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HOOVER, John David, 1947-
SEPARATION AND CHARACTERIZATION OF TWO ISOENZYMES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO SUSPENSION CULTURE WR-132.

The University of Oklahoma, Ph.D., 1975
Chemistry, biological

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

SEPARATION AND CHARACTERIZATION OF TWO ISOENZYMES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO SUSPENSION CULTURE WR-132

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

JOHN DAVID HOOVER

Norman, Oklahoma

1975

SEPARATION AND CHARACTERIZATION OF TWO ISOENZYMES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TOBACCO SUSPENSION CULTURE WR-132

APPROVED BY

Eddie Carol Smith

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DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

The author would like to thank Dr. Eddie C. Smith for his patient guidance and encouragement in the completion of this problem. His presence and influence will always be remembered. Special appreciation is given to Dr. Simon H. Wender whose vast knowledge and teaching skills contributed greatly to the development of the author. Appreciation is expressed to Dr. Ralph A. Jacobson, Dr. R.E. Lehr and Dr. D.C. Cox for their assistance and suggestions in the preparation of this manuscript.

Thanks are expressed to fellow graduate students, especially Kerry Sublett and Bernie Powell for their advice and many years of friendship.

The author also thanks Susan Walker for typing this manuscript and assistance in maintaining the suspension cultures.

DEDICATION

To Sandy

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ABSTRACT

Utilizing fractional ammonium sulfate precipitation and DEAE-cellulose chromatrography, two anodic isoenzymes of glucose-6-phosphate dehydrogenase were isolated from tobacco suspension culture WR-132. The pH optimum of isoenzyme Band I was determined to be 9.0 and for Band IV it was 8.0 - 8.3. Isoenzyme Band I exhibited Michaelis-Menten kinetics for both substrates, glucose-6-phosphate and $NADP^+$, with Michaelis constants of 0.22 mM and 0.06 mM respectively. Band IV exhibited Michaelis-Menten kinetics for glucose-6-phosphate with a Michaelis constant of 0.31 mM. The $NADP^+$ double reciprocal plot contained an abrupt transition between two linear sections. This transition corresponds to an abrupt increase in the apparent K_M and V_{Max} values with increasing $NADP^+$, denoting negative cooperativity. The two Michaelis constants for high and low $NADP^+$ concentrations were 0.06 mM and 0.015 mM, respectively.

Molecular weights of the isoenzymes as determined by SDS disc gel electrophoresis were 85,000 - 89,000 for Band I and 54,000 - 59,000 for Band IV. Gel filtration chromatography on Sephadex G-150 yielded values of 91,000 for Band I and 115,000 for Band IV. A probable dimeric structure for Band IV is suggested with two $NADP^+$ binding sites.

Effector studies with these two isoenzymes revealed that both are inhibited markedly by certain coumarin derivatives but not by various phenolic acids tested. The glucosylated coumarins were much more inhibitory

than their nonglucosylated forms. The enzymatic reaction catalyzed by Band I is accelerated by two coumarins, scopoletin and esculetin. This activation is not observed for Band IV. Chlorogenic acid inhibited both Band I and Band IV almost completely. The activation of Band I by scopoletin and esculetin may explain the enhanced activity of the hexose monophosphate pathway that has been reported when plants are subjected to stress conditions.

CHAPTER I

INTRODUCTION

Phenolic compounds are second in abundance only to carbohydrates in plants. The phenolic compounds include the mono- and dihydric phenols, phenolic glycosides, flavonoids, anthocyanins, aromatic amino acids, coumarin derivatives and lignin. The exact role of these phenolics in plant metabolism is still not understood though there have been some notable efforts made toward elucidating their role (24,74).

Many phenolics, such as scopolin, scopoletin and chlorogenic acid, form a relatively large pool in plant tissue with a significant portion of the pool being metabolically inactive (41). The pool could serve as a reservoir for waste products, but this seems unlikely because it would be more advantageous to the plant to degrade these compounds for use in the metabolism of the plant. Alternately, the phenolics may serve as a defense against infection due to viruses, bacteria or fungi. This role is advocated by many investigators (21,41,65).

The pathway responsible for the production of phenolic compounds in microorganisms and higher plants is the shikimic acid pathway (fig 1). Since chorismic acid serves as a precursor for phenolic compounds, the pathways for the synthesis of various phenolics become unique after chorismic acid (33). The phenolic acids, ferulic acid, caffeic acid,

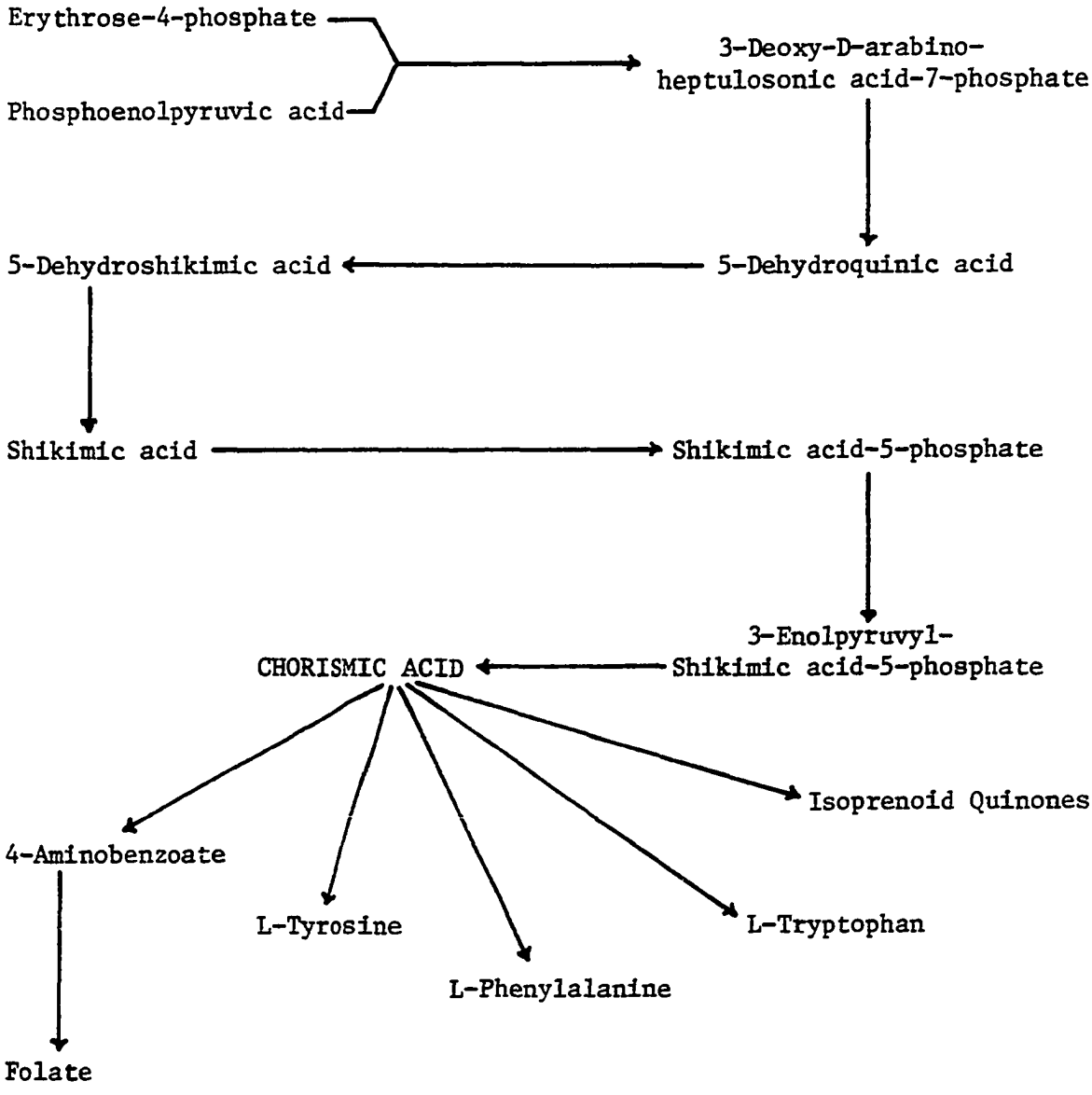


FIGURE 1 AN OUTLINE OF THE SHIKIMIC ACID PATHWAY

para-coumaric acid and *trans*-cinnamic acid as well as lignin are synthesized in one well characterized branch pathway (fig 2). The phenolic acids are incorporated into lignin by a pathway which has not been well-characterized. Gross *et al.* (30) have shown that *trans*-cinnamic acid, *para*-coumaric acid and ferulic acid are incorporated into lignin, and Innerarity *et al.* (34) were able to demonstrate the incorporation of radioactive scopoletin, an intermediate of the pathway, into scopolin, fabiatrin and a lignin-like substance.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, E.C. 1.1.1.49), with its coenzyme, niacinamide adenine dinucleotide phosphate ($NADP^+$), is the first enzyme of the hexose monophosphate pathway, catalyzing the conversion of glucose-6-phosphate (G-6-P) into 6-phosphogluconolactone. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase are the controlling enzymes of the hexose monophosphate pathway due to the limited availability of $NADP^+$ (60). One of the intermediates of this pathway is erythrose-4-phosphate. This sugar phosphate can combine with phosphoenolpyruvic acid, an intermediate in glycolysis, to initiate the shikimic acid pathway. Because of the plausible involvement of the hexose monophosphate pathway in phenolic biosynthesis (fig 3), a change in the phenolic concentration could be postulated to influence the activity of the enzyme glucose-6-phosphate dehydrogenase.

It is an established fact that under stress conditions, the concentrations of many phenolics change in higher plants. There are numerous reported instances in which the phenolic concentration changes with infection due to bacteria, viruses or fungi. Usually, there is an accumulation of

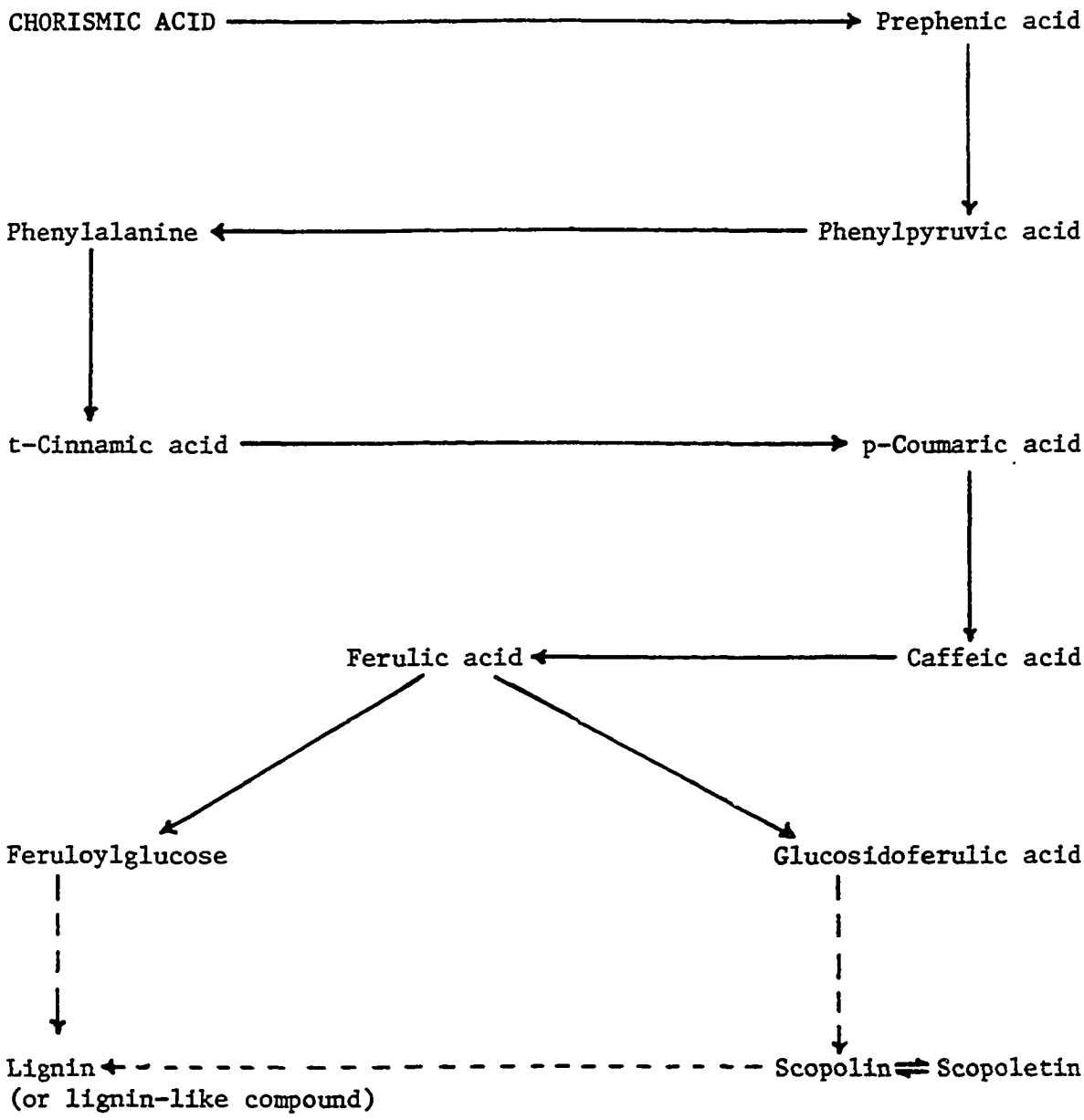


FIGURE 2 THE SHIKIMIC ACID PATHWAY FROM CHORISMIC ACID TO LIGNIN. Solid lines ⟶ represent one step metabolic sequences. Dashed lines - - - → represent possible metabolic sequences, which may or may not be one step processes.

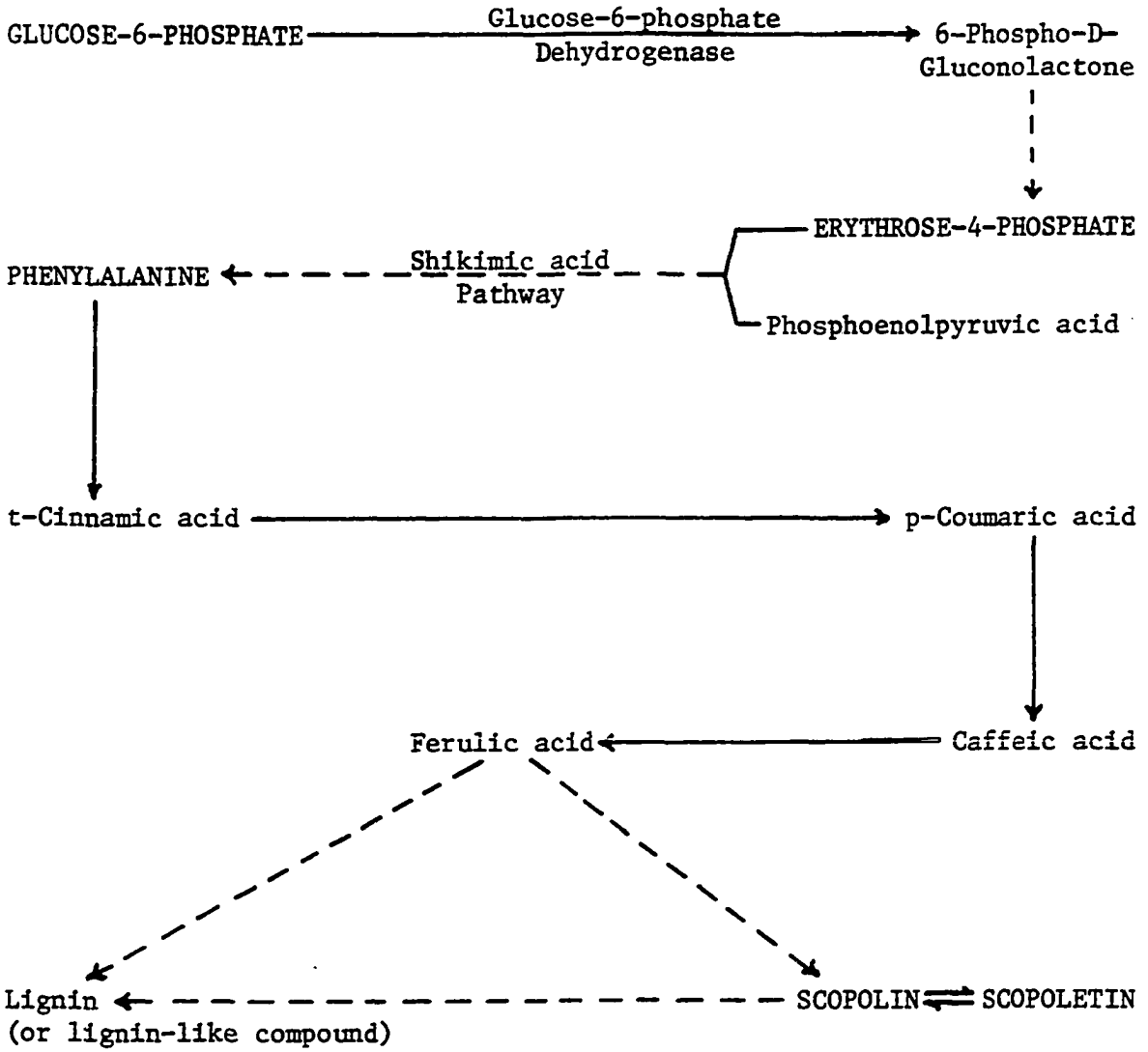


FIGURE 3 AN ABBREVIATED OUTLINE CONNECTING THE HEXOSE MONO-PHOSPHATE PATHWAY AND THE SHIKIMIC ACID PATHWAY. Solid lines ——→ represent one step metabolic sequences. Dashed lines - - - → represent possible metebolic sequences.

phenolic compounds around the location of stress (21). In tobacco plants infected with *Pseudomonas solanacearum*, there is an increase in the scopoletin (6-methoxy-7-hydroxycoumarin) and scopolin (7-glucoside of scopoletin) concentration around the infected regions (61). The rapid increase in the scopoletin concentration is not due to hydrolysis of scopolin. Other reported stress conditions which lead to an accumulation of scopolin and/or scopoletin include: boron deficiency in tobacco plants (68); treatment of tobacco plants with 2,4-dichlorophenoxyacetic acid (2,4-D) (15,24,70), 4-amino-3,5,6-trichloropicoline acid (70), maleic hydrazide (71), ultraviolet irradiation (39) and X-ray irradiation (2). In grapefruit peel, gamma irradiation also caused the accumulation of scopoletin and scopolin (57).

Another phenolic compound undergoing a concentration change during certain changes in environment is chlorogenic acid (3-O-caffeoyl-quinic acid). Tobacco plants when grown with a mineral deficiency of nitrogen showed an increase in chlorogenic acid (5). Tobacco plants grown at daytime temperatures of 5.5°C, instead of the control temperature of 29°C, accumulated chlorogenic acid (39). Ultraviolet irradiation, however, caused only a slight increase in the chlorogenic acid concentration.

An enhancement of the shikimic acid pathway might be responsible for the accumulation of phenolic compounds. However, no direct evidence is available to support this supposition. An alternative course would be through an increased utilization of the hexose monophosphate pathway. Godin (28) originally reported that it is an enhancement of the hexose monophosphate pathway that leads to an accumulation of phenolic compounds. Neish (48) observed that it does not require the complete operation of

the hexose monophosphate pathway for increased phenolic biosynthesis. In tobacco tissue surrounding lesions produced by tobacco mosaic virus, the activity of glucose-6-phosphate dehydrogenase increased 300% over the control (64). In tobacco leaves infected with potato virus (16) and in potato tuber cells after slicing (36), the same enzyme, glucose-6-phosphate dehydrogenase, increased in activity. In general, it can be stated that with an increase in phenolic biosynthesis due to infection, there is an accompanying increase in the activity of the hexose monophosphate pathway (35,63,67) as well as glucose-6-phosphate dehydrogenase.

Thus, there appears more than a casual relationship between the phenolic accumulation in plant tissue and the activity of the enzyme glucose-6-phosphate dehydrogenase. In the present study, two isoenzymes of glucose-6-phosphate dehydrogenase have been isolated from a tobacco suspension culture. The kinetic and physical properties of both isoenzymes were measured. Effector studies were performed to ascertain what, if any, effect particular phenolic compounds have upon the activity of a mixture of isoenzymes as well as the two separated isoenzymes.

CHAPTER II

MATERIALS AND METHODS

WR-132 TOBACCO SUSPENSION CULTURE

The source material for the isoenzymes used in the following studies was a tobacco suspension culture line (*Nicotiana tabacum* L., var. Xanthi). The suspension culture was obtained from Dr. A.C. Olson, U.S. Department of Agriculture, Albany, California. The cells were collected by centrifugation and then resuspended so that in 10 ml of suspension there were 2 grams of tobacco cells. Ten ml were then used as an inoculum and added to 40 ml of medium in a 125 ml erlenmeyer flask. All transfers were made in a Laminar flow hood (Agnew-Higgins) utilizing sterile techniques. The suspended cells were then grown at room temperature in continual subdued light (1 foot candle) on a New Brunswick Model "V" gyrotary shaker (190 rpm). The cells were harvested after 10 days of growth. The medium used was a revised medium of Linsmaier and Skoog (43) in which 2,4-dichlorophenoxyacetic acid was substituted for indole-3-acetic acid, and kinetin was deleted (Table 1).

ELECTROPHORESIS

A) Anodic Disc Gel Electrophoresis

Polyacrylamide gel electrophoresis was used to separate the anodic isoenzymes according to the procedures of Ornstein and Davis (51).

Table 1

COMPOSITION OF REVISED WR-132 CULTURE MEDIUM

COMPOUND	mg/l	COMPOUND	mg/l
sucrose	3000.0	H_3BO_3	6.2
KNO_3	1900.0	glycine	2.0
NH_4NO_3	1650.0	KI	0.83
$CaCl_2 \cdot 2H_2O$	440.0	niacin	0.5
$MgSO_4 \cdot 7H_2O$	370.0	pyridoxine-HCl	0.5
KH_2PO_4	170.0	2,4-dichlorophenoxy-acetic acid	0.5
myo-inositol	100.0	$Na_2MoO_4 \cdot 2H_2O$	0.25
Na_2EDTA	37.3	thiamine-HCl	0.10
$FeSO_4 \cdot 7H_2O$	27.8	$CuSO_4 \cdot 5H_2O$	0.025
$MnSO_4 \cdot 4H_2O$	22.3	$CoCl_2 \cdot 6H_2O$	0.025
$ZnSO_4 \cdot 4H_2O$	8.6		

A Buchler Polyanalyst Disc Electrophoresis Apparatus was utilized for the separations. The running pH was 9.3 with bromophenol blue used as the tracking dye. The electrophoresis gels were 7.5% acrylamide and 0.2% N,N'-methylene bisacrylamide. After completion of electrophoresis, the isoenzymes were visualized according to a modified procedure of Barnes, Kuehn and Atkinson (6) by placing the gels in 100 mM tris-HCl buffer (pH 8.0), 5 mM glucose-6-phosphate, 5 mM Mg^{+2}, 0.5 mM $NADP^+$, 0.04 mM nitroblue tetrazolium and 0.02 mM phenazine methosulfate.

B) Cathodic Disc Gel Electrophoresis

Cathodic electrophoresis was performed in the same manner as anodic electrophoresis except that the electrical current was reversed. The running pH was 4.3 with methyl green used as the tracking dye.

Potassium persulfate was used as the gel polymerization catalysis instead of ammonium persulfate. After completion of electrophoresis the isoenzymes were visualized according to the same procedure as that used for anodic isoenzymes.

C) Starch Gel Electrophoresis

Starch gel electrophoresis was performed utilizing the procedure of Brewer (8). The gel was 10% electrostarch (Electrostarch Co.) in 5 mM histidine (pH 7.0). Electrophoresis was performed for approximately 4½ hours at a constant voltage of 400 volts. The staining procedure was that used to visualize enzyme bands on polyacrylamide gels.

