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I. EFFECT OF KINETIN AND INDOLEACETIC ACID ON THE ACTIVITIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE. II. KINETIC AND PHYSICAL PROPERTIES OF TWO ISOENZYMES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM TOBACCO TISSUE CULTURE W-38.

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SUMMARY

Cultures of tobacco tissue Nicotiana tabacum W-38 were grown on medium containing various amounts of kinetin and 3-indoleacetic acid. The activities of glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the cell extracts were assayed at the end of three and four weeks of growth. At all the IAA levels, the activities of both G6PD and 6PGD were found to increase with an increase in the kinetin concentration. However, the influence of combinations of IAA and kinetin on the activities of these two enzymes was not identical in every case.

Several phenolic compounds were found to inhibit the activity of 6PGD. Chlorogenic acid at 0.4 mM completely inhibited the activity of both 6PGD bands. The correlation between the activation of 6PGD and G6PD by kinetin, application, and the inhibition of 6PGD by the phenolic compounds studied will be discussed.

Glucose 1,6 diphosphate also has an inhibitory effect on these isoenzymes over low range 6-phosphogluconate concentrations. On the other hand, 2,3 diphosphoglycerate activated the enzyme up to 200% of its original activity.

Anodic polyacrylamide gel electrophoresis revealed the presence of two major zones of 6PGD. The slow anodic

CHAPTER I

INTRODUCTION

Plant phenolics are a structurally diverse group of compounds synthesized by all higher plants. Scopoletin has been reported to occur in all solanaceous plants (Harborne and Simonds 1964). Scopolin, the 7-glucoside of scopoletin (6-methoxy-7-hydroxycoumarin) is apparently the most commonly occurring glycoside of scopoletin.

Numerous reports have indicated that the major part of scopoletin present in healthy tobacco plants is present as derivatives, mainly scopolin (Steck 1967).

Under stress conditions, free scopoletin is present in larger amounts. Sequeira (1967) found an increase in both scopolin and scopoletin in tobacco plants infected with *Pseudomonas solanacearum*. In boron-deficient tobacco plants (Watanabe 1961), scopolin increases over twenty fold compared to control plants. Tobacco plants sprayed with 2, 4-dichlorophenoxyacetic acid and Tordon (4-amino 3, 5, 6-trichloropicolinic acid) have shown an increased concentration of scopolin and scopoletin. Maleic hydrazide-treated tobacco plants were found to accomulate scopolin and scopoletin as compared with corresponding controls (Winkler 1967). Other stress conditions

known to result in the accumation of scopolin include mineral deficiency (Armstrong 1968), cold treatment (Koeppe 1968), ultraviolet irradiation (Frey-Wyssling 1957, Koeppe 1968), and X-ray irradiation (Koeppe 1968).

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP+ 1-oxidoreductase, EC 1.1.1.49)(G6PD) is the first enzyme of the pentose phosphate pathway. This enzyme and 6-phosphogluconate dehydrogenase (6PGD) (D-6-phospho-gluconate: NADP+ 1-oxidoreductase EC 1.1.1.44) have been suggested as controlling enzymes of the pathway (Schnarren-berger 1973). The activities of these dehydrogenases may be limited by the availability of NADP+ and inhibited by NADPH (Ashihara 1975). The inhibition of the activity of G6PD and 6PGD may be important for the control of the pentose phosphate pathway.

Erythrose 4-phosphate (E4P), an intermediate of the pathway, can combine with phosphoenolpyruvic acid to initiate the shikimic acid pathway, which is used by many plants in the biosynthesis of their phenolic compounds. Ashihara (1964) has suggested a possible role of the pentose phosphate pathway as a supplier of building blocks for phenolic compound biosynthesis. Godin (1955) reported that the phenolic accumulation results from the enhancement of the pentose phosphate pathway activity. Farkas (1962) has discussed possible relations between the pentose phosphate

pathway activity and the enhanced synthesis of phenolic compounds. In fact, plant infection not only increases the phenolic biosynthesis but also the pentose phosphate pathway activity as well (Oritani 1955, Shaw 1957, Kahl 1973). Wender (1970) has reviewed which pathways might be enhanced to achieve accumulation of the phenolic compounds. The shikimic acid pathway is one such pathway. The pentose phosphate pathway is not only the pathway which supplies erythrose-4-phosphate for the phenolic compound biosynthesis through the shikimic acid pathway; it is also used for the formation of pentoses for biosynthesis of nucleic acids, for glucose oxidation; and for production of the reducing power of the cell, NADPH (Figures 1 and 2). Furthermore, the formation of phenolics has been affected by changes in the pool of phenylalanine (Sugano 1978, Berlin 1978). The pentose phosphate pathway has control over this aromatic amino acid through the shikimic acid pathway.

In some plant systems, hormones increase the activities of the pentose phosphate pathway (Black 1962). However, the increase of glucose 6-phosphate dehydrogenase activity to account for the pentose phosphate pathway participation in growth was not sufficiently clarified by kinetin application (Akemine 1975, Komamine 1975 and Scott 1964). Furthermore, marked increase in the activities of (G6PD) and (6PGD) were observed during culture of tobacco callus under shoot-forming conditions (Thorpe 1973), while these activities

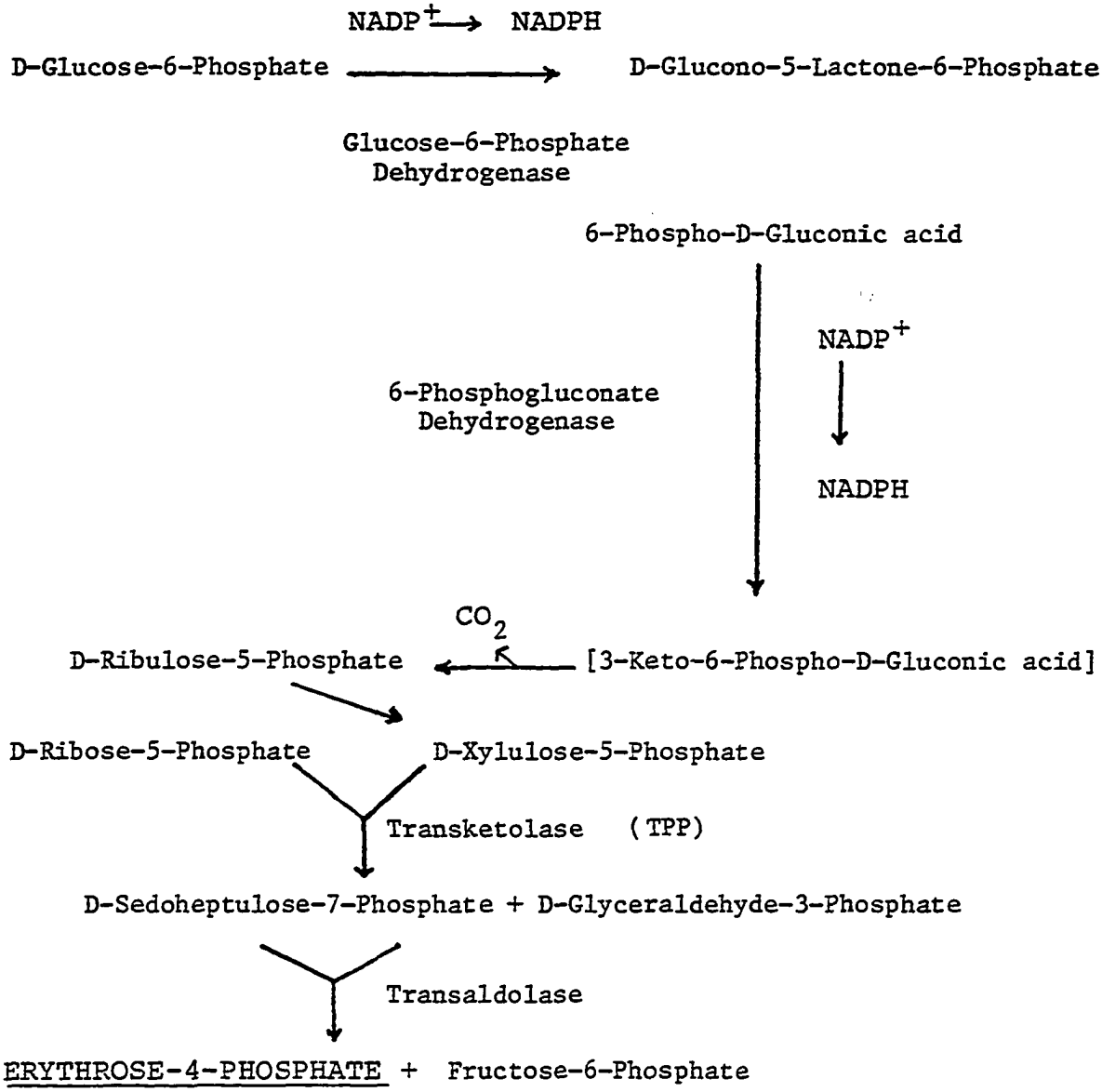

Fig. 1 Pentose phosphate pathway as a source of erythrose-4-phosphate, pentose-5-phosphate and NADPH. (Wender 1970)

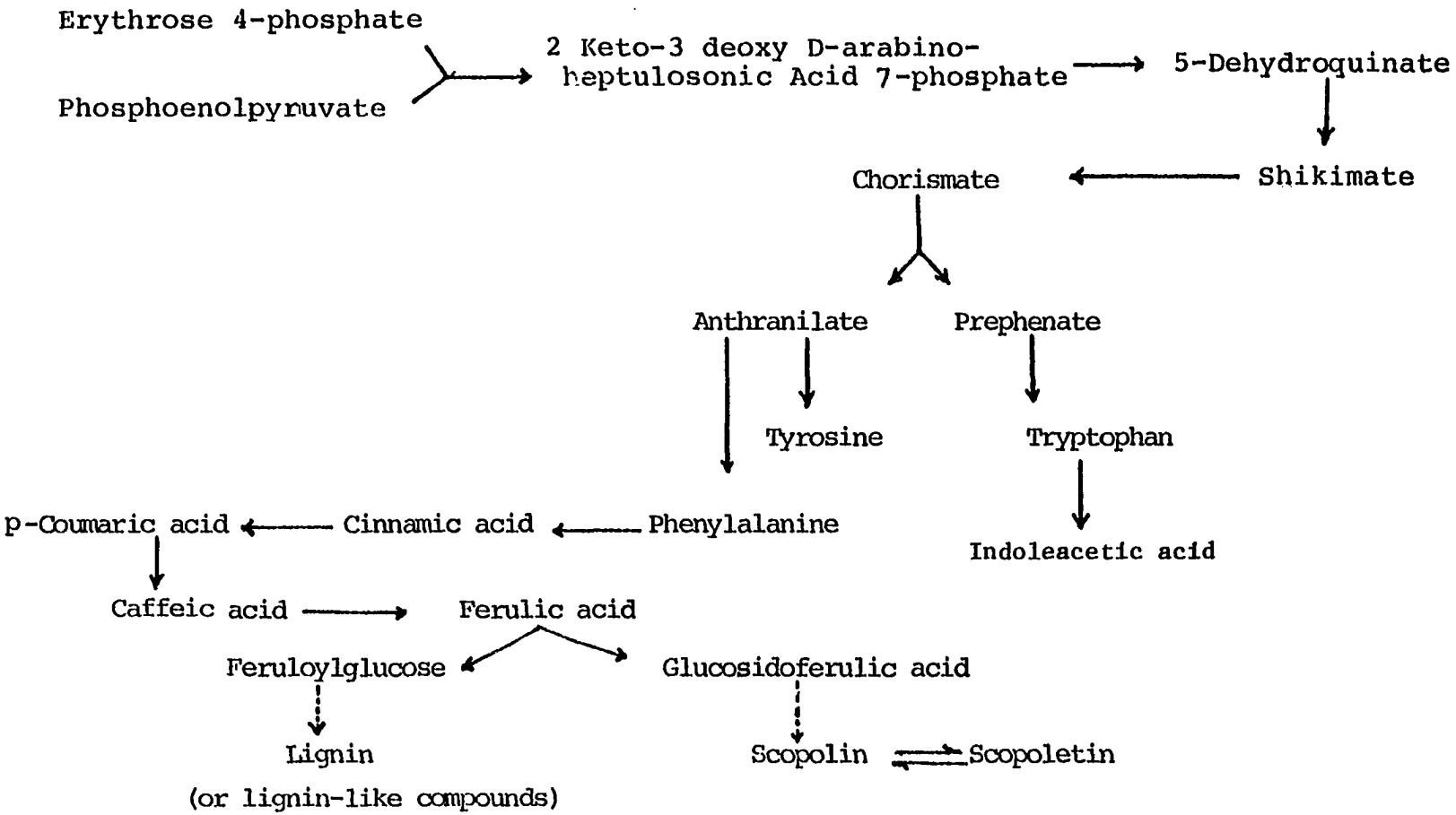

Fig. 2 Utilization of erythrose 4-phosphate for biosynthesis of aromatic amino acids, indoleacetic acid, scopolin and scopoletin via shikimic acid pathway.

in normal callus culture decreased upon increasing growth rate (Scott 1964).

3-Indoleacetic acid, a plant growth hormone whose biosynthesis includes the shikimic acid pathway, is known to increase upon plant infection; moreover, the increase of 3-indoleacetic acid seems to depend on the activation of of its biogenesis and not on the inhibition of its degradation (Matta 1970). It is of interest that bacterial or virus infections which may raise the free auxin levels in plants also appear to cause accumulation of scopoletin. Disease resistance and susceptibility of tobacco callus tissue are found to depend on 3-indoleacetic acid and kinetin levels in the tissue (Hoberlach 1978). Skoog (1961) and Sargent (1960) have found that the level of scopolin and scopoletin attained in the tissue is dependent on kinetin and indoleacetic acid concentrations in the growth medium. This concentration change in phenolic compounds is utilized to study the activity of the key enzymes of the pentose phosphate pathway. In such a study the relationship between scopolin and scopoletin concentrations and the pentose phosphate pathway may be clarified.

Since there appears to be some relationship between the accumulation of the phenolic compounds scopolin, scopoletin and chlorogenic acid in some plant tissue, and the activity of the pentose phosphate pathway during stress conditions, an investigation of the effect of these and

other phenolic compounds on the activity of two 6PGD isoenzymes has been investigated. Such experiments should contribute to the understanding of the relationship between scopolin and scopoletin, on one hand, and the pentose phosphate pathway on the other. Also, it should contribute to the understanding of the role of phenolic compounds in plant metabolism.

CHAPTER II

MATERIALS AND METHODS

W-38 Tobacco Tissue Culture

The source material for the enzymes in this work was *Nicotiana tabacum* W-38 callus tissue culture. The tissue was grown on a revised medium of Linsmaier and Skoog (1965) with 2 mg/l IAA, 200 ug/l of kinetin, unless otherwise stated. The tissue was grown at room temperature under continual subdued light (1-foot candle). Three pieces of tissue approximately 5x5x3 mm were cut from 3-week-old stock tissue and placed in 50 ml of the following medium. All operations were carried out in a laminar flow hood utilizing sterile techniques.

W-38 Culture Medium	Per 1 liter
Skoog's salt solution	100 ml
Fe++-EDTA solution	5 ml
Sucrose	30 gm
Deionized water	865 ml
Adjust the pH to 5.6 with 0.1 N NaOH	
Inositol	100 mg
IAA solution	20 ml
Kinetin solution	2 ml
Thiamine solution	10 ml

Skoog Salt Solution	1 liter
$NH_4\ NO_3$	16.5 gm
$CaCl_2.2H_2O$	4.4 gm
KNO_3	19.0 gm
$MgSO_4.7H_2O$	3.7 gm
KH_2PO_4	1.7 gm
H_3BO_3	62 mg
$MnSO_4.H_2O$	169 mg
$ZnSO_4.4H_2O$	86 mg
Minor salt solution	10 ml

Minor Salt Solution	1 liter
KI	830 mg
$Na_2MoO_4.2H_2O$	250 mg
$CuSO_4.7H_2O$	25 mg
$CoCl_2.6\ H_2O$	25 mg

Fe++ - EDTA Solution	1 liter
$FeSO_4$	5.52 gm
EDTA	3.24 gm

IAA Solution

IAA 10 mg / 100 ml H_2O

Thiamine Solution

Thiamine hydrochloride 10 mg / 100 ml H_2O

Kinetin suspension

Kinetin 10 mg / 100 ml H_2O

Dispense 50 ml of medium into a 125 ml Erlenmeyer flask containing 0.5 gm agar. Autoclave 15 minutes at 121°C and 18 psi. Shake the flasks before solidification.

Disc Gel Electrophoresis

Enzyme solutions were analyzed for isoenzyme components by polyacrylamide disc gel electrophoresis according to the method of Ornstein and Davis (1962). Gels were composed of 7.5% acrylamide and 0.2% N, N-methylene bisacrylamide. Runs were made at pH 9.3. Bromophenol blue was added to the upper buffer to serve as a tracking dye. After completion of electrophoresis, the isoenzymes were visualized according to the procedures described by Schnarrenberger (1973) by placing the gels in solution containing 40 mM tris-chloride at pH 8.8, 5 mM $MgCl_2$, 250 µM $NADP^+$, 1 mM 6-phosphogluconate, 0.5 mg/ml p-nitroblue tetrazolium, and 25 µg/ml phenazine methosulfate.

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 (1964). 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 60 x 1.5 cm. The above buffer was used as the eluting buffer with a flow rate adjusted to 5 ml/hr. Two ml fractions were collected using a Gilson model FC-80K fractionator, and the absorbance at 280 mm was followed to detect the presence of protein.

Enzymatic activity was measured to determine the elution volume of the isoenzymes. 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 (1972). Seven cm gels with an acrylamide concentration of 7.5% were prepared. Two mg of each standard protein and samples of Band I and Band II were dissolved in 10 mm sodium phosphate (pH 7.0), and one part of this solution was mixed with nine parts of 10 mm sodium phosphate (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. This solution was incubated in a boiling 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 to facilitate layering. One hundred µl of this final protein solution

(approximately 0.1 mg of protein) was applied to the top of each electrophoresis gel.

After electrophoresis (8 mA/gel) for 4-1/2 hours, gels were removed and placed in a staining solution of 0.25% wt/vol, Coomassie Brilliant Blue in 45% (v/v) methanol and 9% (v/v) acetic acid for 4 - 6 hours. The gels were destained with 5% (v/v) methanol in 7.5% (v/v) acetic acid until dark protein bands were visible. Mobilities were calculated using the equation:

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

Molecular weights were calculated from a plot of log molecular weight versus mobility.

