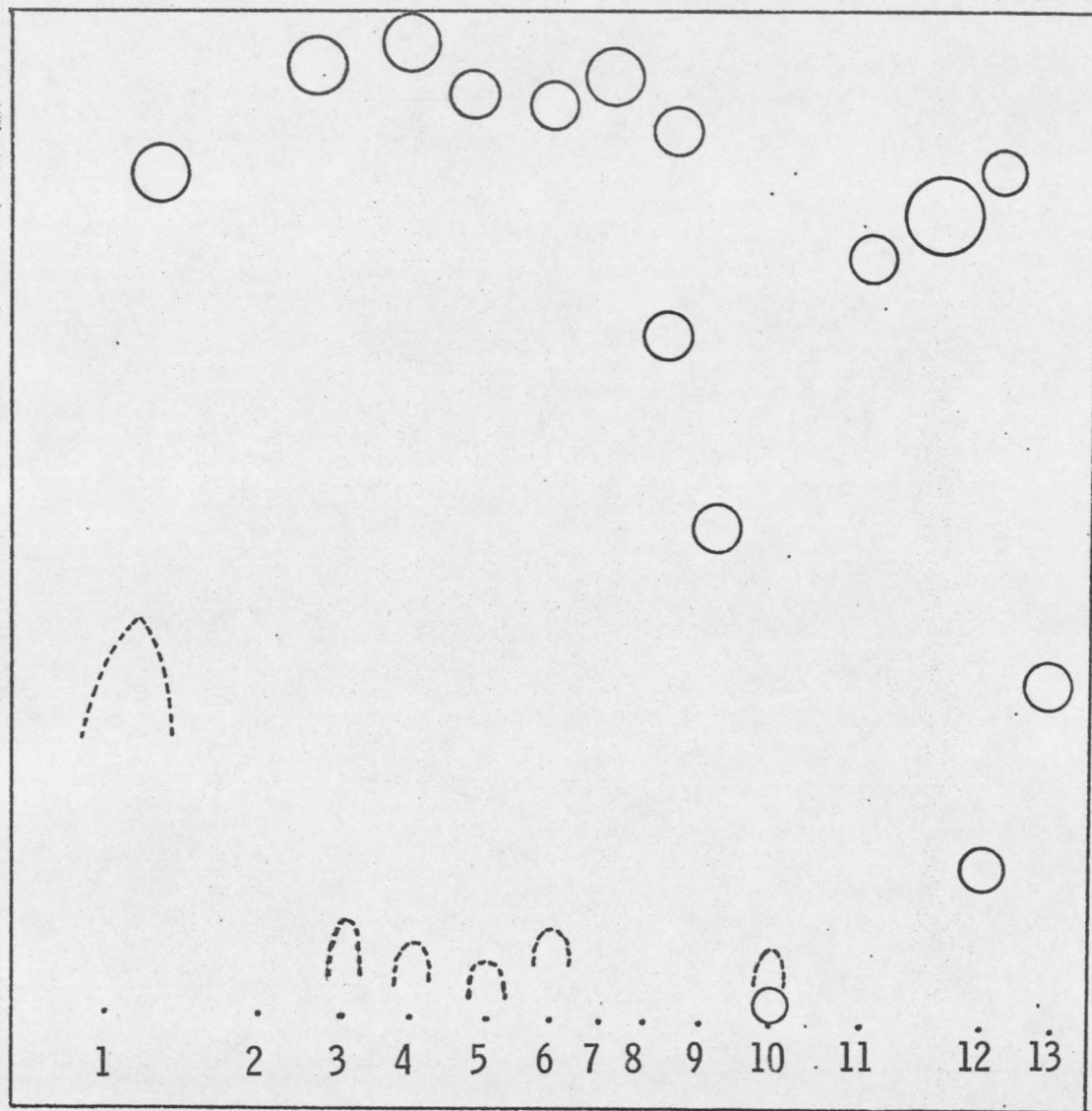


Figure 20

Silica gel thin layer chromatography of N-terminal dansyl derivatives of Escherichia coli B glucose-6-phosphate dehydrogenase, and amino acid dansyl standards. The solvent system employed was benzene 80 parts, pyridine 20 parts, and acetic acid 5 parts. The dansyl derivatives spotted on the thin layer plate were as follows;

1. Escherichia coli B glucose-6-phosphate dehydrogenase; 2. valine;
3. isoleucine; 4. proline; 5. phenyl alanine; 6. leucine; 7. tyrosine;
8. hydroxyproline; 9. histidine; 10. tryptophan; 11. alanine; 12. methionine and 13. serine. Open circles represent yellow fluorescent spots due to the N-terminal derivatives of the amino acids, and broken circles blue fluorescent spots due to the dansyl reagent.

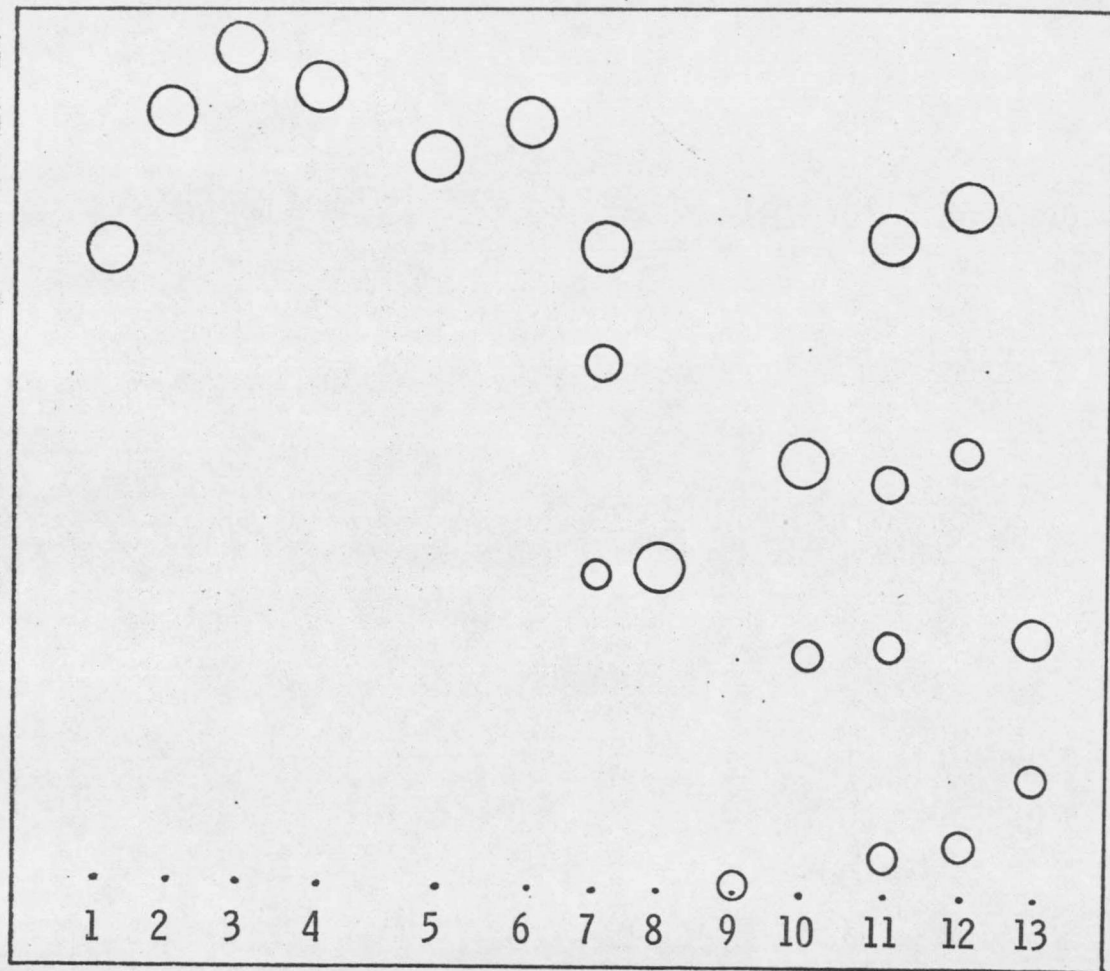


Figure 21

Silica gel thin layer chromatography of N-terminal dansyl derivative of Escherichia coli B Glucose-6-phosphate dehydrogenase, and amino acid dansyl standards. The solvent system employed was chloroform 38 parts, ethanol 9 parts, and acetic acid 3 parts. The dansyl derivatives spotted on the thin layer plate were as follows: 1. alanine; 2. Escherichia coli B Glucose-6-phosphate dehydrogenase; and 3. methionine.

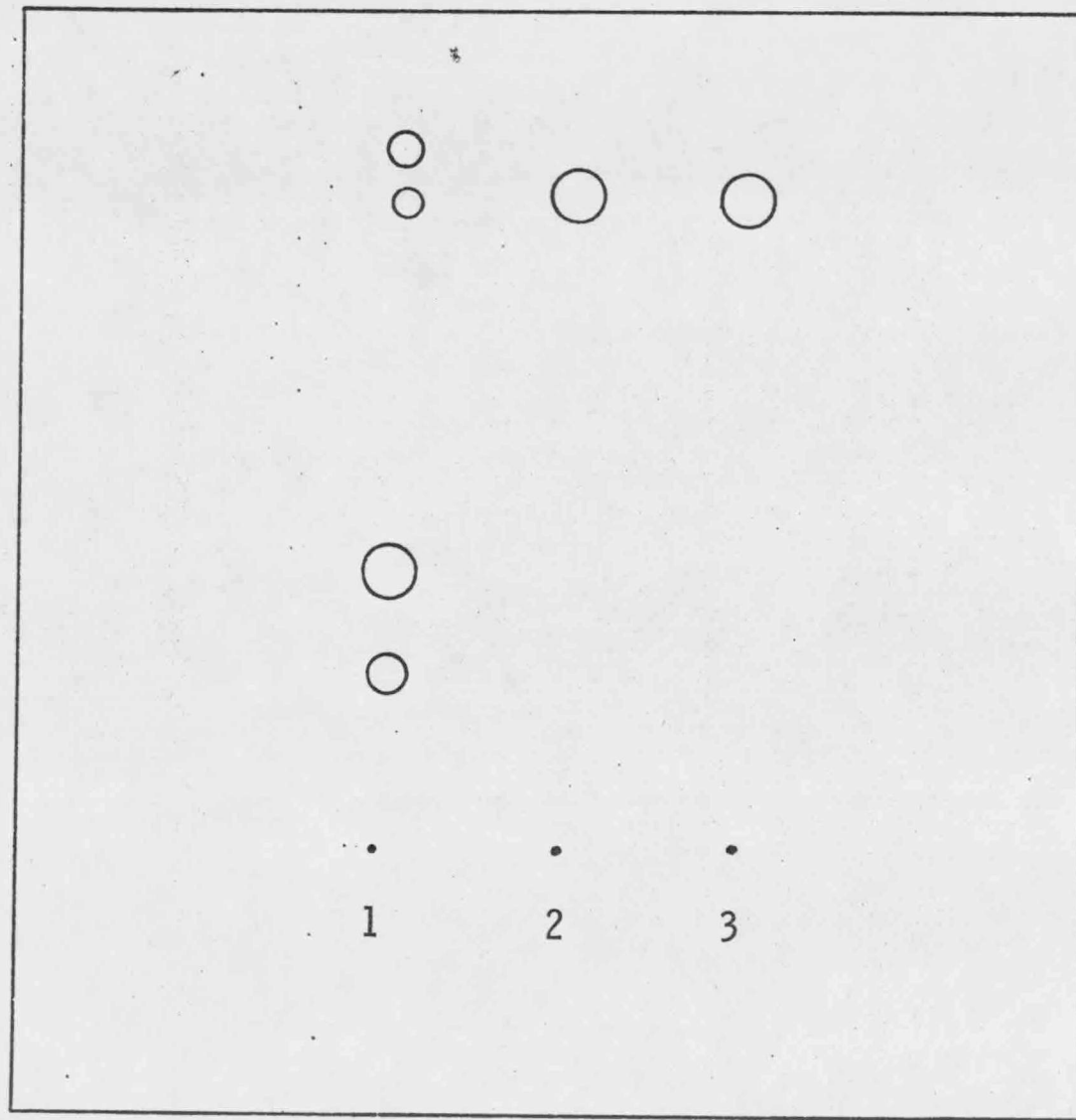


Figure 22

Silica gel thin layer chromatography of the N-terminal dansyl derivative of Escherichia coli B glucose-6-phosphate dehydrogenase, and amino acid dansyl standards. The solvent system employed was benzene 80 parts, pyridine 20 parts, and acetic acid 5 parts. The dansyl derivatives spotted on the thin layer plates were as follows: 1. alanine; 2. Escherichia coli B glucose-6-phosphate dehydrogenase; and 3. methionine. Open circles represent yellow fluorescent spots due to N-terminal dansyl derivatives of the amino acids, broken circles represent blue fluorescent spots due to the dansyl reagent.