MOLECULAR WEIGHT DETERMINATIONS

A) Gel Filtration Chromatography

Molecular weights of the isoenzymes were determined by gel filtration chromatography using Sephadex G-150 according to the procedure of Andrews (4). Sephadex G-150, equilibrated in 30 mM β-mercaptoethanol, 10^{-5} M $NADP^+$ and 100 mM imidazole-HCl buffer (pH 6.5), was packed in an Ace Glass chromatography column so that the bed was 58 X 1.5 cm. The above buffer was used as the eluting buffer with the flow rate adjusted to 20 ml/hr. Two ml fractions were collected using a Gilson Escargot Fractionator, Model SC-15, and the absorbance at 280 nm was followed to detect the presence of protein. Enzymatic activity was measured to determine the elution volume of the isoenzymes. For one of the isoenzymes, a different eluting buffer had to be used. The buffer was a 100 mM KCl and 50 mM tris-HCl buffer (pH 7.5). Using this buffer the absorbance at 230 nm was used to detect the presence of proteins. Molecular weights of the isoenzymes were calculated using a semi-logarithmic plot of

molecular weight versus elution volume.

B) SDS Polyacrylamide Gel Electrophoresis

A second method used for determining molecular weights was sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis utilizing the procedure of Weber, Pringle and Osborn (69). Seven cm gels with an acrylamide concentration of 7.5% were prepared. Two mg of each standard protein as well as 2 mg of lyophilized isoenzyme powder or a concentrated isoenzyme solution were mixed with 0.01 M sodium phosphate buffer (pH 7.0) containing 1% β-mercaptoethanol and 1% SDS. Sufficient buffer was used so that the weight ratio of SDS to protein was at least 3:1. This solution was incubated in a 100°C water bath for 2 minutes and cooled to room temperature. One ml of this solution was mixed with 0.04 ml of 0.05% bromophenol blue and 0.04 ml of β-mercaptoethanol. A few crystals of sucrose were added for increased density. One hundred fifty µl of this final protein solution (approximately 0.4 mg of protein) was applied to the tops of the electrophoresis gels using a micropipette.

Electrophoresis (8 mA/gel) was performed at room temperature for approximately 5 hours. After completion of electrophoresis, the gels were removed from the glass tubes and placed in an aqueous solution of 0.25% wt/vol Coomassie Brilliant Blue 45.4% vol/vol in methanol and 9.2% vol/vol in glacial acetic acid for 4 hours. The gels were destained by diffusion with 5% vol/vol methanol and 7.5% vol/vol glacial acetic acid solution. The dark protein bands were then visible against the light destained background. Mobilities (M) were calculated using the equation:

$$M = \frac{\text{Distance of protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Gel length before staining}}{\text{Distance of dye migration}}$$

Molecular weights were calculated using a semi-logarithmic plot of molecular weight versus mobility.

ENZYME ASSAYS

The assay procedure of Brown and Wray (10) was used to measure enzymatic activity. The conversion of glucose-6-phosphate to 6-phosphogluconolactone was followed by measuring the increase in absorbance at 340 nm due to the production of reduced $NADP^+$. The reaction was initiated by the addition of $NADP^+$, producing linear rates of reaction for the first 4-6 minutes.

Each assay consisted of 100 mM tris-HCl buffer (pH 8.0), 5 mM Mg^{+2} plus enzyme in a total volume of 3 ml. The concentrations of G-6-P and $NADP^+$ were varied depending upon the situation. Saturating levels of G-6-P and $NADP^+$ were 5 mM and 1 mM respectively.

A Varian Techtron Model 635 UV-Visible Recording Spectrophotometer was used for all enzyme assays.

THIN LAYER CHROMATOGRAPHY

Thin layer plates were prepared using a Desaga-Brinkmann spreader set to a thickness of 0.375 nm. Avicel SF was suspended in water (22.2 g per 100 ml) and mixed in a blender for 30-45 seconds. The suspension was set aside for 15 minutes to remove any large air bubbles, and then used to prepare the thin layer plates. Ascending chromatography was utilized to develop the chromatograms. Four different solvent systems were employed to develop the chromatograms. The solvents employed were: methylisobutyl ketone, formic acid and water 14:3:2 (KFW); benzene, ethyl acetate, formic acid and water 9:21:6:3 (BzEFW); benzene, acetic acid, water and nitromethane 34:32:5:18 (BzAWN) and ethyl acetate, pyridine and water 2:1:1 upper layer

(EPW). The developed chromatograms were viewed under a UV light (366 nm Black Ray B-100). Plates were also passed over NH_4OH to increase the intensity of fluorescence of the spots.

POLYCLAR AT

The Polyclar AT used in the homogenization was washed with several solvents prior to use. First, 250 grams of Polyclar AT was soaked in 1000 ml of deionized distilled water for 1 hour and suction-filtered. The Polyclar AT was placed in the following solvents consecutively to be soaked for 30 minutes, washed with deionized distilled water and suction-filtered. The solvents were: 385 ml dimethylformamide, 770 ml glacial acetic acid, 2310 ml deionized distilled water, 1540 ml redistilled methanol and finally 2000 ml deionized distilled water. The wet Polyclar AT was air-dried. This dry Polyclar AT was hydrated for 30 minutes prior to use in the homogenization mixture.

CHEMICALS

All the chemicals, except those noted below, were obtained from Sigma Chemical Company.

Chemical	Source
Ferulic acid	Aldrich Chemical Company
para-Coumaric acid	Calbiochem
Caffeic acid	California Foundation for Biochemical Research
Esculetin Chlorogenic acid	Fluka AG
Avicel SF (microcrystalline cellulose)	Americal Viscose Division of FMC Corporation
Polyclar AT (polyvinylpolyprrolidone)	GAF Corporation Chemical Division

Chemical	Source
DEAE-cellulose (DE-52 preswollen microgranular)	Reeve Angel Corporation
Sephadex G-150	Pharmacia Fine Chemicals Incorporated
β-galactosidase Creatine kinase Polyphenol oxidase Glyceraldehyde-3-phosphate dehydrogenase	Worthington Biochemical Corporation

The phenolic solutions used in the effector studies were prepared just prior to use and kept wrapped in aluminum foil. It was found that the phenolic solutions were stable for only a few days. This was determined in initial studies by subjecting the solutions to extensive study through use of thin layer chromatography.

CHAPTER III

ISOLATION OF ISOENZYMES AND GROWTH OF CELLS

PREPARATION OF ENZYME

Crude glucose-6-phosphate dehydrogenase as isolated from a tobacco suspension culture exists as 4 anodic isoenzymes. Visualized on polyacrylamide gel electrophoresis (fig 4), the 4 isoenzymes are designated as Band I, II, III and IV with the corresponding mobilities of 0.12, 0.20, 0.28 and 0.38. Cathodic polyacrylamide gel electrophoresis of the same extract indicated that no cathodic glucose-6-phosphate dehydrogenase isoenzymes were present. Starch gel electrophoresis also confirmed the existence of only 4 anodic isoenzymes with no cathodic bands.

The isoenzymes of glucose-6-phosphate dehydrogenase were separated utilizing DEAE-cellulose chromatography. The separation procedure is summarized in table 2.

Sixty grams of WR-132 tobacco tissue was mixed with 30 grams washed glass beads, 30 grams Polyclar AT which had been hydrated 1/2 hour prior to use and 120 ml of 2 mM EDTA, 30 mM β-mercaptoethanol, 100 mM tris-HCl buffer (pH 8.5). The β-mercaptoethanol was necessary in the buffer to maintain enzymatic activity. The Polyclar AT was present to absorb any phenolic compounds released from the cells. This mixture was homogenized in a Sorvall Omnimixer at 5000 rpm for 5-1/2 minutes. The Omnimixer cup was immersed in an ice bath during the homogenization with all subsequent

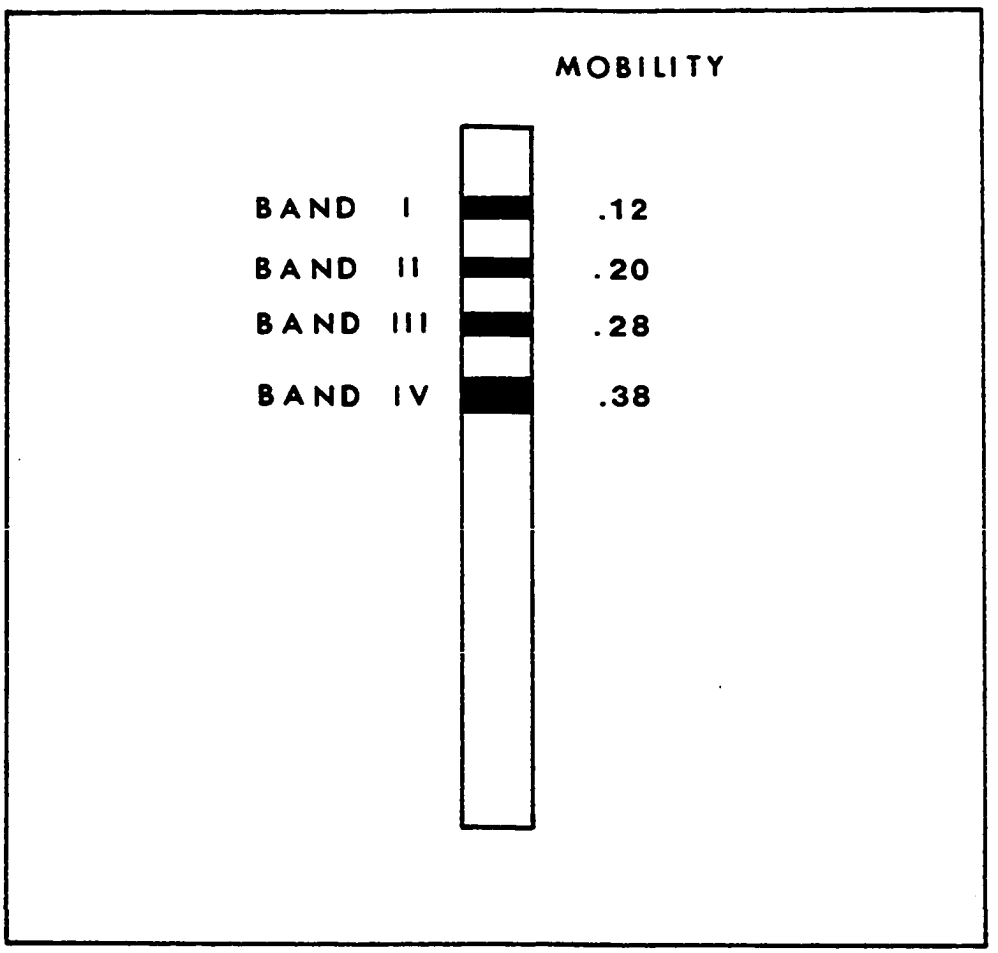


FIGURE 4 ANODIC POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES FROM TOBACCO SUSPENSION CULTURE WR-132. Mobilities are relative to bromophenol blue. Bands were visualized with: 100 mM tris-HCl buffer (pH 8.5), 5 mM G-6-P, 5 mM $NADP^+$, 0.04 mM nitroblue tetrazolium and 0.02 mM phenazine methosulfate and 5 mM Mg^{+2}.

TABLE 2

ISOLATION PROCEDURE FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES BAND I AND BAND IV

STEP 1

Homogenize WR-132 tobacco tissue in 2 mM EDTA, 30 mM β-mercaptoethanol, 100 mM tris-HCl buffer(pH 8.5) for 5-1/2 minutes at 5000 rpm. Filter through 4 layers of cheesecloth. Centrifuge 15 minutes at 34,800 x g. Save the supernatant.

STEP 2

Bring the supernatant to 30% saturation with solid $(NH_4)_2SO_4$. Centrifuge 10 minutes at 34,800 x g. Save the supernatant.

STEP 3

Bring the supernatant to 70% saturation with solid $(NH_4)_2SO_4$. Centrifuge 10 minutes at 34,800 x g. Dissolve the pellet in a small volume of 30 mM β-mercaptoethanol, 100 mM imidazole-HCl buffer (pH 6.5). Dialyze overnight against 100 volumes of the same buffer.

STEP 4

DEAE-cellulose chromatography. Elute isoenzymes using buffer made 10^{-5} in $NADP^+$ and 50 mM NaCl.

operations carried out at 4°C. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 34,000 X g for 15 minutes. The supernatant was saturated to 30% with solid $(NH_4)_2SO_4$, centrifuged for 10 minutes at 34,000 X g and the pellet discarded. The resulting supernatant was saturated to 70% with solid $(NH_4)_2SO_4$ and centrifuged at 34,000 X g for 10 minutes. The pellet which contained the enzyme was dissolved in a small volume of 30 mM β-mercaptoethanol, 100 mM imidazole-HCl buffer (pH 6.5). The crude enzyme preparation was dialyzed against 100 volumes of the same imidazole-HCl buffer.

The dialyzed enzyme preparation was applied to the top of a DEAE-cellulose column equilibrated with 10^{-5} M $NADP^+$, 30 mM β-mercaptoethanol and 100 mM imidazole-HCl buffer (pH 6.5). Isoenzyme Band I eluted from the column with the elution buffer, while Band IV was eluted from the column using the elution buffer made 50 mM in NaCl. The flow rate was maintained at 1 ml per minute with 10 ml fractions collected using a Gilson Escargot Fractionator. A typical elution pattern from a DEAE-cellulose column is shown in fig 5. The major activity peak for Band I is tube no. 14 and the activity peak for Band IV is centered around tube no. 24. Band I usually was contaminated with other proteins while Band IV was found to be contamination free of other proteins as determined by anodic polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brilliant Blue. After DEAE-cellulose chromatography, Band I was extremely unstable, usually losing all catalytic activity within 36-48 hours. Therefore, all kinetic measurements on Band I had to be performed immediately. However, Band IV posed no such problem and usually exhibited sufficient catalytic activity at the end of a week of storage

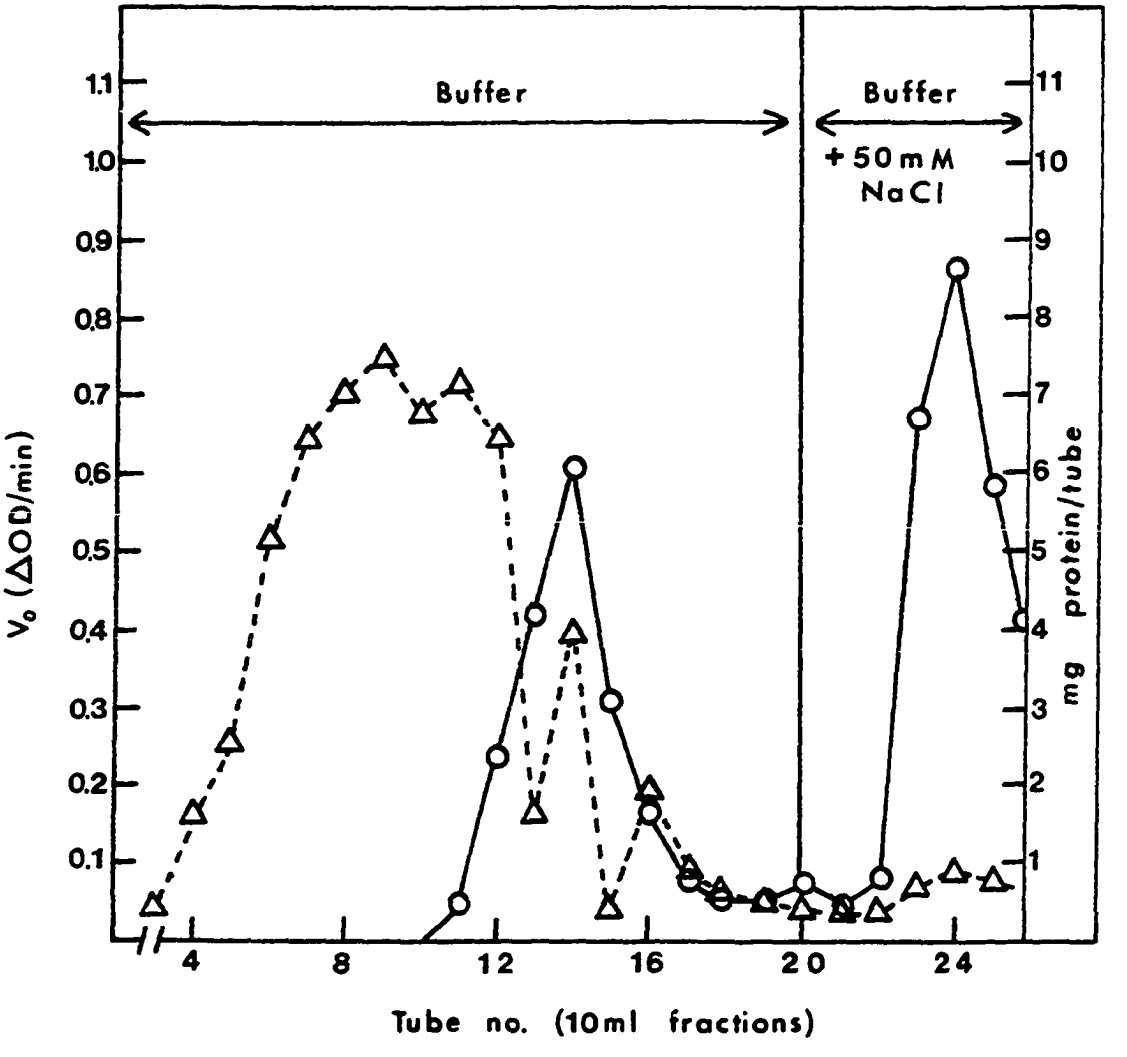


FIGURE 5 ELUTION PROFILE OF BAND I AND BAND IV FROM A DEAE-CELLULOSE COLUMN. The buffer is 10^{-5} M $NADP^+$, 30 mM β-mercaptoethanol and 100 mM imidazole-HCl (pH 6.5). Δ- -Δ mg protein/tube at 280 nm O——O glucose-6-phosphate dehydrogenase activity

at 4°C to perform kinetic studies.

A crude preparation of isoenzymes usually had an activity of approximately 0.232 μmoles of NADPH formed/min with a specific activity of 0.0211 μmoles of NADPH formed/min/mg of protein. Band I as isolated from a DEAE-cellulose column usually exhibited an activity of approximately 0.1514 μmoles of NADPH formed/min with a corresponding specific activity of 0.0757 μmoles of NADPH formed/min/mg of protein. Band IV, after DEAE-cellulose chromatography, had an activity of 0.4148 μmoles of NADPH formed/min. The specific activity of this Band IV preparation was 0.5926 μmoles of NADPH formed/min/mg of protein. A typical purification scheme for Band I and Band IV would start with approximately 80,000-100,000 total counts of enzymatic activity, where one count was defined as a concentration change of 0.001 μmoles of NADPH/min. After DEAE-cellulose chromatography, Band I had approximately 7,600 total counts and Band IV had approximately 20,800 total counts of activity. Band I and Band IV therefore accounted for 9% and 25% respectively of the initial total activity. The purification scheme for Band I and Band IV resulted in a 3.6 and 28.1 fold purification respectively. Acticities expressed in ΔOD/min were converted into μmoles NADPH formed/min by using the following equation:

$$\mu\text{moles NADPH formed/min} = \Delta\text{OD/min} \times 3/6.22$$

Kajinami (37) had reported that Polyclar AT was not necessary in the initial cell homogenization in order to obtain enzymatic activity. However, in this study, as well as others (22,40), it was essential that Polyclar AT be in the homogenization step. When tobacco cells were broken without Polyclar AT there was no enzymatic activity. When the inactive

preparation was run through a Sephadex G-25 column equilibrated in the homogenization buffer, the effluent did exhibit activity, indicating that the inhibitor was a small molecular weight compound. The two extracts, with and without activity, were subjected to ascending thin layer chromatography utilizing 4 different solvent systems to develop the chromatograms. In each of the developed chromatograms, there appeared three major phenolic compounds from the inactive preparation which were not visible in the active preparation (fig 6). In the solvent system KFW, the three phenolic compounds had R_f values of 50, 27 and 20. In BzEFW, the R_f values were 71, 61 and 54; in BzAWN the R_f values were 89, 84 and 72. The R_f values in EPW were 38, 28 and 14. The three phenolic compounds could be extracted with methanol, but not butanol. The methanol extracts of the tobacco cells were streaked on Avicel SF thin layer plates and developed in BzAWN and BzEFW. The three spots were extracted from the Avicel using 100 mM imidazole-HCl buffer (pH 6.5). When 0.3 ml of the extracted phenolics were present in a glucose-6-phosphate dehydrogenase assay, there was a 35-40% inhibition of the control activity.

Five and one-half minutes was also selected as the optimum breaking time. An increase in the breaking time did not increase activity. It was determined that 5½ minutes was the optimum homogenization time producing a preparation with maximum activity and one which retained the greatest amount of glucose-6-phosphate dehydrogenase activity after storing at $4^{\circ}C$.

GROWTH OF WR-132 TOBACCO CELLS

A study was undertaken to observe the growth of WR-132 tobacco suspension cultures to ascertain whether the age of the cells altered the

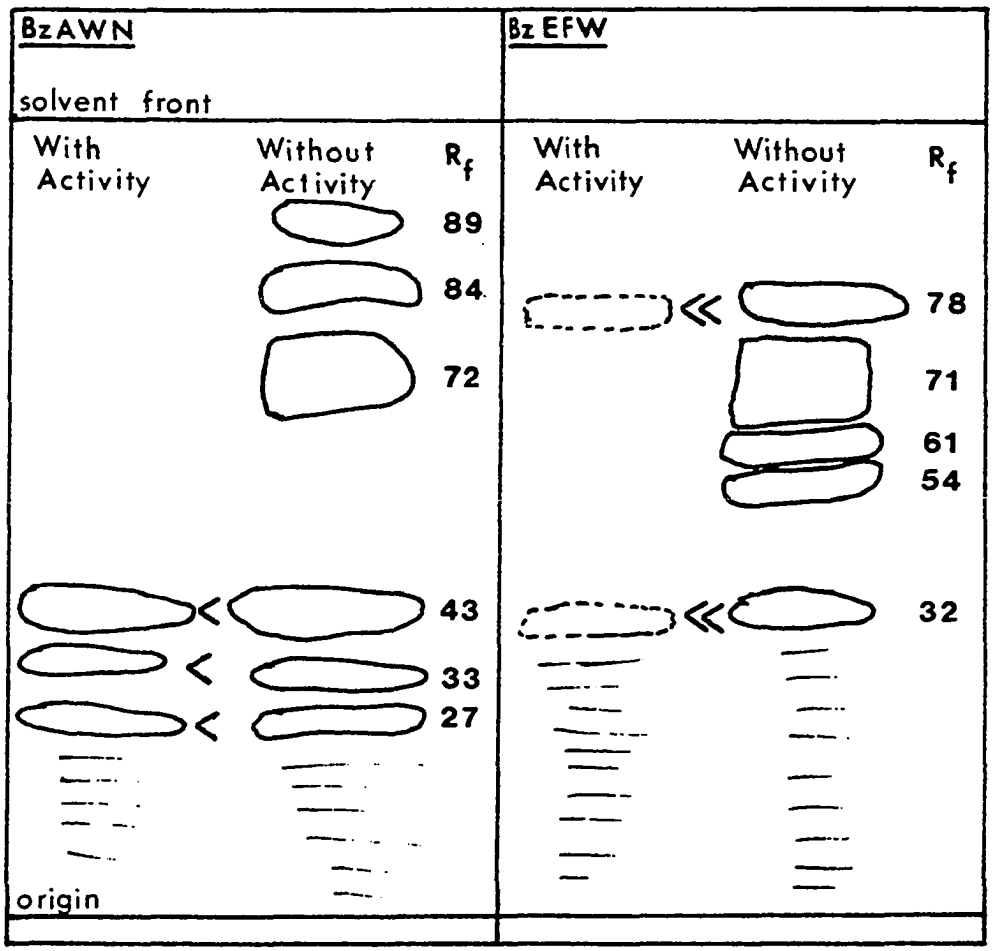


FIGURE 6 THIN LAYER CHROMATOGRAM OF CRUDE ENZYME EXTRACTS.
BzAWN - benzene, acetic acid, water and nitromethane 34:32:5:18.
BzEFW - benzene, ethyl acetate, formic acid and water 9:21:6:3

relative percentages of the 4 isoenzymes of glucose-6-phosphate dehydrogenase. The cells were aseptically transferred as usual and harvested. The weight of cells, volume of medium and pH of the medium were recorded. The cells followed an exponential growth curve with the volume of the culture medium decreasing proportionally (figs 7 and 8). The pH of the culture medium increased from an initial pH of 4.3 at the time of transfer to a maximum pH of 6.15 at day 8 followed by a slight drop in pH for 9 and 10 day old medium (fig 9). Concurrently, relative amounts of the isoenzymes of glucose-6-phosphate dehydrogenase were examined (Table 3).