C) Finger Printing Related Methods

Two-dimensional chromatography and high voltage electrophoresis were performed according to the method of Helinski and Yanofsky (1962). Descending chromatography was done, using a solvent of iso-butanol: formic acid (90%): water (70:0.9:2.1) for approximately 10 hours with methyl red as a mobile indicator.

High voltage electrophoresis was performed, using the apparatus manufactured by Savant, for 75 minutes at 2,400 V. The solvent consisted of pyridine: acetic acid: water (1:10:300), pH 3.7. Quinine sulfate was used as an indicator. The chromatogram was visualized by spraying with 0.5% ninhydrin in

acetone, and heating at 100°C for 15 minutes.

Sample preparations were performed as described by Kim (1978), where 5 mg of each isoenzyme was dissolved in 1 ml of 0.05 M ammonium carbonate buffer, pH 8.3. The vials containing the isoenzymes were then immersed in a boiling water bath for 30 seconds. Eight µg of trypsin (Sigma) in 0.8 ml of the above buffer was added to each sample and incubated at 30°C in a water bath for 2 hours. The reaction mixture was placed in a boiling water bath for 5 minutes to stop the reaction and denature the unhydrolyzed proteins as well as trypsin. The samples were then centrifuged at 27,000 xg for 15 minutes. The resulting supernatant fluid was collected and lyophilized. The lyophilized sample was dissolved in 0.1 ml deionized water and then spotted on Whatman 3 MM filter paper.

Enzyme Assays

a. Glucose 6-phosphate dehydrogenase. The assay procedure of Hohorst (1965) was used with slight modification. All assays were performed with a Varian techtron model 635 uv-visible recording spectrophotometer. Each assay consisted of 100 mM tris.HCl buffer (pH 7.6), 5 mM Mg^{++}, 1 mM glucose-6-phosphate, 0.39 mM $NADP^{+}$ plus enzyme in a total volume of 3 ml.

b. 6-Phosphogluconate dehydrogenase. The assay procedure of Hohorst (1965) was used as mentioned above. The assay mixture contain 100 mM tris-HCl buffer (pH 7.6), 5 mM Mg^{++}, and

different concentration of 6-phosphogluconate and NADP+ varied depending on the assay. Saturating levels of 6-phosphogluconate and $NADP^+$ were 2.62 mM and 0.40 mM, respectively.

Enzyme Preparations

Two parts of tissue were mixed with one part of glass beads, one part of washed hydrated polyclar AT and two parts of extraction solution. The extraction solution consisted of 100 mM tris-HCl buffer pH (8.5), 2 mM EDTA and 30 mM β-mercaptoethanol. The above mixture was homogenized in a Sorvall Omnimixer at 8000 rpm for 6 minutes. The Omnimixer cup was immersed in an ice path during homogenization. The homogenate was filtered through four layers of cheesecloth and centrifuged at 34,000 x g for 15 minutes. The supernatant was used as a source of glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase.

Polyclar AT

The polyclar AT used in the homogenization was washed with several solvents prior to use. First, 250 grams of polyclar AT were soaked in 1000 ml of deionized distilled water for 1 hour and suction-filtered. The polyclar AT was placed in the following solvents consecutively and soaked for 30 minutes, then 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
Polyclar AT (polyvinylpolypyrrolidone)	GAF Corporation, Chemical Division
DEAE-cellulose (preswollen), CM-cellulose	Reeve Angel Corporation
Sephadex G-150	Pharmacia Fine Chemicals Incorporated
Creatine kinase	Worthington Biochemical Corporation

The phenolic solutions used in the effector studies were prepared just prior to use and the container flasks were kept wrapped in aluminum foil. It was found that the phenolic solutions were stable for only a few days (Hoover 1977).

CHAPTER III

EFFECT OF KINETIN AND INDOLEACETIC ACID ON THE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, 6-PHOSPHOGLUCONATE DEHYDROGENASE AND ON GROWTH OF W-38 TOBACCO CELLS

Evidence presented earlier pointed to the possible disruption of the scopoletin synthesis or degradation system associated with normal plant anabolism under conditions of disease or injury. The level of scopoletin and scopolin in tobacco tissue has been shown to be markedly influenced by the supply of 3-indoleacetic acid (IAA) and kinetin in the nutrient medium (Skoog 1961 and Sargent 1960). If the pentose phosphate pathway is involved in phenolic biosynthesis, it can be assumed that a change in phenolic concentration could be related to a change in the key enzymes controlling such a pathway.

Cultures of tobacco tissue were grown on medium containing various combinations of kinetin (0-1.0 mg/l) and IAA (0-50) mg/l). Following three and four weeks of growth period, the cells were harvested and G6PD and 6PGD were assayed in the cell extract.

Table 1 shows G6PD activities after 21 days of cellular growth. In general, at any constant IAA, the activity of G6PD

TABLE 1

ACTIVITIES OF G-6-P DEHYDROGENASE (μ MOLES NADPH FORMED/ MIN/GM FRESH WEIGHT) AFTER 21 DAYS OF GROWTH

(± represents standard deviation)

KINETIN CONC. (mg/l)	3-INDOLEACETIC ACID CONC. (mg/l)					
	0	0.1	2.0	10.0	25.0	50.0
0.0	0.19±.04	0.21±	0.18±.02	0.44±.04	0.33±.04	0.35±.01
0.001	0.22±.01	0.34±.06	0.31±.07	0.37±.02	0.42±.07	0.49±.11
0.010	0.34±.04	0.36±.09	0.41±.06	0.40±.07	0.41±.06	0.59±.12
0.100	0.44±.06	0.48±.02	0.58±.08	0.48±.07	0.59±.07	0.69±.12
0.200	0.46±.07	0.62±.11	0.58±.08	0.65±.06	0.59±.07	0.75±.14
1.00	0.74±.07	0.74±.13	0.83±.13	0.93±.12	0.82±.07	0.90±.15

seems to increase as the amount of kinetin added to the growth medium was increased. This trend of G6PD activity increase holds at most of the kinetin and IAA combinations tested. At any constant kinetin levels, the amount of IAA added to the growth medium influences the G6PD activity. For example, among the IAA combination used, the lowest G6PD activities were obtained when the cells were grown on a medium containing no IAA. Moreover, the increase in IAA added to the growth medium either increase or maintain a high G6PD activity. In general, G6PD activity increase may be accounted for in combinations containing high IAA. Those combinations of IAA and kinetin that did not give rise to G6PD activity increase may indicate a summation of different effects of IAA and kinetin on the cell metabolic system. Moreover, the increase of G6PD activities due to higher IAA combinations became less pronounced as the kinetin level added was increased. The activities of 6PGD at the end of three weeks of growth period are shown in Table 2. In general, this enzyme like G6PD is increased in activity as the amount of kinetin added to the growth medium was increased. The 6PGD activity increase was observed at most IAA and kinetin combinations used.

At a constant kinetin level in the growth medium, the increase in the amount of IAA added influences 6PGD activity. This activity change may be divided into three groups, at low (0-0.1 mg/l), intermediate (2-10 mg/l) and high (25-50 mg/l) IAA combinations. The combinations containing intermediate levels of IAA caused a slight decrease in 6PGD activity in

TABLE 2

ACTIVITIES OF 6-PG DEHYDROGENASE (μ MOLES NADPH FORMED/ MIN/GM FRESH WEIGHT) AFTER 21 DAYS OF GROWTH

(± represents standard deviation)

KINETIN CONC. (mg/l)	3-INDOLEACETIC ACID CONC. (mg/l)					
	0	0.1	2.0	10.0	25.0	50.0
0.0	0.32±.05	0.35±.03	0.31±.05	0.31±.02	0.40±.02	0.28±.02
0.001	0.38±.08	0.40±.03	0.29±.05	0.33±.08	0.40±.07	0.41±.07
0.010	0.39±.03	0.41±.07	0.36±.04	0.38±.08	0.40±.11	0.50±.12
0.100	0.43±.07	0.55±.13	0.49±.07	0.43±.07	0.58±.02	0.61±.16
0.200	0.51±.03	0.56±.12	0.54±.03	0.52±.06	0.58±.04	0.73±.11
1.00	0.52±.02	0.70±.13	0.73±.13	0.79±.06	0.80±.12	0.91±.07

comparison to low and high IAA combinations. Moreover, the increase in IAA from 0 mg/l to 0.1 mg/l IAA within the low IAA combinations caused an increase in 6PGD activity. At intermediate IAA combinations, no significant change in 6PGD activity was observed in changing the IAA level from 2.0 mg/l to 10 mg/l.

Comparing G6PD activities with 6PGD activities in the cell extract at the end of three weeks, higher 6PGD activities were obtained only at low IAA and low kinetin concentrations.

Table 3 shows the activities of G6PD at the end of four weeks of growth period. At a constant IAA concentration, increasing the kinetin level produced increased G6PD activity in the cell extract in most combinations of IAA and kinetin used. At any constant kinetin level in the growth medium, the increase in the amount of IAA added, influence G6PD activity. The IAA influence on G6PD at 28 days of growth may be divided into three groups, low (0 - 1 mg/l), intermediate (2-10 mg/l) and high (25-50 mg/l) IAA combinations. As for the low IAA combinations, the G6PD activity was increased when IAA concentration increased from 0 mg/l to 0.1 mg/l IAA. The same conclusion was drawn from the high IAA combinations, where an increase in IAA from 25 mg/l to 50 mg/l, increase G6PD activity. On the other hand, a slight increase or decrease of G6PD activity was obtained as the level of IAA changes within the intermediate IAA combinations.

TABLE 3

ACTIVITIES OF G-6-P DEHYDROGENASE (μ MOLES NADPH FORMED/ MIN/GM FRESH WEIGHT) AFTER 28 DAYS OF GROWTH

(± represents standard deviation)

KINETIN CONC. (mg/l)	3-INDOLEACETIC ACID CONC. (mg/l)					
	0	0.1	2.0	10.0	25.0	50.0
0.001	0.23±.05	0.32±.02	0.28±.02	0.21±.02	0.44±.08	0.63±.07
0.010	0.25±.01	0.45±.07	0.30±0	0.36±.05	0.51±.04	0.63±.13
0.100	0.26±0	0.44±.04	0.59±.05	0.49±.04	0.56±.10	0.75±.02
0.200	0.41±.01	0.55±.10	0.59±.02	0.53±.04	0.55±.08	0.87±.07
1.00	0.46±.02	0.63±.06	0.85±.06	0.86±.07	0.91±.07	1.1±.02

The 6PGD activities in the cell extract at the end of four weeks growth period are shown in Table 4. In general, it was found that at constant IAA level, as the amount of kinetin added to the growth medium was increased, 6PGD activity was increased. This was found to be true on most of IAA and kinetin combinations tested. At any constant kinetin level, high (25-50 mg/l) IAA combinations increase the 6PGD activity relative to low (0-0.1 mg/l) or intermediate (2-10 mg/l) IAA combinations. Comparing the 6PGD activities within the range of low and high IAA combinations, reveal the increase in the 6PGD activities as the IAA concentration increases from the lower limit to the higher limit of such combinations.

Comparing G6PD activities at 21 days and 28 days, higher G6PD activities were more frequent at 0 mg/l IAA combinations than any other combination. Moreover, about the same activities of G6PD at 21 and 28 days were generally obtained at other than 0 mg/l IAA combinations. The variation of each value under the different combinations of IAA and kinetin plays a significant role in arriving to such conclusion. The same conclusion may be drawn when 6PGD activities were compared at 21 and 28 days.

A time study was performed on G6PD and 6PGD activities of cells grown under two combinations of IAA and kinetin levels. One combination, designated as the control, was 0.2 mg/l kinetin plus 2.0 mg/l IAA. The other combination, designated

TABLE 4

ACTIVITIES OF 6-PG DEHYDROGENASE (μ MOLES NADPH FORMED/ MIN/GM FRESH WEIGHT) AFTER 28 DAYS OF GROWTH

(± represents standard deviation)

KINETIN CONC. (mg/l)	3-INDOLEACETIC ACID CONC. (mg/l)					
	0	0.1	2.0	10.0	25.0	50.0
0.001	0.25±.01	0.38±.02	0.28±.02	0.22±.03	0.32±.04	0.54±.04
0.010	0.21±.03	0.39±.02	0.311±.01	0.28±.03	0.39±.03	0.54±.01
0.100	0.27±.06	0.41±.04	0.41±.02	0.35±.06	0.41±.05	0.69±.01
0.200	0.40±.01	0.41±.01	0.52±.02	0.39±.06	0.52±.02	0.88±.01
1.00	0.54±.03	0.46±.04	0.75±.02	0.87±.04	0.88±.04	0.97±.06

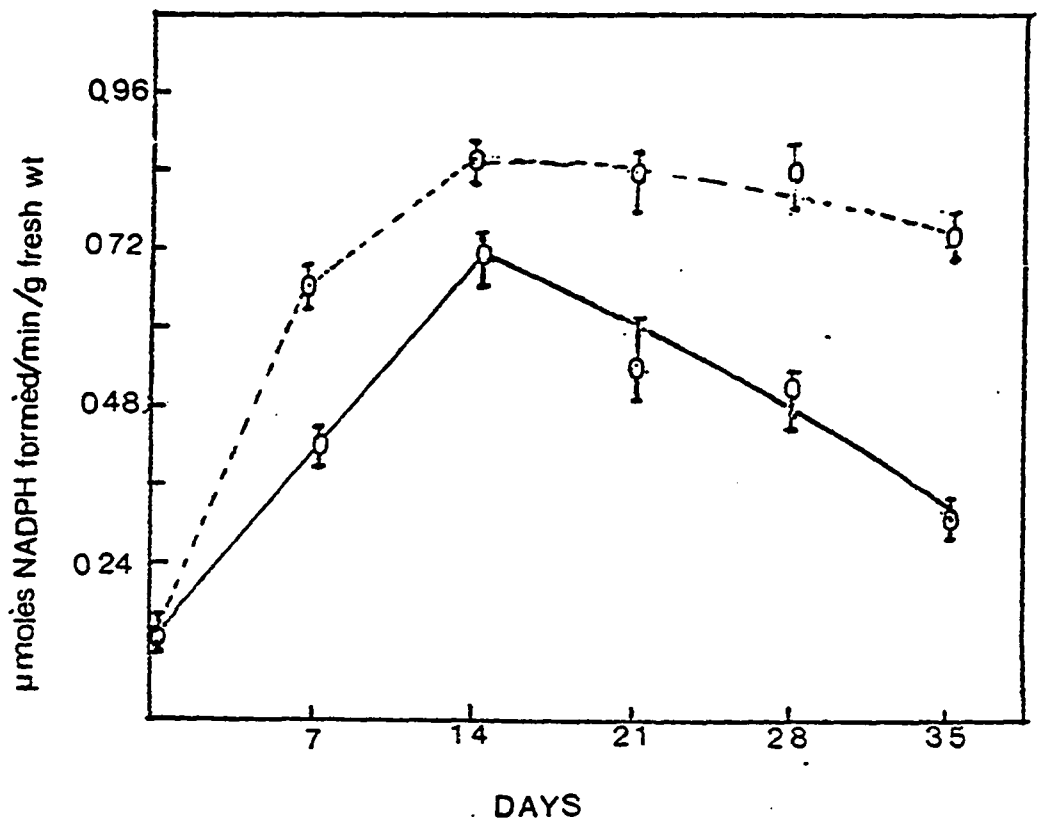

Figure 3. Activities of G6PD (moles NADPH formed /min /g. fresh weight) at various times of growth. Deviations from the mean value are shown.
O——O Control O----O High kinetin.

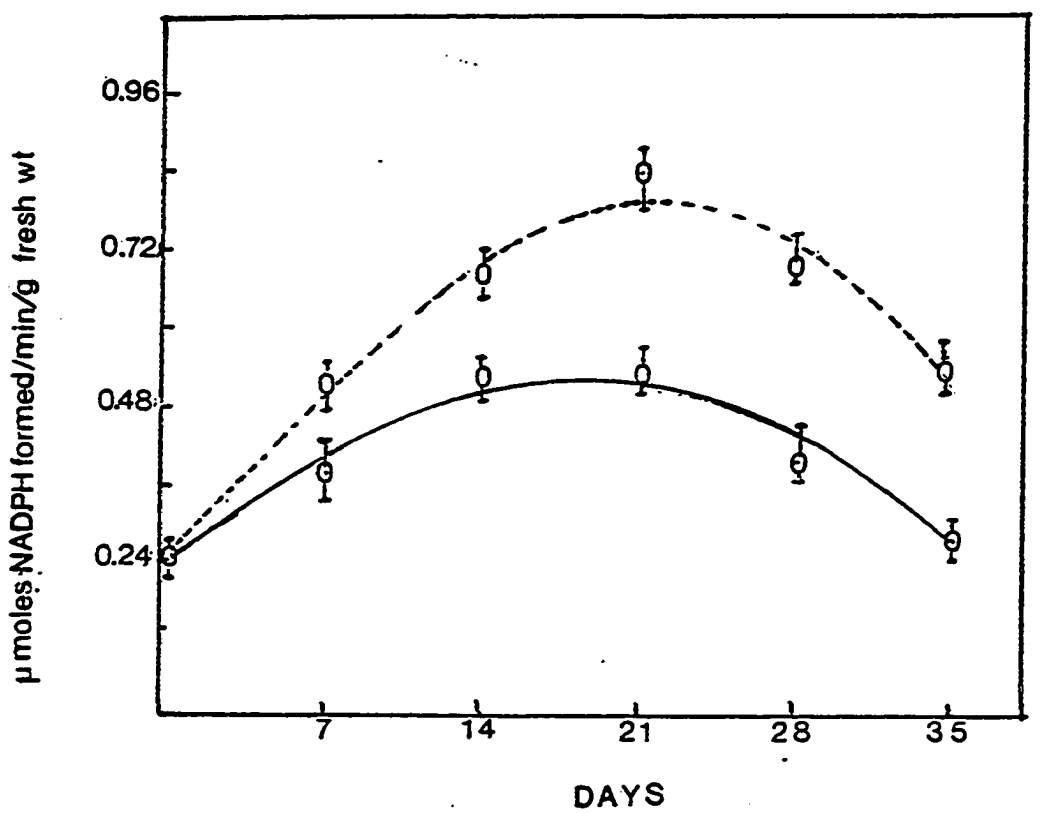

Figure 4. Activities of 6PGD (µmoles NADPH formed /min /g. fresh weight) at various times of growth. Deviations from the mean value are shown.
O——O Control O·····O High kinetin.

as high kinetin, contained 1.0 mg/l kinetin plus 0.1 mg/l IAA. As shown in Figures 3 and 4, the high kinetin cells maintained higher G6PD and 6PGD activities than those of the control over a period of 5 weeks. Such results may indicate a long term effect of kinetin over G6PD and 6PGD.