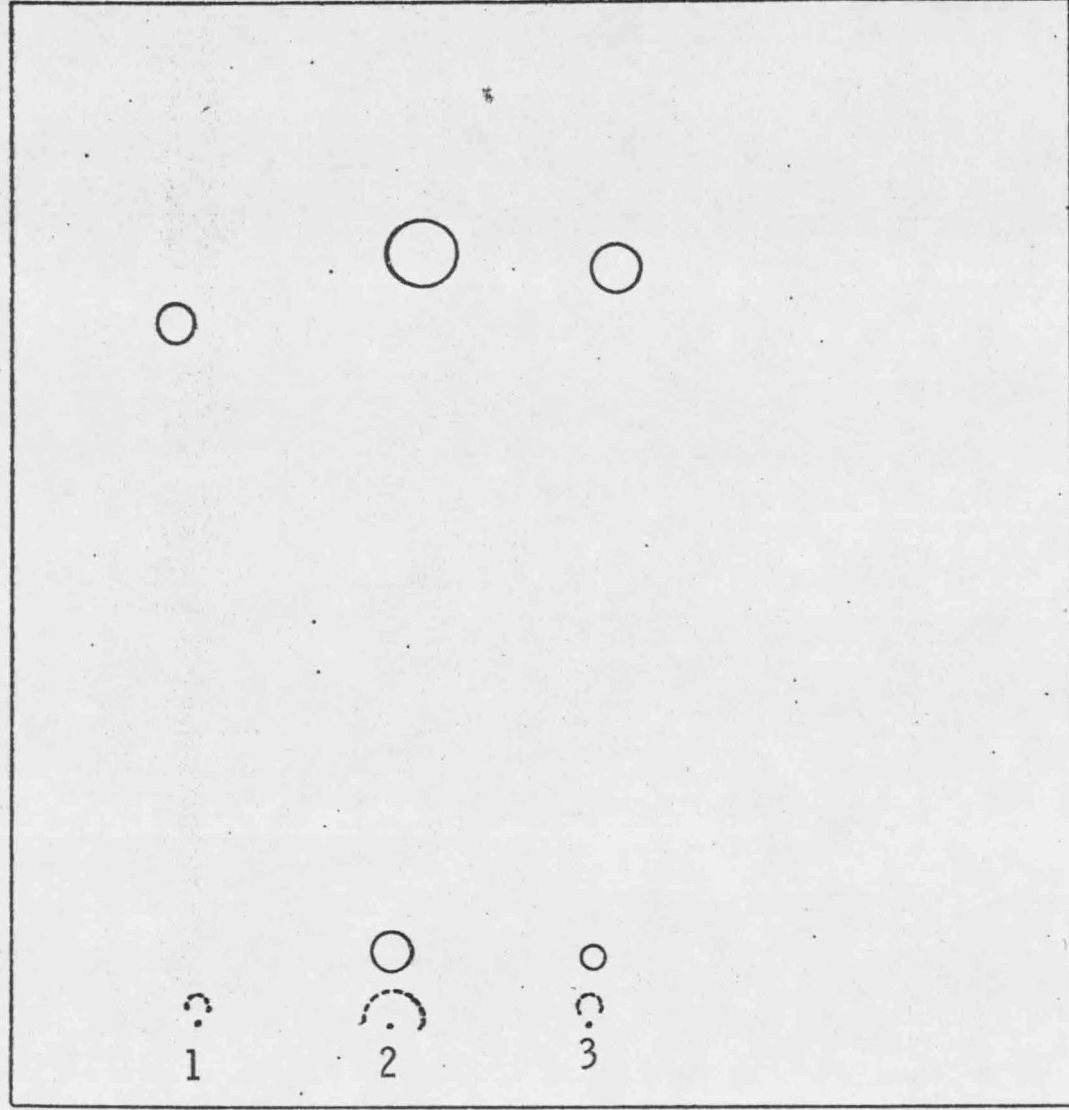
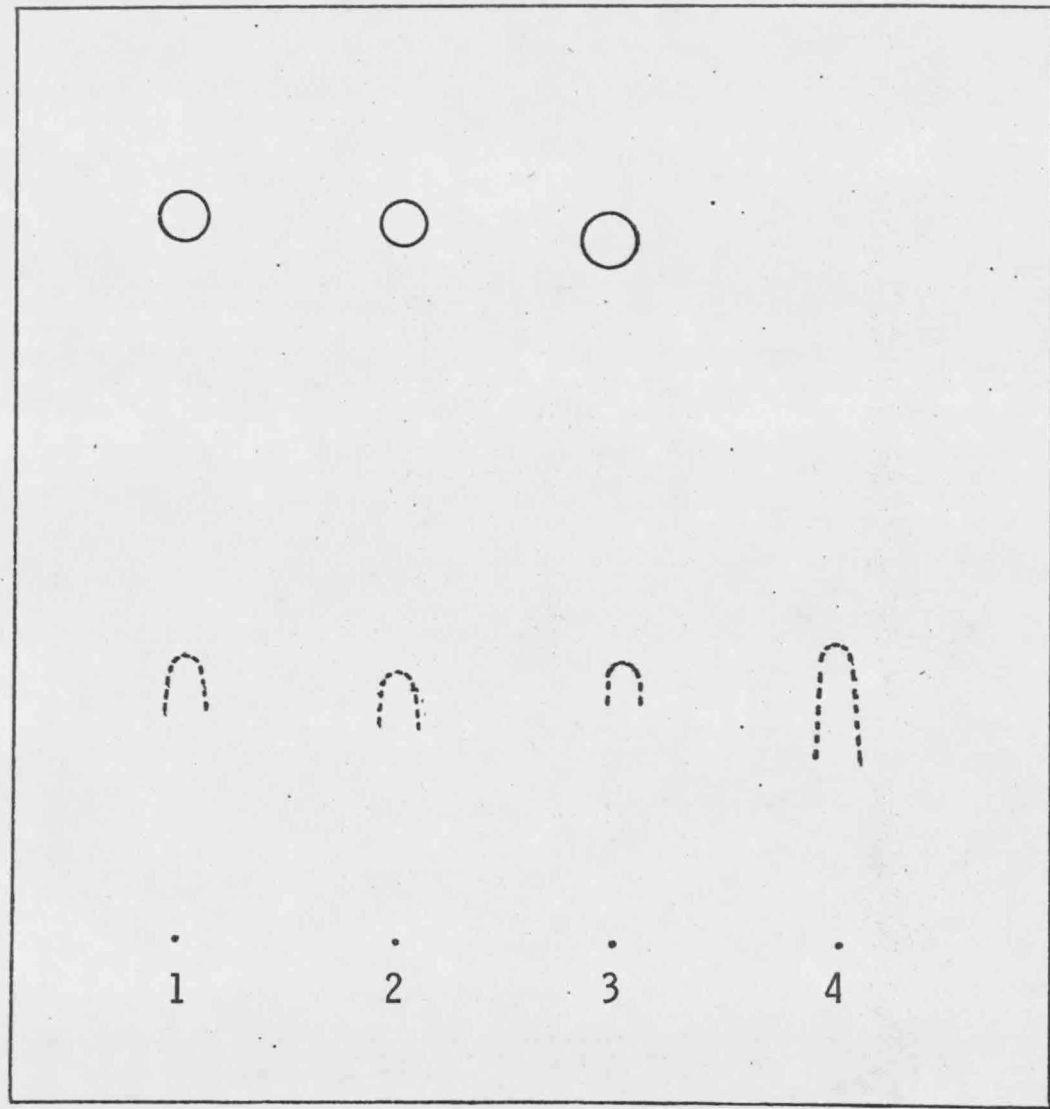


Figure 23

Silica gel thin layer chromatography of the N-terminal dansyl derivative of Escherichia coli B Glucose-6-phosphate dehydrogenase, and amino acid dansyl standards. The solvent system employed was benzene 80 parts, pyridine 20 parts, and acetic acid 5 parts. The dansyl derivatives spotted on the thin layer plate were as follows: 1. methionine; 2. Escherichia coli B Glucose-6-phosphate dehydrogenase; 3. methionine and Escherichia coli B Glucose-6-phosphate dehydrogenase; and 4. reagent (dansyl amine and hydroxide).