TABLE 3

RELATIVE INTENSITY OF ISOENZYMES WITH RESPECT TO AGE

	Age of tobacco cells								
BAND	2	3	4	5	6	7	8	9	10
I	5	5	5	4	4	3	3	1	0
II	5	4	5	3	4	3	3	2	1
III	2	2	2	3	4	3	4	4	5
IV	2	2	3	3	4	4	5	4	5

0-5 represent the relative intensities of the various isoenzymes as observed on anodic polyacrylamide gel electrophoresis. 5 is the most intense band with 0 meaning that a band was not visible.

It was observed that with young tobacco cells, Band I and Band II were the most prominent bands. As the cells aged, Bands III and IV became the most prominent bands. This feature was used in later isolations of Bands I and IV. When Band I was the object of investigation, younger cells were harvested. The weight of cells per flask was less than after

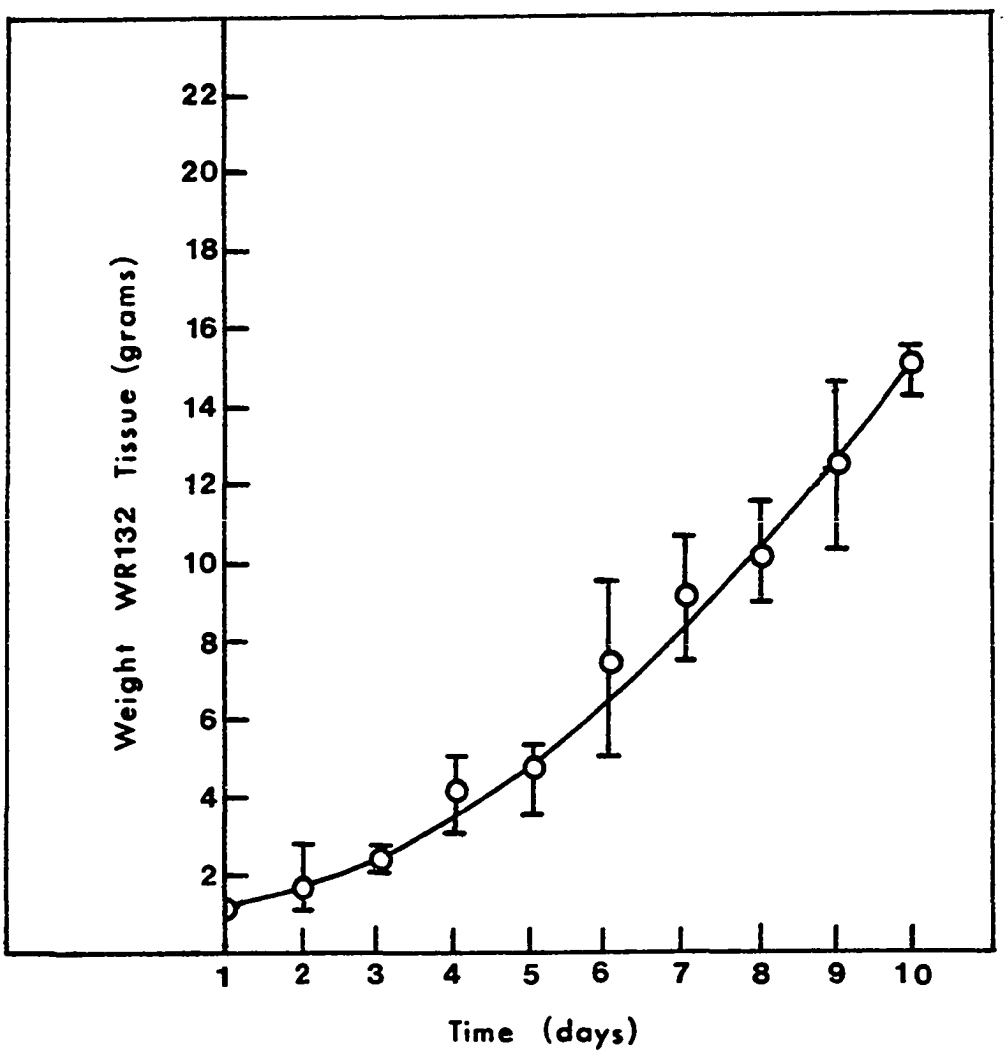


FIGURE 7 GROWTH CURVE OF WR-132 TOBACCO SUSPENSION CULTURE. The weight of cells is a wet weight. The deviations in weight are shown.

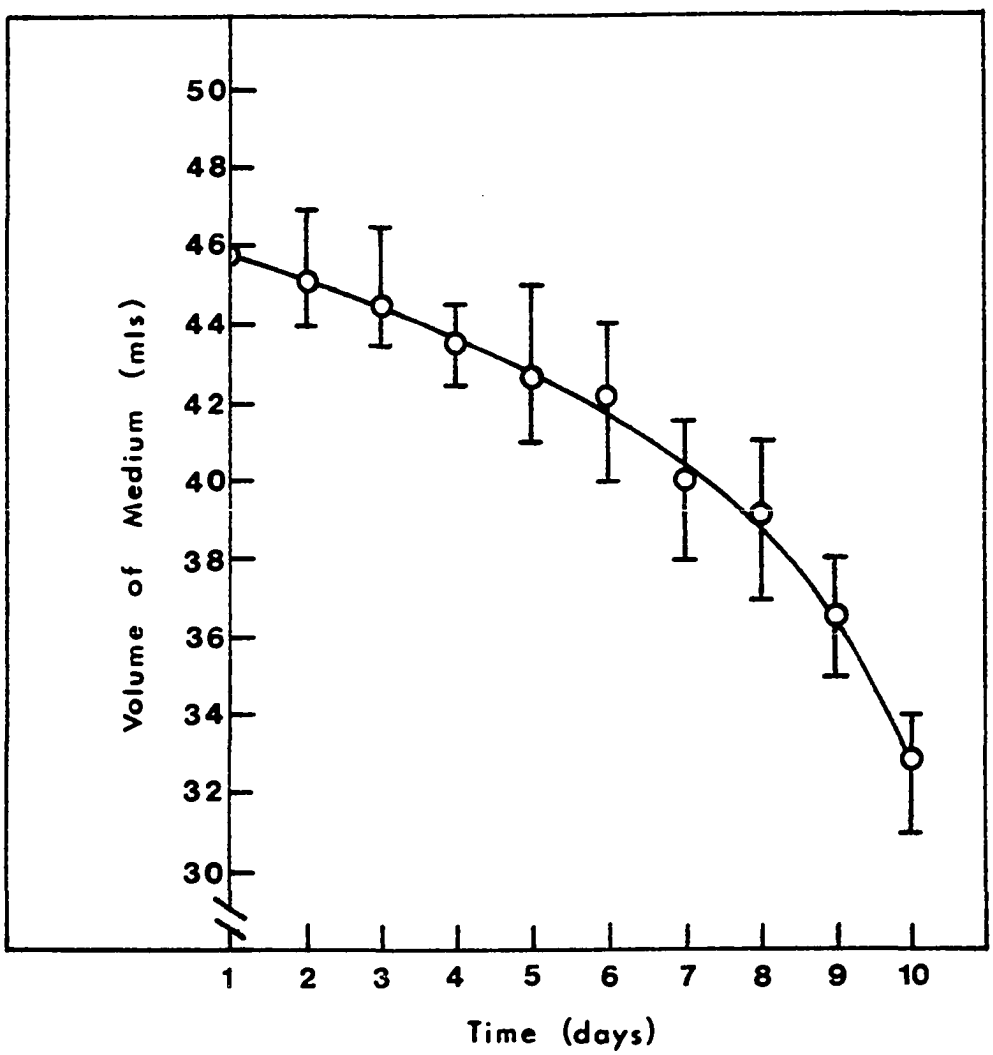


FIGURE 8 CHANGE IN THE VOLUME OF WR-132 CULTURE MEDIUM WITH RESPECT TO AGE OF THE CELLS. Deviations from the average are shown.

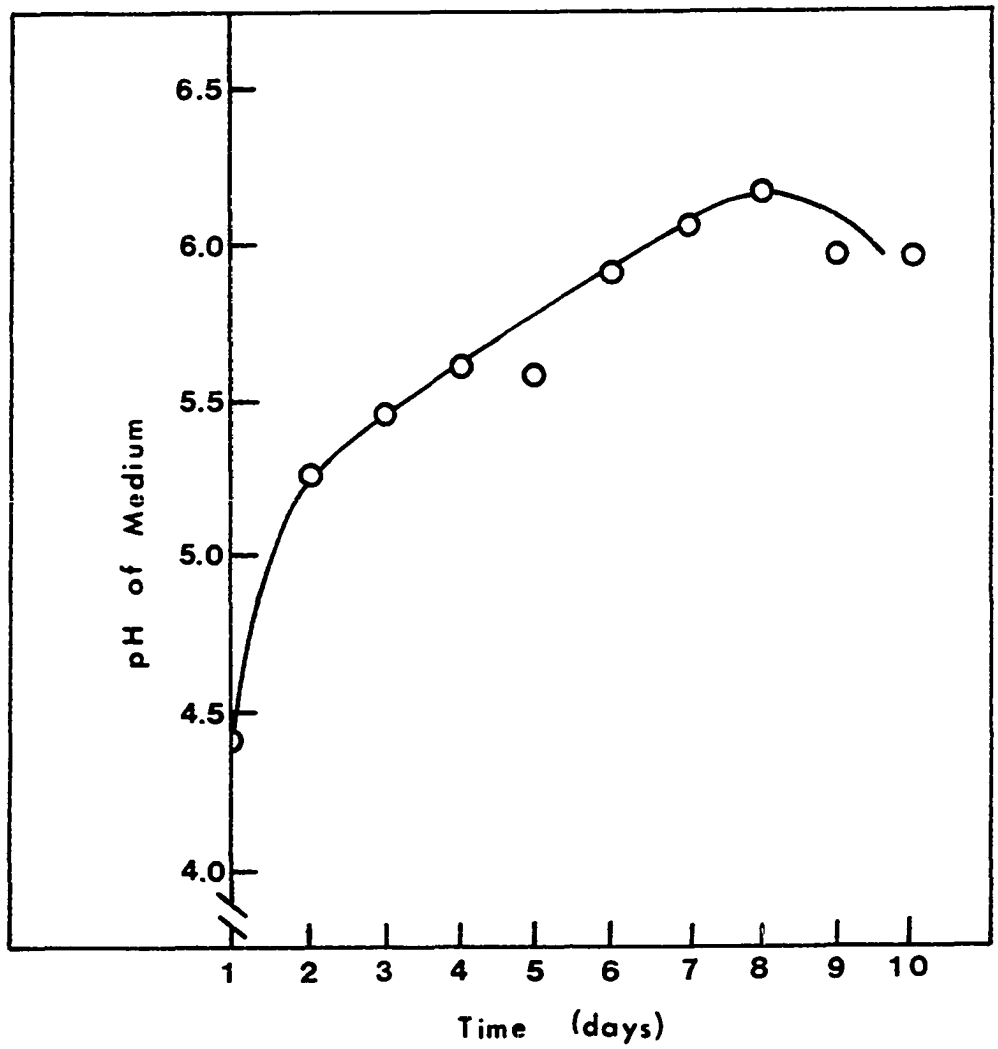


FIGURE 9 CHANGE IN THE pH OF WR-132 CULTURE MEDIUM WITH RESPECT TO AGE OF THE CELLS.

10 days of growth, but this was compensated for by increasing the number of flasks transferred. Alternately, when Band IV was being studied, much older cells were harvested. In this manner, the relative concentration of Band I was decreased with a much higher percentage of Band IV present.

CHAPTER IV

KINETIC AND PHYSICAL PROPERTIES OF ISOENZYMES BAND I AND BAND IV

Initial kinetic studies of glucose-6-phosphate dehydrogenase involved enzyme preparations containing all 4 isoenzymes. A plot of reaction velocity versus pH revealed a broad activity peak with a maximum between a pH of 7.5 and 8.5 (fig 10). All the buffers used for the plot were adjusted with NaCl to the same ionic strength (300 mM) to alleviate any problems that might arise because of variances in activity due to ionic strength differences. The activity peak, instead of being a sharp peak usually associated with a single enzyme, is a broad activity peak. With isoenzymes present, each exhibiting it's own pH optimum, a curve could be produced exhibiting the combination of pH optimums.

A plot of the reaction velocity versus glucose-6-phosphate concentration is shown in fig 11. The curve is not typical of an enzyme exhibiting Michaelis-Menten kinetics because of the intermediary plateau region. However, as with the pH profile, a mixture of isoenzymes is present. The saturation curve that could be generated from a mixture of enzymes could exhibit an intermediary plateau due to the four isoenzymes, each with its own Michaelis constant. A Lineweaver-Burk plot (44) of the data (fig 12) reveals a break in the line giving rise to 2 different Michaelis constants: 0.38 mM for low glucose-6-phosphate and 0.90 mM

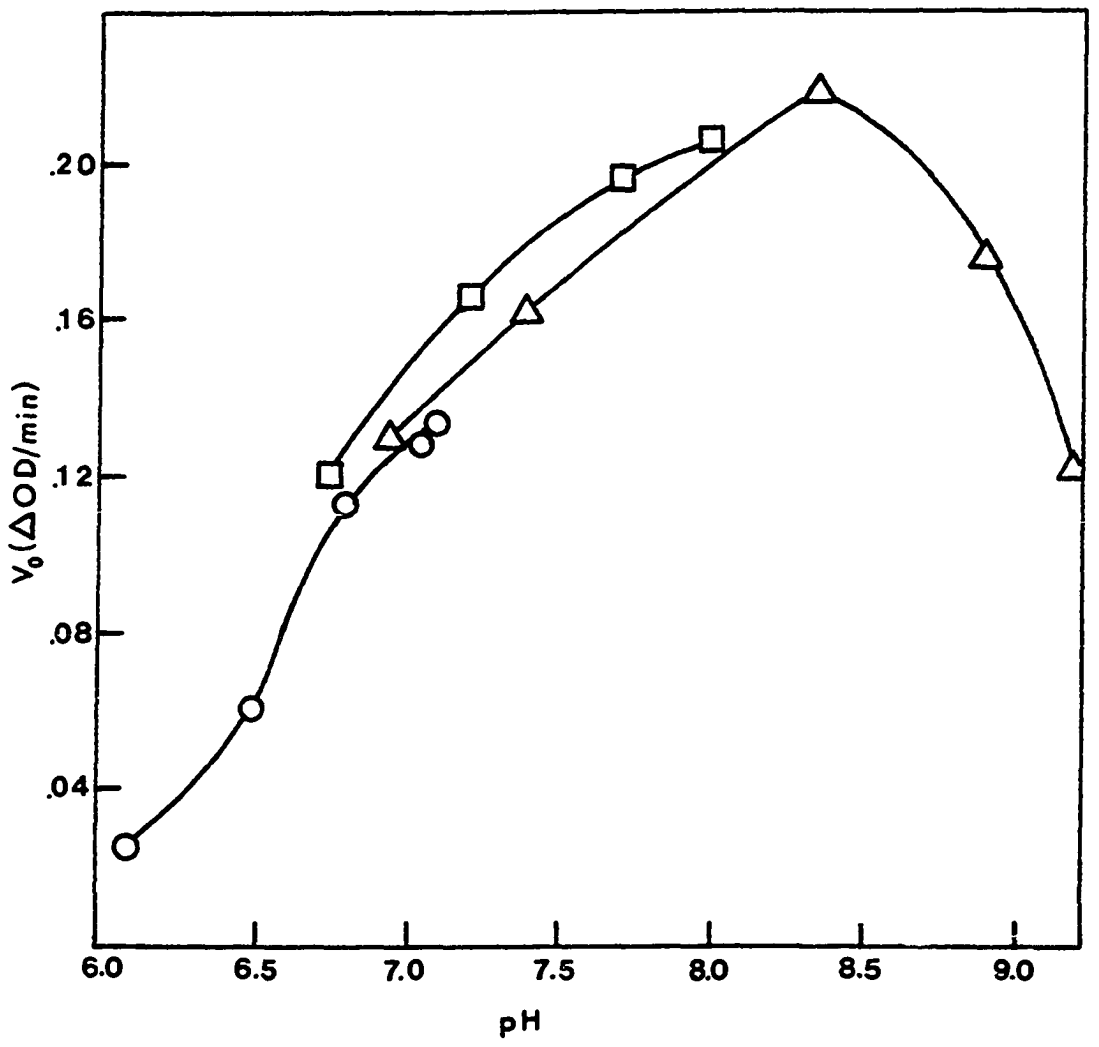


FIGURE 10 pH PROFILE FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME MIXTURE. The assays contained 5 mM G-6-P, 5 mM Mg^{+2} and 0.5 mM $NADP^{+}$ in the following buffers: O—O phosphate, □—□ tris-HCl and Δ—Δ glycine-NaOH.

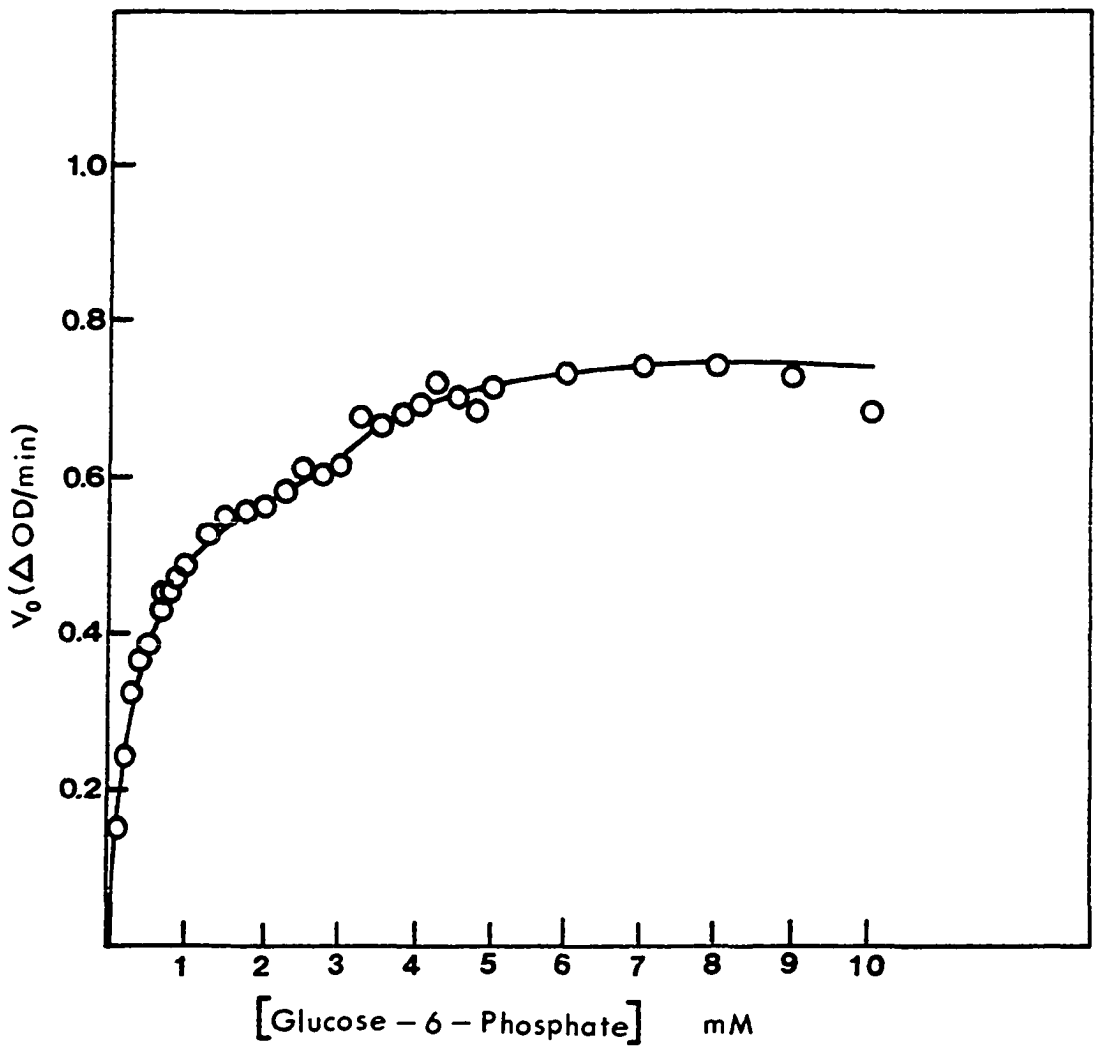


FIGURE 11 GLUCOSE-6-PHOSPHATE SATURATION CURVE FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES. Each assay consisted of 5 mM Mg^{+2}, and 0.5 mM $NADP^{+}$ in 100 mM tris-HCl buffer (pH 8.0). G-6-P was varied for the curve.

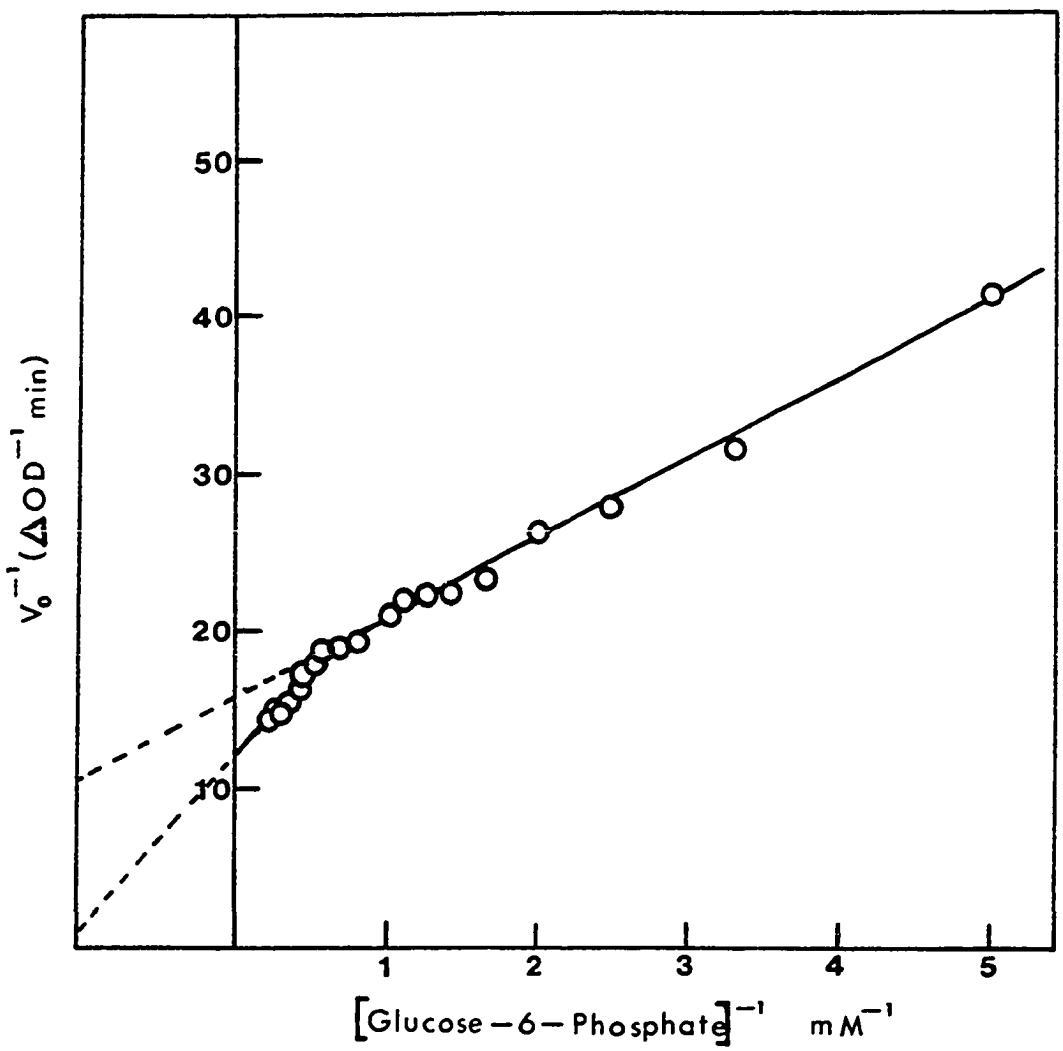


FIGURE 12 GLUCOSE-6-PHOSPHATE DOUBLE RECIPROCAL PLOT FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES.