Growth of W-38 Tobacco Cells

Table 5 shows the effect of different combinations of IAA and kinetin on the fresh weight after 21 days. It is important to indicate that the fresh weight obtained in each flask depends also on the surface area of the inoculum exposed to the growth medium. Under our conditions, maximal growth per flask was obtained at 2-10 mg/l (IAA) in the growth medium. At any IAA level increasing the kinetin concentration in the growth medium tended to reduce the tissue fresh weight. Low kinetin concentrations, however, tended to induce loose cell aggregation. Optimal cellular growth with the least loose aggregation may be obtained at any combination of 0.1-10 mg/l IAA and 0.1-.200 mg/l kinetin. The effect of kinetin and IAA on cellular fresh weight after 28 days of growth period is shown in Table 6. The effects that were observed after 21 days of growth were observed again after a growth period of 28 days.

In correlating the growth rates with G6PD and 6PGD activities, Scott (1964) found that the activities of those enzymes decreased upon increasing growth rate. Our findings

TABLE 5

G/FLASK FRESH WEIGHT OF W-38 TOBACCO CELLS AFTER 28 DAYS OF GROWTH

(± represents standard deviation)

Kinetin Conc. mg/l	3-Indoleacetic Acid Conc. (mg/l)					
	0	0.1	2.0	10.0	25.0	50.0
0.001	5.64 ±.52	5.7 ±1.0	13.9 ±.9	14.0 ±.63	3.66 ±. 5	4.51 ±.55
0.010	3.85 ±.35	5.65 ±.35	10.6 ±1.4	9.85 ±.60	5.4 ±.8	5.43 ±.93
0.100	2.89 ±.36	4.78 ±.92	7.75 ±2.25	5.00 ±.15	5.45 ±.95	2.95 ±.45
0.200	4.2 ±.3	4.65 ±1.15	5.92 ±.29	8.53 ±1.89	5.30 ±.80	3.75 ±.75
1.00	3.2 ±.5	3.87 ±.13	3.28 0.70	2.62 0.70	1.79 ±.27	2.21 ±.34

TABLE 6

G/FLASK FRESH WEIGHT OF W-38 TOBACCO CELLS AFTER 21 DAYS OF GROWTH

(± represents standard deviation)

Kinetin Conc. mg/l	3-Indoleacetic Acid Conc. (mg/l) 0	0.1	2.0	10.0	25.0	50.0
0.001	3.64 ±.30	3.94 ±.20	6.90 ±0.10	5.67 ±.47	3.30 ±.32	3.58 ±.32
0.010	3.46 ±.30	5.70 ±.80	5.35 ±.35	6.45 ±1.15	5.27 ±.50	2.12 ±.22
0.100	2.54 ±.13	4.23 ±.51	4.73 ±.23	4.9 ±1.5	3.14 ±.33	2.07 ±.19
.200	3.20 ±.40	3.91 ±.22	4.67 ±.07	3.93 ±.70	3.01 ±.15	1.78 ±.48
1.00	2.22 ±.30	2.68 ±.20	1.93 ±.41	1.41 ±.10	2.1 ±.28	1.20 ±.10

came in agreement with such results. However, this was found to be true in correlating growth rates and activities of G6PD and 6PGD at a constant IAA concentration with kinetin concentrations between 0.001-1.00 mg/l.

CHAPTER IV

ISOLATION OF BAND I AND BAND II OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

6-Phosphogluconate dehydrogenase activity isolated from tobacco tissue culture W-38 exists as two anodic bands when observed after polyacrylamide gel electrophoresis (Figure 5). The slow migrating band toward the anode is designated as Band I and the fast migrating band is designated as Band II. The corresponding mobilities of Band I and Band II on 7.5% polyacrylamide gels were 0.26 and 0.40, respectively. In order to determine the metabolic role of these 6-phosphogluconate dehydrogenase, the individual bands have been isolated, and their physical and kinetic properties have been determined.

When the kinetic studies were the object of investigation, Band I was separated from Band II using a DEAE-cellulose column equilibrated with 0.1 M imidazole buffer pH 6.5 . Included in the buffer were 30mM β-mercaptoethanol and 10^{-5} M NADP+ to maintain enzymatic activity. When the object was to get pure Band I and Band II for peptide mapping, a buffer which was 50 mM imidazole, pH 6.5, made in 30 mM β-mercaptoethanol in 10% glycerol V/V was

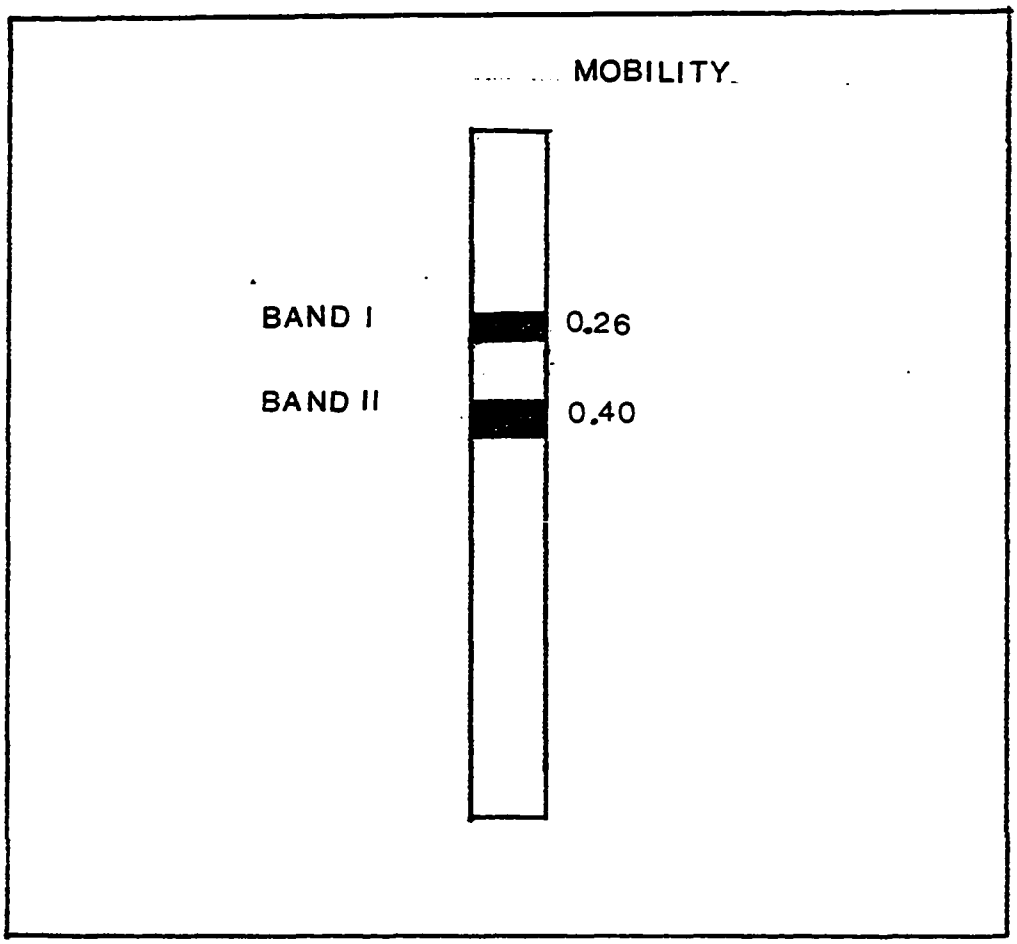

Fig. 5 Anodic polyacrylamide disc gel electrophoresis of 6-phosphogluconate dehydrogenase from W-38 tobacco tissue culture. Mobilities are relative to bromophenol blue. Bands were visualized with a solution containing 40 mM tris.MCl at pH 8.8, 5 mM Mg^{++}, 250 µM $NADP^{+}$, 1 mM 6-phosphogluconate, 0.5 mg/ml p-nitroblue tetrazolium, and 25 µg/ml phenazine methosulfate.

used. The addition of glycerol maintained the enzymatic activity for one month (at 4°C) without any significant loss. All purification steps were carried out at 4-6°C and summarized in Table 7.

Crude Enzyme Preparation

Purification of 6PGD was begun with 350 grams of W-38 tobacco tissue. The cells were mixed with 175 grams of glass beads, 175 grams of polyclar AT (which had been hydrated for 1/2 hour prior to use) and 700 ml of the 4-6°C cold extraction solution. The extraction solution was 100 mM.tris.HCl buffer pH (8.5) containing 2 mM EDTA and 30 mM β-mercaptoethanol. The polyclar AT was present to adsorb any phenolic compound released from the cells during homogenization. The above mixture was homogenized in a Sorvall Omnimixer at 8000 rpm for six minutes. The Omnimixer cup was immersed in an ice bath during the homogenization. The homogenate was filtered through four layers of cheesecloth and centrifuged at 34,000 x g for 15 minutes.

$(NH_4)_2SO_4$ Precipitation

The supernatant was saturated to 25% with solid $(NH_4)_2SO_4$ and centrifuged at 34,000 x g for 15 minutes. The resulting supernatant was saturated to 70% with solid $(NH_4)_2SO_4$ and centrifuged for 15 minutes.

TABLE 7

ISOLATION PROCEDURE FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE ISOENZYMES BAND I AND BAND II

STEP 1

Homogenize W-38 tobacco tissue in extraction solution for 6 minutes at 8000 rpm. Filter through 4 layers of cheesecloth. Centrifuge at 34,000 x g for 15 minutes. Save the supernatant.

STEP 2

Bring the supernatant to 25% saturation with solid $(NH_4)_2SO_4$. Centrifuge 10 minutes at 34,000 x g. Save the supernatant.

STEP 3

Bring the supernatant to 70% saturation with solid $(NH_4)_2SO_4$. Centrifuge 15 minutes at 17,000 x g. Dissolve the pellet in a small volume of 20 mM phosphate buffer pH (7.0) made in 10% glycerol V/V. Dialyze for two hours against 4 liters of the same buffer.

STEP 4

Mix with CM-cellulose equilibrated against the same buffer. Centrifuge at 10,000 x g for 10 minutes. Save the supernatant.

STEP 5

Bring the supernatant to 70% saturation with solid $(NH_4)_2SO_4$. Centrifuge for 20 min. at 28,000 x g. Dissolve the pellet in a small volume of 30 mM β-mercaptoethanol, 50mm imidazole-HCl buffer (pH 6.5) in 10% glycerol V/V. Dialyze against the same buffer overnight.

TABLE 7, Continued

STEP 6

Use a DEAE-cellulose column equilibrated in 50 mM imidazol buffer in 30 mM β-mercaptoethanol (pH 6.5) made in 10% glycerol V/V. Elute Band I in the equilibrium buffer. Elute Band II using buffer made in 100 mM NaCl. Concentrate Band I and Band II separately by ultrafiltration cell equipped with Pellicon PSAC membrane.

STEP 7

Bring the enzyme preparations of Band I and Band II to 75% saturation using solid $(NH_4)_2SO_4$. Centrifuge at 28,000 x g for 15 min. Dissolve the pellet in a small volume of 50 mM tris HCl buffer (pH 8.5) in 30 mM β-mercaptoethanol made in 10% glycerol. Dialyze for two hours in the same buffer.

STEP 8

Use a Sephadex G-150 column (90 x 2.5) equilibrated with 50mM tris.HCl buffer (pH 8.5) in 30 mM β-mercaptoethanol made in 10% glycerol V/V. Elute Band I or Band II using the same buffer. Concentrate by ultrafiltration as mentioned earlier. Dialyze four hours against the same buffer.

STEP 9

Use an affinity column equilibrated in 50 mM trisHCl buffer (pH 8.5) in 30 mM β-mercaptoethanol made in 10% glycerol. Elute Band I or Band II using equilibrium buffer made in 50 mM pyrophosphate. Concentrate Band I or Band II by ultrafiltration. Dialyze against deionized water using the same filter.

CM-cellulose Treatment

This pellet which contained the enzyme was dissolved in a small volume of 20 mM phosphate buffer (pH 7.0) made in 30 mM β-mercaptoethanol and 10% glycerol V/V.

The enzyme preparation was dialyzed for 12 hours against 4 liters of the phosphate buffer. CM-cellulose equilibrated against the same buffer was added to the enzyme preparation and mixed for 30 minutes. Then the CM-cellulose was removed by centrifugation at 10,000 x g for 10 minutes. The supernatant was then subjected to 70% saturated $(NH_4)_2SO_4$ and centrifuged for 20 minutes at 28,000 x g. The pellet was dissolved in a small volume of a solution containing 30 mM β-mercaptoethanol, and 50 mM imidazole HCl buffer (pH 6.5) in 10% glycerol V/V.

DEAE cellulose Chromatography

The enzyme preparation was dialyzed against four liters of the above mentioned 50 mM imidazole buffer pH 6.5 for 12 hours. The enzyme preparation was layered on the top of a DEAE-cellulose column equilibrated with the same 50 mM imidazole buffer pH 6.5 buffer. Band I was eluted in the equilibrium buffer. Band II was eluted using the equilibrium buffer made in 100 mM NaCl. The flow rate was maintained at 30 ml per hour with 9.6 ml fractions being collected. The most active fractions of each band were pooled separately and concentrated to 10-15 ml using an Amicon Model 50 or 52 ultrafiltration cell equipped with a Pellicon PSAC membrane filter under a stream of nitrogen gas. Of the total enzyme activity that has been

eluted from the column, Band I represented 40%, while Band II represented 60%.

The solution containing each band was brought to 75% $(NH_4)_2SO_4$ saturation and centrifuged at 28,000 x g for 15 minutes. The pellet of each band was dissolved in 50 mM tris.HCl buffer (pH 8.5) in 10% glycerol V/V made in 30 mM β-mercaptoethanol and dialyzed for two hours against the same buffer.

Sephadex G-150 Chromatography

The dialyzed enzyme preparation of Band I or Band II was layered on the top of a Sephadex G-150 column (90 x 2.5 cm) equilibrated with the above mentioned 50 mM tris.HCl buffer (pH 8.5). The enzyme was eluted with the equilibrium buffer, located and concentrated to 5-10 ml using ultrafiltration cell as mentioned. The concentrated enzyme solution was dialyzed for two hours using the same 50 mM tris.HCl buffer (pH 8.5).

NADP+ agarose Affinity Column Chromatography

Two ml at a time of the enzyme preparation was layered on the top of a 15 ml NADP+ agarose affinity column equilibrated against the 50 mM tris.HCl buffer (pH 8.5) mentioned earlier. Ligand concentration varied from 2-3.6 μM NADP+/g moist weight of the column material in each bach purchased. An equal amount of the 50 mM tris.HCl buffer (pH 8.5) was used to wash the enzyme into the column. After the column was

washed with 50 ml of the 50 mM tris.HCl buffer (pH 8.5) mentioned, the enzyme was eluted from the column using the 50 mM tris.HCl buffer (pH 8.5) made in 50 mM pyrophosphate. The column flow rate was adjusted to 20 ml per hour and 4.5 ml fractions were collected. The most active fractions were pooled, concentrated and dialyzed against deionized water using the ultrafiltration cells mentioned earlier. The dialysis was necessary to get rid of all the glycerol contained in the buffer. A sample of the enzyme preparation of each band was analyzed by polyacrylamide gel electrophoresis and gave a single band when stained for protein or enzyme activity. The enzyme preparation of Band I or Band II which was free of other proteins was lyophilized and prepared for finger printing. The affinity column was washed with 100 ml of 6 mM urea solution and equilibrated again for reuse. These same procedures applied in the affinity column have been successfully used in the purification of 6-phosphogluconate dehydrogenase from sheep liver (Griffiths 1977) and rabbit mammary glands (Betts 1975).

CHAPTER V

KINETIC AND PHYSICAL PROPERTIES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE ISOENZYMES

All kinetic studies have been done on a partially purified preparation of Band I and II. After the 25-70% $(NH_4)_2SO_4$ fractionations described in Table 7, Band I was isolated from Band II using DEAE-cellulose column equilibrated with 0.1 M imidazole buffer (pH 6.5). Included in the buffer were 30 mM β-mercaptoethanol and 10^{-5}M NADP+ to maintain enzymatic activity. Due to stability problems, Band I assays had to be performed no later than 24 hours after isolation. Band II could be assayed within a week of the isolation without any significant loss of activity.

For Band I the plot of pH versus velocity yields an optimal pH value above 7.0. No change in the maximum activity was noted over the pH range from 7-9 (Figure 6). The same result was obtained for Band II (Figure 7). For Band I, a saturation curve with respect to 6-phosphogluconate as a substrate is shown in Figure 8. The corresponding double reciprocal plot is shown in Figure 9. A smooth saturation curve and a linear Lineweaver-Burk plot yields

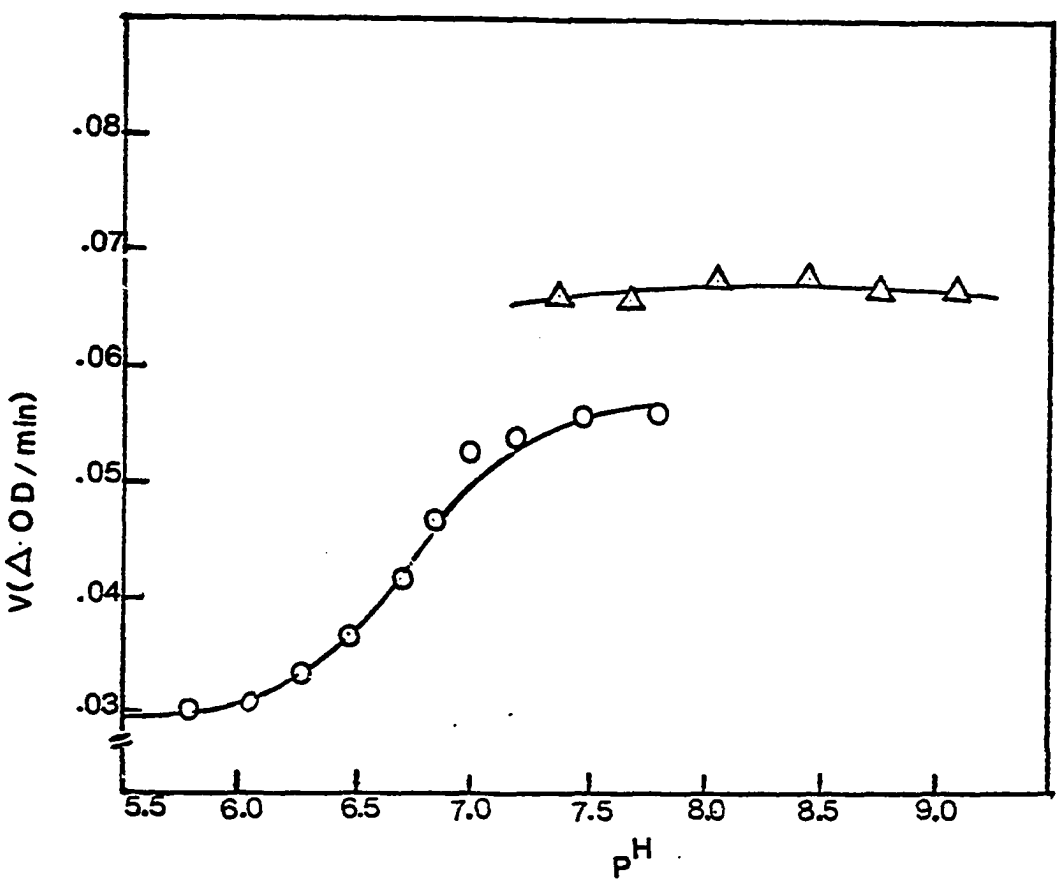

Fig. 6 pH profile for 6-phosphogluconate dehydrogenase band I. The assays contained 2.7 mM 6-phosphogluconate, 3.4 mM Mg^{+2} and 0.4 mM NADP+ in the following buffers, O—O .1 M Tris.malate, Δ—Δ .1M Tris.HCl.