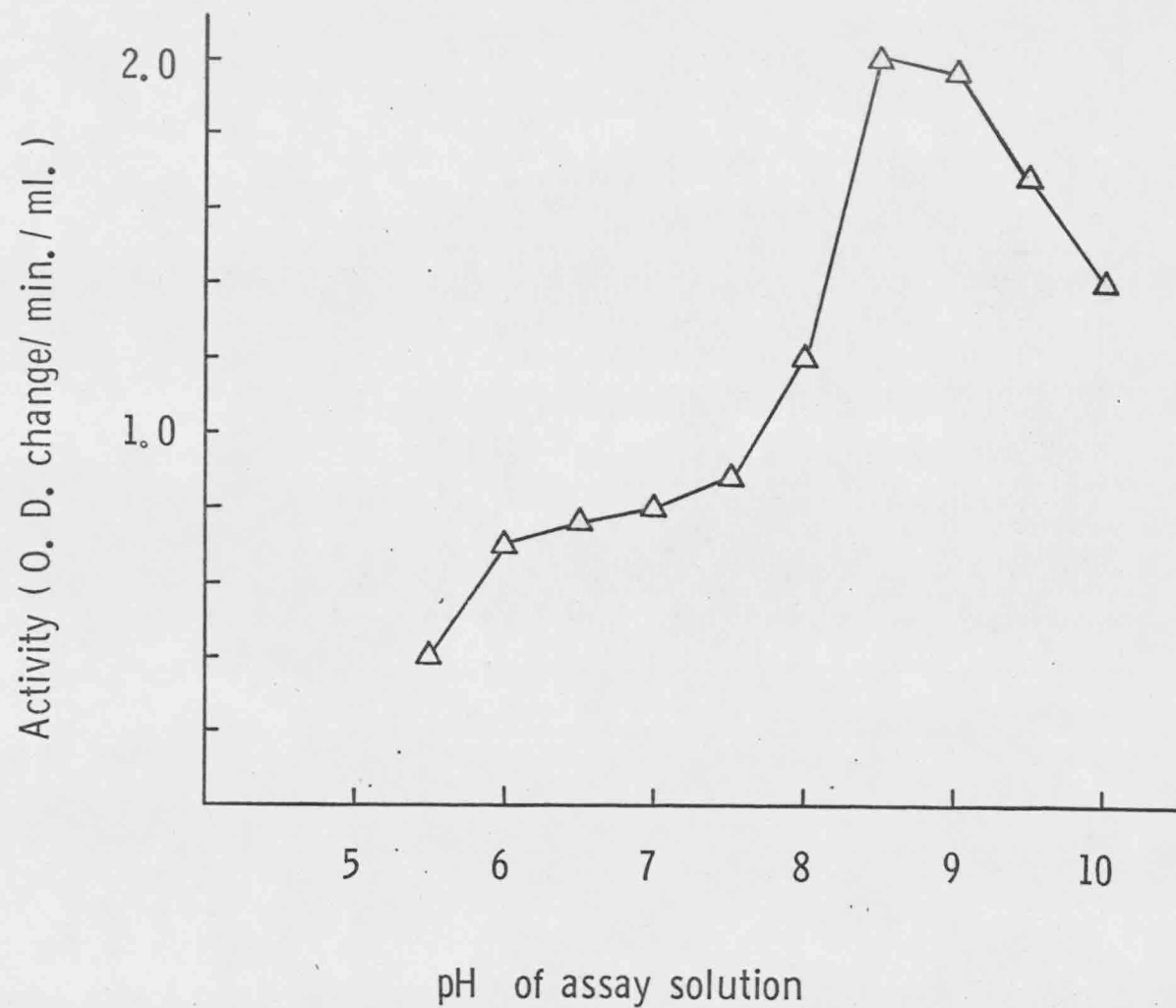


produce multiple spots, but these could be eliminated by using only freshly prepared N-dansyl-methionine samples. Upon storage of the sample, or failure to remove all of the oxygen in the Carrius tube prior to hydrolysis, several spots were produced. The N-terminal dansyl amino acid derivatives fluoresced yellow under an ultra-violet light, and the blue fluorescent spots were due to the dansyl-amine and -hydroxide produced by the acid hydrolysis of excess dansyl-chloride. In standards that produced several spots, the R_f value of the fastest moving component, which was also the most intense spot, was reported in Table XVI.

On the basis of the three thin layer chromatograms it was decided that the N-terminal amino acid of E. coli B G6PDH was methionine or alanine. Thin layer chromatograms were developed for the N-terminal dansyl derivative of G6PDH chromatographed with methionine and alanine dansyl standards (Figures 21 and 22). On the basis of these chromatograms it was decided that methionine was the N-terminal amino acid of G6PDH. The N-terminal dansyl derivative of G6PDH was then co-chromatographed with a standard dansyl methionine sample (Figure 23). The mixed G6PDH derivative and methionine standard chromatographed as a single fluorescent spot, confirming methionine as the N-terminal amino acid of E. coli B G6PDH. In addition, the production of only one fluorescent spot with G6PDH derivatives provided another criterion of purity of the enzyme preparation, and also supported the previous evidence in favor of a single subunit species.

Figure 24

Effect of pH on the enzyme activity of Escherichia coli B glucose-6-phosphate dehydrogenase. Enzyme activity was reported as the increase in absorbance at 3390 Å per minute, per milliliter of enzyme solution. Enzyme assays were performed as described under the Materials and Methods section.



pH Activity Studies

The enzyme activity of E. coli B G6PDH as a function of the pH of the assay solution was investigated as outlined in the Materials and Methods section. Figure 24 illustrates the results of this study. The optimum pH for G6PDH activity was determined to be 8.5. At this pH the activity of the dehydrogenase was 227% of the value obtained when assayed at pH 7.4. In the reversible ($\Delta G^\circ = -0.1$ kcal/mole) G6PDH catalysed oxidation of G6P to δ -phospho- δ -gluconolactone, hydrogen ions are produced; consequently a more basic enzyme environment facilitated the removal of these hydrogen ions and favored the oxidation of substrate. Specific activities of purified G6PDH measured at pH 8.5 were in the range 160 units/mg to 180 units/mg. However, throughout this investigation the enzyme activity was measured at pH 7.4.

The subunit molecular weight of E. coli B G6PDH was found to be 26.50×10^3 daltons (cf. sodium dodecyl sulphate gel electrophoresis, and high speed sedimentation equilibrium ultracentrifuge determinations of subunit molecular weight). If it was assumed that G6PDH contained one active center per 26.50×10^3 dalton subunits, it was possible to express the activity of the purified dehydrogenase in terms of the turnover number. This measure of activity was the number of molecules of substrate (G6P or NADP^+) reacting per active center per minute, and gave a measure of the rate of G6PDH oxidation of G6P. At a pH of 7.4 the turnover number was calculated to be $2.173 \times 10^3 \text{ min}^{-1} \text{ active center}^{-1}$;

Table XV

Stability of Glucose-6-phosphate dehydrogenase as a Function of Incubation pH

Incubation pH	Activity = Δ OD min/ml Incubation Time (hours)						Percentage Control in 120 hours
	0	24	48	72	96	120	
10.0	3.0	1.6	0.8	0.6	0.6	0.5	16.7
9.5	3.2	2.4	1.2	1.0	0.8	0.8	23.4
9.0	3.5	3.4	2.4	1.3	1.4	1.3	35.7
8.5	4.8	4.8	3.4	2.8	2.6	2.5	52.1
8.0	4.6	4.6	3.8	3.6	3.3	3.2	69.6
7.5	4.4	4.4	3.6	3.2	2.8	2.7	61.4
7.0	4.4	4.0	3.4	3.2	2.8	2.7	61.4
6.5	4.0	4.0	3.2	3.0	2.7	2.7	67.5
6.0	3.6	3.6	3.2	3.0	2.6	2.6	72.2

when activities were measured at pH 8.4 the value of the turnover number was $4.933 \times 10^3 \text{ min}^{-1} \text{ active center}^{-1}$.

pH Stability Studies

Time stability studies of G6PDH as a function of the incubation solution pH were investigated as described under Materials and Methods. Table XV portrays the results of this stability study. The data indicated that G6PDH was most stable when stored in solutions of pH 8.0 or lower. Thus, the stability pH optimum does not parallel the activity pH optimum. Consequently, during the preparation and assay of G6PDH, activities were measured at pH 7.4 where the stability was maximal, and not at the most active value, pH 8.5.

Investigation of Sulphydryl Groups Involved in Enzyme Activity

Purified E. coli B G6PDH, specific activity 85 units/mg, was incubated with the sulphydryl reagents N-ethyl maleimide (NEM) and p-chloromercuribenzoate (PCMB) as described in the Materials and Methods section. The protein solution employed contained 0.077 mg/ml G6PDH. Tables XVI, XVII and XVIII illustrate the results of these incubations. The data clearly implicates the existence of one or more sulphydryl groups, from cysteine residues, necessary for G6PDH activity. The results indicate that sulphydryl groups are located at the active center of the dehydrogenase. Several explanations can be formulated to explain the functions of the one, or more active sulphydryl groups. Cysteine residues may be directly involved in the enzymic activity by binding substrate moieties

Table XVI

Activity of Glucose-6-phosphate dehydrogenase incubated in the presence of 5 millimolar N-ethyl maleimide at 4° C for 30 hours. The activities are expressed as percentages of the control.

Tube #	G6PDH Solution (0.077 mg/ml) (ml)	0.1 M Tris-HCl, pH 7.1 (ml)	7.5 mM NADP ⁺ (ml)	12 mM ATP (ml)	10 mM NEM (ml)	Percentage Control
1 (control)	0.25	0.75				100 %
2	0.25	0.25			0.50	35.7
3	0.25		0.25		0.50	80.7
4	0.25			0.25	0.50	62.5
5	0.25		0.125	0.125	0.50	102.5

Table XVII

Activity of Glucose-6-phosphate dehydrogenase incubated in the presence of 5 millimolar N-ethylmaleimide at 25° C for 24 hours.. The activities are expressed as percentages of the control.

Tube #	G6PDH Solution (0.077 mg/ml) (ml)	0.1 M Tris-HCl pH 7.1 (ml)	12 mM G6P (ml)	12 mM ATP (ml)	10 mM NEM (ml)	Percentage Control
1 (control)	0.25	0.75				100 %
2	0.25	0.25			0.50	0
3	0.25			0.25	0.50	73.6
4	0.25		0.25		0.50	4.7
5	0.25		0.125	0.125	0.50	23.3

Table XVIII

Activity of Glucose-6-phosphate dehydrogenase incubated in the presence of 1.0 millimolar p-chloro-mercuribenzoic acid at 4° C for 1.5 hours. The activities are expressed as percentages of the control.

Tube #	G6PDH Solution (0.077 mg/ml) (ml)	0.1 M Tris-HCl pH 7.1 (ml)	7.5 mM NADP ⁺ (ml)	12 mM G6P (ml)	12 mM ATP (ml)	2 mM PCMB (ml)	Percentage Control
1 (control)	0.25	0.75					100 %
2	0.25	0.25				0.5	0
3	0.25		0.25			0.5	25.5
4	0.25				0.25	0.5	0
5	0.25			0.25		0.5	0
6	0.25		0.125		0.125	0.5	71.4

or by an involvement in the catalytic mechanism and subsequent formation of the transition state complex. Consequently, chemical modification of the thiol results in the inability of the dehydrogenase to catalyse the oxidation of G6P. On the other hand, the presence of the amino acid cysteine at the active site may be necessary for the maintenance of the tertiary structure essential for enzymic activity, and the thiol residue would then not participate in the bonding of substrates or the transition state complex. Thus, reaction of the thiol residue with N-ethylmaleimide or p-chloromercuribenzoic acid results either in the destruction of the required tertiary structure, or a steric hindrance to the binding of the substrate, G6P, and coenzyme NADP^+ . Another possible explanation is the localization of a sulphydryl group (cysteine residue) at an allosteric site on the dehydrogenase. Reaction of the thiol residue with the sulphydryl reagents would then result in the loss of enzymic activity by modification of this allosteric site with a resultant loss of the conformation required for enzymic activity.

The sulphydryl moieties of G6PDH reacted with both N-ethyl maleimide (NEM) and p-chloromercuribenzoic acid; however, the reaction with PCMB proceeded much more rapidly. When G6PDH was incubated in 1 mM NEM, pH 7.1, 25° C, the enzyme was 93% active, as compared to control, after 24 hours. This compares with a 100% loss of G6PDH activity when incubated 1.5 hours in 1 mM PCMB, pH 7.1, at 4° C. Riordan and Valee³⁹ have noted that only the most reactive sulphydryl

groups of proteins combine with NEM to yield thiol ethers. Five mM NEM was necessary to effect 100% inactivation of G6PDH in 24 hours; at 4° C, 30 hours of treatment in 5 mM NEM produced 64.3% inactivation. These data suggest that the sulphydryl groups on G6PDH are not readily accessible for NEM modification. This low reactivity may be due to steric hindrance, or interaction of the thiol with other functional groups. Reaction with 1 mM PCMB proceeds much more rapidly (Table XVIII). Enzyme activity could be restored to the PCMB inactivated G6PDH by addition of excess cysteine. This result clearly implicates the role of a sulphydryl group in enzymic activity, and eliminates the possibility of PCMB interaction with groups other than thiols.