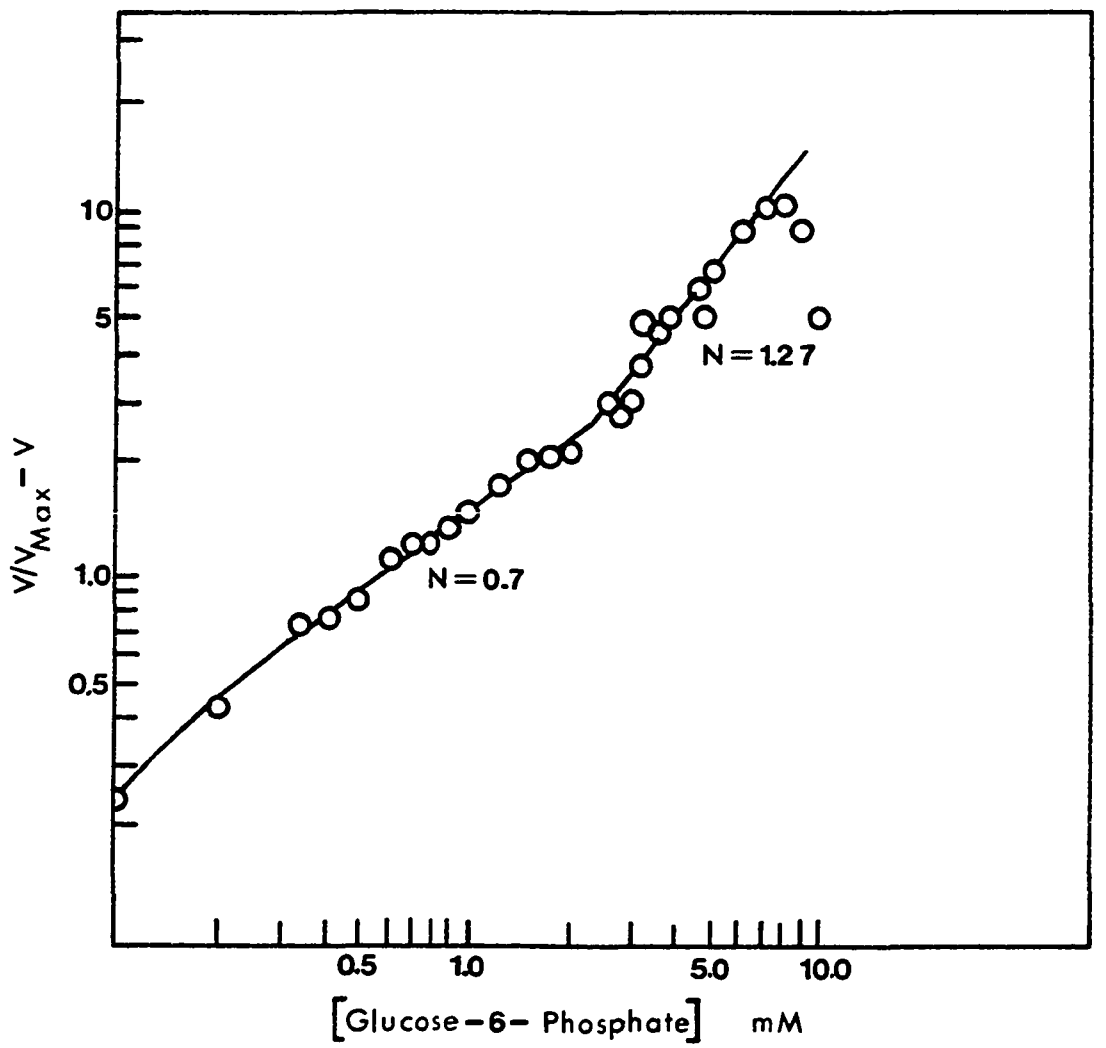


FIGURE 13 GLUCOSE-6-PHOSPHATE HILL PLOT FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES.

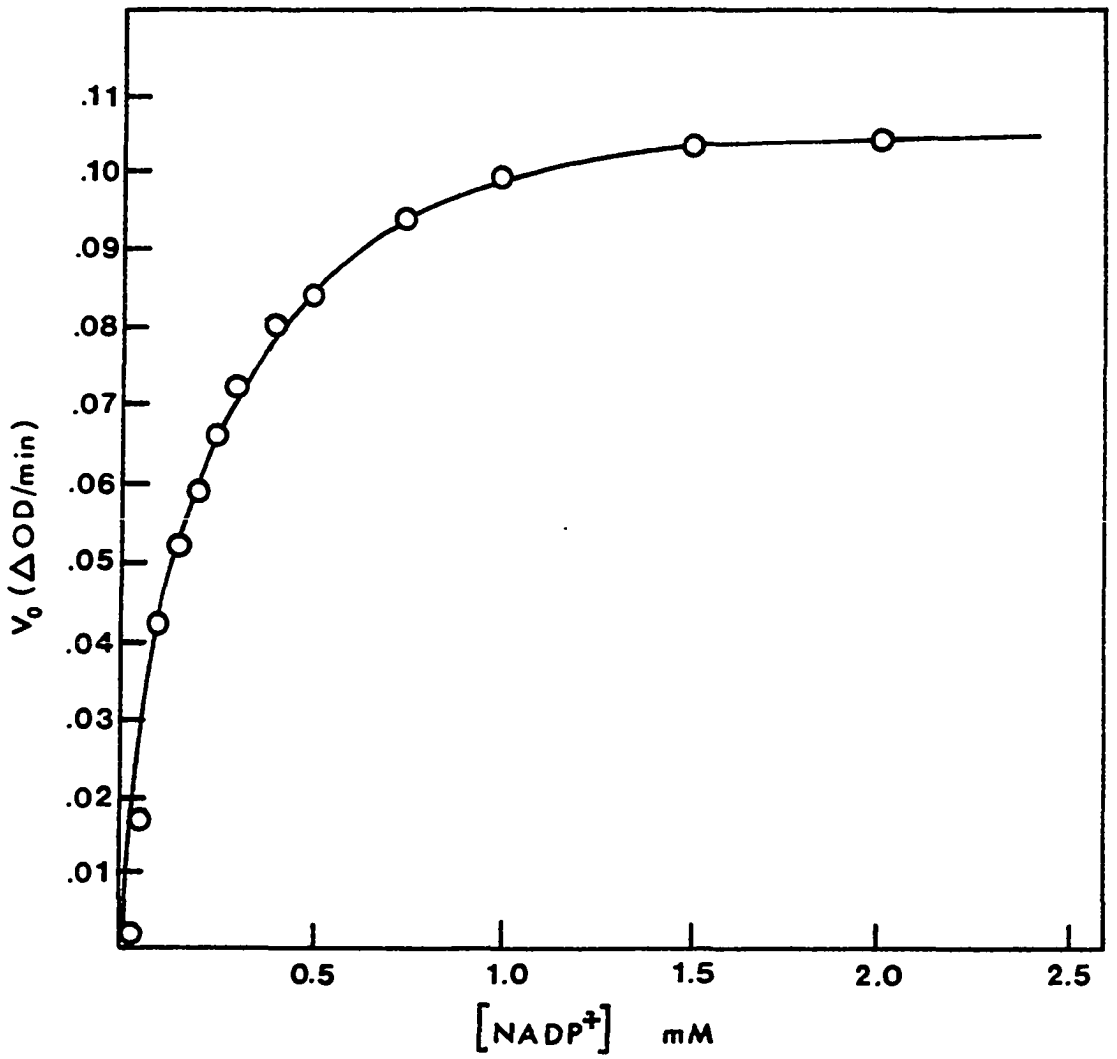


FIGURE 14 $NADP^+$ SATURATION CURVE FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES. Each assay consisted of 5 mM G-6-P and 5 mM Mg^{+2} in 100 mM tris-HCl buffer (pH 8.0). $NADP^+$ was varied for the curve.

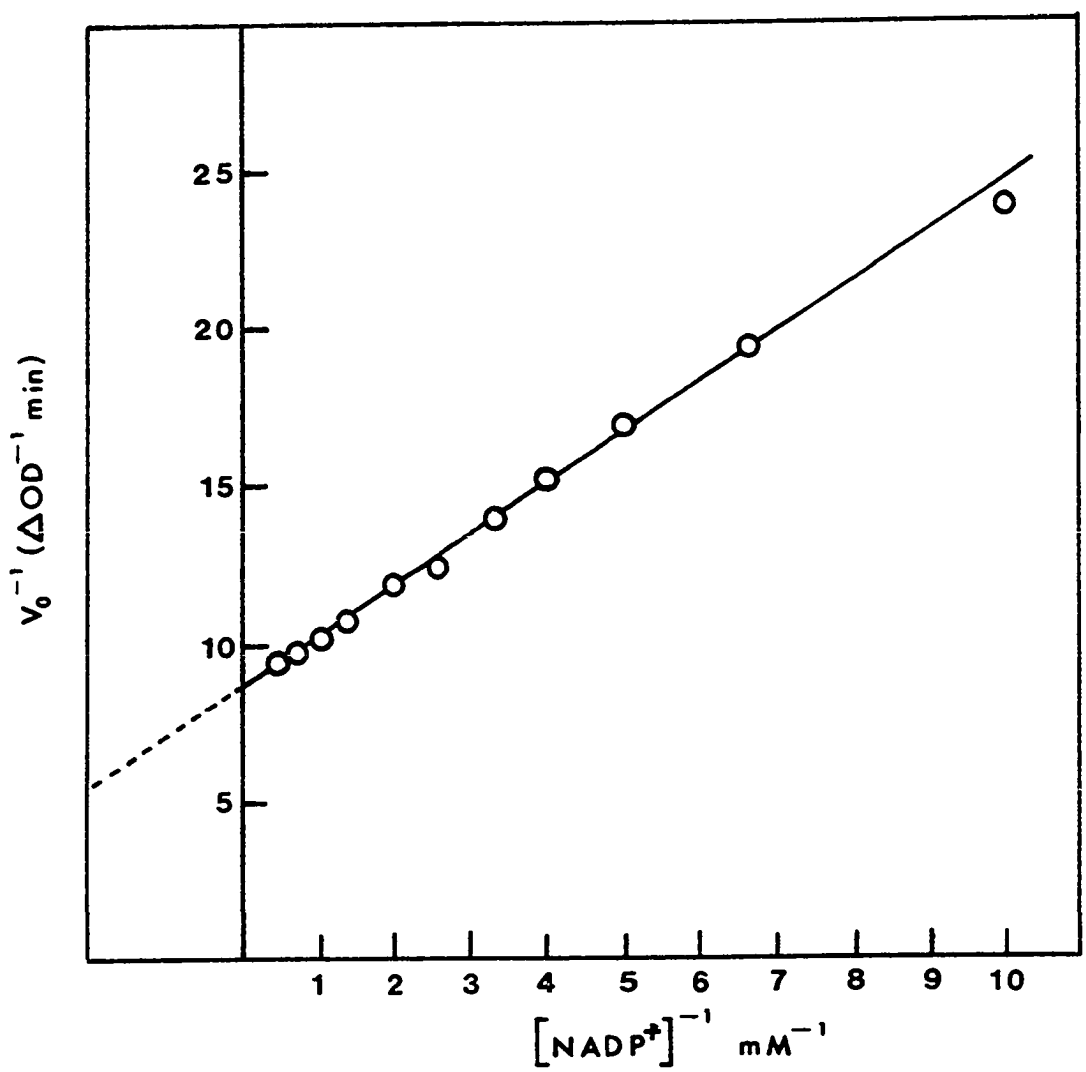


FIGURE 15 $NADP^+$ DOUBLE RECIPROCAL PLOT FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES.

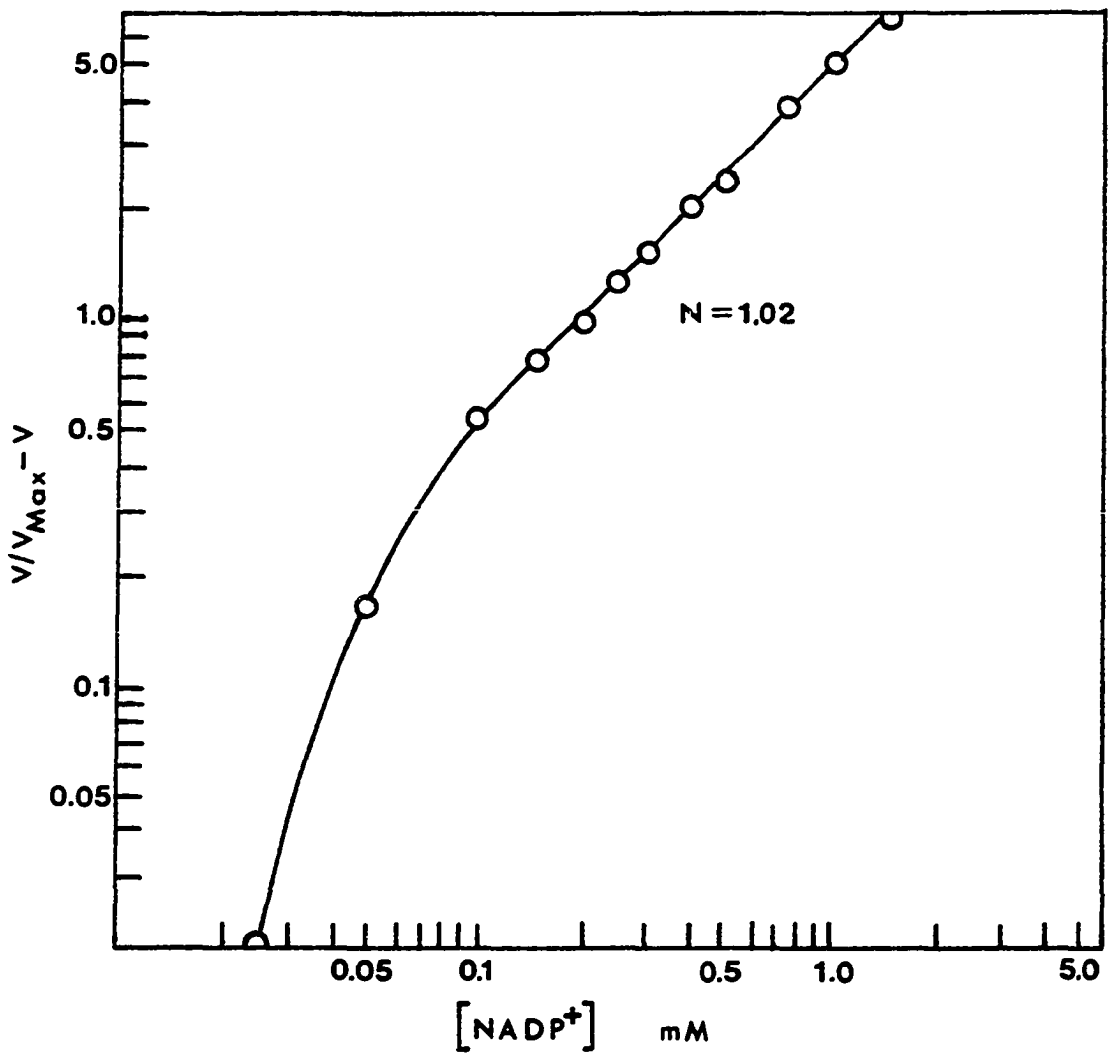


FIGURE 16 $NADP^+$ HILL PLOT FOR A MIXTURE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES.

for high glucose-6-phosphate concentrations. The corresponding Hill plot (fig 13) (46) has a change in slope, changing from a slope of 0.7 for low substrate concentrations to a slope of 1.27 for high substrate concentrations. Fig 14 illustrates the $NADP^+$ saturation curve for a mixture of isoenzymes. A double reciprocal plot (fig 15) is linear with an $S_{0.5}$ value of 0.19 mM. The Hill plot (fig 16) has a slope of 1.02 denoting one $NADP^+$ binding site per enzyme molecule. Kajinami *et al.* (38) reported Michaelis constants, K_M, for glucose-6-phosphate and $NADP^+$ as 0.30 mM and 0.045 mM respectively. The same source material was used for the isolation of glucose-6-phosphate dehydrogenase. An explanation for the discrepancy might be the relative concentrations of the 4 isoenzymes. The age of the tobacco cells affects the isoenzyme concentration. Kajinami's preparation could have contained a higher concentration of one isoenzyme, giving different K_M values.

Band I was separated from the other glucose-6-phosphate dehydrogenase isoenzymes by DEAE-cellulose chromatography. The pH profile of this isoenzyme yields a sharp peak with a maximum activity at a pH of 9.0 (fig 17). A saturation curve with respect to glucose-6-phosphate as a substrate is shown in fig 18 with the corresponding double reciprocal plot shown in fig 19. Band I exhibits Michaelis-Menten kinetics as denoted by the smooth saturation curve and the linear Lineweaver-Burk plot. The $S_{0.5}$ value obtained from the double reciprocal plot is 0.22 mM. A Hill coefficient of 1.01 calculated from the Hill plot (fig 20) indicates that there is one glucose-6-phosphate binding site per enzyme molecule.

$NADP^+$ also gives a smooth Michaelis-Menten type of saturation curve (fig 21). The calculated $S_{0.5}$ value from the double reciprocal plot (fig 22) is 0.06 mM. The Hill plot (fig 23) is linear with a slope

of 1.04.

The molecular weight of Band I was determined by 2 different methods. The first method was that of Andrews (4) utilizing a Sephadex G-150 column. The column was first standardized using γ-globulin (MW 150,000), lipoxidase (MW 108,000), creatine kinase (MW 80,000), serum albumin (MW 68,000), ovalbumin (MW 43,000) and α-chymotrypsinogen-A (MW 25,700). The standards were dissolved in 2 ml of elution buffer to which a few crystals of sucrose had been added to increase the density. The entire 2 ml was applied to the top of the column bed using a Pro pipettor. After the column was standardized, 2 ml of Band I enzyme preparation from a DEAE-cellulose column was applied to the column in the same manner. Glucose-6-phosphate dehydrogenase activity was checked to determine the elution volume of Band I. The molecular weight obtained from a semi-logarithmic plot of molecular weight versus elution volume (fig 24) was 91,000.

The fractions from the Sephadex G-150 column containing Band I were saved for molecular weight determinations utilizing the SDS electrophoresis procedure of Weber, Pringle and Osborn (69). Three or four samples were obtained, combined and concentrated by lyophilization. After concentration, the sample was dialyzed extensively against 3000 volumes of deionized distilled water. The water was changed several times. The sample was lyophilized again and the lyophilized powder was used for the molecular weight determination. The standards used for SDS gel electrophoresis were β-galactosidase (subunit MW 130,000), lipoxidase (MW 108,000), serum albumin (MW 68,000), catalase (MW 58,000), ovalbumin (MW 43,000) and α-chymotrypsinogen-A (MW 25,700). A molecular weight of 85,000 ± 4000 was obtained from a semi-logarithmic plot of molecular weight versus mobility (fig 25).

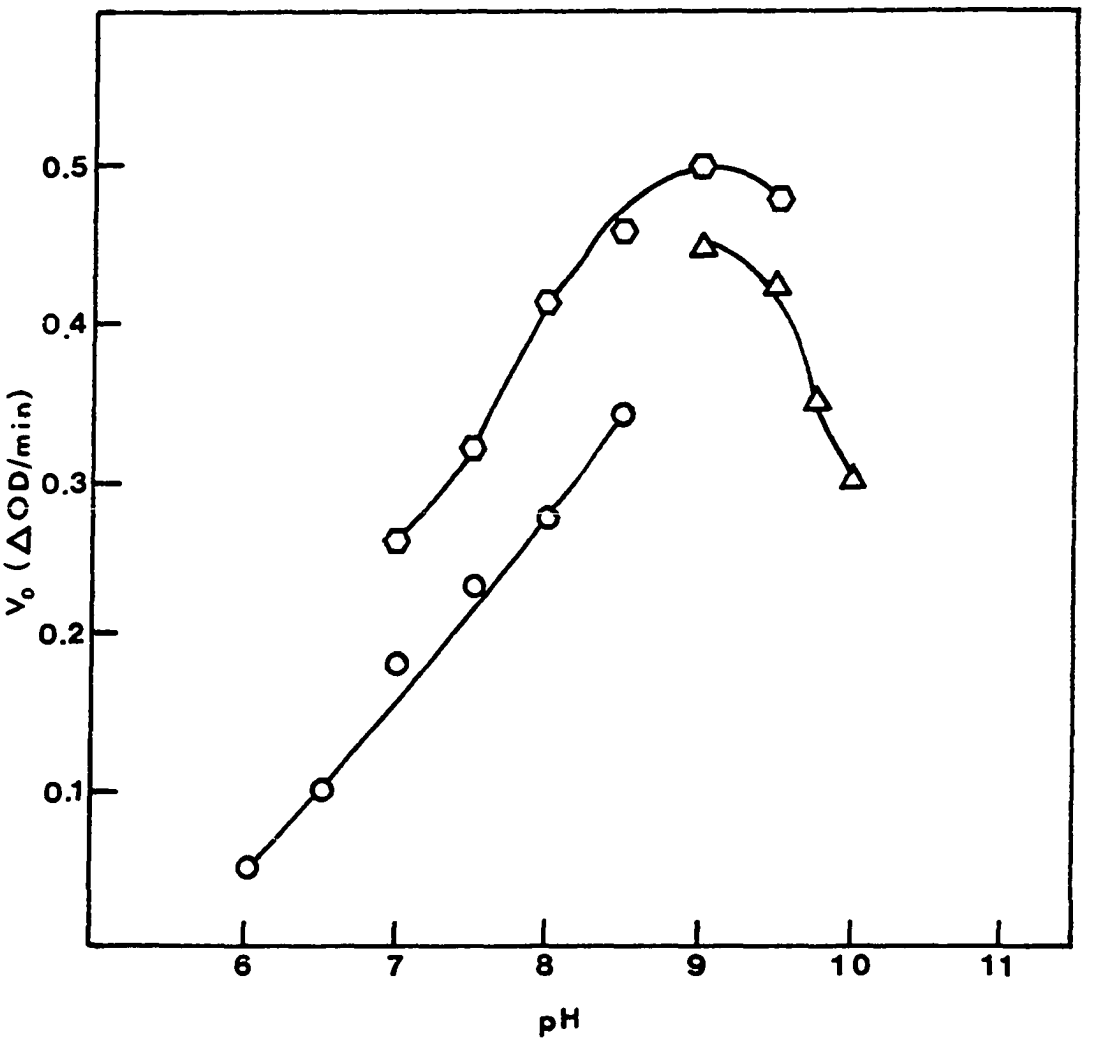


FIGURE 17 pH PROFILE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I. Assays contained 5 mM G-6-P, 5 mM Mg^{+2}, 0.5 mM $NADP^{+}$ and enzyme in the following buffers: O——O phosphate, ⬡——⬡ tris-HCl and △——△ glycine-NaOH.