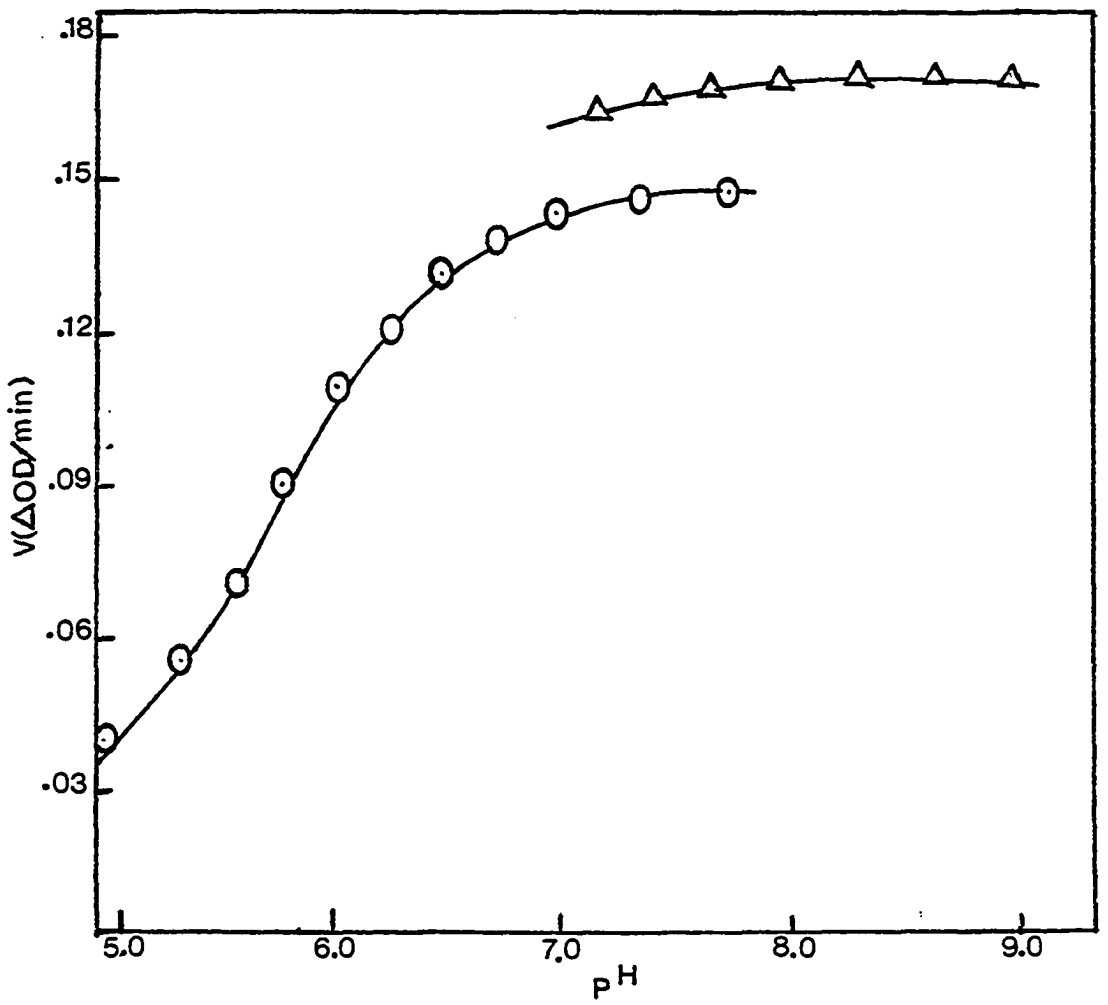

Fig. 7 pH profile for 6-phosphogluconate dehydrogenase isoenzyme band II. The assays contained 2.7 mM 6-phosphogluconate, 3.4 mM Mg^{+2}, and 0.4 mM NADP+ in the following buffers, Δ—Δ .1M tris malate O—O .1M tris.HCl.

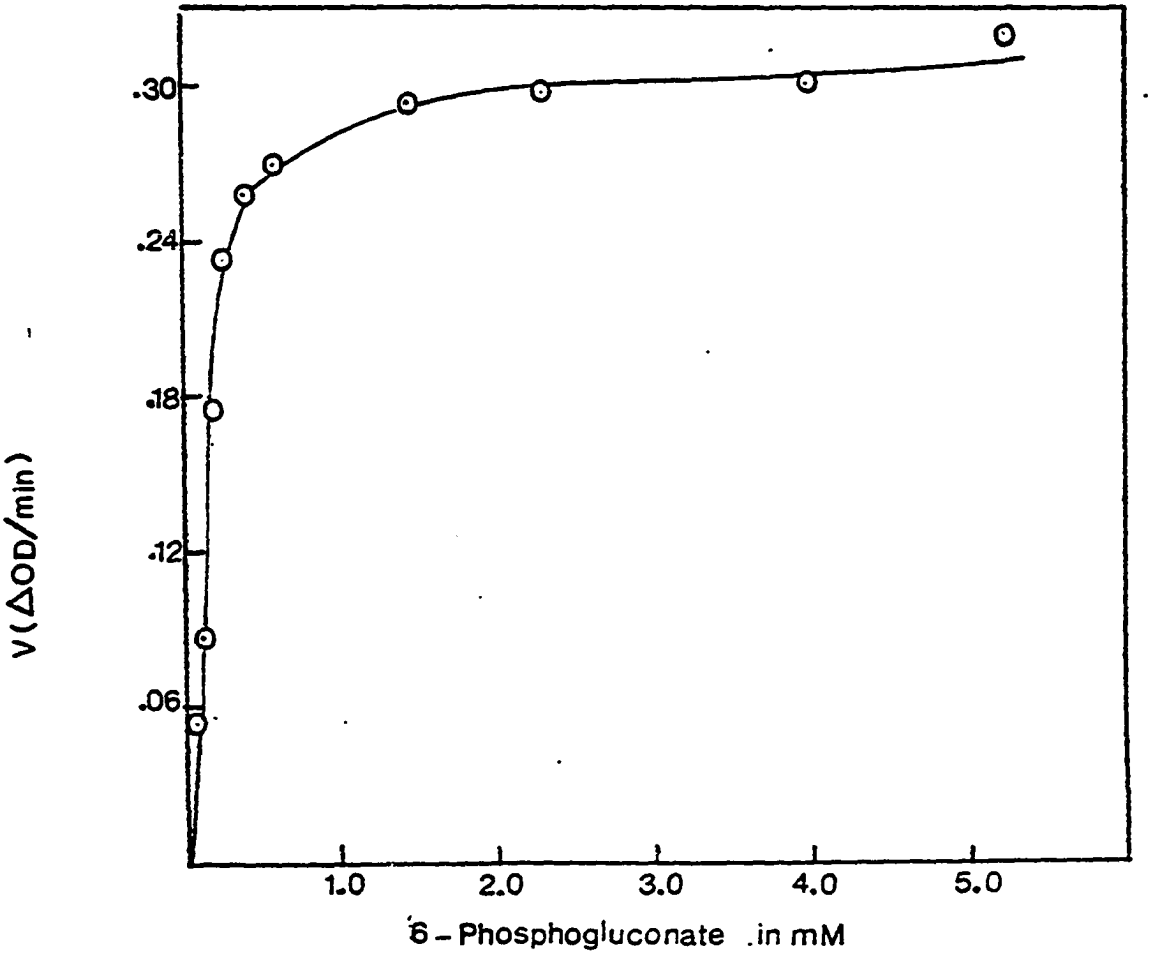

Fig. 8 6-Phosphogluconate saturation curve for 6-phosphogluconate dehydrogenase band I. Each assay contained 3.4 mM Mg++, 0.4 mM NADP+ in 100 mM tris. HCl buffer, pH 7.8 . 6-phosphogluconate was varied.

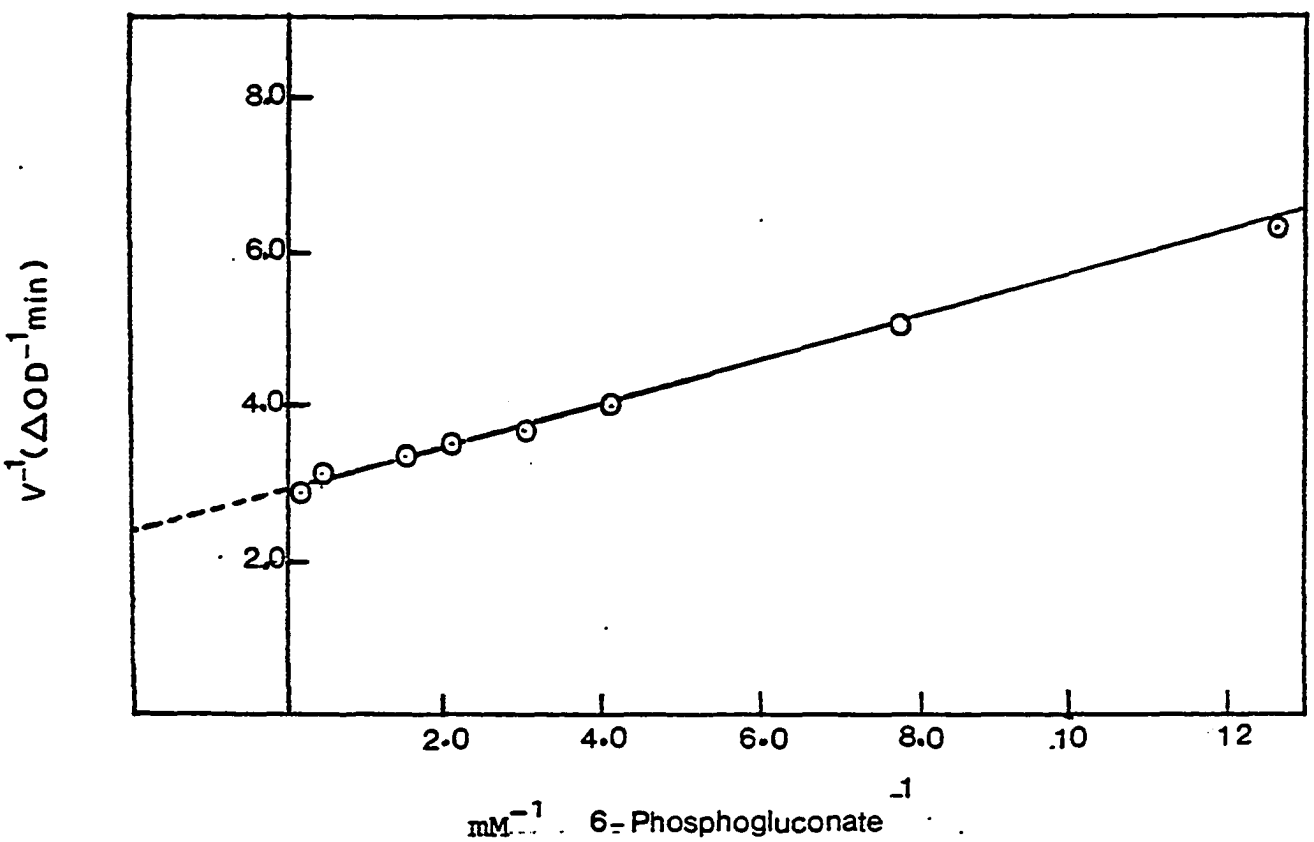

Fig. 9. 6-Phosphogluconate double reciprocal plot for 6-phosphogluconate dehydrogenase band I.

an $S_{0.5}$ value of 0.087 mm. A Hill coefficient of 0.96 calculated from the Hill plot (Fig. 10) indicates Michaelis-Menten type enzyme molecule.

Band II saturation curve (Fig. 11) and Lineweaver-Burk plot (Fig. 12) indicate an $S_{0.5}$ value of 0.078 mM of 6-phosphogluconate. Band I has a Hill coefficient of 0.97 as denoted from the Hill plot (Fig. 13), this indicates Michaelis-Menten type enzyme molecule. With respect to NADP+, as a substrate, Band II saturation curve (Fig. 17) is a smooth Michaelis-Menten type of saturation curve. The double reciprocal plot (Fig. 18) is linear and yields an $S_{0.5}$ value of 3.0 µM. A Hill plot (Fig. 19) gives a slope of 1.15 and may indicate some kind of interaction of the binding site of NADP+. The nature of such interaction is not known at present. Band I saturation curve (Fig. 14) and double reciprocal plot (Fig. 15) reveal an $S_{0.5}$ value of 2.7 µM. A Hill plot (Fig. 16) yields a slope of 1.0

The molecular weights of Bands I and II were determined by two different methods. A Sephadex G 150 column prepared by the method of Andrews (1964) in a 60 x 1.5 cm Ace glass column was used. The equilibrating and eluting buffer was 0.1 imidazole (pH 6.5) containing 30 mM β-mercaptoethanol and 10^{-5}M NADP+. The column was first standardized using lipoxidase (M W 108,000), creatine kinase (M W 80,000), serum albumin (M W 68,000) ovalbumin (M W 43,000) and α-chymotrypsinogen-A, (M W 25,700). The

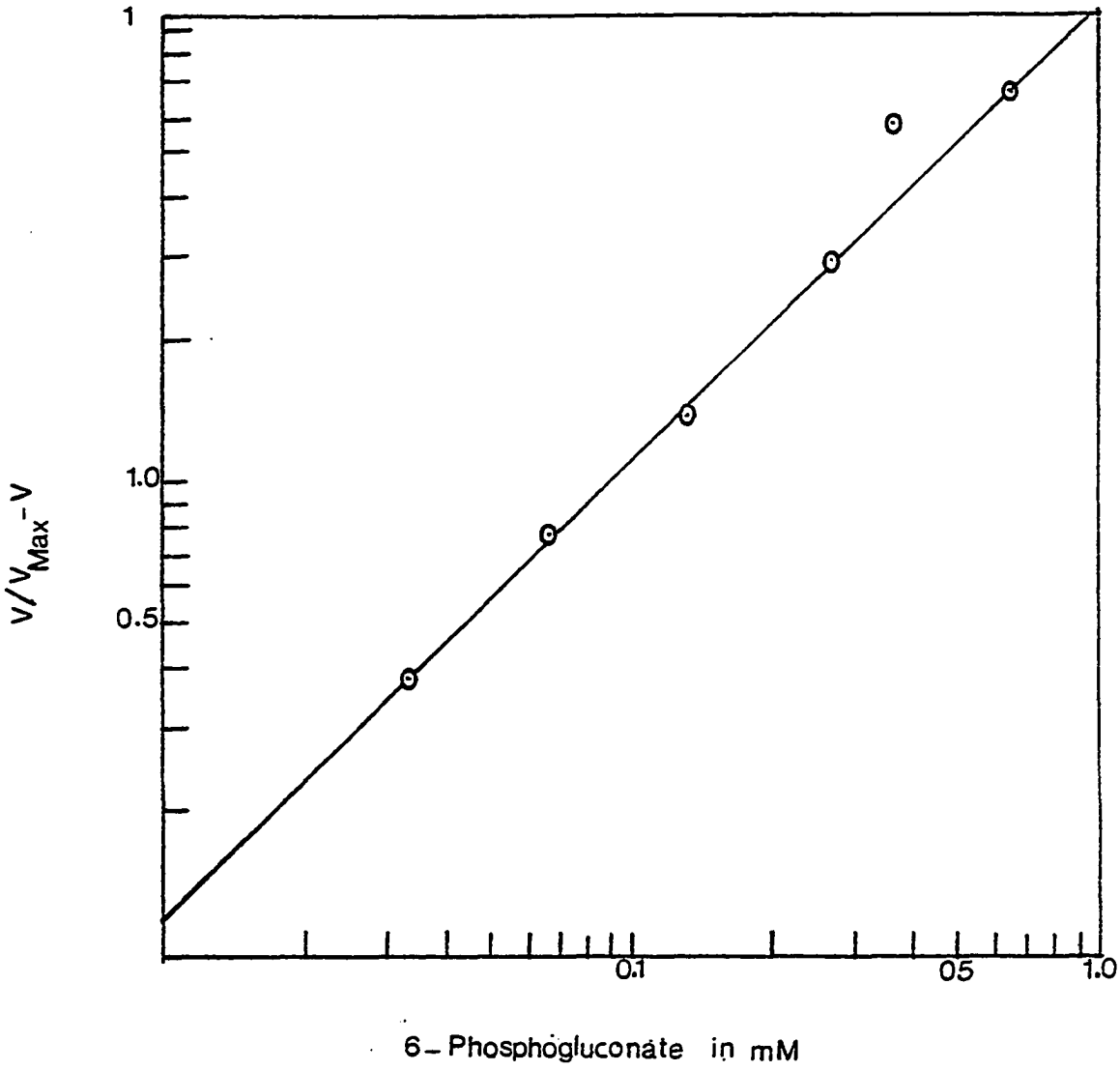

Fig. 10 6-Phosphogluconate Hill plot for 6-phosphogluconate dehydrogenase band I.