When G6PDH was incubated with 1 mM PCMB (Table XVIII) both G6P and ATP failed to provide any protection to mercaptide formation and concomitant loss of enzymic activity. However, 2.75 mM NADP^+ provided a 25.5 % protection, and incubation in the presence of 1.9 mM NADP^+ and 3 mM ATP provided a 71.4% protection. Kinetic studies have suggested that ATP is a competitive inhibitor for the G6P binding site. Consequently ATP was chosen as a substrate analogue and thus made feasible the study of synergistic effects in the presence of NADP^+ , without product formation. The protection provided by NADP^+ , NADP^+ /ATP incubations suggested that the sulphydryl group is located at the active site of G6PDH. In addition, since neither G6P or ATP afforded any protection, the data imply that the thiol residue is situated close to the

NADP⁺ binding site.

Incubation of G6PDH with 5 mM NEM (Tables XVI and XVII) indicated that NADP⁺ again provided better protection than any other single compound. This further supports the localization of the cysteine residue close to the NADP⁺ binding site. Incubations in the presence of NADP⁺ and ATP provided complete protection from NEM inactivation (Table XVI). The data in Table XVII show that ATP provided 73.6% protection and G6P provided only 4.7% protection from 5 mM NEM inactivation. When G6PDH was incubated in the presence of both ATP and G6P, 23.3% protection resulted. These data suggest that ATP and G6P do bind to exactly the same site on G6PDH as was suggested by the kinetic data.

In both NEM and PCMB inactivation of G6PDH incubation in the presence of NADP⁺/ATP mixtures afforded the most effective protection. These data can be accounted for in two ways. The sulphydryl residue may be located in the active center of G6PDH close to the NADP⁺ binding site, or a sulphydryl may be present at the site of ATP binding. It is possible that there are several sulphydryl groups necessary for G6PDH activity; these moieties may be located at several positions on the enzyme surface.

Kinetic Studies

Kinetic investigations were performed using purified E. coli B G6PDH, specific activity 65-80 units/mg, as described in the Materials and Methods section.

Table XIX illustrates the most important features of the kinetic results. Michaelis-Menten constants, K_m , were calculated from the intercepts and slopes of the reciprocal velocity/substrate concentration plots. The values of the slopes and intercepts of these plots were obtained from computer calculated least square analyses of the kinetic data. The nature of kinetic investigations required the measurements of enzyme reactions over a range of substrate concentrations varying from a saturating concentration to a value well below saturation. Consequently, the major errors in kinetic investigations arose from measurements of the initial reaction rates ($\Delta OD_{3390} \text{ } ^\circ \text{ min}^{-1}$), especially in measurements at low concentrations of substrates. ΔOD values would be estimated to 0.002 units, resulting in a maximum error in velocity measurements of approximately 20%.

Figures 25 and 26 illustrate the hyperbolic contour of plots of velocity versus substrate concentration obtained when each substrate was the variable substrate. Figure 27 illustrates a typical double reciprocal plot (Lineweaver-Burk plot) of velocity versus substrate concentration obtained when NADP^+ was the variable substrate. The Michaelis-Menten constant, K_m , for NADP^+ was calculated to be

Figure 25

Velocity of Escherichia coli B glucose-6-phosphate dehydrogenase catalysis as a function of nicotinamide adenine dinucleotide phosphate concentration. Experimental conditions are as described under the Materials and Methods section.

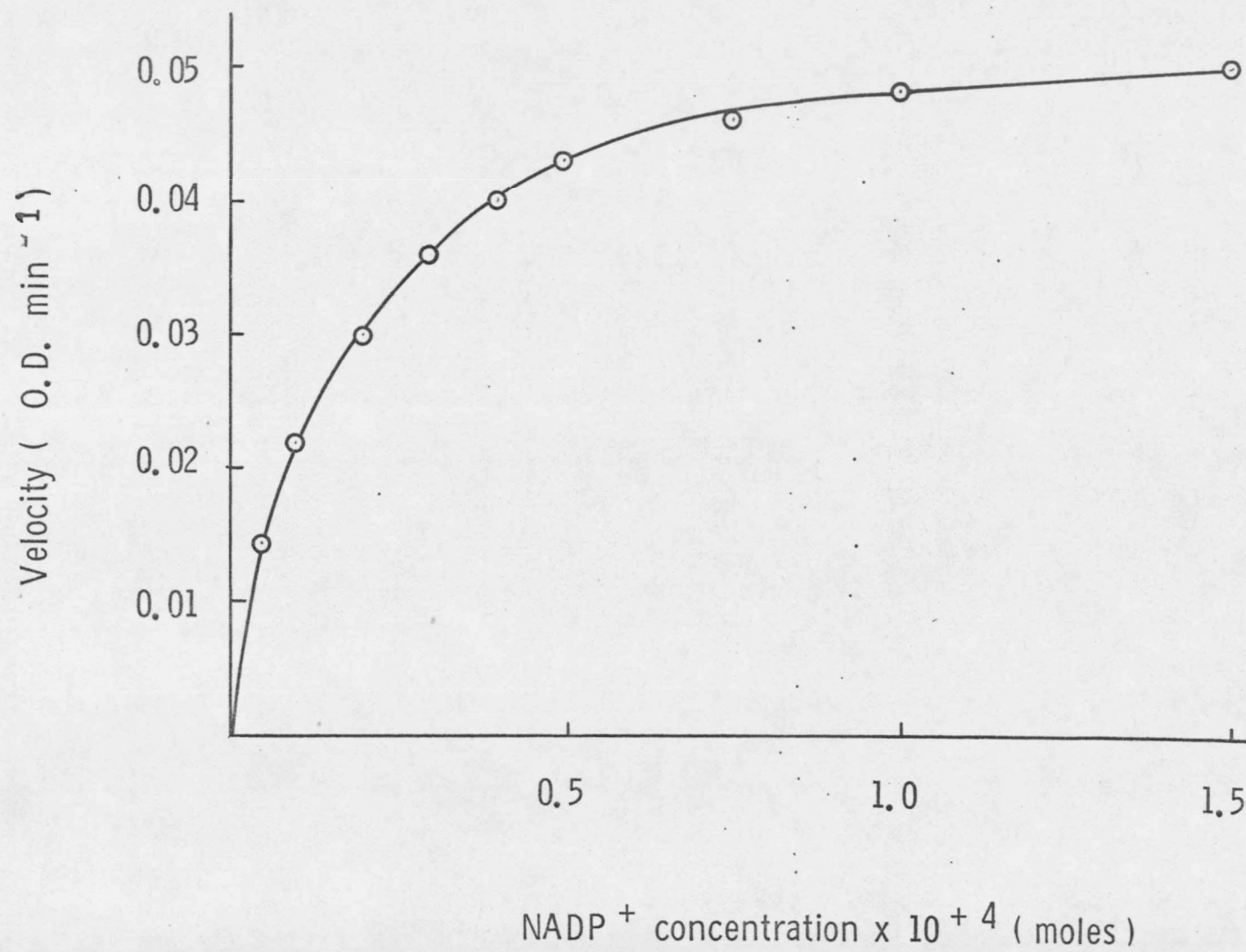


Figure 26

Velocity of Escherichia coli B Glucose-6-phosphate dehydrogenase catalysis as a function of glucose-6-phosphate concentration.

Experimental conditions are as described under the Materials and Methods section.

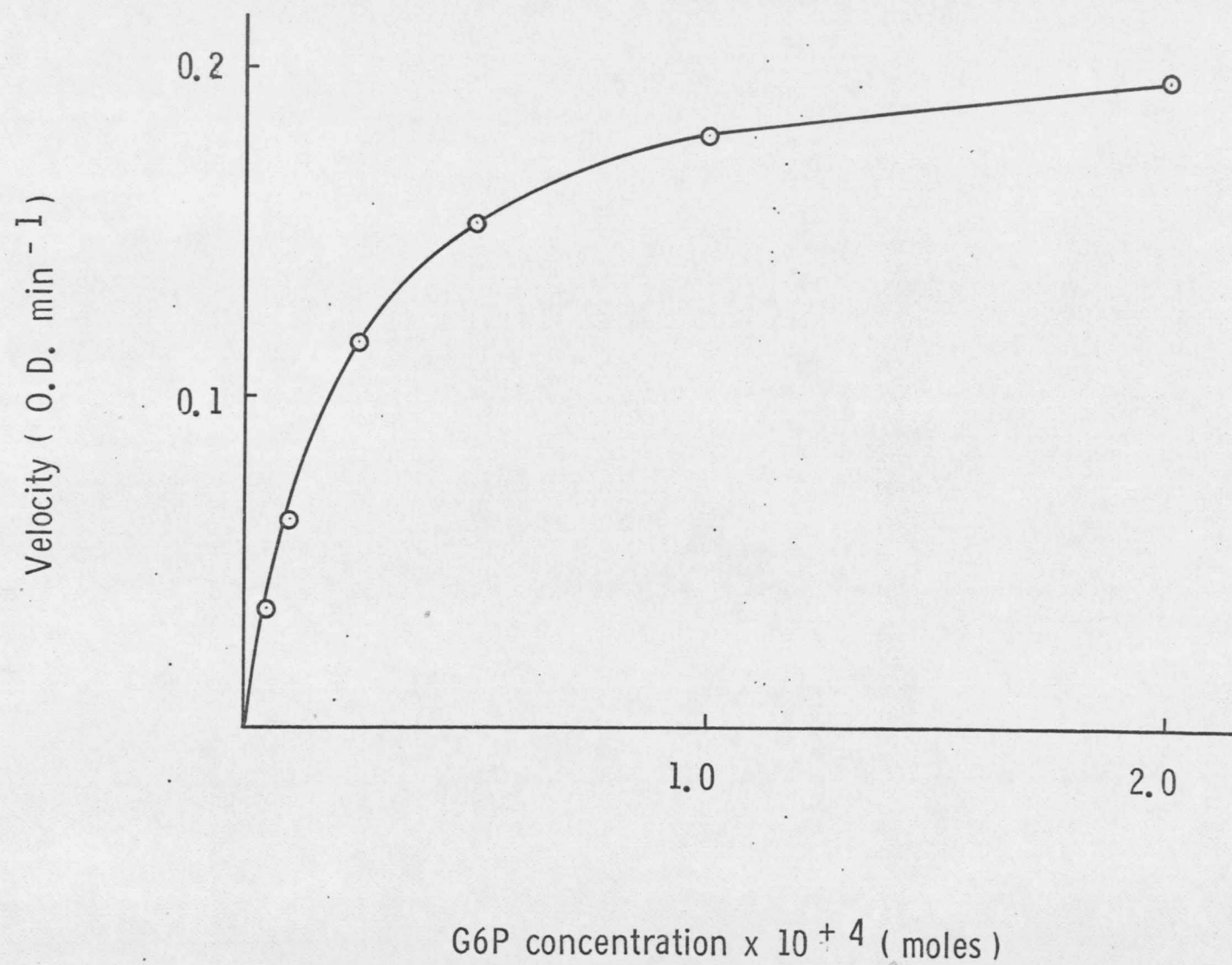


Table XIX

Kinetic Studies of Glucose-6-phosphate dehydrogenase Catalysis

Variable Substrate	Non-Variable Substrate	Inhibitor	Type of Inhibition
NADP^+	G6P	NAD^+	Non-competitive
G6P	NADP^+	NAD^+	Non-competitive
NADP^+	G6P	ATP	Uncompetitive
G6P	NADP^+	ATP	Competitive
G6P	NADP^+	AMP	None
NADP^+	G6P	NADH	None
NADP^+	G6P	NADPH	Competitive

Figure 27

Double reciprocal plots of the velocity of glucose-6-phosphate dehydrogenase catalysis as a function of nicotinamide adenine dinucleotide phosphate concentration. Experimental conditions are as described under the Materials and Methods section.