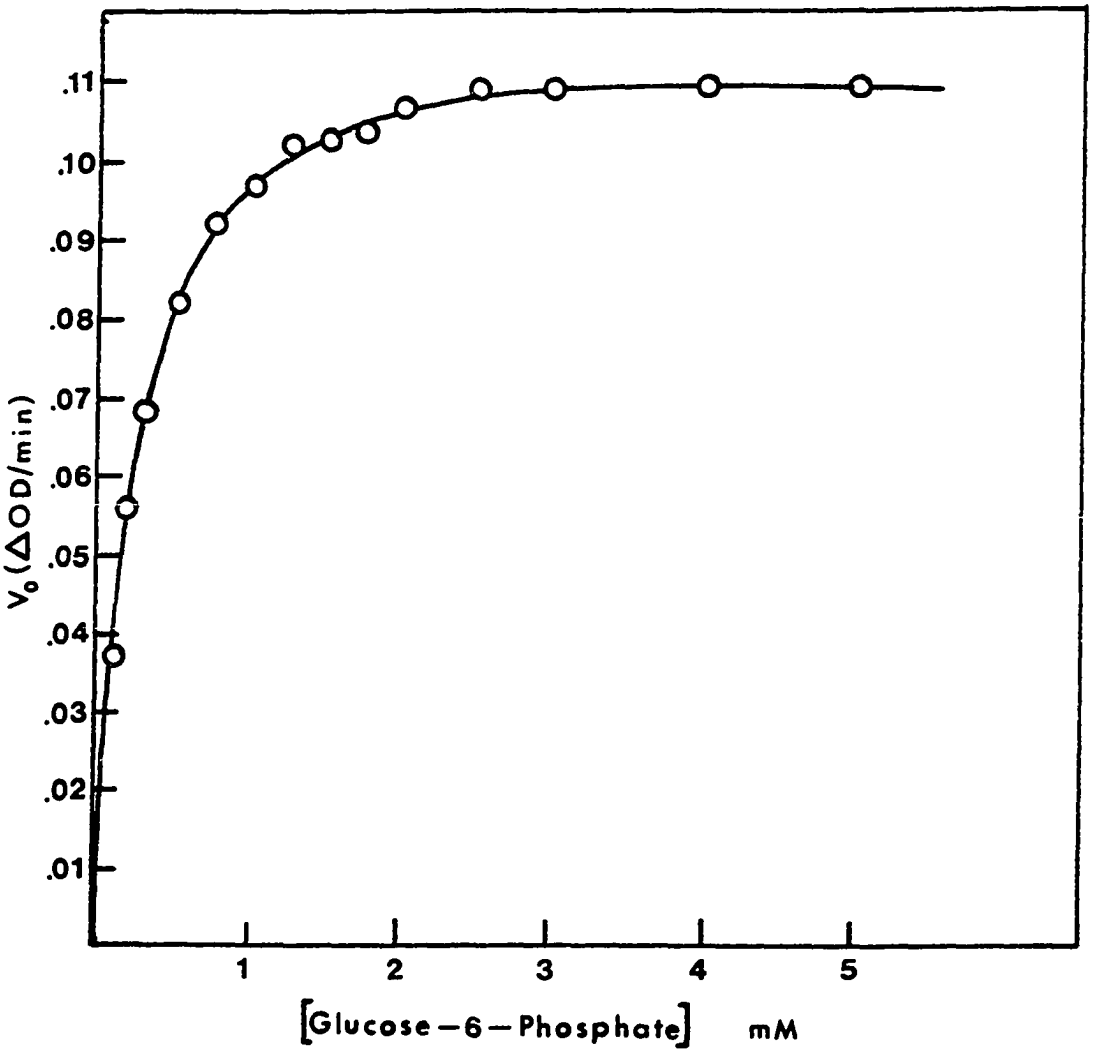


FIGURE 18 GLUCOSE-6-PHOSPHATE SATURATION CURVE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I. The assays contained 5 mM Mg^{+2} and 0.5 mM $NADP^{+}$ in 100 mM tris-HCl buffer (pH 8.0). G-6-P was varied.

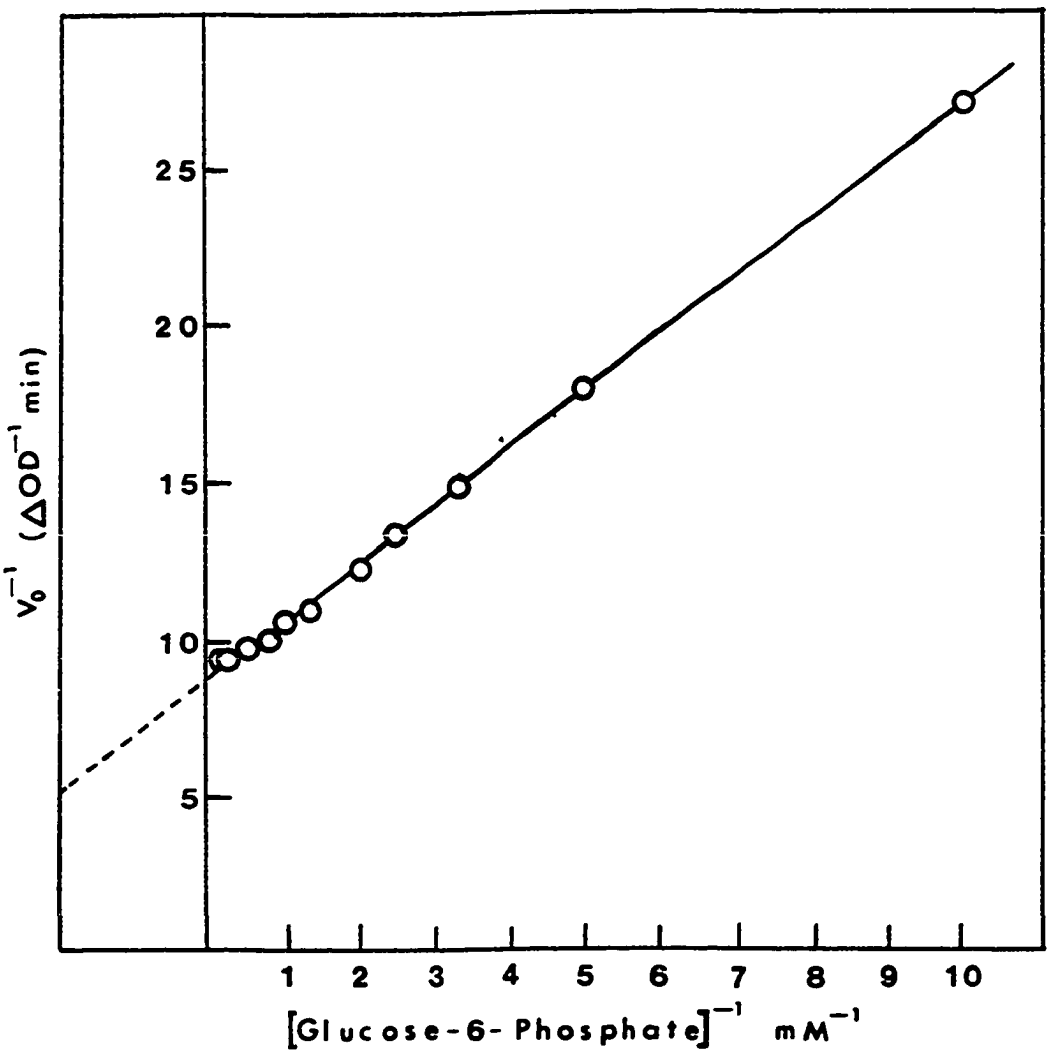


FIGURE 19 GLUCOSE-6-PHOSPHATE DOUBLE RECIPROCAL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I.

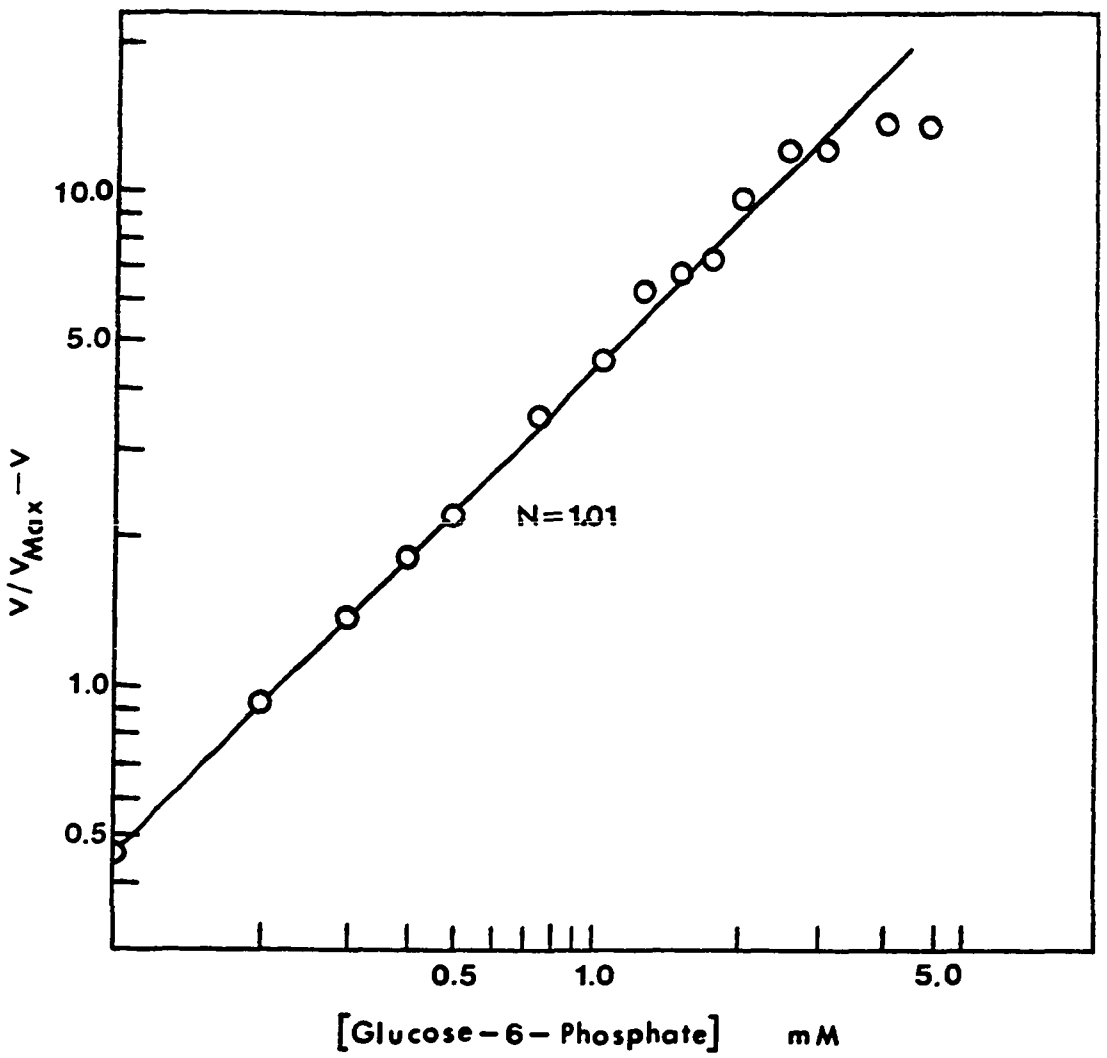


FIGURE 20 GLUCOSE-6-PHOSPHATE HILL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I.

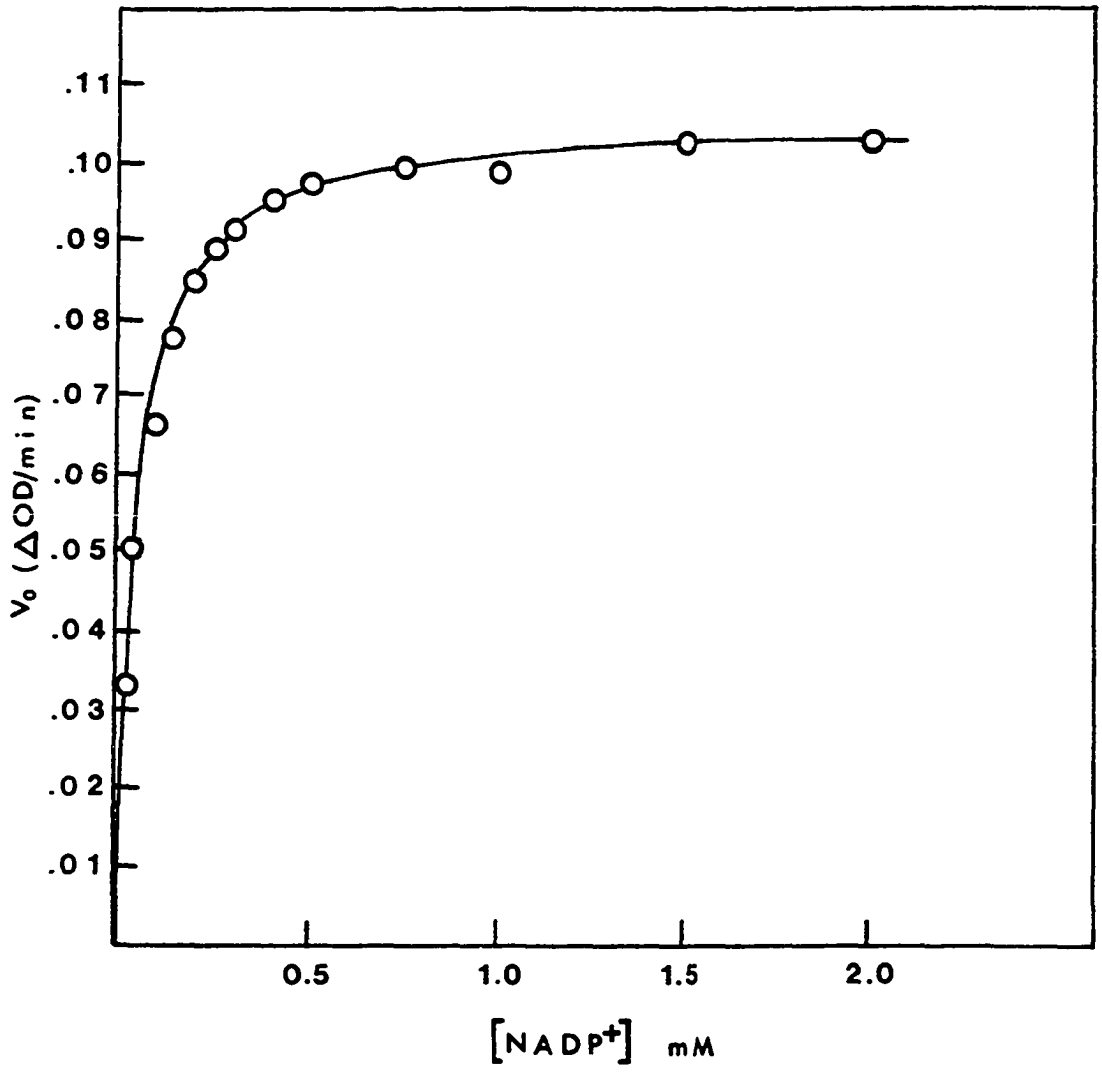


FIGURE 21 $NADP^+$ SATURATION CURVE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I. The assays contained 5 mM G-6-P and 5 mM Mg^{+2} in 100 mM tris-HCl buffer (pH 8.0). $NADP^+$ was varied.

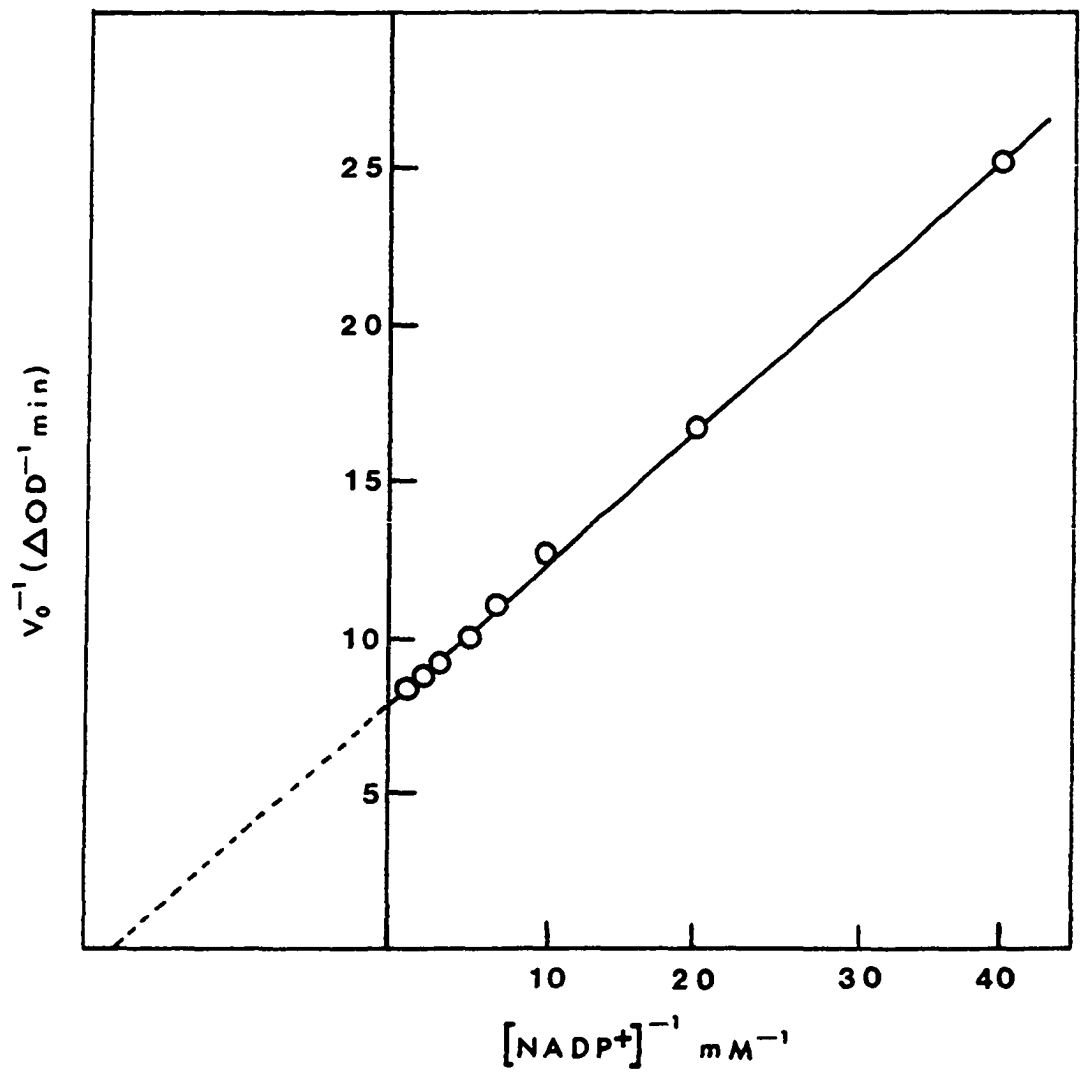


FIGURE 22 $NADP^+$ DOUBLE RECIPROCAL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I.

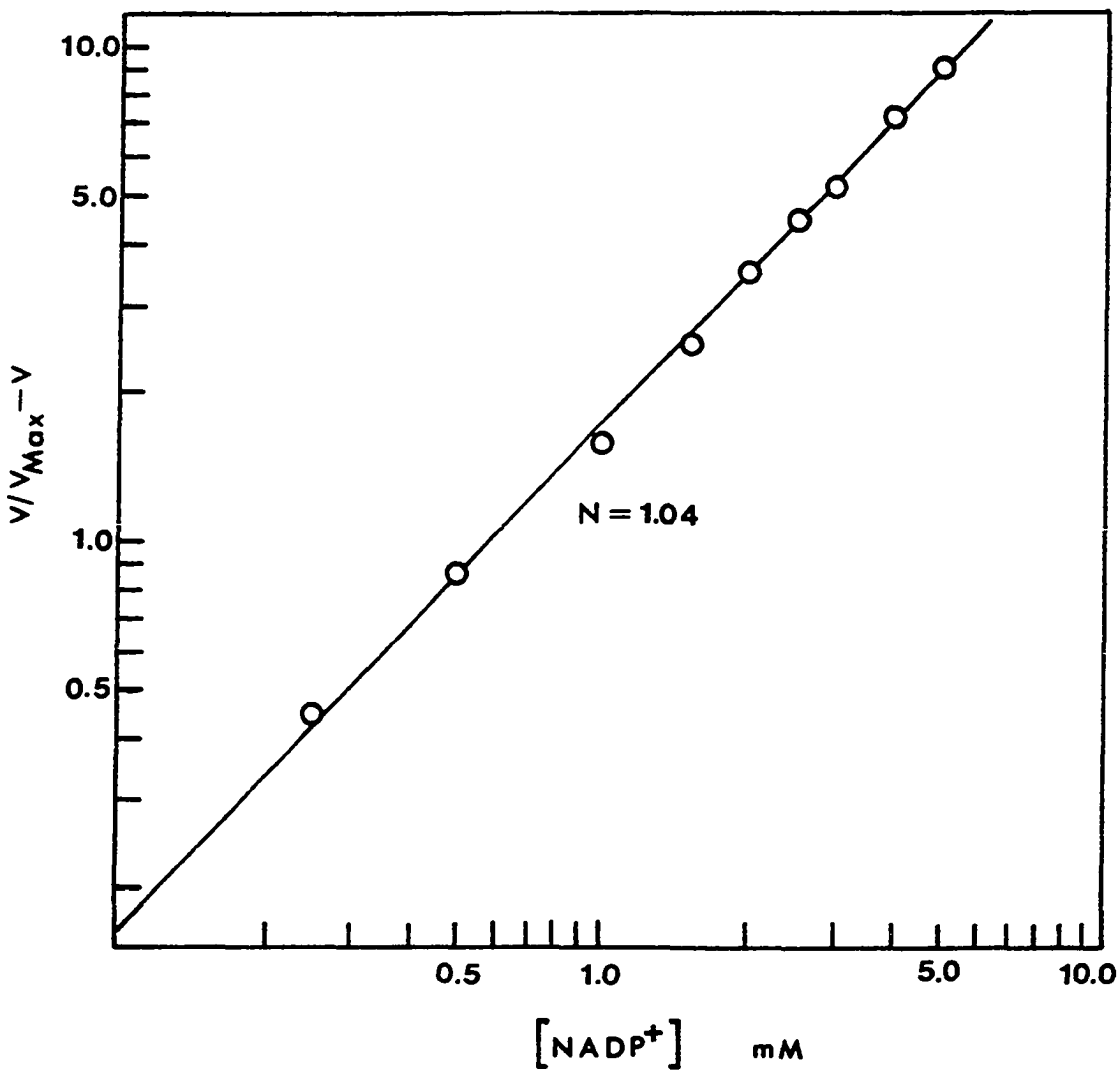


FIGURE 23 $NADP^+$ HILL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I.

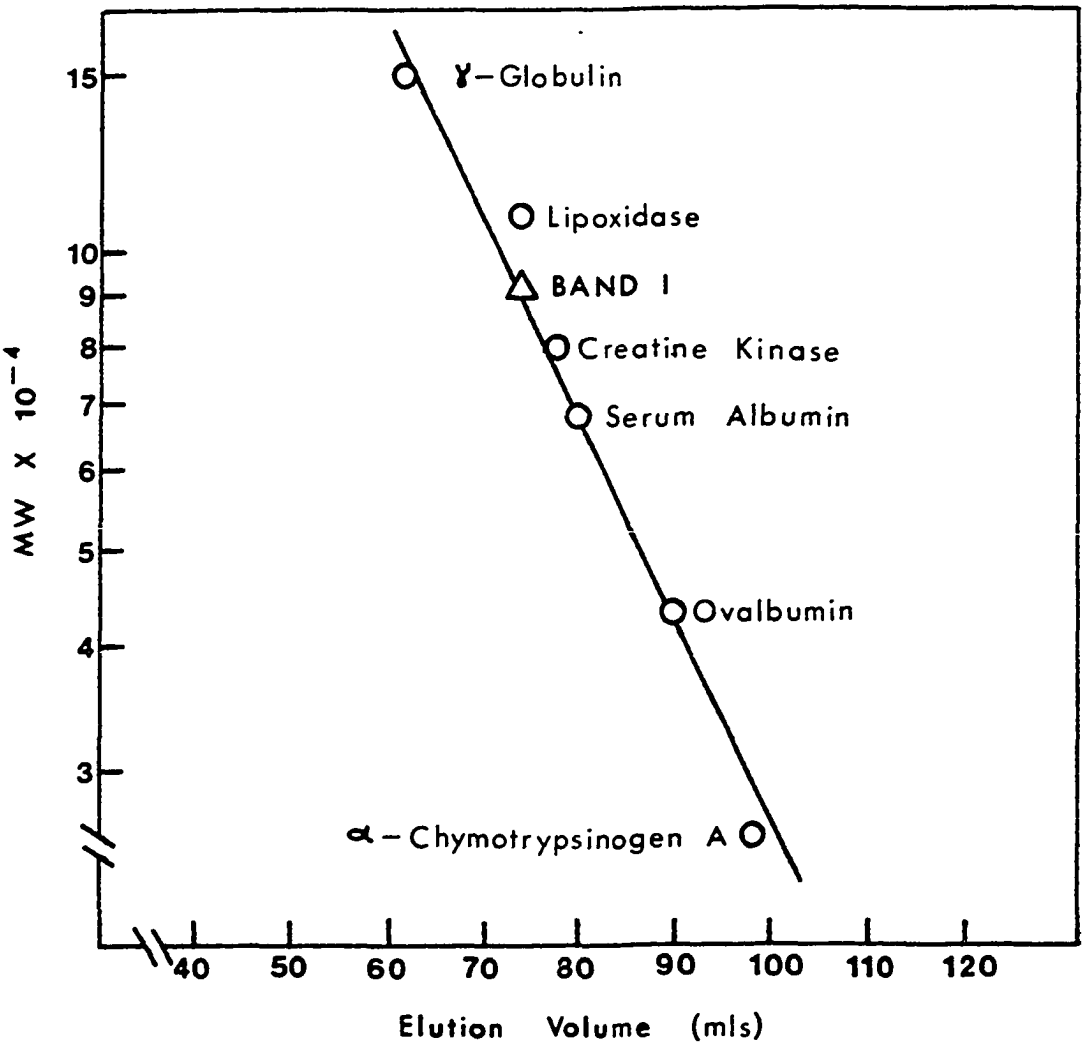


FIGURE 24 DETERMINATION OF THE MOLECULAR WEIGHT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-150. The elution buffer was 100 mM KCl and 50 mM tris-HCl (pH 7.5).