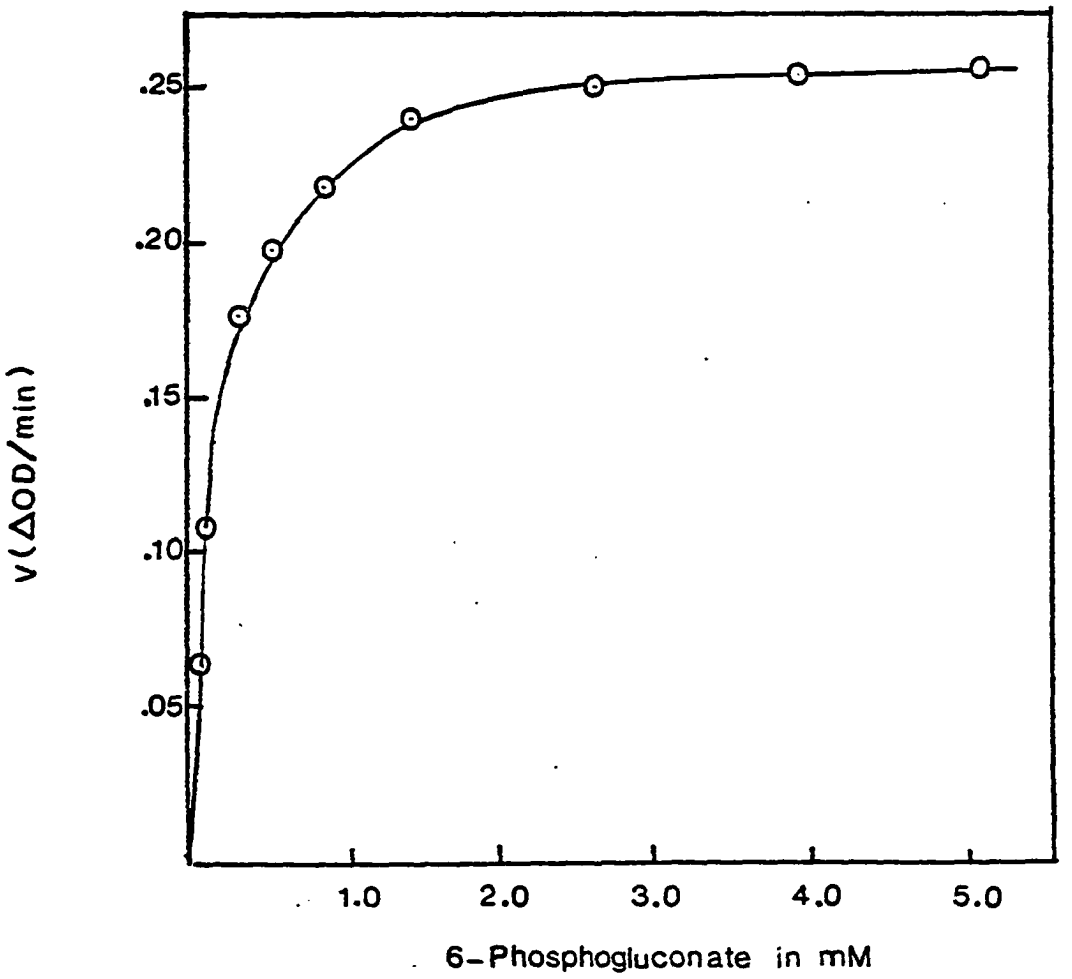

Fig.11 6-Phosphogluconate saturation curve for 6-phosphogluconate dehydrogenase band II. Each essay consisted of 3.4 mM Mg++, 0.4 mM NADP+ and various amount of 6-phosphogluconate in 100 mM tris.HCl buffer pH 7.8 .

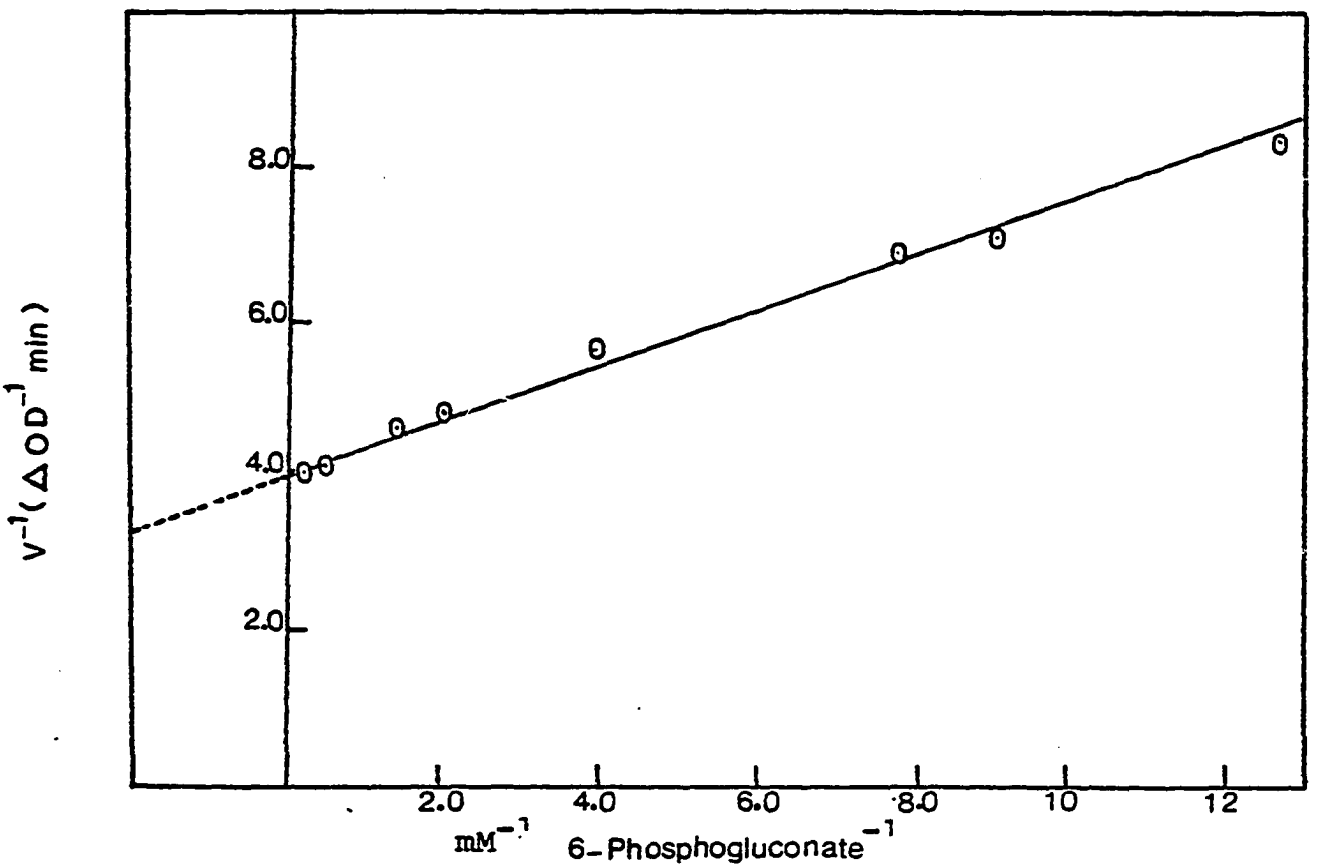

Fig. 12 6-Phosphogluconate double reciprocal plot for 6-phosphogluconate dehydrogenase band II.

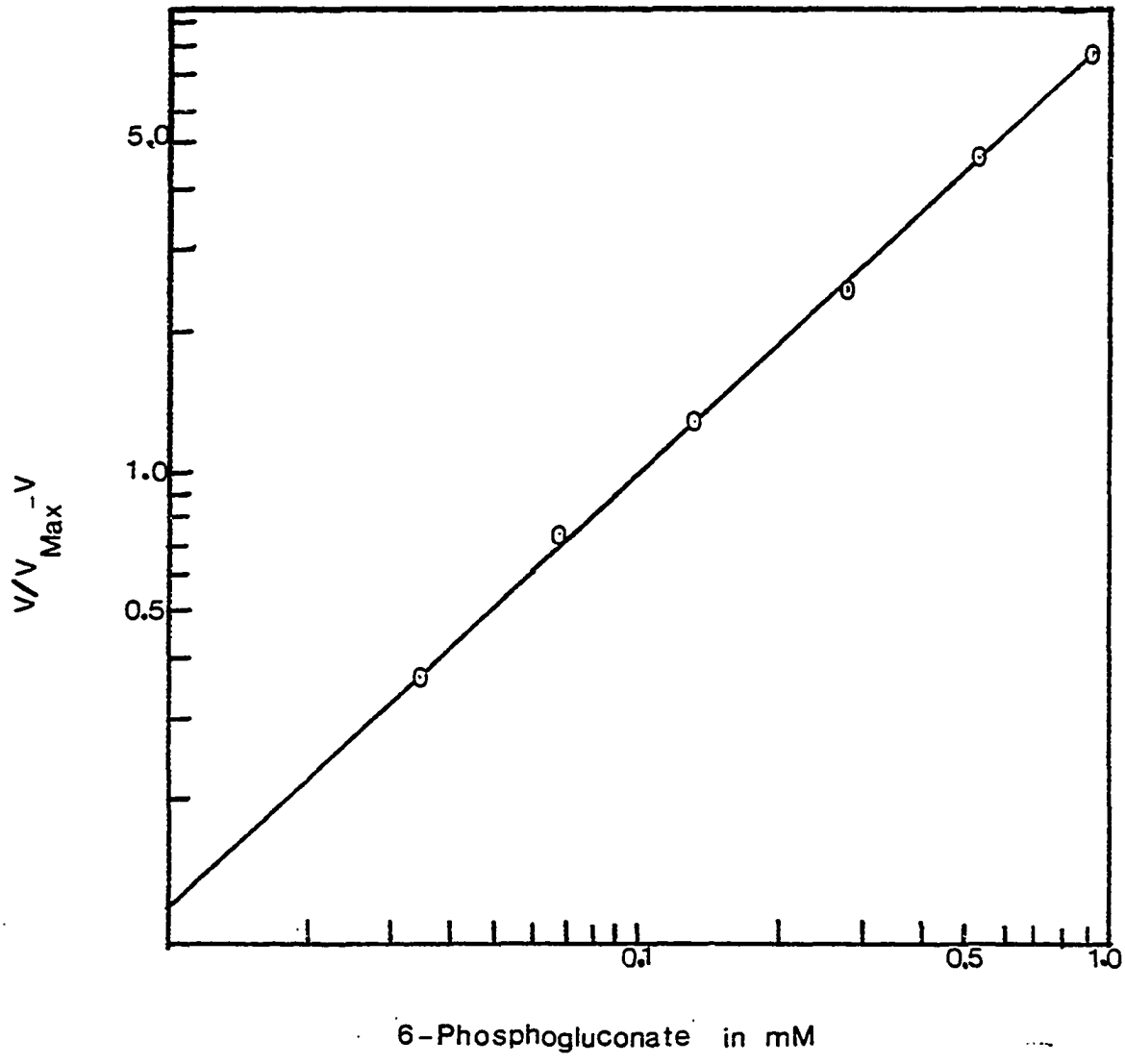

Fig. 13 6-Phosphogluconate Hill plot for 6-phosphogluconate dehydrogenase band II.

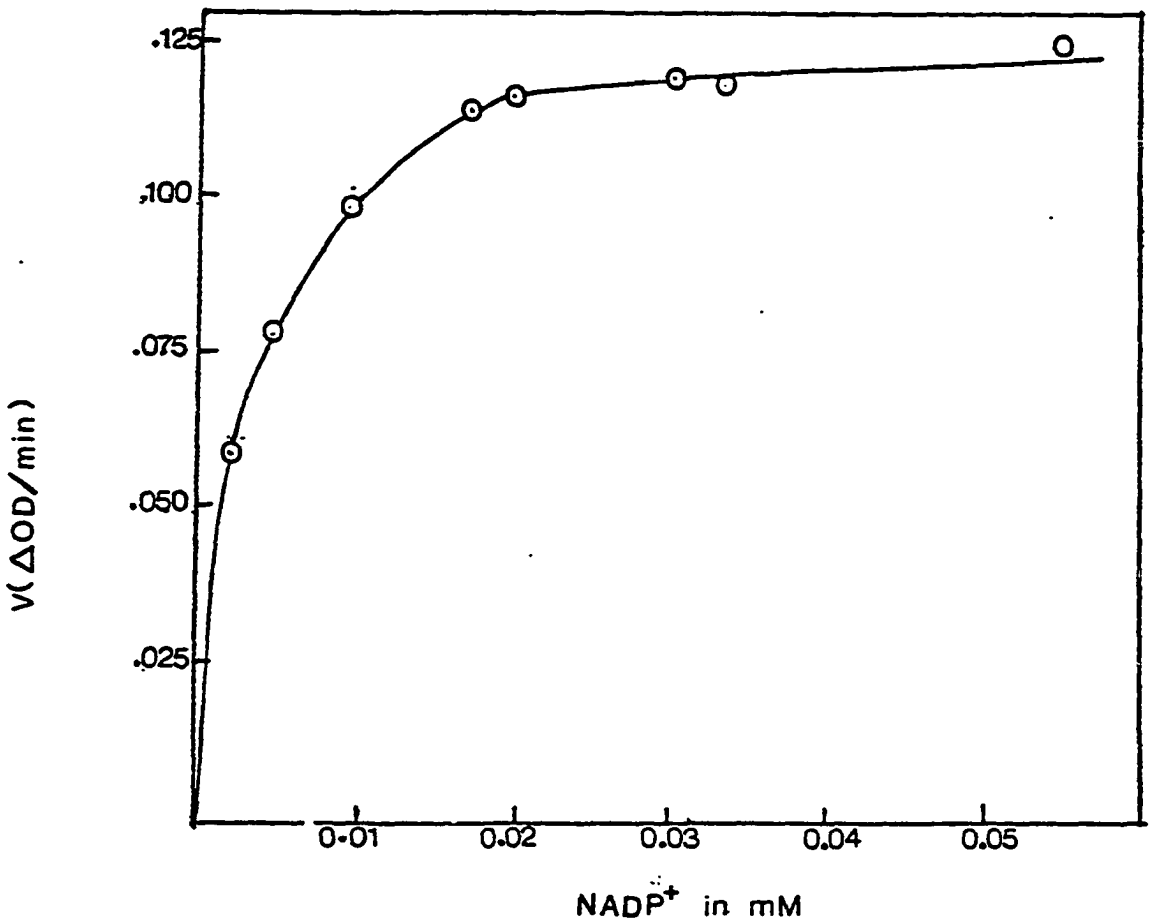

Fig. 14 NADP+ saturation curve for 6-phosphogluconate dehydrogenase band I. Each assay consisted of 2.7 mM 6-phosphogluconate, 3.4 mM NADP+, and various amounts of NADP+ in 100 mM tris buffer pH 7.8 .

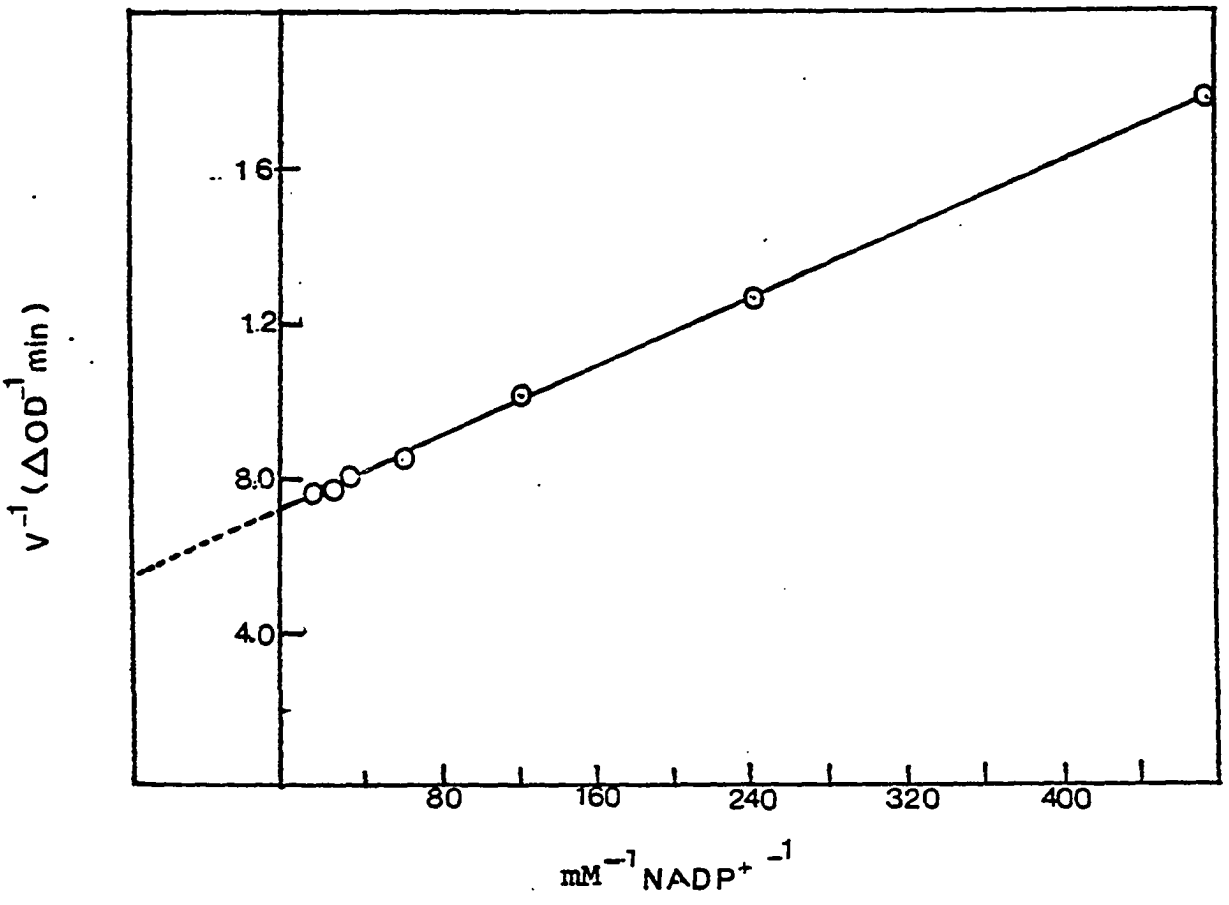

Fig. 15 NADP+ double reciprocal plot for 6-phosphogluconate dehydrogenase band I.