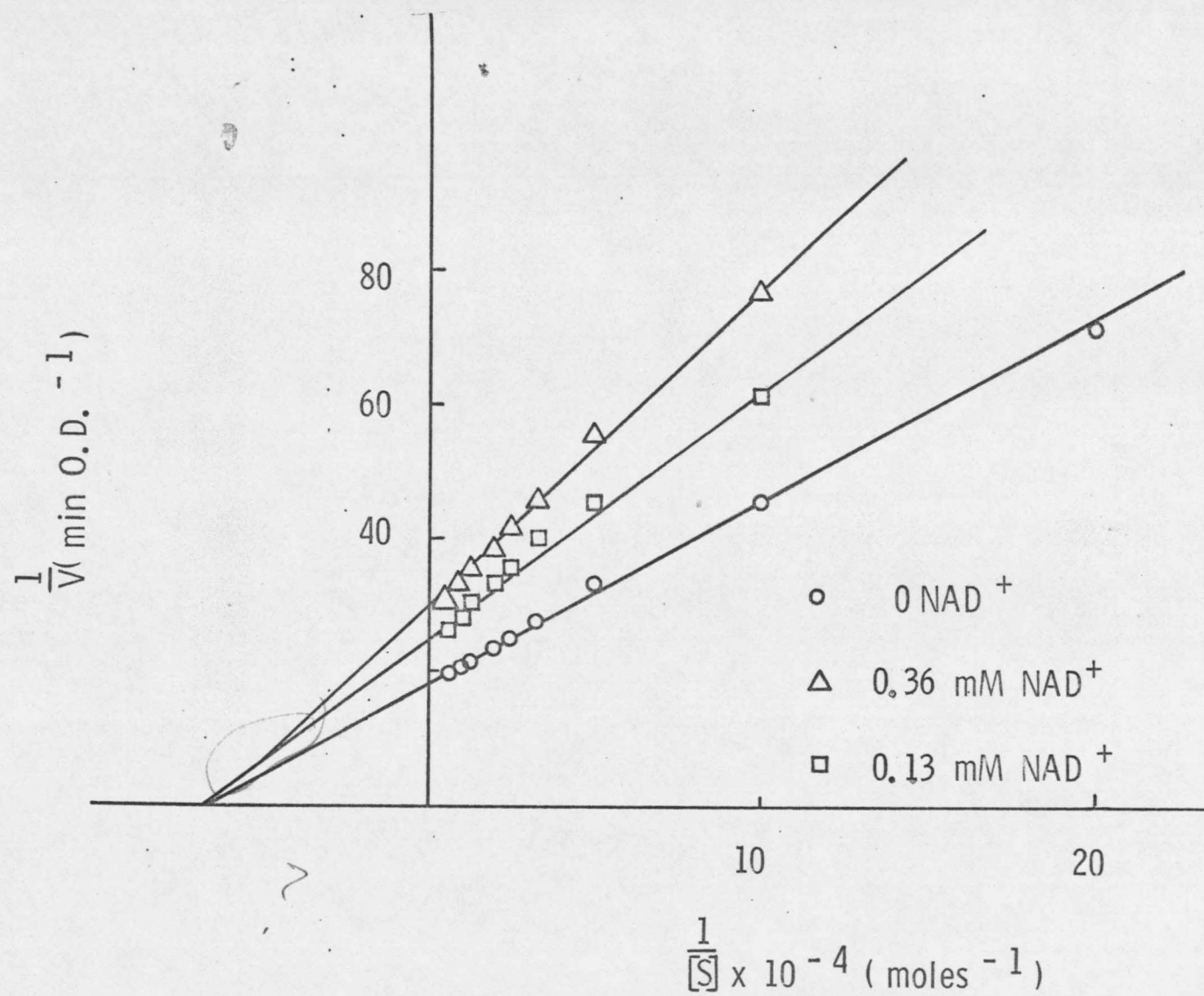
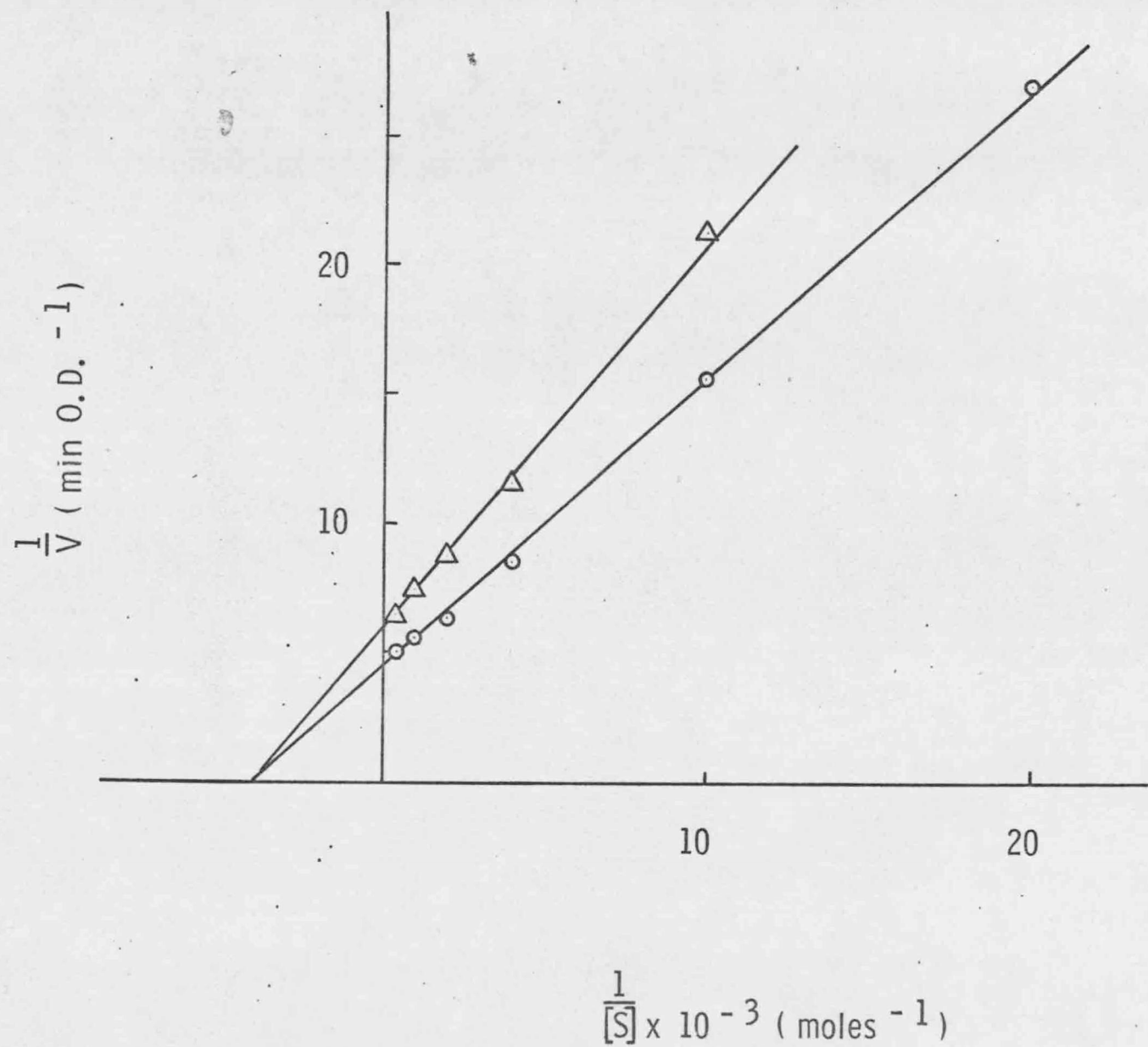


Figure 28

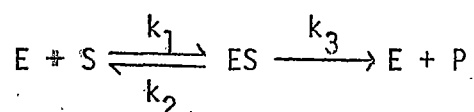
Double reciprocal plots of the velocity of glucose-6-phosphate catalysis versus glucose-6-phosphate concentration. The experimental conditions are as described under the Materials and Methods section.



1.80×10^{-5} moles. Double reciprocal plots of velocity versus substrate concentration, for NADP^+ as the variable substrate, in the presence of NAD^+ (Figure 27) indicated that the dinucleotide inhibited G6PDH catalysis. The intercept of these plots on the $1/S$ axis indicated that NAD^+ inhibition was non-competitive. This result suggests that the dinucleotide coenzyme, NAD^+ , binds to a site on G6PDH other than that responsible for binding NADP^+ , the coenzyme necessary for enzyme activity. *E. coli* B G6PDH catalysis is specific for NADP^+ and the enzyme exhibits no activity when NAD^+ is substituted for NADP^+ . Figure 28 illustrates a typical Lineweaver-Burk plot obtained when G6P was the variable substrate. The Michaelis-Menten constant, K_m , for G6P was calculated to be 2.23×10^{-4} moles. In the presence of 1.5 mM NAD the enzyme catalysis again exhibited non-competitive inhibition. The results of the NAD^+ inhibited G6PDH catalysis (Figures 27 and 28) suggested that NAD^+ does not bind at the active site of the enzyme, and implicates the role of an allosteric site for NAD^+ binding. In several enzyme preparations NAD^+ was found to be an inhibitor of G6PDH activity. In one experiment, performed under saturating concentrations of both NADP^+ and G6P, a 3 mM solution of NAD^+ afforded 62.5% inhibition of activity. Ten mM NAD^+ completely inhibited the G6PDH activity. These results suggest that NAD^+ may be a negative allosteric effector of *E. coli* B G6PDH activity.

In order to further investigate the implied allosteric role of

NAD⁺ on E. coli B G6PDH activity, the kinetic data was plotted according to Hill⁴¹ and Atkinson.⁴² This treatment of the kinetic data, termed the empirical Hill⁴¹ equation and further developed by Atkinson,⁴² provides a convenient method for the study of the role of an allosteric effector. In the simple Michaelis-Menten approach to enzyme catalysis, the rate limiting step is assumed to be product formation from the enzyme substrate complex.



The Michaelis-Menten equation derived from this model is

$$V = \frac{V_{\max} [S]}{K_m + [S]}$$

where V is the initial velocity of the reaction, $V_{\max}$ the maximum velocity, $[S]$ the substrate concentration and K_m the Michaelis-Menten constant = $\frac{k_2 + k_3}{k_1}$.

In this approach it is assumed that there is only one substrate-binding (interacting) site per enzyme molecule. Hill⁴⁰ and Atkinson⁴¹ have developed a treatment of enzyme kinetics for an enzyme with n interacting sites for the substrate molecule. In this treatment $V = k[ES_n]$ when the concentration of all complexes containing fewer than n molecules of substrate are negligible.

$$V = k[ES_n]$$

$$\text{then } V_{\max} = k[E_T]$$

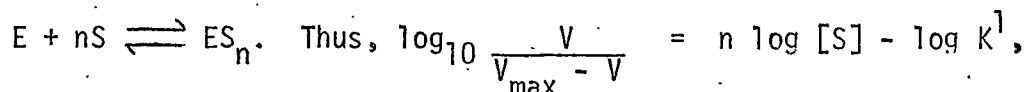
where E_T = total enzyme concentration

$$[E] = [E_T] - [ES_n]$$

and thus, $k[E] = V_{\max} - V$ where k = rate constant, V is the reaction rate, and $V_{\max}$ is the maximum rate attained when the enzyme is saturated with substrate.

$$\text{Thus, } \frac{[ES_n]}{[E]} = \frac{V}{V_{\max} - V} = \frac{[S]^n}{K^1}$$

where $K^1 = \frac{[E][S]^n}{[ES_n]}$, the equilibrium constant of the reaction



the Hill equation.