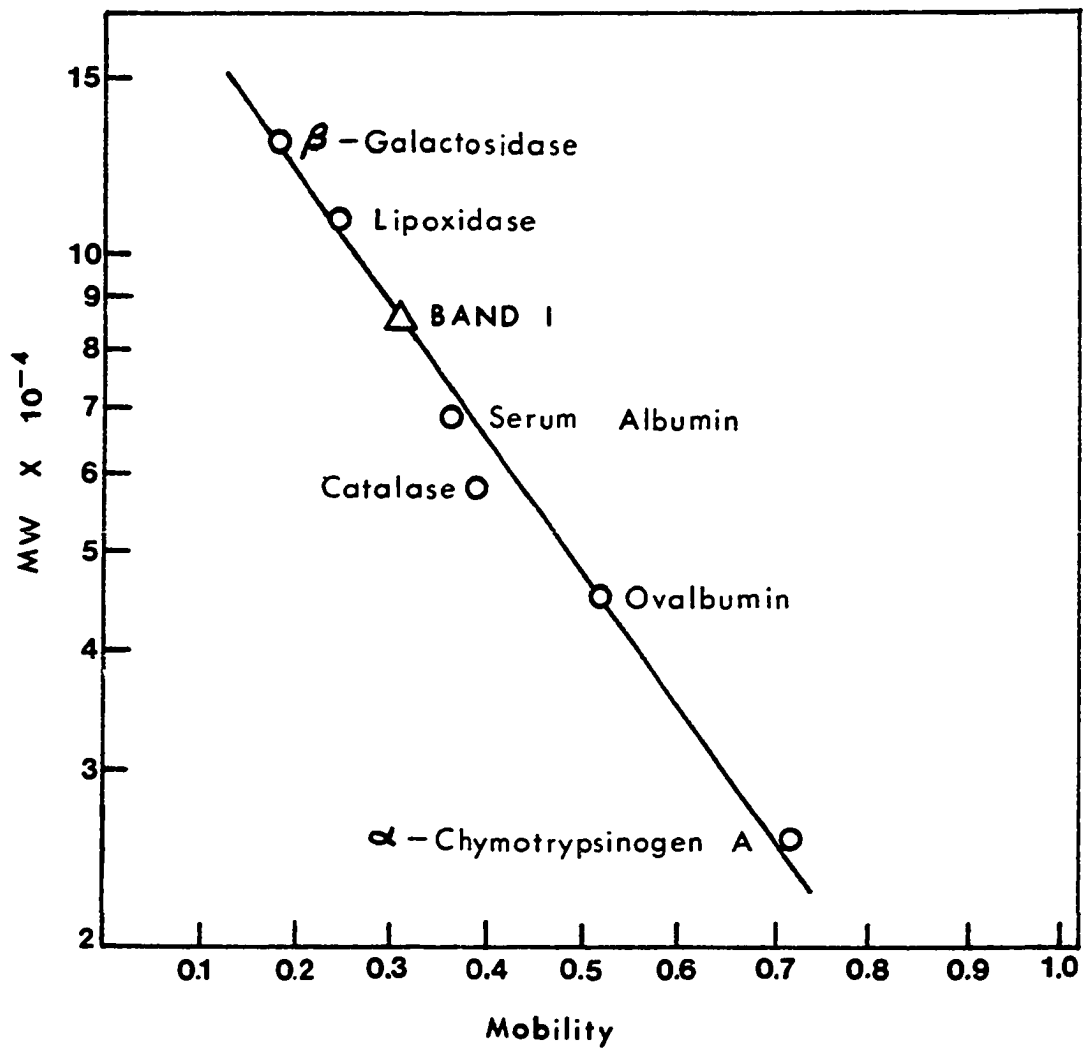


FIGURE 25 DETERMINATION OF THE MOLECULAR WEIGHT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND I BY ELECTROPHORESIS ON SDS POLYACRYLAMIDE GELS.

Band IV as obtained from a DEAE-cellulose column was usually free from other detectable contaminating proteins as determined by polyacrylamide gel electrophoresis. The effect of pH on enzymatic activity of Band IV is shown in fig 26. The pH optimum appears to be 8.0-8.3. The glucose-6-phosphate saturation curve is shown in fig 27 with the corresponding Lineweaver-Burk plot in fig 28. The curves are that of a Michaelis-Menten enzyme with an $S_{0.5}$ value of 0.31 mM. A Hill plot (fig 29) has a slope of 0.98 indicating one glucose-6-phosphate binding site.

The $NADP^+$ saturation curve for Band IV is shown in fig 30. When the double reciprocal data are plotted (fig 31), there is an abrupt transition between 2 linear sections. Teipel and Koshland (66) attribute this type of phenomena to a multi-site enzyme in which the relative magnitude of the binding constants of the sites first decrease and then increase as saturating levels of substrate are reached. This transition corresponds to an abrupt increase in the apparent K_M and V_M values with increasing $NADP^+$. The 2 apparent Michaelis constants are 0.06 mM and 0.015 mM. Using the V_M value which corresponds to the $S_{0.5}$ value of 0.06 mM, a Hill plot was constructed (fig 32). Again, there is a change in slope at 0.1 mM $NADP^+$. For $NADP^+$ concentrations greater than 0.1 mM the Hill coefficient is 1.05. With $NADP^+$ concentrations less than 0.1 mM, the line is nonlinear. Regraphing the Hill plot using the small V_M and only $NADP^+$ concentrations less than 0.1 mM, a straight line is generated with a slope of 1.09. This type of phenomena is not uncommon, being reported for glutamate dehydrogenase from pig heart (29), ox liver (13,19,20) and yeast (23), and for glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (11,14) as well as glucose-6-phosphate dehydrogenase from human erythrocytes (55), yeast (3) and sweet potato (47).

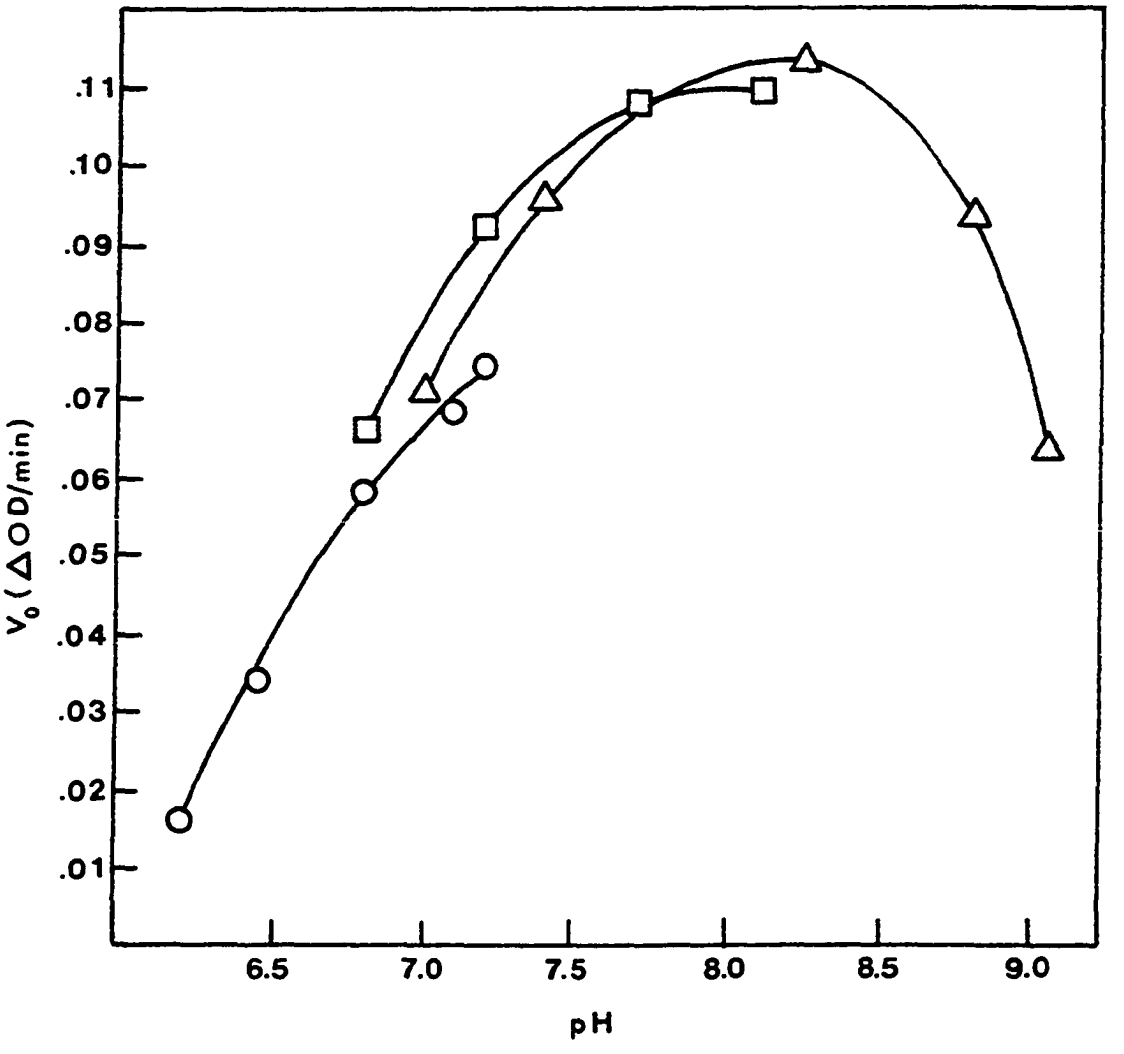


FIGURE 26 pH PROFILE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV. The assays contained 5 mM G-6-P, 5 mM Mg^{+2} and 0.5 mM $NADP^{+}$ in the following buffers: O——O phosphate, □——□ tris-HCl and △——△ glycine-NaOH.

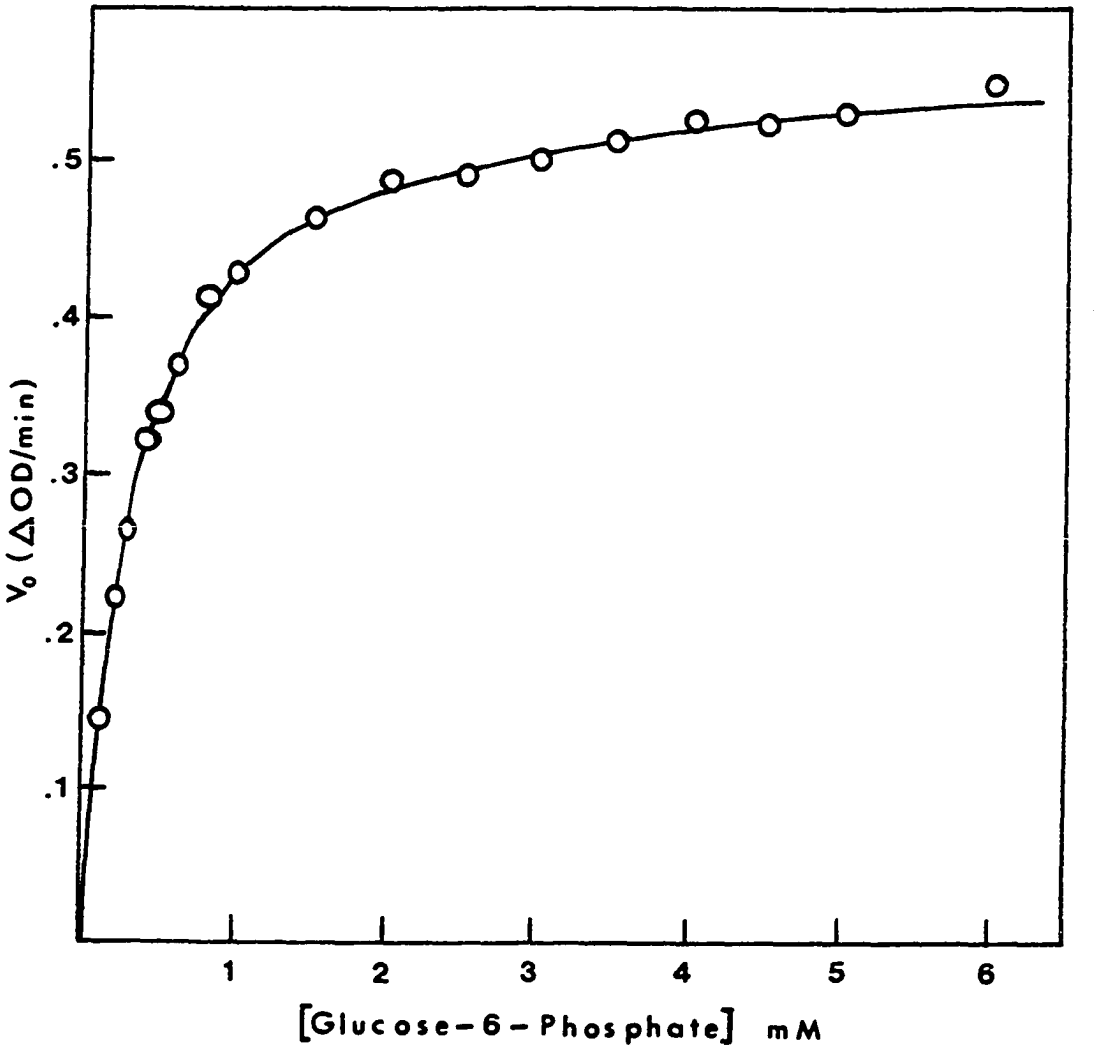


FIGURE 27 GLUCOSE-6-PHOSPHATE SATURATION CURVE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV. The assays contained 5 mM Mg^{+2} and 0.5 mM $NADP^+$ in 100 mM tris-HCl buffer (pH 8.0). G-6-P was varied.

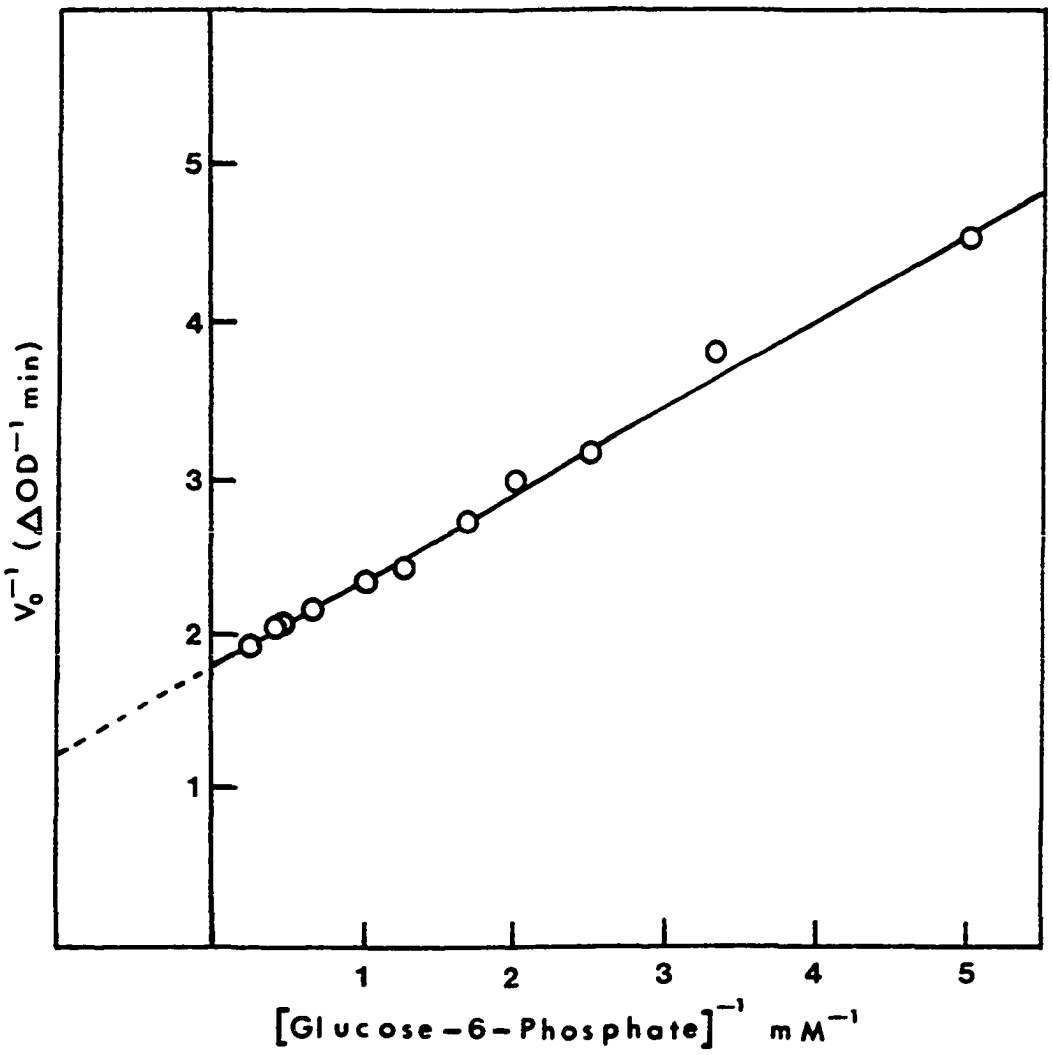


FIGURE 28 GLUCOSE-6-PHOSPHATE DOUBLE RECIPROCAL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV.

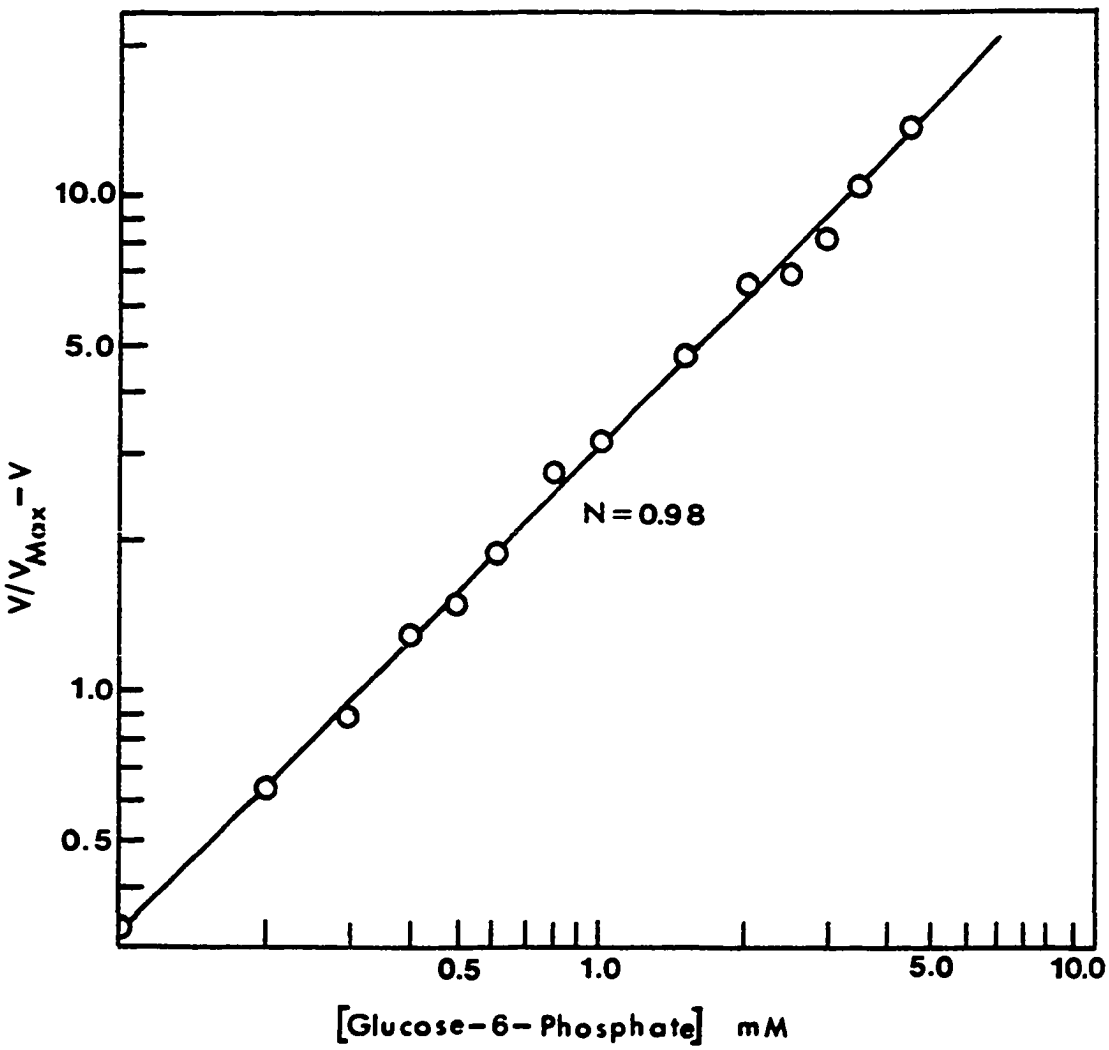


FIGURE 29 GLUCOSE-6-PHOSPHATE HILL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV.

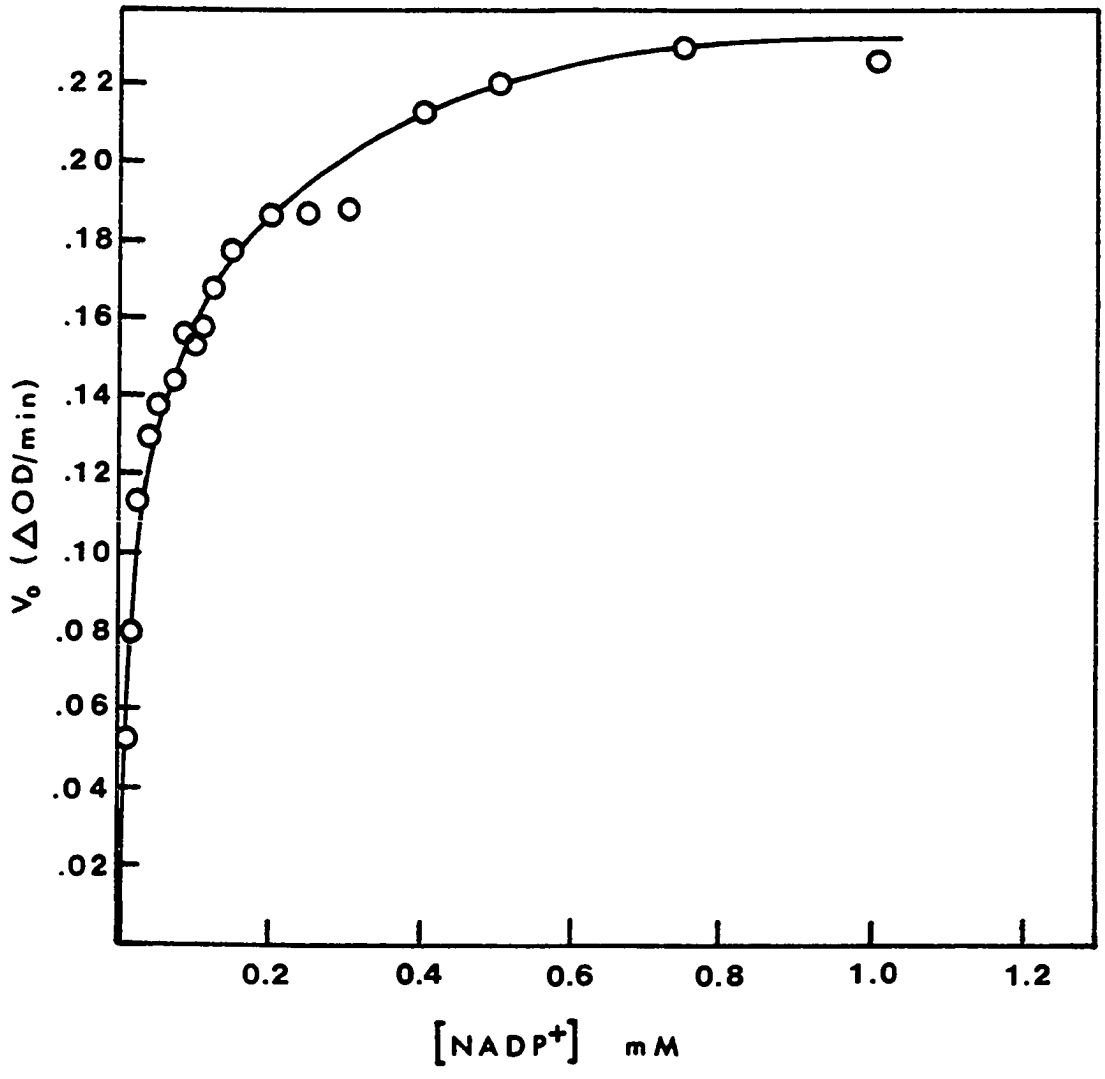


FIGURE 30 $NADP^+$ SATURATION CURVE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV. The assays contained 5 mM G-6-P and 5 mM Mg^{+2} in 100 mM tris-HCl buffer (pH 8.0). $NADP^+$ was varied.