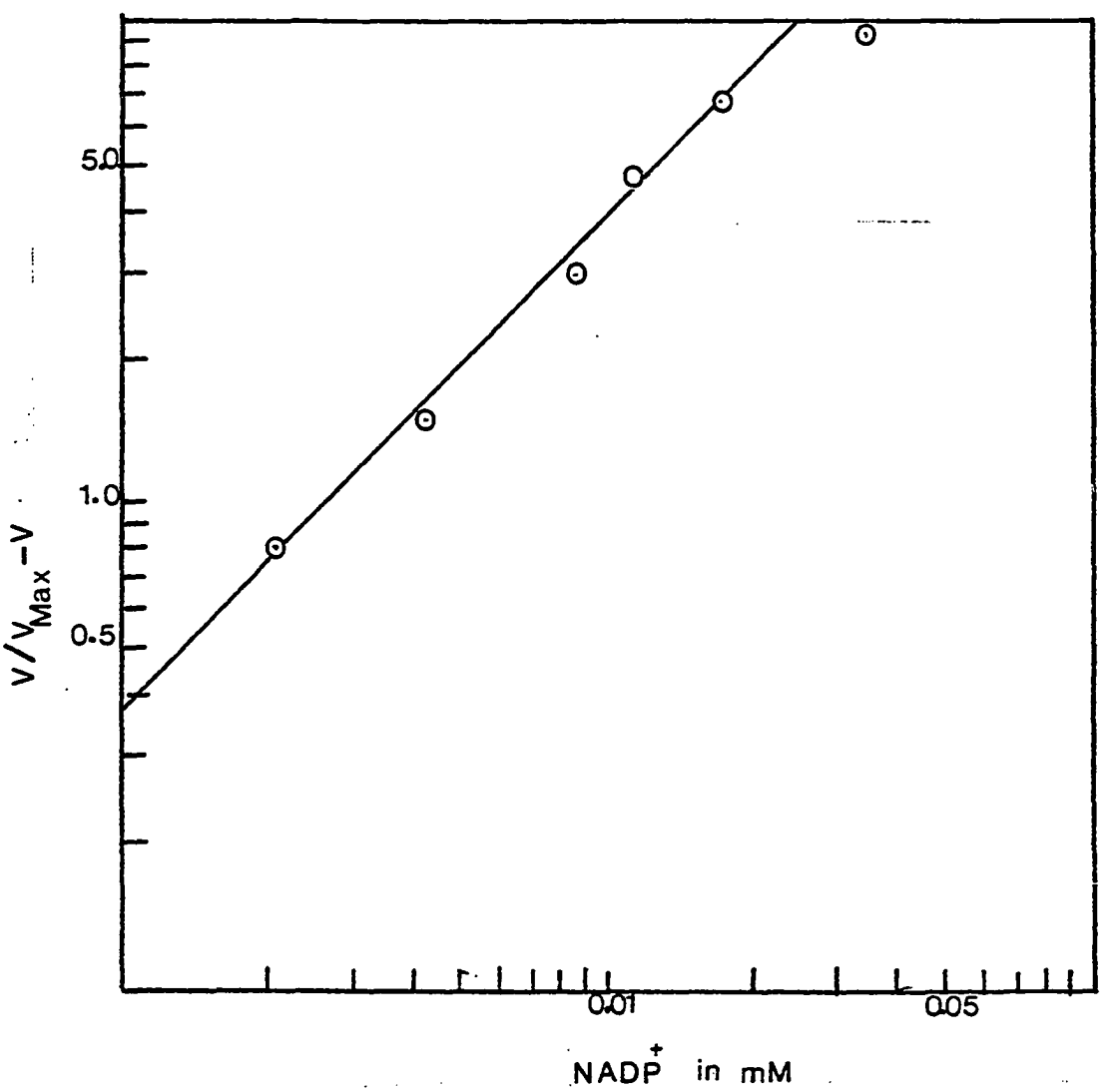

Fig. 16 NADP+ Hill plot for 6-phosphogluconate dehydrogenase band I.

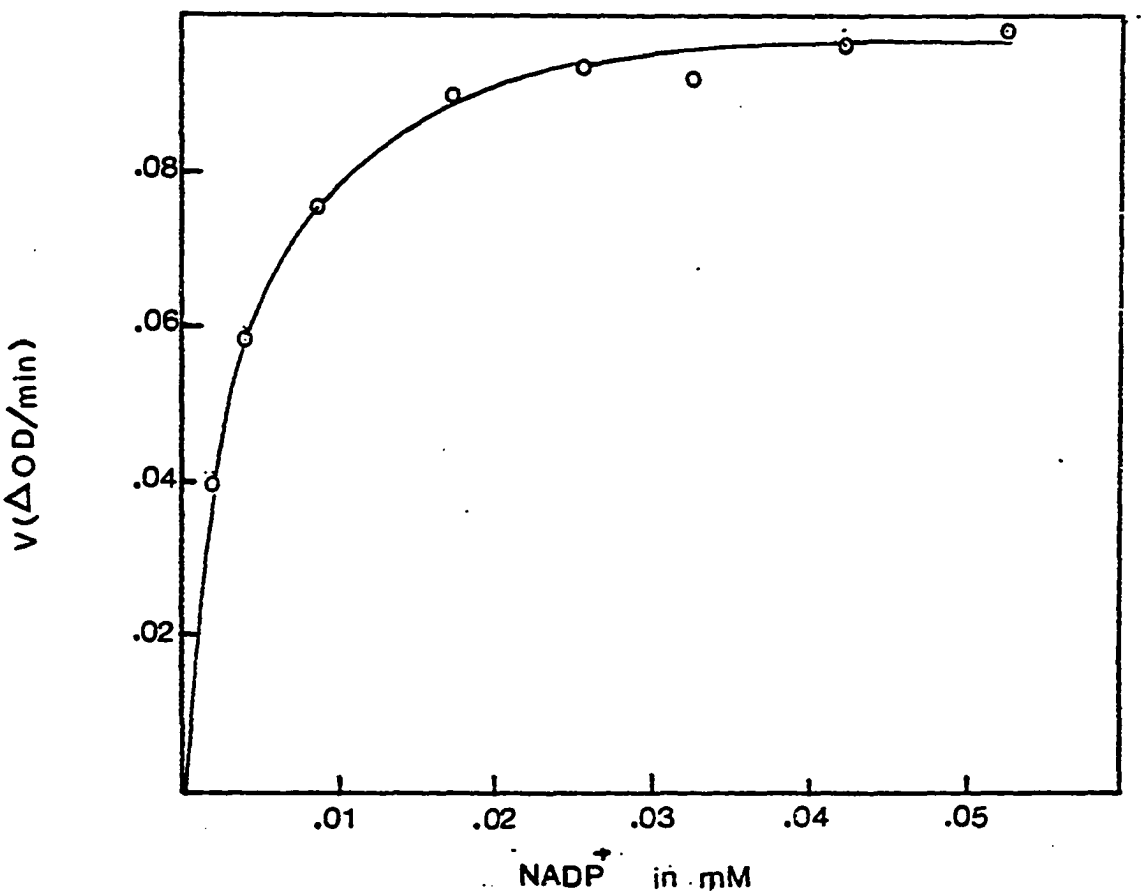

Fig. 17
NADP+ saturation curve for 6-phosphogluconate dehydrogenase band II. Each assay contained 3.4 mM Mg++, 2.7 mM 6-phosphogluconate and various amounts of NADP+ in 100 mM tris.HCl buffer pH 7.8 .

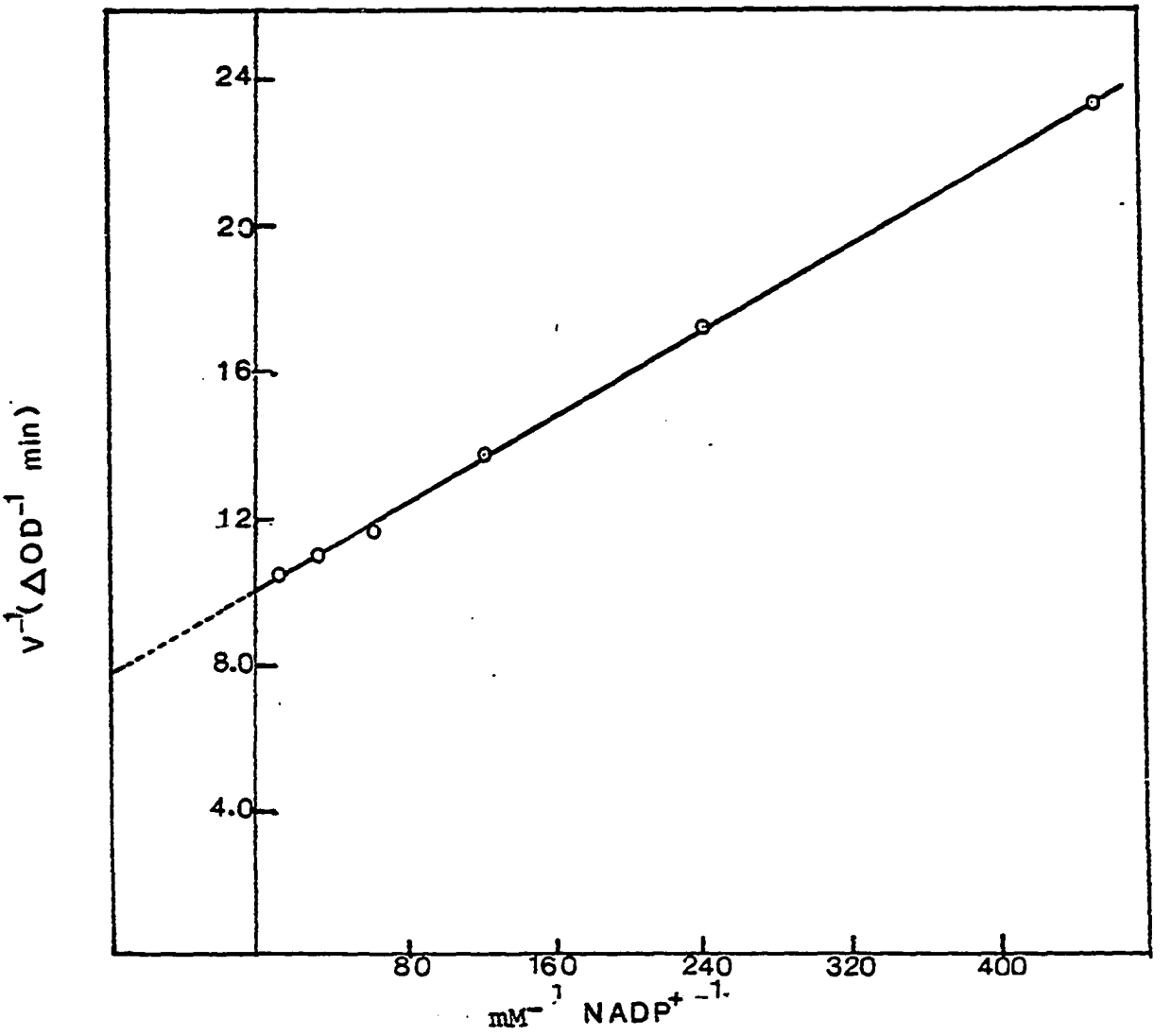

Fig. 18 NADP+ double reciprocal plot for 6-phosphogluconate dehydrogenase band II.

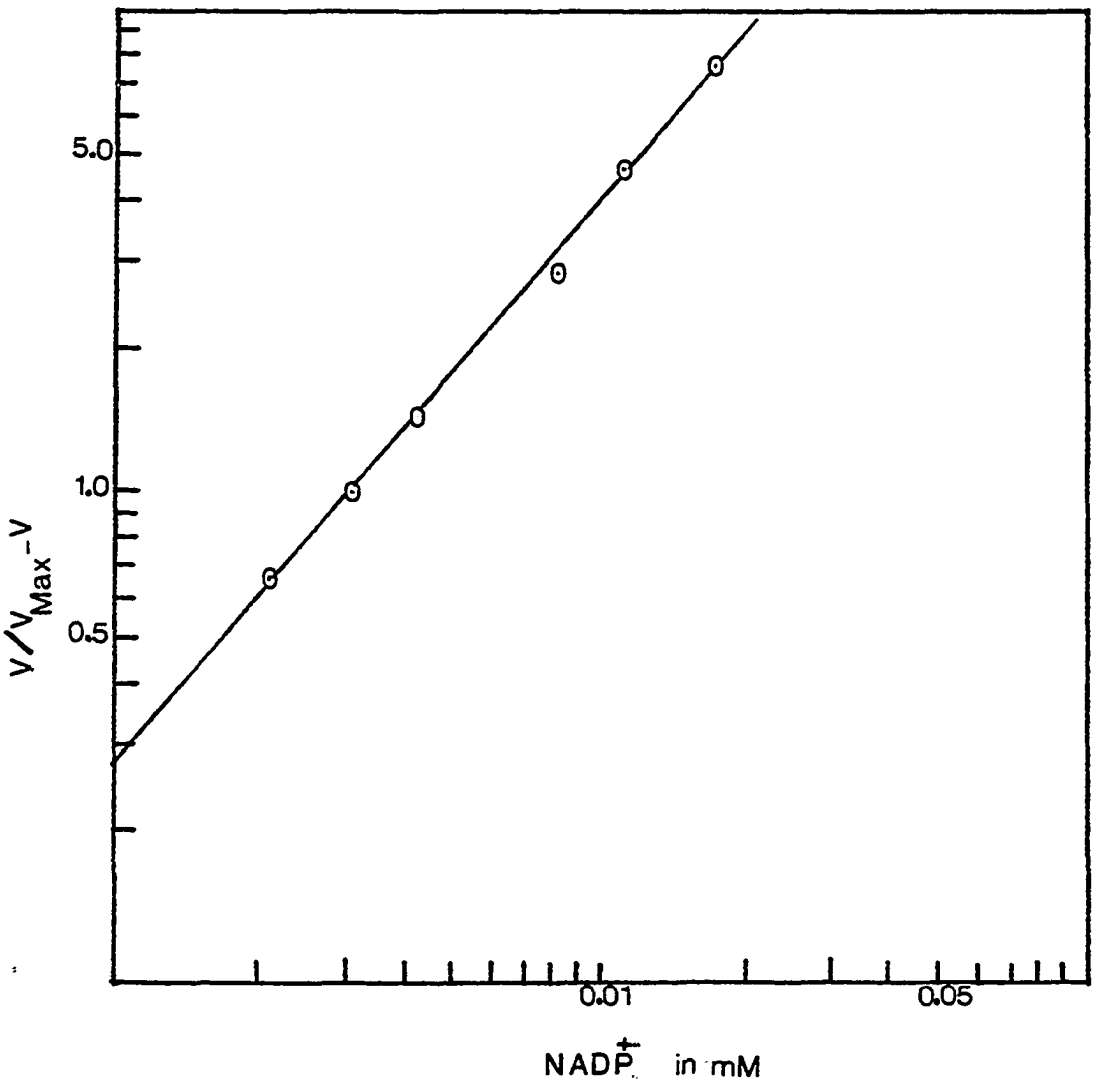

Fig. 19 NADP+ Hill plot for 6-phosphogluconate dehydrogenase band II.

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 loaded on the column bed using a propipettor. Separate columns were used for each isoenzyme. The flow rate was adjusted to approximately 5 ml/hr and 2 ml fractions were collected. The absorbance of each fraction at 280 nm was measured to determine the presence of protein. The 6-phosphogluconate dehydrogenase activity was measured to determine the elution value of Band I and Band II. The molecular weight obtained from a semi-logarithmic plot of molecular weight versus elution was 72,000 $\pm$ 5000 for Band I (Fig. 20) and 70,000 $\pm$ 6,000 for Band II (Fig. 21).

Molecular weights were determined by a second method, the SDS gel electrophoresis procedure of Weber, Pringle and Osborn (1972). A purified lyophilized powder was used for molecular weight determination. The standards used for SDS gel electrophoresis were β-galactosidase (subunit M W 130,000) serum albumin (M W 68,000), ovalbumin (M W 43,000) and α-chymotrypsinogen-A, (M W 25,700). A molecular weight of 69,000 $\pm$ 5,000 obtained from a semi-logarithmic plot of molecular weight versus mobility for Band I and II, respectively (Fig. 22, 23).

The two bands appear to contain no subunit structure, as both gel filtration and SDS electrophoresis indicate molecular weights of 69,000 - 72,000 for each band.