In this equation the value of n , the Hill coefficient is a function of the number of interacting substrate-binding sites per enzyme molecule and of the strength of the interaction.⁴¹ The Hill equation also provides a convenient method for the study of the effect of an allosteric effector on the cooperativity (or interaction) between the substrate-binding sites in the presence of the allosteric modifier. When $n = 1$ no cooperativity between substrate-binding sites is indicated, and the Hill equation reduces to the simple

Michaelis-Menten treatment. When $n > 1$ positive cooperativity is observed, in this case the allosteric effector molecule has a positive, or activating, effect on the substrate-binding (interacting sites). When $n < 1$ negative allosterism or inhibition is suggested.

Figure 29

Hill plots and coefficients for nicotinamide adenine dinucleotide phosphate concentration as the variable substrate. Experimental conditions are as described under the Materials and Methods section.

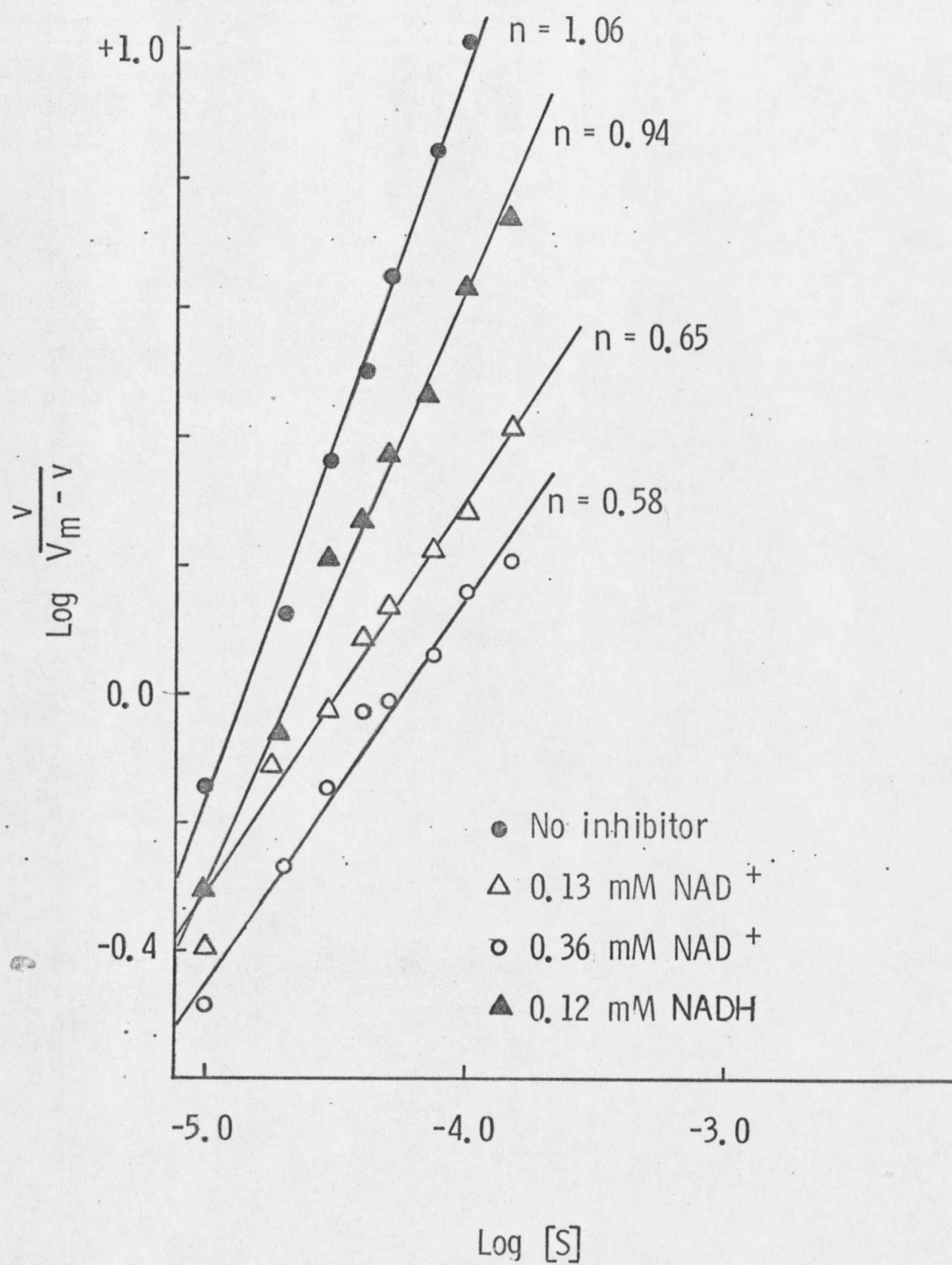


Figure 30

Hill plots and coefficients for glucose-6-phosphate concentration as the variable substrate. Experimental conditions are as described under the Materials and Methods section.

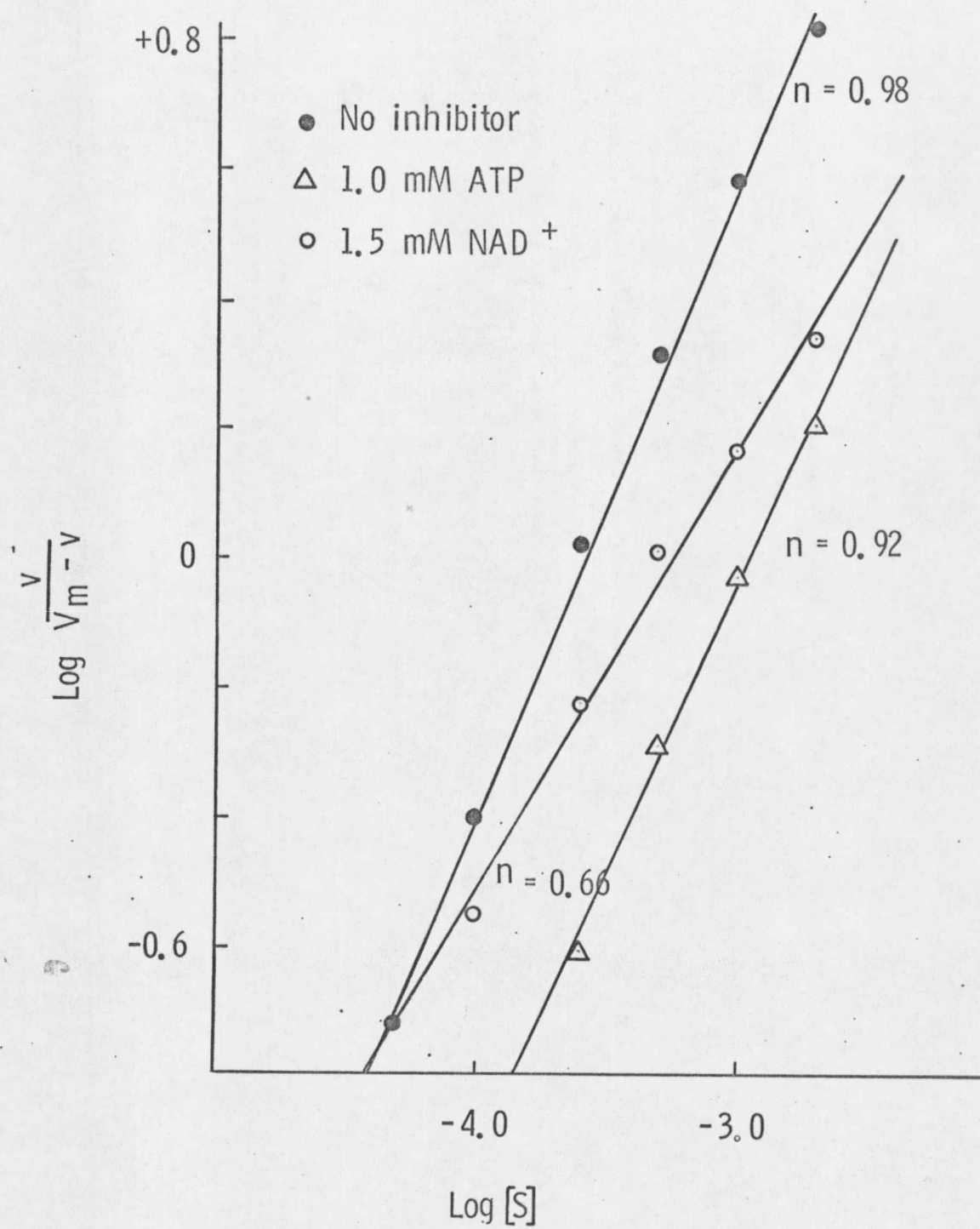
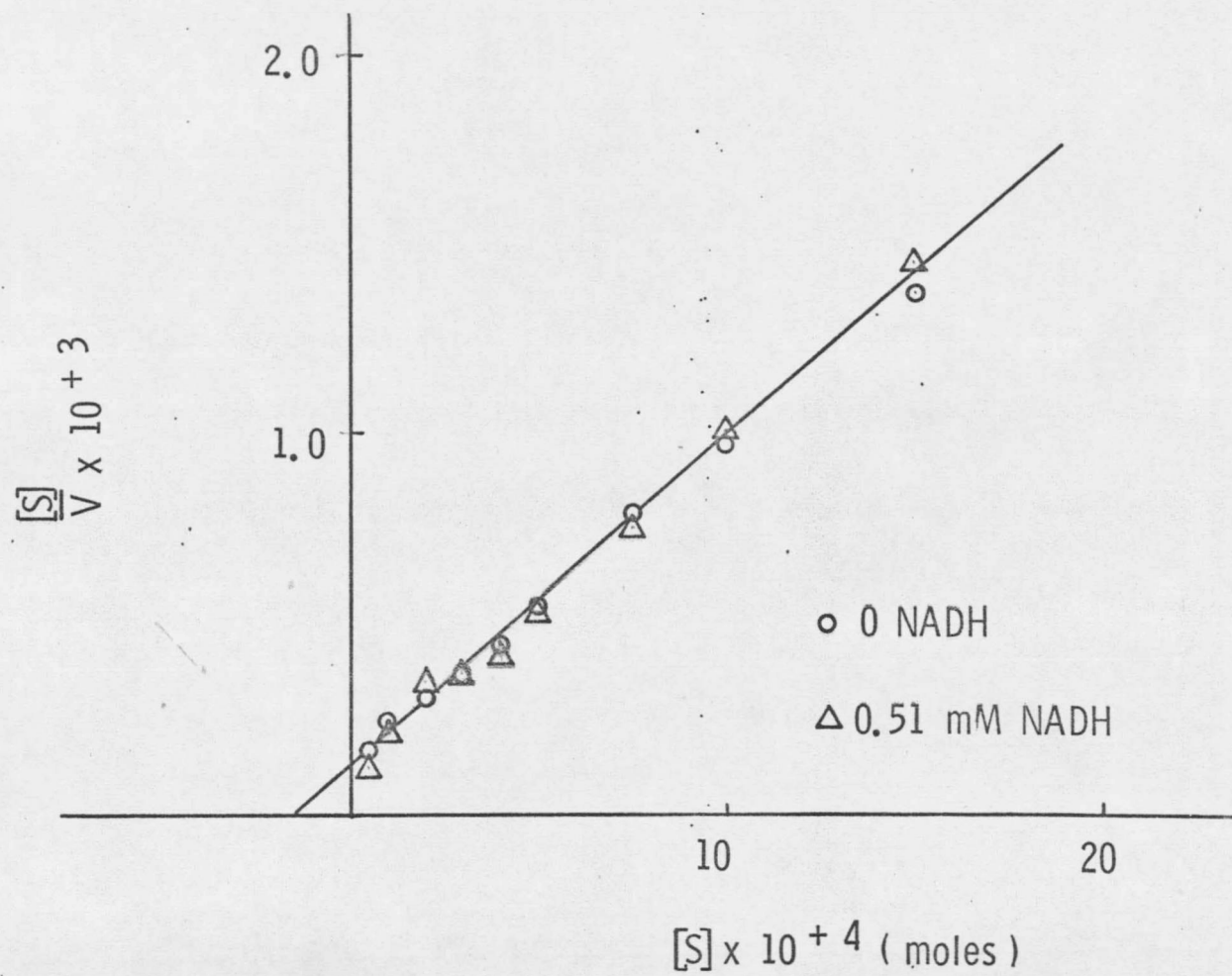


Figure 31

Woolf plots ($\frac{[S]}{V}$ versus $[S]$) for nicotinamide adenine dinucleotide phosphate concentration as the variable substrate. Experimental conditions are as described under the Materials and Methods section.