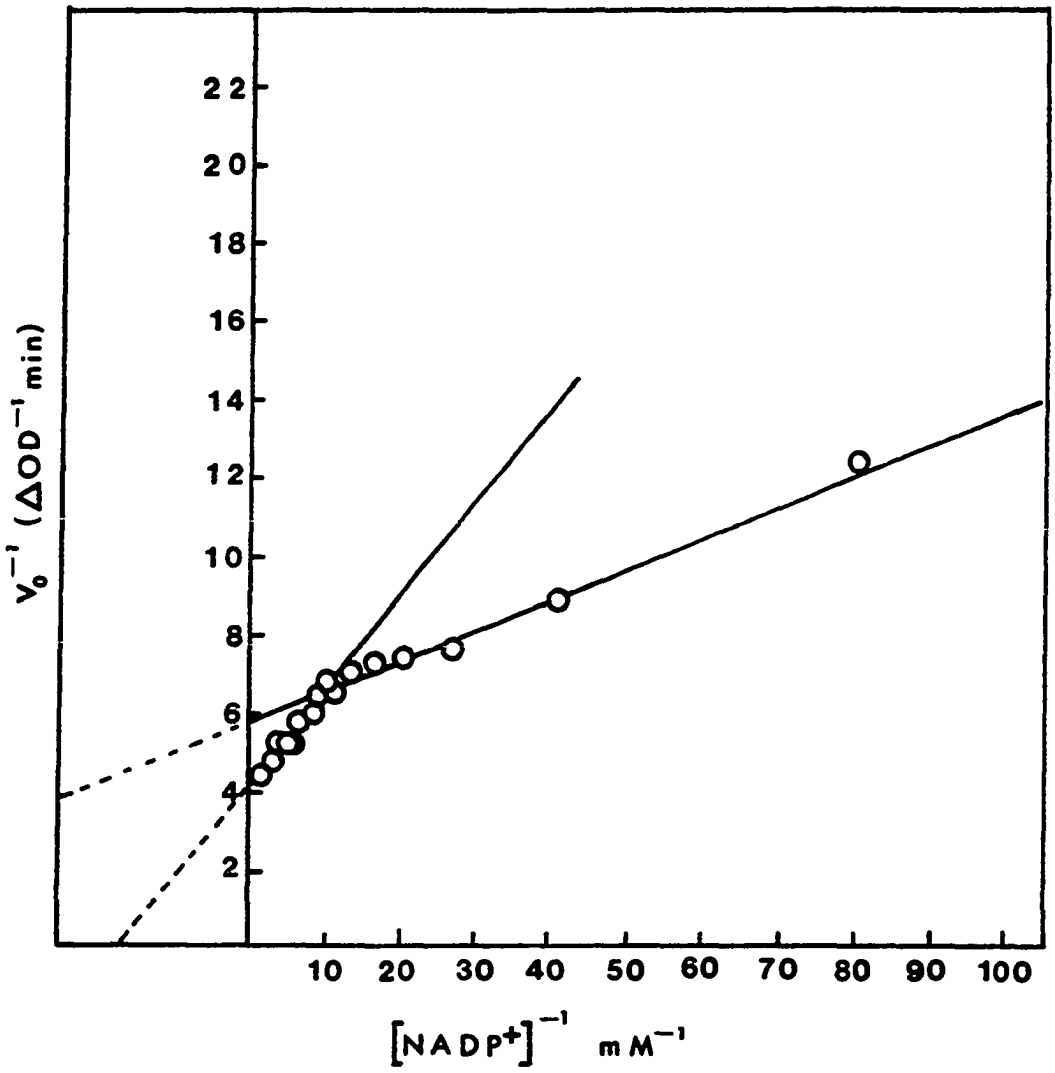


FIGURE 31 $NADP^+$ DOUBLE RECIPROCAL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV.

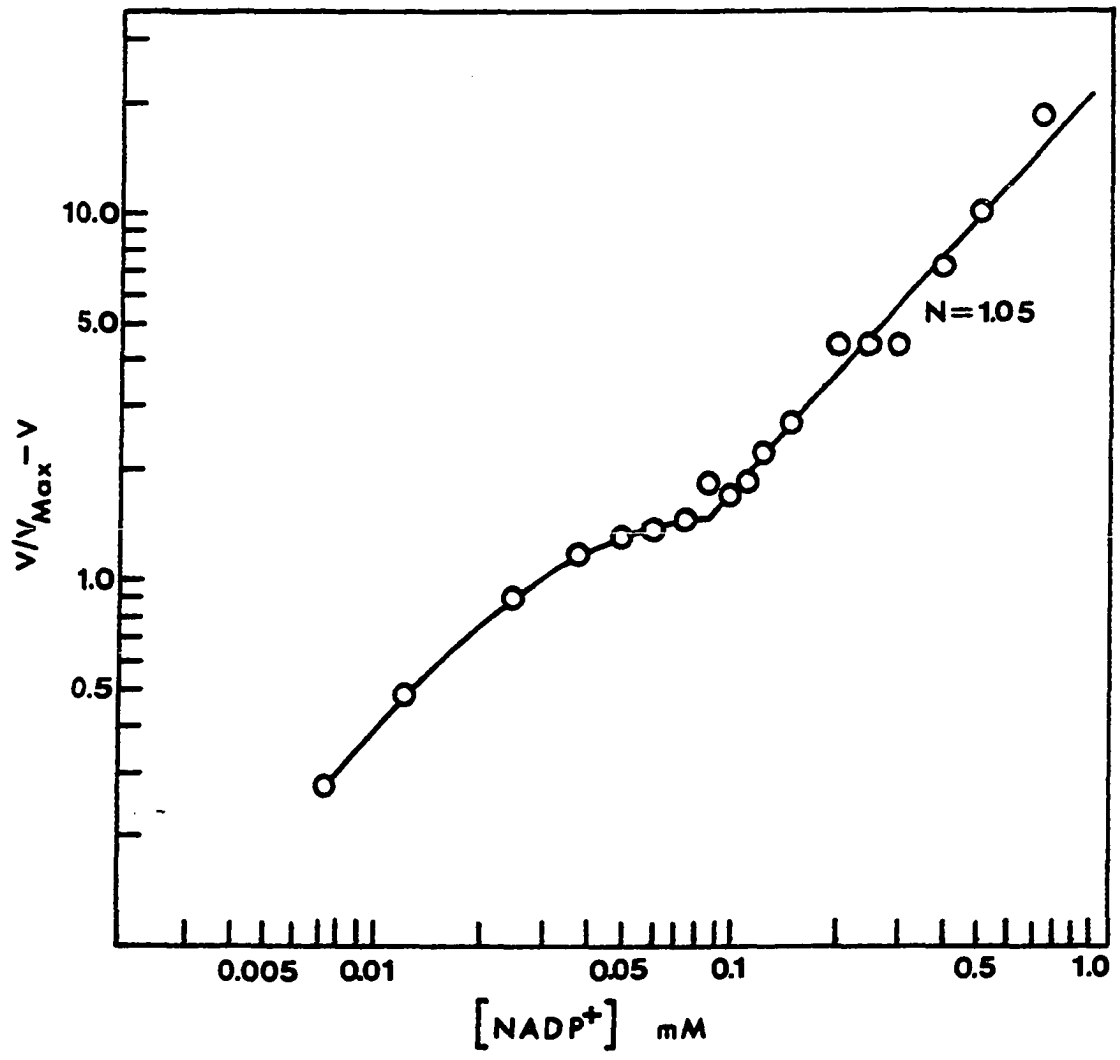


FIGURE 32 $NADP^+$ HILL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV. The larger V_{Max} is used to plot the graph.

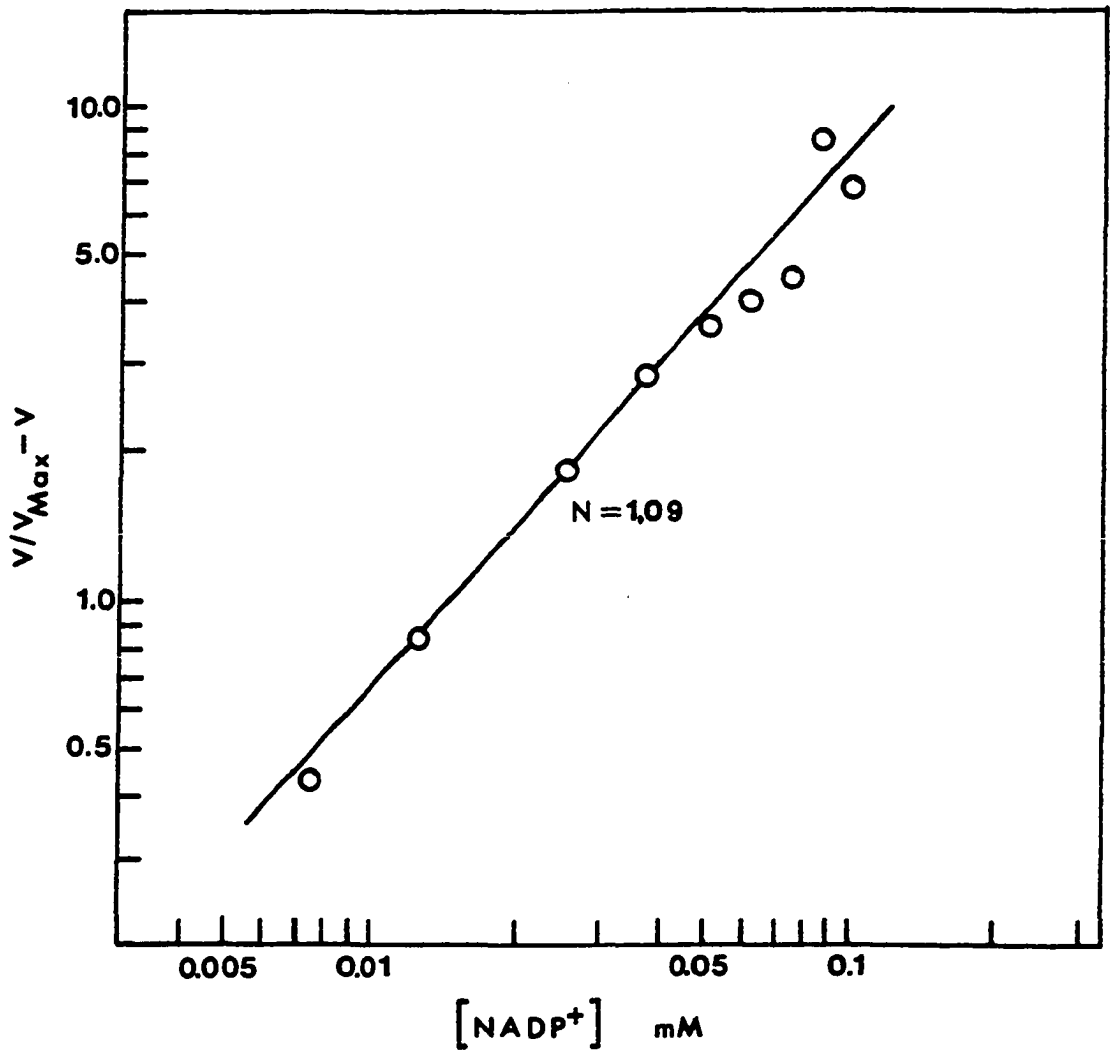


FIGURE 33 $NADP^+$ HILL PLOT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV. $NADP^+$ concentrations less than 0.1 mM and the smaller V_{Max} are used to plot the graph.

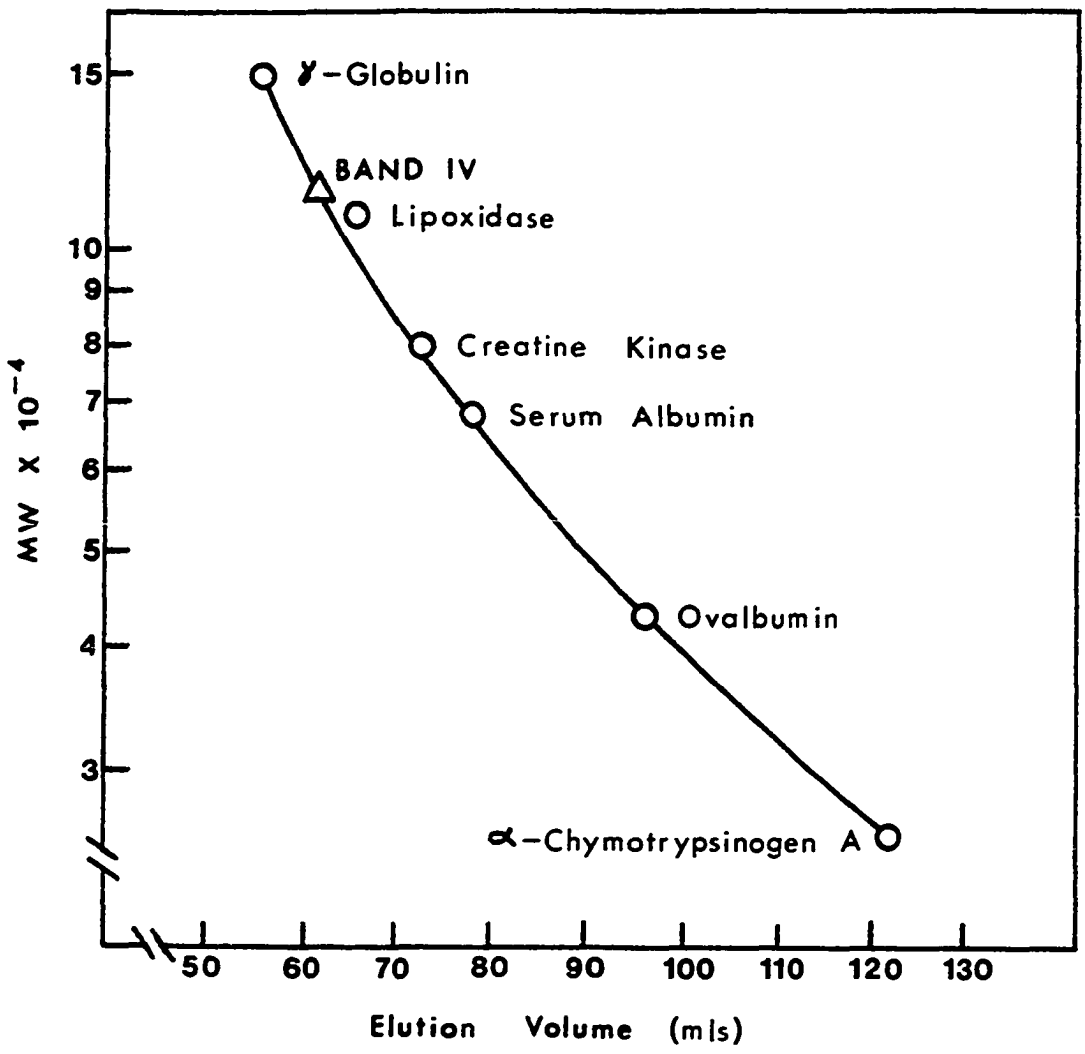


FIGURE 34 DETERMINATION OF THE MOLECULAR WEIGHT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-150..The elution buffer is 10^{-5} M $NADP^+$, 30 mM β-mercaptoethanol and 100 mM imidazole-HCl (pH 6.5).

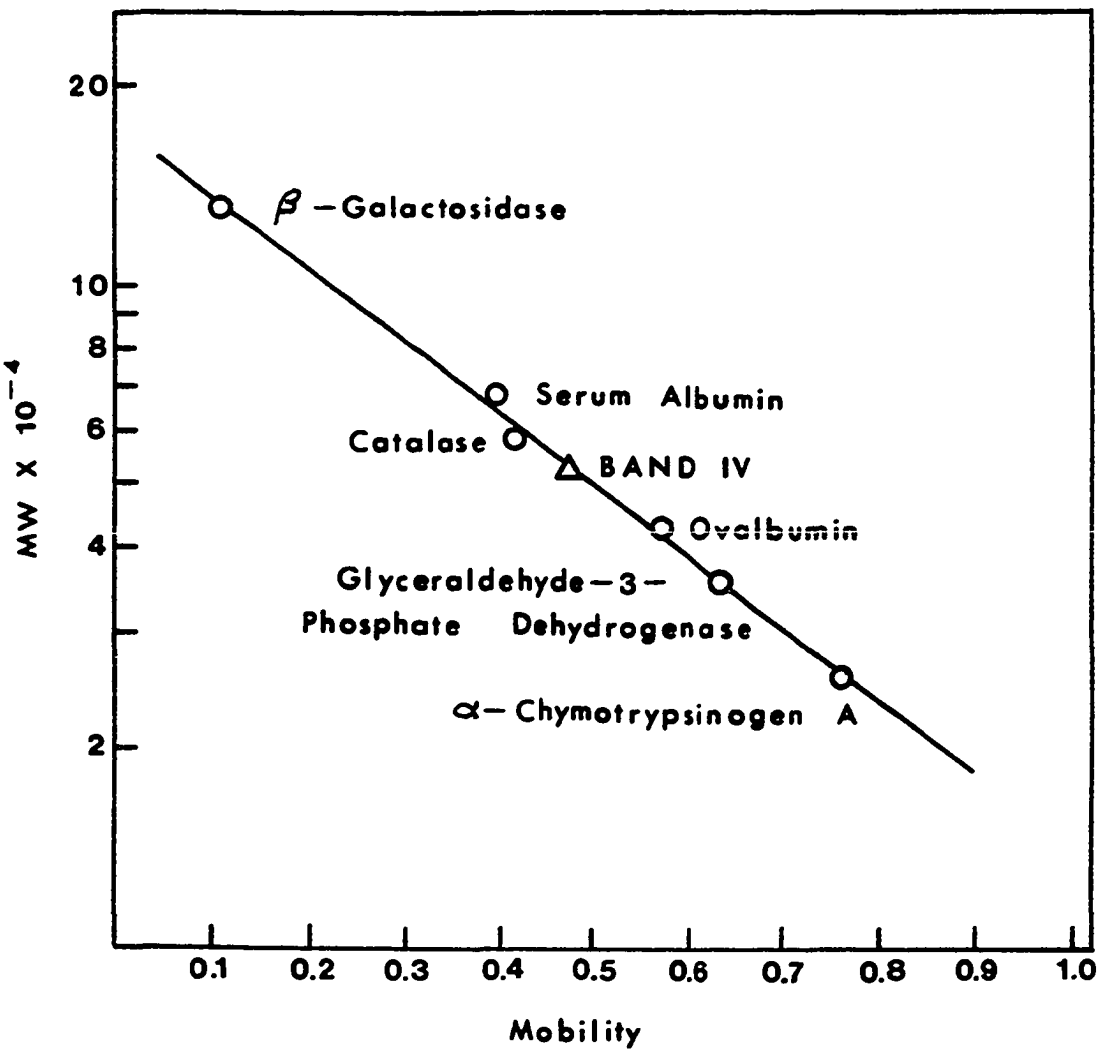


FIGURE 35 DETERMINATION OF THE MOLECULAR WEIGHT OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME BAND IV BY ELECTROPHORESIS ON SDS POLYACRYLAMIDE GELS.

The data suggest there are at least 2 $NADP^+$ binding sites, one with a much lower K_M than the other site. The abrupt increase in the apparent K_M and V_M values with increasing $NADP^+$ concentrations corresponds to negative cooperativity.

The molecular weight of Band IV was determined by the same procedures as were used for Band I. The results of the first technique, Sephadex G-150 column chromatography, are shown in fig 34. The standards are the same as those used for Band I. The molecular weight calculated from the curve is 115,000 ± 9,000. However, using SDS gel electrophoresis, a procedure known to disrupt subunit structure in proteins, a single band was visible with a MW of 54,000-59,000 (fig 35). Since the value of 115,000 obtained by Sephadex G-150 column chromatography is approximately twice the value obtained from SDS gel electrophoresis, a probable dimeric structure of identical subunits for Band IV is suggested.

It is difficult to make comparisons of the data from the crude mixture of isoenzymes with data for glucose-6-phosphate dehydrogenase from other sources. The crude mixture did contain isoenzymes, whose relative concentrations changed with the age of the tobacco tissue and, therefore, the apparent K_M values for glucose-6-phosphate and $NADP^+$ would change with the age of the tissue harvested. A comparison can be made using Bands I and IV. In Table 4, the apparent K_M values obtained from Bands I and IV are somewhat similar in several cases to those reported for glucose-6-phosphate dehydrogenase from other sources.

Band I exhibits Michaelis-Menten kinetics for both substrates, glucose-6-phosphate and $NADP^+$, with one binding site for each substrate. The enzyme appears to contain no subunit structure, as both gel filtration

TABLE 4

COMPARISON OF MICHAELIS CONSTANTS (K_M) OF
GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM VARIOUS SOURCES

SOURCE	MICHAELIS CONSTANTS	SUBSTRATE	REFERENCE
Tobacco suspension culture WR-132			
Band I	6×10^{-5} M	$NADP^+$	-
	2.2×10^{-4} M	G-6-P	-
Band IV	6.0×10^{-5} M	High $[NADP^+]$	-
	1.5×10^{-5} M	Low $[NADP^+]$	-
	3.1×10^{-4} M	G-6-P	-
Sweet potato	2.97×10^{-5} M	$NADP^+$	44
	5.27×10^{-4} M	High [G-6-P]	
	1.27×10^{-4} M	Middle [G-6-P]	
	3.25×10^{-5} M	Low [G-6-P]	
Spinach leaves	4.0×10^{-4} M	Chloroplastic G-6-P	59
	3.3×10^{-4} M	Cytoplasmic G-6-P	
Yeast	1.9×10^{-5} M	$NADP^+$	3
	8.0×10^{-5} M	High [G-6-P]	
	3.4×10^{-5} M	Low [G-6-P]	
Yeast *Candida utilis*	6.7×10^{-5} M	$NADP^+$	16
	2.3×10^{-4} M	G-6-P	

TABLE 4 (cont.)

SOURCE	MICHAELIS CONSTANTS	SUBSTRATE	REFERENCE
Bacteria *Leuconosroc mesenteroides*	5.69×10^{-6} M	$NADP^+$	47
	8.1×10^{-5} M	G-6-P	
Acetobacter xylium	4.0×10^{-5} M	$NADP^+$	7
	1.64×10^{-3} M	G-6-P	
Rat mammary gland	8.9×10^{-6} M	$NADP^+$	39
	3.0×10^{-5} M	G-6-P	
Cow adrenal cortex	5.6×10^{-6} M	$NADP^+$	11
	4.2×10^{-5} M	G-6-P	
Human erythrocytes	4.4×10^{-6} M	$NADP^+$	72
	3.9×10^{-5} M	G-6-P	

and SDS gel electrophoresis indicate a molecular weight of 85,000 to 91,000. Furthermore, Band I is not a glycoprotein. When Band I was stained for glycoprotein using the method of Glassman and Neville (27), no glycoprotein bands were visible. This was verified using ovalbumin, a well characterized glycoprotein.

Glucose-6-phosphate as a substrate for Band IV exhibits a typical saturation curve with one binding site on the enzyme. However, $NADP^+$ produces an abrupt transition in its Lineweaver-Burk plot indicating negative cooperativity. It is suggested that there are 2 $NADP^+$ binding sites on Band IV, one with a much lower K_M than the other site. The molecular weights suggest that Band IV is a dimer, with a subunit molecular weight of 54,000-59,000 and a native molecular weight of 115,000. When Band IV was checked to determine if it were a glycoprotein, it was found to contain no carbohydrate moieties.

The molecular weights of Band I and IV are compared with glucose-6-phosphate dehydrogenase from other sources in table 5. The molecular weights of the enzymes isolated in this study are consistent with many of those reported in the literature.