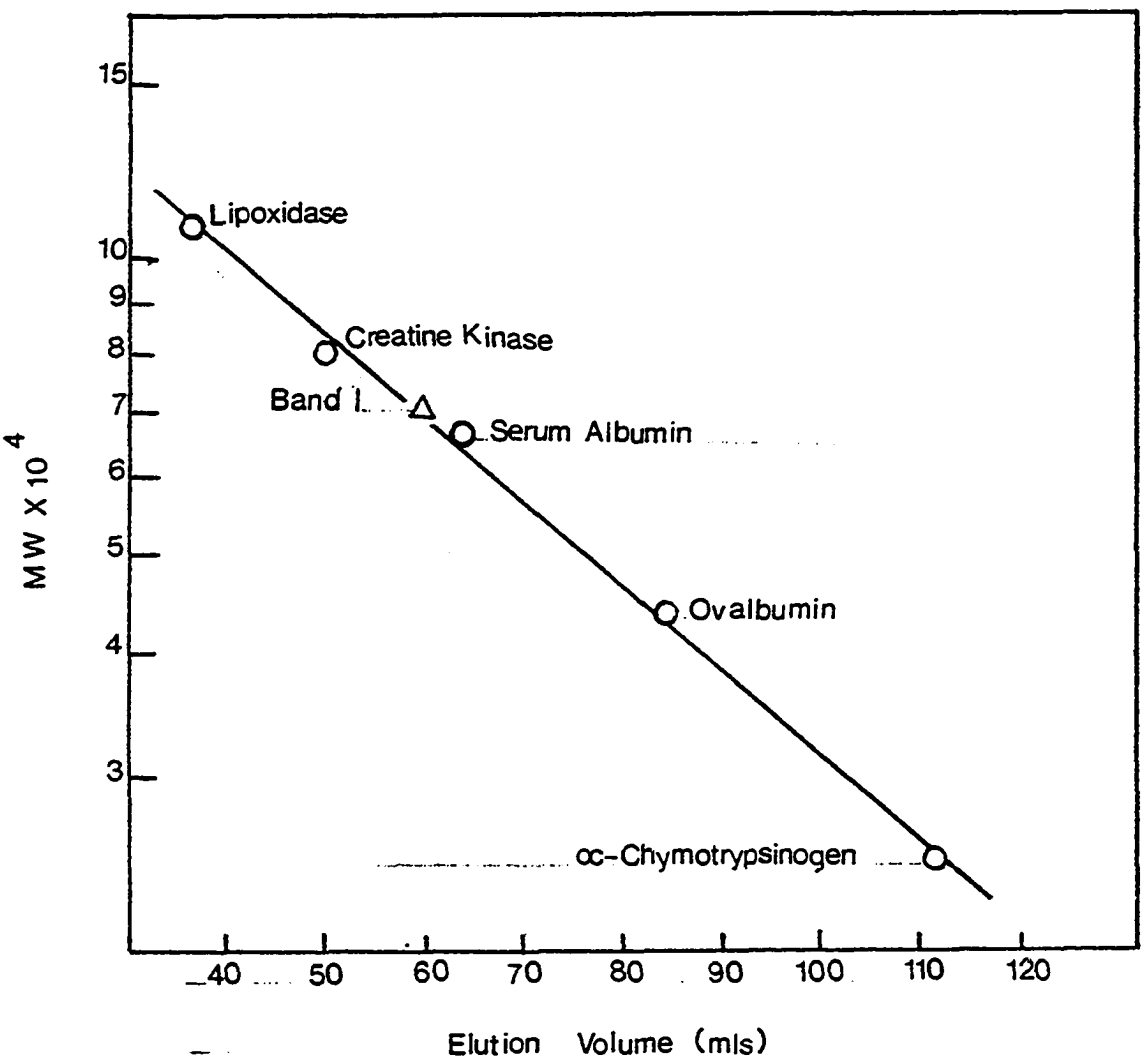

Fig. 20. Determination of molecular weight of 6-phosphogluconate dehydrogenase band I by gel filtration chromatography on Sephadex G-150. The elution buffer was 10^{-5}M NADP+, 30 mM β-mercaptoethanol and 100 mM imidazole-HCl buffer pH 6.5.

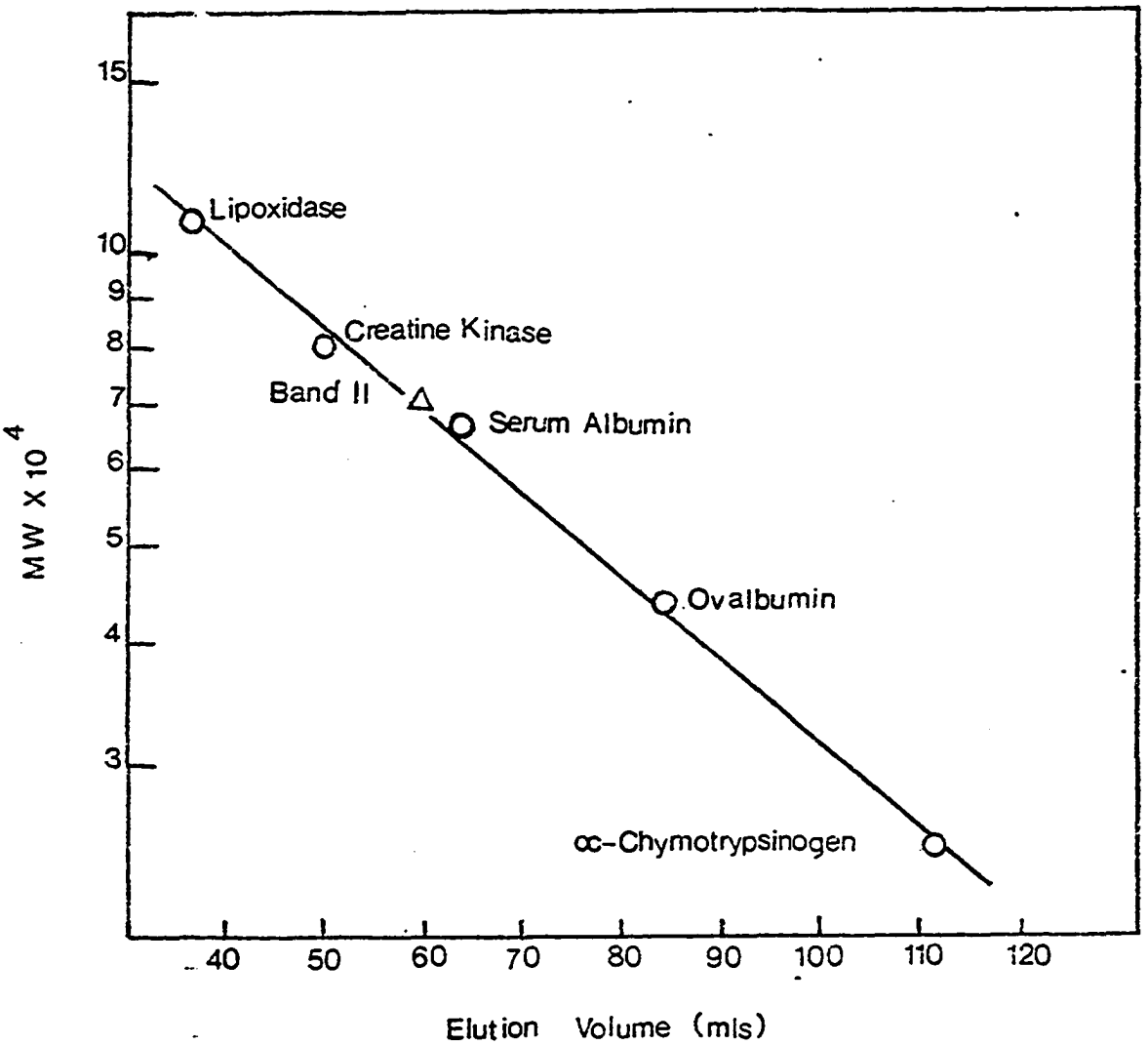

Fig. 21 Determination of molecular weight of 6-phosphogluconate dehydrogenase band II by gel filtration chromatography on Sephadex G-150.

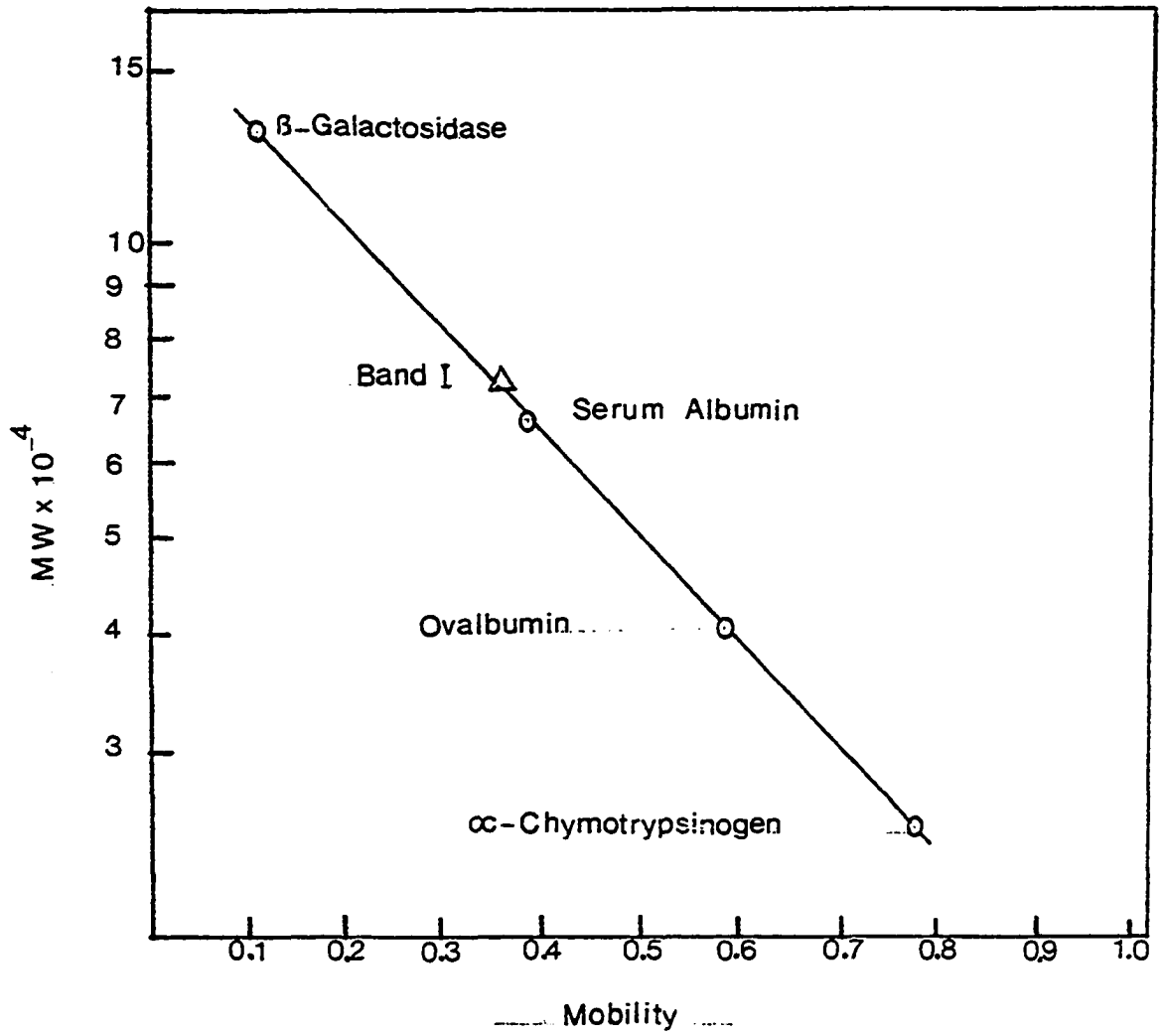

Fig. 22 Determination of molecular weight of 6-phosphogluconate dehydrogenase band I by electrophoresis on SDS polyacryladmide gels.

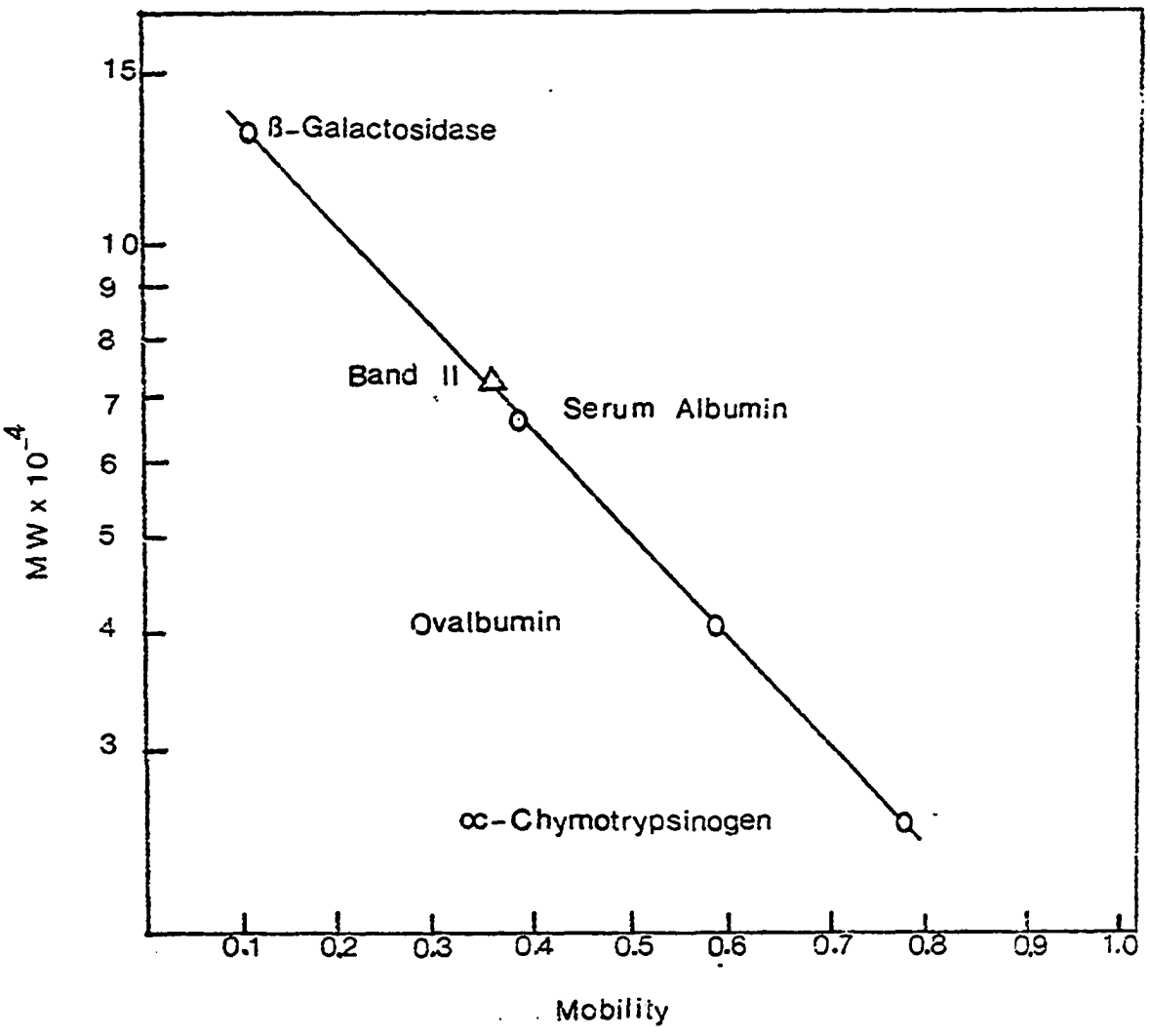

Fig. 23 Determination of molecular weight of 6-phosphogluconate dehydrogenase band II by electrophoresis on SDS polyacrylamide gels.

Tryptic peptide maps of highly purified preparations of Band I and Band II are shown in Figures 24 and 25, respectively. At least 29 peptide fragments may be common between the two bands as judged from the chromatograms developed by ninhydrin spray. The peptide fragments have been put into groups to facilitate comparison. A match group may be defined as the group that has all its members in one band overlap the same members in the other band. Looking at Band I chromatogram, peptide fragments, 1-10, 12-16, 17-19, 20-22, 23-25 and 26-29 can be classified as match groups. Several discrepancies have been cited, however. For example, looking at peptide fragments 4-8 in Band I, it becomes clear that peptide 37 is absent. Moreover, peptide fragments 34 and 35 located close to peptide fragments 8 and 6 are found in Band I but not in Band II. Peptide fragment 32 located between fragments 12 and 17, and peptide fragment 33 are also present only in Band I. On the other hand, peptide fragment 36 located close to fragment 20, and peptide fragment 18a located close to fragment 14 are found in Band II but not in Band I. Several peptide fragments are located in different positions; for example, peptide fragment 11 in Band I moved more slowly than the peptide fragment 11a in Band II. Peptide fragment 31a in Band II also moved faster than peptide fragment 31 of Band I. Moreover, peptide fragment 30 located between fragment 19 and 28 in Band I occupies a different location from peptide fragment 30a of Band II.

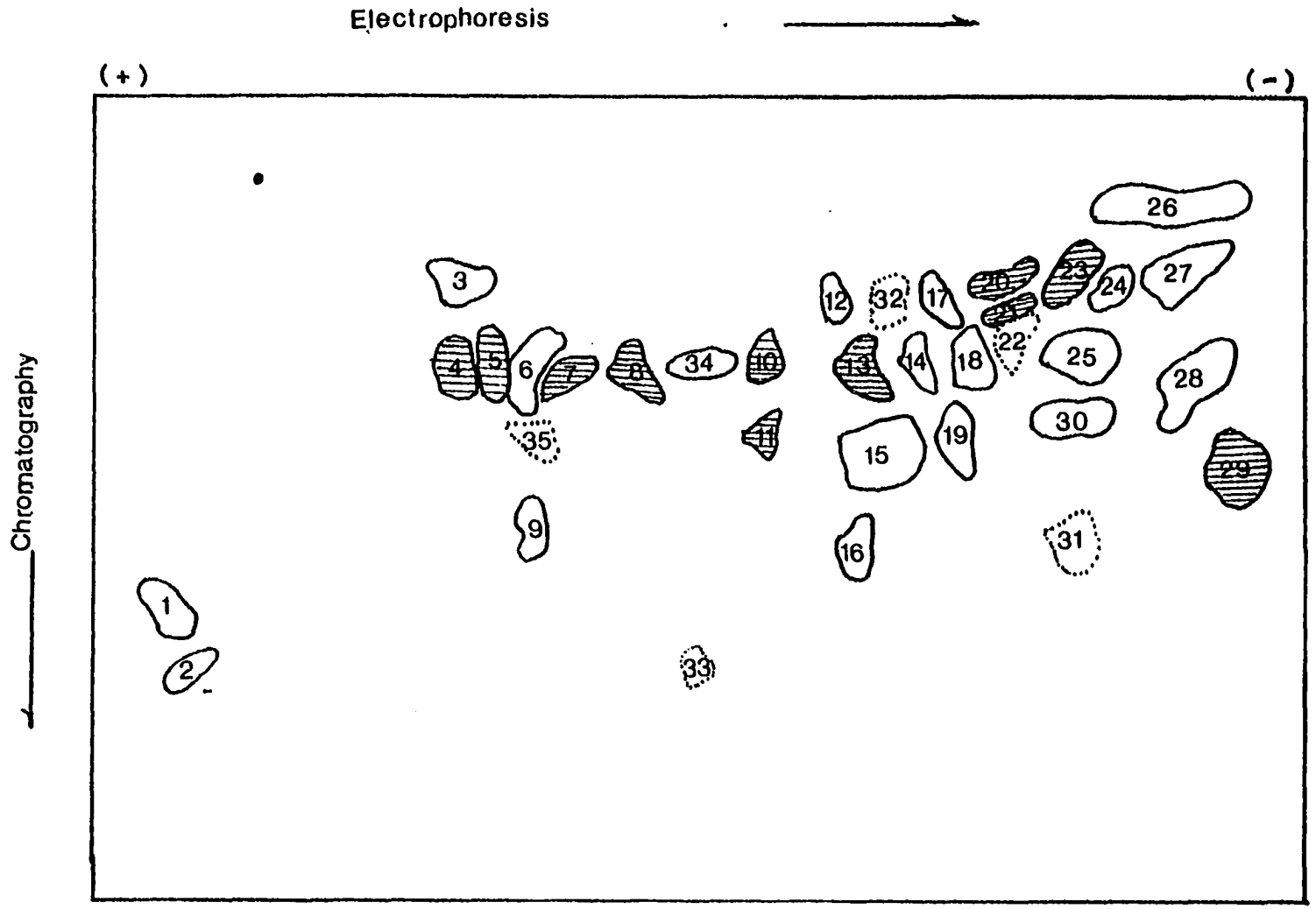

Fig. 24 Tryptic peptide pattern of 6-phosphogluconate dehydrogenase Band I.
Major ninhydrin spots.
Medium intensity ninhydrin spots.
Barely visible ninhydrin. • origin.