Figures 29 and 30 illustrate the Hill plots obtained in the presence of NAD^+ for experiments where NADP^+ and G6P were the variable substrates. The values obtained for the Hill coefficient when NADP^+ was the variable substrate were 0.65 in the presence of $1.33 \times 10^{-4} \text{ M NAD}^+$, and 0.58 in the presence of $3.6 \times 10^{-4} \text{ M NAD}^+$. Computer least square treatment of the Hill plot gave the estimated standard deviations of the Hill coefficients as 0.035 and 0.037 respectively. The Hill coefficient in the presence of 1.5 mM NAD^+ was 0.66 when G6P was the variable substrate, the estimated standard deviation for this value was not calculated. These values suggested that NAD^+ is a negative allosteric effector for E. coli B G6PDH. The values of the Hill coefficient in the absence of NAD^+ were calculated to be 1.06 when NADP^+ was the variable substrate, and 0.903 when G6P was the variable substrate.

The non-competitive nature of NAD^+ inhibition of G6PDH activity, and the values of the Hill coefficients in the presence of NAD^+ suggested that NAD^+ is a negative allosteric effector for G6PDH activity.^{40,41} These kinetic experiments did not prove the existence of an NAD^+ affected allosteric site on G6PDH, but suggested that one or more such sites may exist on E. coli B G6PDH.

Sanwall¹⁵ has reported that NADH is a negative allosteric effector of E. coli B G6PDH activity. Figure 31 illustrates a Woolf plot ($\frac{[S]}{V}$ versus $[S]$) and shows that NADH has no effect on the G6PDH catalysis. Kinetic investigations in the presence of NADH presented

experimental problems. In kinetic experiments the initial rate of G6PDH was measured as $\Delta OD \text{ min}^{-1}$ measured at 3390 Å. However, NADH also absorbed at this wavelength and consequently velocities of the enzyme reaction in the presence of NADH necessitated measuring ΔOD values against a large, background reading. Similar problems were encountered in kinetic experiments in the presence of NADPH. The allosteric effect of NADH reported by Sanwall¹⁵ was not demonstrated in this study, and the Hill coefficient in the presence of $1.2 \times 10^{-4} \text{ M}$ NADH was calculated to be 0.98, a value which does not suggest any allosterism (Figure 29).

When NADP^+ was the variable substrate, and NADPH was added to the enzyme assay solution, double reciprocal plots of velocity versus NADP^+ concentration (Figure 32) indicated that the reduced coenzyme (NADPH) was a competitive inhibitor for the NADP^+ site on G6PDH.

Figure 33 illustrates the Lineweaver-Burk plot of velocity versus G6P concentration in the presence and absence of ATP. The intercept on the $1/V$ axis indicates that ATP was a competitive inhibitor for the G6P site. Figure 34 illustrates the kinetics in the presence of ATP when NADP^+ was the variable substrate. The parallel slopes obtained from the $1/V$ versus $1/S$ plots indicated that the inhibition was uncompetitive in nature. This effect was produced by ATP binding to the G6P site of G6PDH which resulted in a lower effective enzyme concentration. Sanwall¹⁵ has reported that ATP does not affect E. coli B G6PDH catalysis, and this result may be due to the employment of

Figure 32

Double reciprocal plot of velocity versus nicotinamide adenine dinucleotide phosphate concentration as the variable substrate. Experimental conditions are as described under the Materials and Methods section.

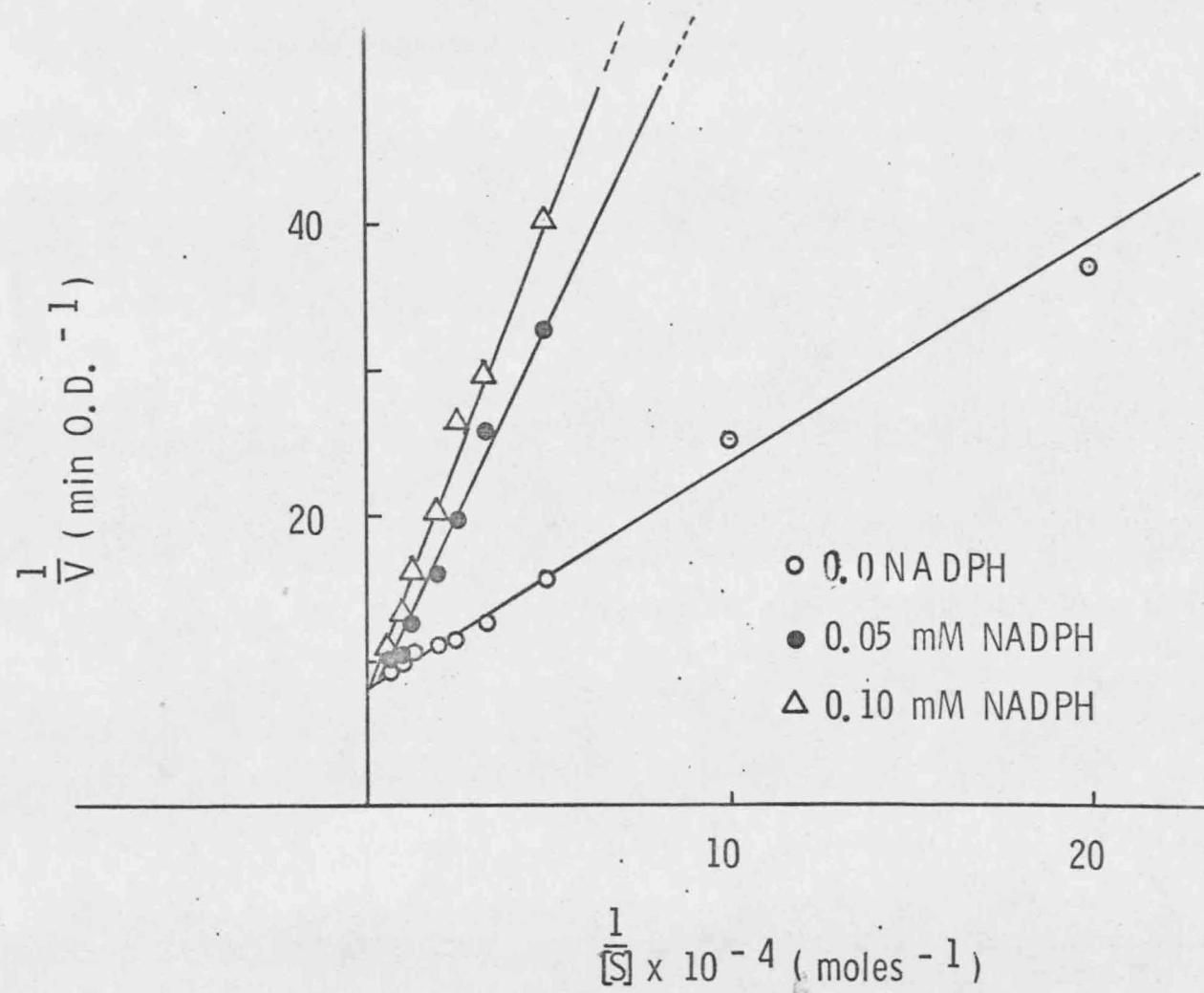


Figure 33

Double reciprocal plot of velocity versus glucose-6-phosphate concentration as the variable substrate. Experimental conditions are as described under the Materials and Methods section.