TABLE 5

MOLECULAR WEIGHTS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM VARIOUS SOURCES

SOURCE	MOLECULAR WEIGHT	REFERENCE
Tobacco suspension culture WR-132		
Band I	85,000-91,000	-
Band IV	115,000 - dimer	-
	54,000-59,000 - subunit	-
Sweet potato roots	110,000	44
Potato tubers	260,000 - with $NADP^+$	24
	130,000 - without $NADP^+$	
Spinach leaves	105,000	59
Brewer's yeast	101,600	73
Yeast *Candida utilis*	104,000	16
Bacteria *Leuconostoc mesenteroides*	135,000 - without $NADP^+$	48

TABLE 5 (cont.)

SOURCE	MOLECULAR WEIGHT	REFERENCE
Neurospora crassa	206,000 - tetramer	60
	104,000 - 114,000 - dimer	
	57,000 - subunit	
Rat mammary gland	241,000 - with $NADP^+$	39
Cow adrenal cortex	236,000 - with $NADP^+$	11
Bovine adrenals	130,000	57
Human erythrocytes	240,000 - with $NADP^+$	72
	123,000 - without $NADP^+$	
	43,000 - subunit	

CHAPTER V

EFFECTS OF VARIOUS COMPOUNDS ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

To ascertain the significance of glucose-6-phosphate dehydrogenase in the metabolism of phenolic compounds in tobacco tissue, several phenolic compounds as well as other compounds were tested for their effect on the enzyme. The compounds tested were: scopoletin, scopolin, esculetin, ferulic acid, *trans*-cinnamic acid, *para*-coumaric acid, caffeic acid, chlorogenic acid, indole-3-acetic acid and erythrose-4-phosphate. Indole-3-acetic acid was studied because it is an *in vivo* growth hormone whose biosynthesis includes the shikimic acid pathway. Erythrose-4-phosphate was of interest because it is one of two compounds which initiate the shikimic acid pathway. The concentrations of the phenolic compounds in the assays varied from 0.04 mM to 0.4 mM. The glucose-6-phosphate concentration was also varied using saturating, $S_{0.5}$ and $S_{0.25}$ (1/3 K_M) concentrations. The reactions were 5 mM in Mg^{+2} and 0.5 mM in $NADP^{+}$. All the reactions were initiated by the addition of $NADP^{+}$, due to a lag period associated with glucose-6-phosphate when it was used to initiate the assays. For the mixture of isoenzymes, the results are summarized in table 6. $S_{1.0}$ refers to saturating levels of glucose-6-phosphate (5 mM G-6-P), $S_{0.5}$ refers to the substrate concentration at 1/2 Vm or Km (0.38 mM G-6-P) and $S_{0.25}$ refers to the substrate concentration at 1/4 Vm or 1/3 Km (0.127 mM G-6-P).

TABLE 6

EFFECT OF VARIOUS COMPOUNDS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYME MIXTURE

EFFECTOR / EFFECTOR CONC.		PER CENT OF CONTROL ACTIVITY				
		0.4 mM	0.3 mM	0.2 mM	0.1 mM	0.04 mM
SCOPOLETIN						
	$S_{1.0}$ G-6-P	44	76	82	96	95
	$S_{0.5}$ G-6-P	59	86	94	90	94
	$S_{0.25}$ G-6-P	82	100	100	92	91
ESCULETIN						
	$S_{1.0}$ G-6-P	101	96	102	100	97
	$S_{0.5}$ G-6-P	105	103	97	92	97
	$S_{0.25}$ G-6-P	91	96	100	88	92
SCOPOLIN						
	$S_{1.0}$ G-6-P	13	39	81	99	97
	$S_{0.5}$ G-6-P	12	45	82	91	88
	$S_{0.25}$ G-6-P	16	44	76	84	92
ESCULIN						
	$S_{1.0}$ G-6-P	24	66	89	104	98
	$S_{0.5}$ G-6-P	32	71	89	92	95
	$S_{0.25}$ G-6-P	39	74	78	83	93
FERULIC ACID						
	$S_{1.0}$ G-6-P	94	90	96	105	101
	$S_{0.5}$ G-6-P	86	90	89	89	96
	$S_{0.25}$ G-6-P	69	64	64	77	83
CAFFEIC ACID						
	$S_{1.0}$ G-6-P	94	89	92	100	97
	$S_{0.5}$ G-6-P	75	84	81	80	91
	$S_{0.25}$ G-6-P	58	62	67	76	88

TABLE 6 (cont.)

EFFECTOR / EFFECTOR CONC.	PER CENT OF CONTROL ACTIVITY				
	0.4 mM	0.3 mM	0.2 mM	0.1 mM	0.04 mM
TRANS-CINNAMIC ACID					
$S_{1.0}$ G-6-P	103	95	97	105	100
$S_{0.5}$ G-6-P	93	93	93	94	96
$S_{0.25}$ G-6-P	88	85	85	85	89
PARA-COUMARIC ACID					
$S_{1.0}$ G-6-P	106	96	95	106	101
$S_{0.5}$ G-6-P	90	88	88	92	96
$S_{0.25}$ G-6-P	72	63	68	74	89
CHLOROGENIC ACID					
$S_{1.0}$ G-6-P	2	15	76	98	100
$S_{0.5}$ G-6-P	3	28	85	98	95
$S_{0.25}$ G-6-P	6	22	72	79	90
INDOLE-3-ACETIC ACID					
$S_{1.0}$ G-6-P	108	98	98	106	101
$S_{0.5}$ G-6-P	88	95	91	90	93
$S_{0.25}$ G-6-P	103	88	76	82	90
ERYTHROSE-4-PHOSPHATE					
$S_{1.0}$ G-6-P	91	94	94	96	97
$S_{0.5}$ G-6-P	86	88	90	91	93
$S_{0.25}$ G-6-P	127	106	106	94	106
SHIKIMIC ACID					
$S_{1.0}$ G-6-P	120			120	102

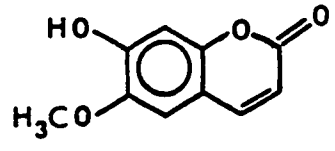
A very low concentration of some of the phenolics will inhibit a mixture of glucose-6-phosphate dehydrogenase isoenzymes. The compound which had the most pronounced effect was chlorogenic acid. Scopoletin, scopolin and esculin have substantial effects on the dehydrogenase reactions. Scopolin, in the experiments performed, was found to be much more inhibitory than scopoletin. The phenolic acids - ferulic, caffeic, *para*-coumaric and *trans*-cinnamic acid, though inhibitory at low glucose-6-phosphate concentrations, are not nearly as potent inhibitors as the coumarin derivatives. Chlorogenic acid, at 0.4 mM inhibits glucose-6-phosphate dehydrogenase almost 100%, with the inhibition decreasing as the concentration of the acid is reduced. It is puzzling as to why this derivative of quinic acid could have an effect of such dimensions. Chlorogenic acid and its isomers, are quite common in plant tissue (32,33,70), yet it will inhibit almost completely an enzyme apparently involved in its synthesis. Chlorogenic acid has been reported to inhibit several other enzymes markedly; in particular, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase from rat lung (53), isoperoxidases C_3 and C_4 from tobacco suspension cultures WR-132 (53,56) and isoperoxidases A_1, A_2 and A_3 from tobacco tissue culture W-38 (52,54,56). Shikimic acid was also tested using the crude mixture. Shikimic acid activated the reaction 120% of control.

Bands I and IV were tested to determine how these phenolics influenced a single isoenzyme. The results are shown in table 7. The compounds are grouped together for structural similarities. Some other compounds tested, but not listed, were glucose, sucrose, shikimic acid, ferulic acid-β-D-glucoside and coumarin. None of these compounds had any significant effect upon the reaction velocities of either Band I or Band IV.

TABLE 7

EFFECT OF VARIOUS COMPOUNDS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE ISOENZYMES BAND I AND BAND IV

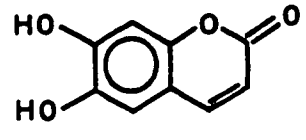
SCOPOLETIN



PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	34	27	42	56	110	45
0.3 mM	76	85	93	103	151	105
0.2 mM	90	101	96	105	140	112
0.1 mM	96	105	97	99	100	103
0.04 mM	98	102	91	94	97	89

ESCULETIN

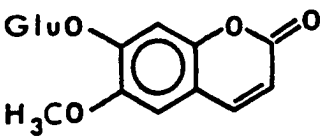


PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	79	68	78	73	158	91
0.3 mM	80	97	77	96	125	105
0.2 mM	90	98	76	100	107	104
0.1 mM	96	102	89	95	100	96
0.04 mM	98	99	91	92	99	92

TABLE 7 (cont.)

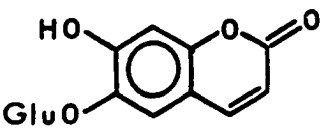
SCOPOLIN



PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	21	14	17	14	24	18
0.3 mM	50	50	49	52	64	50
0.2 mM	82	85	74	84	76	79
0.1 mM	101	101	88	93	90	89
0.04 mM	103	95	96	93	94	85

ESCULIN

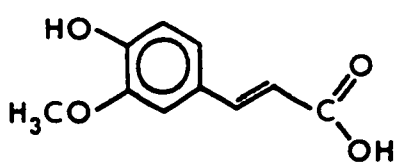


PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	30	21	34	30	50	16
0.3 mM	64	63	38	72	75	68
0.2 mM	90	90	91	94	101	92
0.1 mM	98	98	94	95	94	86
0.04 mM	100	93	92	93	97	86

TABLE 7 (cont.)

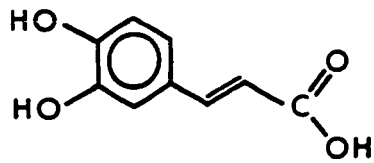
FERULIC ACID



PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	86	89	78	86	67	94
0.3 mM	88	95	82	88	70	96
0.2 mM	91	98	88	87	83	92
0.1 mM	95	95	96	86	91	86
0.04 mM	97	99	100	88	95	92

CAFFEIC ACID

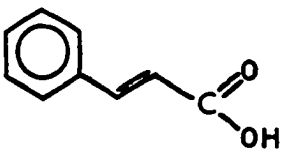


PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	86	90	85	89	80	82
0.3 mM	88	98	96	89	87	83
0.2 mM	93	97	93	90	89	82
0.1 mM	101	97	91	88	92	79
0.04 mM	101	94	93	87	93	87

TABLE 7 (cont.)

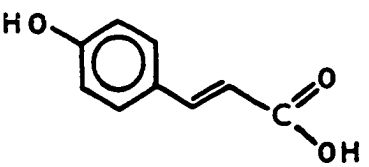
TRANS-CINNAMIC ACID



PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	106	94	108	91	97	100
0.3 mM	114	99	110	96	101	100
0.2 mM	113	97	110	94	103	96
0.1 mM	110	102	106	90	100	94
0.04 mM	111	94	103	96	102	92

PARA-COUMARIC ACID

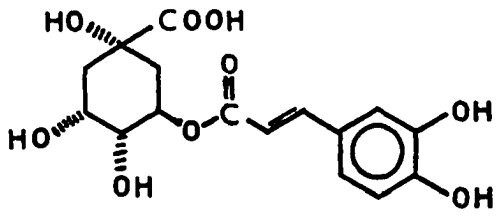


PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	102	90	100	94	94	98
0.3 mM	106	97	100	95	94	95
0.2 mM	106	96	100	94	96	92
0.1 mM	108	101	100	91	101	90
0.04 mM	112	94	100	91	99	90

TABLE 7 (cont.)

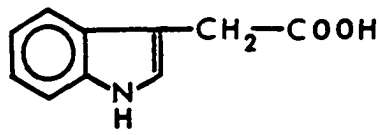
CHLOROGENIC ACID



PER CENT OF CONTROL ACTIVITY

Con. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	1	7	0	4	10	8
0.3 mM	25	28	0	24	23	23
0.2 mM	75	76	60	74	66	69
0.1 mM	90	94	100	85	84	84
0.04 mM	93	95	100	92	85	91

INDOLE-3-ACETIC ACID

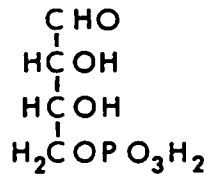


PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	93	86	91	90	94	96
0.3 mM	95	92	96	92	100	95
0.2 mM	95	92	91	92	97	90
0.1 mM	98	93	94	92	93	92
0.04 mM	98	92	100	91	99	92

TABLE 7 (cont.)

ERYTHROSE-4-PHOSPHATE



PER CENT OF CONTROL ACTIVITY

Conc. of Phenolic Compound	$S_{1.0}$ G-6-P		$S_{0.5}$ G-6-P		$S_{0.25}$ G-6-P	
	BAND I	BAND IV	BAND I	BAND IV	BAND I	BAND IV
0.4 mM	89	111	91	91	98	79
0.3 mM	90	110	89	91	96	86
0.2 mM	89	107	93	92	97	100
0.1 mM	93	107	93	104	99	100
0.04 mM	93	112	97	109	100	92

$S_{1.0}$ refers to saturating levels of glucose-6-phosphate (5 mM G-6-P), $S_{0.5}$ refers to K_M concentrations of glucose-6-phosphate (0.22 mM for Band I and 0.31 mM for Band IV) and $S_{0.25}$ refers to 1/3 K_M concentrations (0.073 mM for Band I and 0.103 mM for Band IV). The data for Bands I and IV follow the same trend as that of the mixture of isoenzymes. Chlorogenic acid inhibits the reactions almost completely. The coumarins are inhibitory while the phenolic acids are not. The glucosylated compounds, scopolin and esculin, are much more inhibitory than their hydrolysis products, scopoletin and esculetin, respectively.

Band I is quite different from Band IV in one aspect. At low glucose-6-phosphate concentrations, scopoletin and esculetin accelerate the reaction, 151% of control for scopoletin and 158% for esculetin. There is a threshold effect at the lower substrate levels. Only scopoletin and esculetin exhibit this activation. The ring substitutions and their positioning might be the determining factor for the observed inhibition and activation. Phenolic acids with analogous ring substitutions might then be expected to exhibit the same patterns as the coumarins. Ferulic acid has the same ring substitution pattern as scopoletin, ferulic acid-β-D-glucoside is similar to scopolin and caffeic acid is similar to esculetin. However, these phenolic acids do not inhibit the dehydrogenase reaction the same percentage as the corresponding coumarins. Therefore, more than the ring substitutions could be involved in the inhibition and activation shown by the coumarins.

To ascertain the validity of the observed inhibition activation patterns, three other enzymes were studied. The enzymes were: glucose-6-phosphate dehydrogenase from Torula yeast, Type XII (Sigma Chemical Company), $NADP^+$ specific malate dehydrogenase and $NADP^+$ specific isocitrate dehydrogenase.

The malate and isocitrate dehydrogenases were from crude preparations of WR-132 tobacco suspension cultures. The only phenolic compounds used as effectors were scopoletin, esculetin and chlorogenic acid. The results are shown in table 8. It is interesting that malate and isocitrate dehydrogenase are inhibited much more than glucose-6-phosphate dehydrogenase from either WR-132 tobacco suspension cultures or Torula yeast. All of the enzymes are NADPH producing enzymes and are inhibited by the coumarins, scopoletin and esculetin, as well as the quinic acid derivative, chlorogenic acid. The activation observed with Band I is not observed with any of the other NADPH producing enzymes. However, this is not unexpected because the three enzymes tested contained isoenzymes and from the crude mixture in this study, no activation was observed. Therefore, no conclusions can be drawn between the different NADPH producing enzymes.

To establish that the activation by scopoletin was not caused by a "scopoletin oxidase", several enzyme assays were run using scopoletin and esculetin as substrates. The assays contained either scopoletin or esculetin as substrates, 0.5 mM $NADP^+$, 5 mM Mg^{+2} and enzyme in 100 mM tris-HCl buffer (pH 8.0). Other assays contained permutations of the assay ingredients. No reduction of $NADP^+$ was observed at 340 nm. *In situ* assays were also performed. After completion of electrophoresis of the enzyme solution, the polyacrylamide gels were placed in a dehydrogenase staining solution containing scopoletin or esculetin as substrates instead of glucose-6-phosphate. Again, permutations were tested. There were no visible bands to record. Therefore, the activation observed with scopoletin and esculetin is not due to a "scopoletin oxidase" using scopoletin or esculetin as substrates and $NADP^+$ as a coenzyme.

TABLE 8

EFFECT OF PHENOLICS ON THE ACTIVITY OF THREE DEHYDROGENASE ENZYMES

1. GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM TORULA YEAST, TYPE XII

EFFECTOR / EFFECTOR CONC.	PER CENT OF CONTROL ACTIVITY				
	0.4 mM	0.3 mM	0.2 mM	0.1 mM	0.04 mM
SCOPOLETIN					
$S_{1.0}$ G-6-P	63	76	80	71	77
$S_{0.5}$ G-6-P	53	76	95	82	74
$S_{0.25}$ G-6-P	56	73	80	73	87
ESCULETIN					
$S_{1.0}$ G-6-P	67	69	80	64	68
$S_{0.5}$ G-6-P	68	71	70	64	65
$S_{0.25}$ G-6-P	62	73	60	67	67
CHLOROGENIC ACID					
$S_{1.0}$ G-6-P	7	24	40	52	52
$S_{0.5}$ G-6-P	5	14	45	45	48
$S_{0.25}$ G-6-P	6	7	33	33	20

2. ISOCITRATE DEHYDROGENASE ($NADP^+$) FROM WR-132 TOBACCO SUSPENSION CULTURES

EFFECTOR / EFFECTOR CONC.	PER CENT OF CONTROL ACTIVITY				
	0.4 mM	0.3 mM	0.2 mM	0.1 mM	0.04 mM
SCOPOLETIN					
$S_{1.0}$ G-6-P	5	19	67	88	92
$S_{0.5}$ G-6-P	6	46	69	95	95
$S_{0.25}$ G-6-P	1	75	88	72	100
ESCULETIN					
$S_{1.0}$ G-6-P	49	74	78	85	84
$S_{0.5}$ G-6-P	77	100	95	87	89
$S_{0.25}$ G-6-P	60	57	67	63	93
CHLOROGENIC ACID					
$S_{1.0}$ G-6-P	6	13	37	77	84
$S_{0.5}$ G-6-P	6	16	49	74	89
$S_{0.25}$ G-6-P	0	7	40	75	103

TABLE 8 (cont.)

3.MALATE DEHYDROGENASE ($NADP^+$) FROM WR-132 TOBACCO SUSPENSION CULTURES

		PER CENT OF CONTROL ACTIVITY				
EFFECTOR / EFFECTOR CONC.		0.4 mM	0.3 mM	0.2 mM	0.1 mM	0.04 mM
SCOPOLETIN						
$S_{1.0}$	G-6-P	6	24	61	91	93
$S_{0.5}$	G-6-P	8	40	65	75	86
$S_{0.25}$	G-6-P	17	50	67	88	100
ESCULETIN						
$S_{1.0}$	G-6-P	56	80	67	91	88
$S_{0.5}$	G-6-P	92	100	89	75	100
$S_{0.25}$	G-6-P	92	90	89	88	86
CHLOROGENIC ACID						
$S_{1.0}$	G-6-P	7	13	39	79	93
$S_{0.5}$	G-6-P	23	10	33	62	57
$S_{0.25}$	G-6-P	8	10	44	62	71

CHAPTER VI

DISCUSSION

Glucose-6-phosphate dehydrogenase, as isolated from WR-132 tobacco suspension cultures, exists as 4 isoenzymes. The data from the mixture of isoenzymes are misleading and only emphasize the importance of obtaining single isoenzymes for future investigations.

The molecular weights presented in table 5 indicate that the molecular weight of glucose-6-phosphate dehydrogenase from some sources, namely potato tuber (26), *Neurospora crassa* (61) and human erythrocytes (72), changes in the presence or absence of $NADP^+$. $NADP^+$, in all three cases, causes the enzyme to form either a dimer or a tetramer. It might be assumed that the active catalytic configuration of the enzyme is the form produced in the presence of $NADP^+$. The molecular weight of Band I as determined by gel filtration was 91,000. The elution buffer was 100 mM KCl and 50 mM tris-HCl (pH 7.5). The molecular weight as indicated by SDS gel electrophoresis was 85,000 ± 4,000. It is therefore difficult to conclude whether or not Band I is a dimer because $NADP^+$ was not in the elution buffer for gel filtration.

The elution buffer used in the gel filtration of Band IV did contain $NADP^+$. The molecular weight of 115,000 as determined by this procedure, is approximately twice the value of 54,000 to 59,000 as determined by SDS gel electrophoresis. A probable dimeric structure of identical subunits

for Band IV is suggested.

Grossman and McGown (31) have reported that pH affects the kinetic behavior of glucose-6-phosphate dehydrogenase. At a pH of 6.7, the enzyme exhibits Michaelis-Menten kinetics. However, at a pH above 7.4, the enzyme exhibits sigmoidal kinetics with respect to glucose-6-phosphate. All of the kinetic studies on Band I and Band IV were performed at pH 8.0, except for the pH profiles. The intermediary plateau in the saturation curve for Band IV could be a result of pH. An interesting situation could exist in which pH might influence the kinetics of the enzyme.

In response to phenolic compounds, both isoenzymes are inhibited by certain coumarins tested and chlorogenic acid, but showed little response to the phenolic acids. Scopoletin and esculetin are less inhibitory than their glucosylated forms. Band I is activated at low substrate concentrations by scopoletin and esculetin, while Band IV is not. The activation of Band I by scopoletin appears to be a threshold effect. At high scopoletin and high G-6-P concentrations, the reaction is inhibited; however, at the low G-6-P concentration (1/4 V_M G-6-P levels) the reaction is activated by scopoletin. This situation occurs at 0.4, 0.3 and 0.2 mM concentrations of scopoletin. At concentrations lower than this, namely 0.1 and 0.04 mM, scopoletin will not activate the reaction. Einhellig *et al.* (17) reported that scopoletin also has a threshold effect on the growth of tobacco, sunflower and pigweed. Scopoletin was supplied to the plants in a nutrient culture. At 10^{-3} M, scopoletin was inhibitory to growth. A scopoletin concentration of 10^{-4} M to 10^{-3} M produced no major growth effects, while scopoletin concentrations of 5×10^{-5} M to 10^{-4} M did appear to stimulate growth in all three plants.

There are several reports that two hexose monophosphate pathways exist in plant tissue, one localized in the cytosol and the other in the chloroplasts (1,59,60). The exact location of the shikimic acid pathway is in doubt. The final step, lignin production, is associated with the cell wall, but the location of pathway sequences is unsettled. The chloroplast is the site most mentioned. If the chloroplast is the correct site, then a question of localization of isoenzymes is raised. Band I and Band IV might occupy different subcellular locations in a plant cell. Band IV might be the cytoplasmic enzyme, functioning in a pathway responsible for NADPH and pentose phosphate production. The other isoenzyme, Band I, might be responsible for producing not only NADPH but also erythrose-4-phosphate for phenolic biosynthesis. When a plant is subjected to stress, the phenolic concentration would increase somewhat, creating a demand for precursors, namely erythrose-4-phosphate. This, in return would require the activation of glucose-6-phosphate dehydrogenase. The level of glucose-6-phosphate in the chloroplast probably is quite low. Therefore, an increase in the coumarin concentration would activate the enzyme. Glucose-6-phosphate is not freely permeable through the chloroplast cell wall, but sugar phosphates which are easily interconvertible to glucose-6-phosphate are permeable (32). Because the phenolics are localized in a subcellular organelle, it would be unnecessary for the phenolics to activate Band IV located in the cytosol. At this point, however, these suggested ideas are still speculative.

Peculiar as it may seem, phenolic acids have essentially no effect on the activity of either Band I or IV. Lignin biosynthesis can proceed from the phenolic acids (30,45). Cinnamic acid can be incorporated directly into lignin while hydroxycinnamic acids cannot. These acids include:

para-coumaric acid, caffeic acid and ferulic acid. For these acids to be incorporated into lignin, they first must be reduced to the corresponding aldehydes and alcohols prior to polymerization (9). This reduction is dependent upon ATP, coenzyme A and a reduced pyridine nucleotide. Thus, if glucose-6-phosphate dehydrogenase is responsible for supplying NADPH, it would be unlikely that the phenolic acids would inhibit the reaction, preventing their incorporation into lignin.

The results on two isoenzymes of glucose-6-phosphate dehydrogenase have emphasized the importance of obtaining information on the two other isoenzymes, Band II and III, as well as the other controlling enzyme in the pathway, 6-phosphogluconic acid dehydrogenase.

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