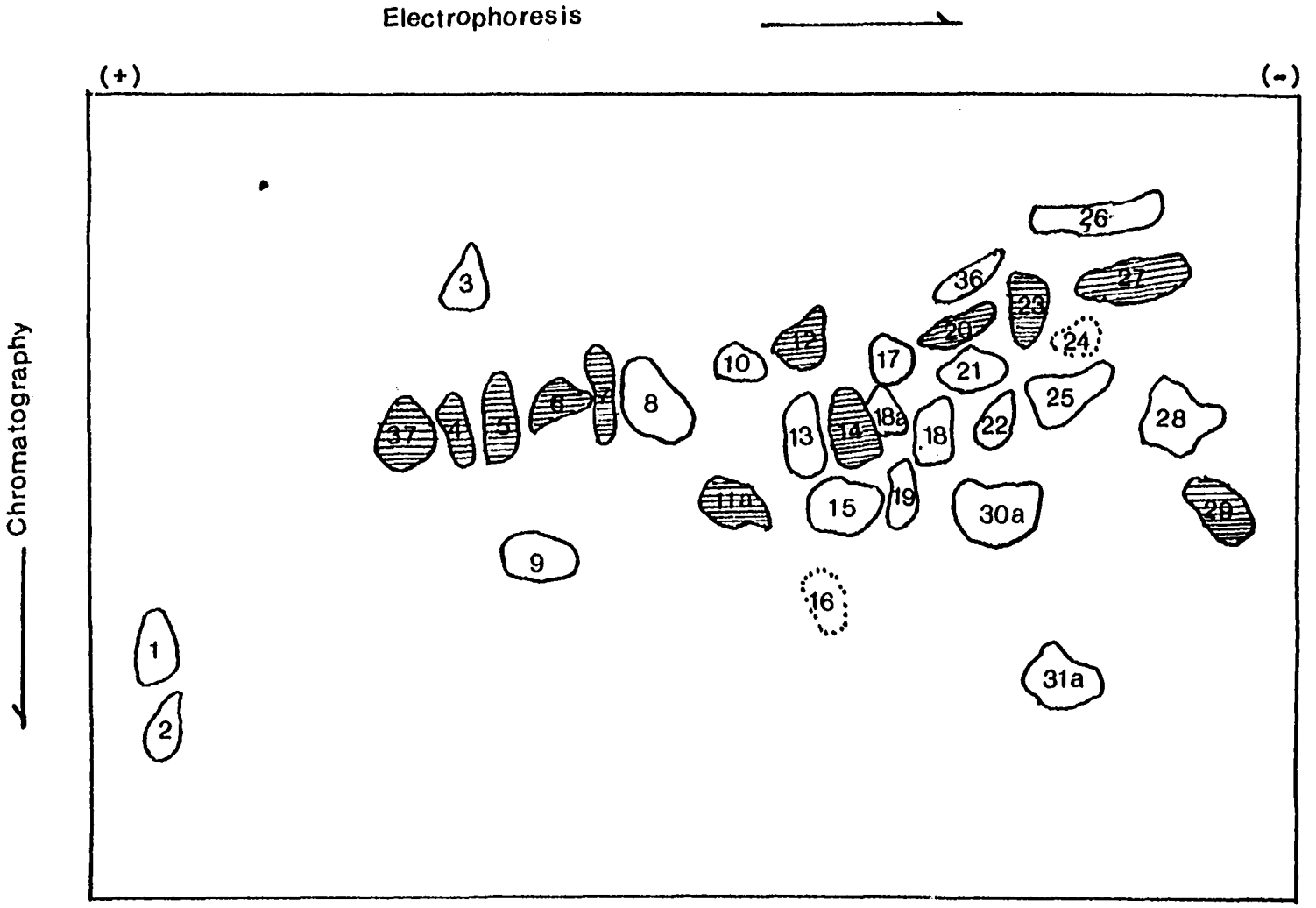

Fig. 25 Tryptic peptide pattern of 6-phosphogluconate dehydrogenase Band II.

Major ninhydrin spots.
Medium intensity ninhydrin spots.
Barely visible ninhydrin. • origin.

In summary, it can be stated that the two bands have about 29 peptide fragments in common, three extra peptide fragments are found only in Band I, namely 32, 33 and 35, two extra peptide fragments, 36 and 37, are found only in Band II and three peptide fragments, 11, 30 and 31, may have been modified in one band or the other. The differences between Band I and Band II peptide fragments are still speculative since the amino acid composition of such peptide fragments were not studied.

The very similar peptide maps of Band I and Band II are not surprising for such isoenzymes with such a similar kinetic properties and molecular weights.

CHAPTER VI

EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

The pentose phosphate pathway is connected through erythrose-4-phosphate to the shikimic acid pathway. Chorismic acid is a precursor for phenolic compounds. The shikimic acid pathway from chorismic acid to lignin and scopolin involves phenolic acid intermediates such as p-coumaric acid, caffeic acid and ferulic acid.

Scopolin and scopoletin, and also chlorogenic acid, undergo a concentration change during certain changes in the environment. An increase in chlorogenic acid (3-O-caffeoylquinic acid) has been detected in tobacco plants when grown with a deficiency of nitrogen (Armstrong 1970) or after cold treatment (Koeppe 1968). Caffeic acid and ferulic acid in carrot cells grown on agar tend to accumulate over a period of 30 days of cellular growth (Sugano 1978).

Scopolin, scopoletin, chlorogenic acid and esculetin were found to inhibit glucose-6-phosphate dehydrogenase (Hoover 1977). 6-Phosphogluconate

dehydrogenase is one of the important enzymes of the pentose phosphate pathway. In a manner similar to that of Hoover (1977), several phenolic compounds were tested for their effect on Band I and Band II of 6-phosphogluconate dehydrogenase. The significance of this study is to elucidate the role of these phenolics in regulating the pentose phosphate pathway. The compounds tested were scopolin, scopoletin, esculin, esculetin, as well as the phenolic acids ferulic, caffeic, p-coumaric and chlorogenic acid. The concentrations of these compounds in the assays were varied from 0.04 mm to 0.4 mm. The 6-phosphogluconate concentration was also varied from full saturation ($S_{1.0}$) to half saturation ($S_{0.5}$) to quarter saturation ($S_{0.25}$) of the enzyme. The reaction solutions were 5 mM in Mg++ and 0.40 mM in NADP+. The data are shown in Table 8. No major difference was found between Band I and Band II in their response to any phenolic compound tested. However, the presence of phenolic compounds has been found to influence Band I and Band II activity. For example, scopoletin was found to have an inhibitory effect on each band of the enzyme at 0.4 mM. Esculetin, too, was found to inhibit Band I and Band II at 0.4 mM. However, at concentrations below 0.3 mM, it was found that esculetin activates Band I, provided the 6-phosphogluconate concentration was low ($S_{0.25}$).

The glucosylated compounds, scopolin and esculin are much more inhibitory than their aglucones, scopoletin

TABLE 8, Continued

SCOPOLETIN

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	100	102	97	95	103	92
0.1 mM	98	102	100	98	103	88
0.4 mM	97	101	94	94	96	86
0.3 mM	88	89	87	95	88	85
0.4 mM	49	67	49	60	51	40

SCOPOLIN

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	93	101	92	98	101	100
0.1 mM	96	100	90	96	97	99
0.2 mM	82	91	78	78	88	80
0.3 mM	33	50	35	50	38	40
0.4 mM	21	21	20	11	13	12

TABLE 8, Continued

ESCULETIN

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	104	100	104	85	110	91
0.1 mM	103	100	107	88	112	84
0.2 mM	104	82	102	87	113	81
0.3 mM	102	89	103	84	110	79
0.4 mM	62	78	64	65	79	60

ESCULIN

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	102	102	96	102	99	98
0.1 mM	97	101	96	95	101	97
0.2 mM	96	93	88	90	97	88
0.3 mM	60	58	58	55	56	55
0.4 mM	22	20	22	18	17	20

TABLE 8

EFFECT OF VARIOUS PHENOLIC COMPOUNDS ON 6-PHOSPHOGLUCONATE DEHYDROGENASE BAND I AND BAND II

FERULIC ACID

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		S_{0-25} 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	99	98	100	95	88	100
0.1 mM	100	89	90	87	81	87
0.2 mM	102	83	84	83	68	72
0.3 mM	90	77	68	72	52	61
0.4 mM	80	67	56	59	45	46

PARA-COUMARIC ACID

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	100	101	90	90	88	91
0.1 mM	98	100	80	88	83	87
0.2 mM	90	95	74	77	70	72
0.3 mM	89	93	64	65	52	67
0.4 mM	72	80	56	51	33	54

TABLE 8, Continued

CHLOROGENIC ACID

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		S_{0-25} 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	107	103	86	93	90	98
0.1 mM	94	88	82	93	83	88
0.2 mM	81	76	63	68	50	68
0.3 mM	17	26	12	23	11	25
0.4 mM	8	0	0	0	4	0

CAFFEIC ACID

Conc. of Phenolic Compound	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	86	95	92	94	96	81
0.1 mM	86	86	92	86	83	75
0.2 mM	82	88	95	77	65	63
0.3 mM	80	83	82	67	54	50
0.4 mM	74	83	79	66	54	49

and esculetin. The same pattern of inhibition was found at all the 6-phosphogluconate levels tested. As for the phenolic acids tested, ferulic acid, p-coumaric acid, and caffeic acid seem to have an inhibitory effect which is dependent on the 6-phosphogluconate concentration. A more potent inhibition is observed at low 6-phosphogluconate levels in each case. Moreover, Band I activity seems to be inhibited to a greater degree (33%) than Band II activity (54%), at 0.4 mM p-coumaric acid, provided low 6-phosphogluconate concentrated. Moreover, chlorogenic acid completely inhibits the enzyme activity at the 0.4 mM concentration.

In an earlier work in this laboratory Hoover (1977) found that these phenolic compounds have an inhibitory effect on glucose 6-phosphate dehydrogenase from WR-132 tobacco tissue culture. However, our data showed that ferulic, caffeic and p-coumaric acid were found to have a more inhibitory effect (up to 50%) on 6-phosphogluconate dehydrogenase from W-38 tobacco tissue culture compared to glucose-6-phosphate dehydrogenase from WR-132 tobacco tissue culture. Moreover, 6-phosphogluconate dehydrogenase has not been activated by scopoletin as glucose 6-phosphogluconate dehydrogenase of WR-132 tobacco tissue culture.

Several non-phenolic compounds have also been tested for their effect on 6-phosphogluconate dehydrogenase (Table 9). Glucose 1,6 diphosphate, which has been found to inhibit 6-phosphogluconate dehydrogenase from yeast and several rat

TABLE 9

EFFECT OF GLUCOSE 1,6 DIPHOSPHATE AND 2,3 DIPHOSPHOGLYCERATE ON 6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY

Conc. of Glucose 1,6 Di Phosphate	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	100	100	100	100	88	95
0.1 mM	100	102	97	95	78	83
0.2 mM	98	100	85	81	60	56
0.3 mM	96	100	64	69	40	43
0.4 mM	89	91	52	59	31	36

Conc. of 2,3 Diphospho Glycerate	PER CENT OF CONTROL ACTIVITY					
	$S_{1.0}$ 6-PG		$S_{0.5}$ 6-PG		$S_{0.25}$ 6-PG	
	BAND I	BAND II	BAND I	BAND II	BAND I	BAND II
0.04 mM	100	104	121	111	134	150
0.1 mM	105	108	138	129	161	166
0.2 mM	108	110	154	147	181	173
0.3 mM	111	112	165	162	190	192
0.4 mM	118	116	169	178	204	192

tissues (Beitner 1979), is one of them. In the studies described here it has been found that this compound inhibits the activity of Band I and Band II of 6-phosphogluconate dehydrogenase. The inhibitory effect is more pronounced at low 6-phosphogluconate concentrations. Surprisingly enough, fructose 1,6 diphosphate has no effect on the two isoenzyme activities. 2,3 Diphosphoglycerate was found to activate the 6-phosphogluconate dehydrogenase up to 200% at low substrate concentration. The activation effect of 2,3 diphosphoglycerate and the inhibition effect of 1,6 diphosphoglucose to 6-phosphogluconate dehydrogenase should be extended to glucose 6-phosphate dehydrogenase before any conclusion can be drawn. At this point, however, it is clear that these two intermediates of the Embden-Meyerhof pathway have some influence on the pentose phosphate pathway. Several compounds were tested and found to have no effect on 6-phosphogluconate dehydrogenase. These compounds were 1,6 diphosphofructose, glucose, glucose 1-phosphate, glucose 6-phosphate and borate.

CHAPTER VII

DISCUSSION

The literature contains numerous reports concerning an increase in the activity of pentose phosphate pathway, and also concerning an accumulation of certain phenolic compounds under certain stress conditions. Skoog and Montaldi (1961) found that application of kinetin to W-38 tobacco tissue culture causes a spectacular rise in scopolin in the tissue. Under our experimental conditions, an increase in the amount of kinetin added to the growth medium causes an increase in the activity of G6PD and 6PGD. It is not known whether this activity increase is caused by the activation of existing enzyme molecules or by the increased synthesis of G6PD and/or 6PGD.

In the studies described in this dissertation scopolin, as well as several other compounds, have been found to inhibit the 6PGD activity *in vitro*. The same phenolic compounds have been implicated in the inhibition of the activity of G6PD from WR-132 tobacco tissue (Hoover 1977). Working with potato tissue culture Kikuta (1977) found that kinetin application caused an increased internal level of NADP+. Increased cellular kinetin apparently results in the accumulation of phenolic compounds,

especially scopolin (Skoog and Montaldi 1961), which in turn has been proven to have an inhibitory effect on G6PD and 6PGD. From a speculative point of view, as a result of this inhibitory effect on scopolin, the internal level of NADP+ could go to a higher level than under normal conditions. As a result, more enzyme molecules might be synthesized to compensate for the inhibitory effect of phenolic compounds and the increasing demand for NADPH.

6-Phosphogluconate dehydrogenase occurs in two forms. Based on studies done by Herbert (1979) on tobacco leaves and other C_3 plants that also have two forms of 6PGD, Band I and Band II may occupy different cellular locations. A cellular extract of 21 day old cells contains 60% of its total 6PGD activity in Band II, while 40% of the activity occurs in Band I. This may indicate that two thirds of the cellular pentose phosphate pathway beyond the 6-phosphogluconate reaction may occur in one location, while 40% of it may occur in the other location. All the proplastic enzymes which have been studied so far have a charge which is more negative than the corresponding soluble isoenzyme (Simcox 1978a and Deluca 1978). It may be concluded that Band II may be located in the chloroplast while Band I is located in the cytosol. The two isoenzymes have been found to be a similar molecular weight. The Km values for both substrates are very similar. Also, no significant differences in the pH optimum were found. Since the physical and kinetic properties of the 6PGD isoenzymes

are similar, the slightly different peptide maps of the two bands may be concerned with directing the isoenzymes into particular cell components (see Simcox, 1978b), although one cannot exclude that such differences may be related to the half life of each isoenzyme.

In response to phenolic compounds, 6-phosphogluconate dehydrogenase has been found to be inhibited by certain phenolic acids and by scopolin, scopoletin, esculin and esculetin. Phenolic acids inhibited the enzyme more effectively at low 6-phosphogluconate concentrations. Scopolin and esculin were more potent inhibitors than their aglucones scopoletin and esculetin, respectively. At low concentrations of these phenolic compounds, essentially no inhibitory effect was noticed. Considering the fact that tobacco cell cultures do not accumulate any intermediates on the pathway of scopoletin biosynthesis (Tsang 1979), this end product inhibition may act as a mechanism for the regulation of pathways involved in biosynthesis of phenolic compounds. The pentose phosphate pathway, the supplier of the building block erythrose 4-phosphate, is such a pathway which may be regulated by these phenolic compounds. One line of evidence which tends to support this idea is the inhibitory activity of scopolin on G6PD from WR-132 tobacco tissue cells (Hoover 1977) and 6PGD (our data) and 0-methyl transferase from W-38 tobacco tissue culture (Tsang 1979). The 0-methyltransferase catalyses the meta methylation of caffeic

acid or mediates the p-methylation of quercetin. More experimental work is needed to correlate the inhibitory effect of scopolin with the change in the activity of glucosyltransferase which mediates the transfer of glucose from UDPG to scopoletin, with the formation of scopolin (Tsang 1979).

Chlorogenic acid too has been found to inhibit 6PGD completely at 0.4 mM. Several enzymes have been found to be inhibited by chlorogenic acid, among such enzymes are G6PD (Hoover 1977), isoperoxidases A_1,A_2,A_3,C_3 and C_4 (Pickering 1973 , Reigh 1974 , Powell 1975 , and Reigh 1974). A_3 isoperoxidase utilized scopoletin as a substrate. The presence of an additional pathway which synthesizes quinic acid (chlorogenic acid derivative) efficiently in higher plants (Tazaki 1979) is of great importance at this stage. More research is needed to pursue this point further, especially its control mechanism in this pathway that starts from glucose.

Glucose 1,6 diphosphate has been found to inhibit 6-phosphogluconate. The same compound has been found by Beitner (1979) to inhibit 6-phosphogluconate dehydrogenase from yeast and rat tissues (1979). Of the study reported here, glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 1,6 diphosphate, and borate separately have been found to have no effect on the enzyme activity. This may indicate that not only are two negative charges necessary for the binding of the glucose moiety, but also the spatial

arrangement of the OH group on the second carbon is critical for reaction. Also the inhibitory effect of glucose 1,6 diphosphate indicates the influence of this moiety on the pentose phosphate pathway.

Activation of 6-phosphogluconate dehydrogenase by 2,3 diphosphoglycerate at low concentrations should be extended to include glucose 6-phosphate dehydrogenase before any conclusion can be made about the regulatory role of this compound in regard to which pathway glucose 6-phosphate may enter.

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