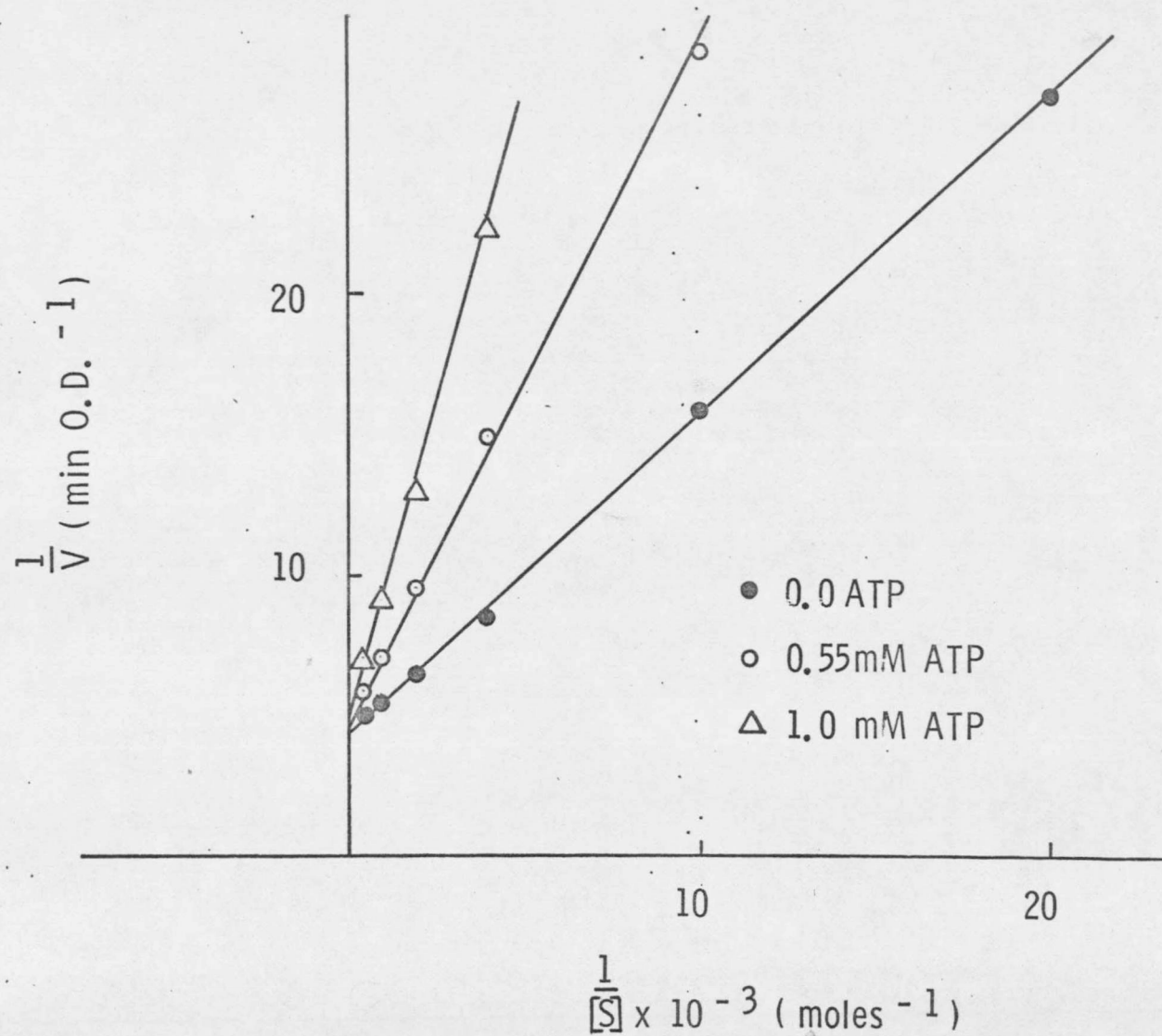
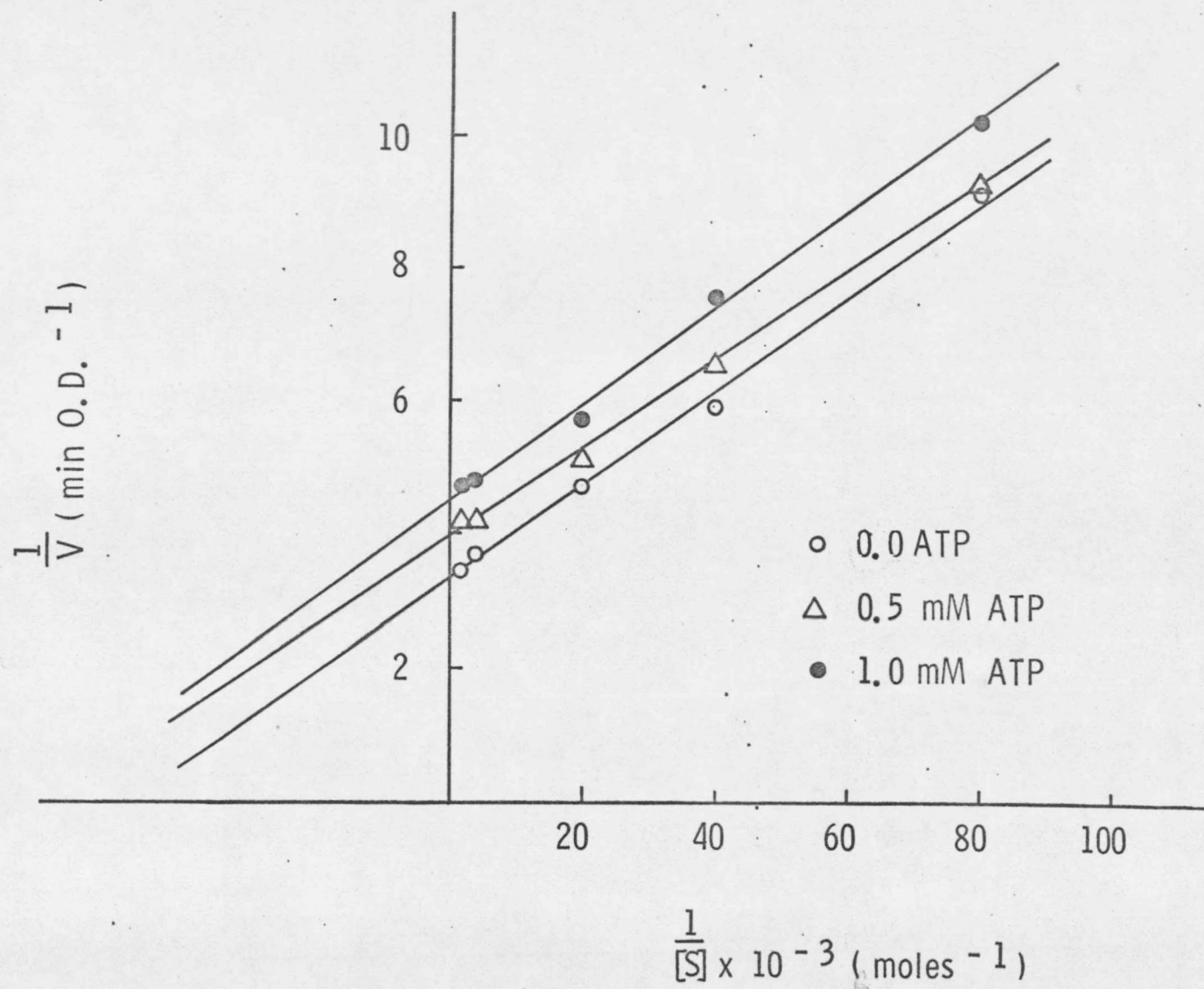


Figure 34

Double reciprocal plots of velocity versus nicotinamide adenine dinucleotide phosphate concentration. Experimental conditions are as described under the Materials and Methods section.



Mg^{++} in the assay solution. Mg^{++} is believed to bind to the β and γ phosphate of ATP and the Mg^{++} -ATP complex might not then be able to bind to the G6P site of G6PDH. In this study Mg^{++} was omitted from the assay solutions employed in kinetic investigations since the enzyme activity of G6PDH was found to be unaffected by Mg^{++} . The data reported by Sanwall¹⁵ was obtained using partially purified E. coli B G6PDH (167-fold purification), and Mg^{++} was reported to be necessary for full activity; in the absence of Mg^{++} , G6PDH exhibited 20% activity. The study reported here employed purified G6PDH (1157-fold purification) and Mg^{++} was found to have no effect on the rate of enzyme catalysis. AMP was also found to have no effect on G6PDH catalysis.

The kinetic data suggested an allosteric role for NAD^+ in controlling the activity of E. coli B G6PDH. This effect may be rationalized in terms of the role of the HMP shunt in E. coli B metabolism. Model and Rittenburg,⁸ in labeling studies with E. coli B, have shown that the major role of the HMP shunt was in providing reduced nicotinic adenine dinucleotide phosphate (NADPH) for biosynthetic purposes. In studies with Neurospora crassa Fuscaldo et al.⁹ have demonstrated that the major role of the first two enzymes of the HMP shunt was in NADPH production. Colonial morphology mutants of Neurospora crassa, involving mutations in G6PDH and 6PGDH, reverted to a wild type morphology upon supplementation of the growth media with an NADPH producing system. No phenotypic reversions to wild type morphology were obtained by supplementation with ribose as a carbon source. These results suggest

that the major role of the HMP shunt is involved in the production of NADPH for biosynthetic purposes. Hansen and Leveille¹¹ have suggested that as much as 50% of the NADPH requirement of Neurospora crassa is provided by the first two enzymes of the HMP shunt. Similar studies on the HMP shunt of Candida utilis, Osmond and Ap Rees¹⁰ have also supported the production of NADPH as the main function of the HMP shunt.

The reduced nicotine adenine dinucleotide phosphate, NADPH, produced by the first two enzymes of the HMP shunt is utilized in the biosynthesis of fatty acids, and in the desaturation of saturated fatty acids. These biosynthetic reactions also require the presence of ATP as an energy source. Kinetic experiments suggested that NAD^+ was a negative allosteric effector for E. coli B G6PDH, and this role can be explained on the basis of the particular requirements of the cell. The primary requirement for E. coli B cells is the production of energy in the form of ATP. This energy is obtained from oxidation of glucose by enzymes of the glycolytic sequence and the Krebs tricarboxylic acid cycle, and results in the production of 38 moles of ATP for each mole of glucose oxidized. The ATP produced by the oxidation of glucose is obtained by direct phosphorylation of ADP, and by oxidative phosphorylation from NADH produced in both glycolysis and the Krebs tricarboxylic acid cycle. Under conditions where E. coli B cells require energy, to sustain the array of metabolic functions, NADH present in the cell is oxidized via oxidative

phosphorylation to yield the necessary ATP. Consequently the cellular level of NAD^+ increases as a result of this utilization of NADH, and this NAD^+ then acts as a negative allosteric effector of G6PDH activity. G6PDH is the first enzyme of the HMP shunt, and the substrate G6P is common to both G6PDH and the glycolytic enzyme phosphoglucoisomerase. Consequently, high cellular levels of NAD^+ inhibit G6PDH activity and insure that glucose metabolism proceeds via glycolysis and the tricarboxylic acid cycle in response to the energy needs of the cell. Under conditions where ATP levels are sufficiently high for the metabolic needs of the cell, the concentration of NAD^+ is low and that of NADH is high since NADH is no longer oxidized by the oxidative phosphorylative sequence. Thus the low levels of NAD^+ do not allow for any allosteric inhibition of G6PDH activity, and under these conditions the NADPH necessary for lipid biosynthesis is produced via the HMP shunt. Further support of this postulate is provided by ATP inhibition of phosphofructokinase, an enzyme of the glycolytic pathway. When cellular levels of ATP are sufficiently high, ATP inhibits phosphofructokinase activity and thus increases the level of G6P in the cell. Consequently, G6P is oxidized by the HMP shunt and results in the production of NADPH. The rationalization presented is based on the primary cellular need for energy (ATP) before any biosynthetic reactions can be effected. Thus, in this case, ATP requirement preempts the need for NADPH necessary for lipid biosynthesis.

CONCLUSIONS

The study reported described a seven stage procedure for the purification of E. coli B G6PDH. The activity was purified 1157-fold to a specific activity of 82 units/mg. The purified dehydrogenase was found to consist of five electrophoretic species, all of which had G6PDH activity. Denaturation with 8 M urea or 1% SDS and 1% β ME resulted in the production of a single protein band upon electrophoresis.

The subunit molecular weight of E. coli B G6PDH was found to be 53×10^3 daltons by SDS polyacrylamide gel electrophoresis. High speed equilibrium sedimentation ultracentrifugation by the method of Yphantis indicated a value of 26.50×10^3 daltons for the subunit molecular weight of G6PDH. The ultracentrifuge data indicated an equilibrium between a 50×10^3 and 25×10^3 dalton species of G6PDH in the denaturant 6 M guanidine-hydrochloride.

The pH optimum for G6PDH activity was found to be pH 8.5, and the enzyme activity was found to be 227% of the value measured at pH 7.4. If it was assumed that G6PDH contained one active center per 26.50×10^3 dalton subunits, the turnover number of enzyme was calculated to be $2.173 \times 10^3 \text{ min}^{-1}$ active center $^{-1}$ at pH 7.4, and $4.933 \times 10^3 \text{ min}^{-1}$ active center $^{-1}$ at pH 8.5. The enzyme was found to exhibit maximum stability when stored at a pH of below 8.0.

N-ethyl maleimide and p-chloro-mercuribenzoate treatment of E. coli G G6PDH indicated that the dehydrogenase contained a cysteine residue located at the active site. The data indicated that this cysteine residue was situated close to the NADP^+ binding site of the enzyme.

Thin layer chromatography of the N-terminal dansyl derivative of E. coli B G6PDH indicated that methionine was the N-terminal amino acid of the enzyme.

Kinetic studies gave values for the Michaelis-Menten constants of NADP^+ and G6P as 1.80×10^{-5} moles and 2.23×10^{-4} moles respectively. ATP was found to be a competitive inhibitor for the G6P binding site of G6PDH, and NADPH competed for binding at the NADP^+ site. The kinetic data indicated that NAD^+ was a noncompetitive inhibitor for both NADP^+ and G6P and suggested an allosteric role for NAD^+ control of G6PDH activity. Analysis of the kinetic data by Hill plots further supported the allosteric effect of NAD^+ on G6PDH activity. The previously reported allosteric effect of NADH on G6PDH activity¹⁵ was not demonstrated in this study. NADH was found to have no effect on the kinetics of G6PDH catalysis. NADP^+ was found to be an obligatory coenzyme for G6PDH, and no enzymic activity was exhibited with NAD^+ as the coenzyme.

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Escherichia coli B
glucose-6-phosphate
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