GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) FROM THE WHITE YAM (D. rotundata) TUBER

BY

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THESIS SUBMITTED IN PARTIAL

FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY
ZARIA, NIGERIA

JUNE, 1 9 8 2

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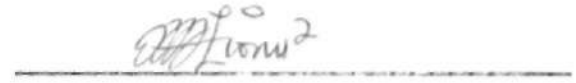
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I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

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D E D I C A T I O N

TO MY MUM FOR BAGGING THE HONOUR OF "BEST MOTHER OF THE DECADE".

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ACKNOWLEDGEMENT

I am ever grateful and thankful to God almighty for giving me the strength, determination, patience, goodluck and goodhealth during the research. I thank you GOD.

I am deeply indebted to my supervisor, Dr. C.O. Ikediobi, who provided the basic requirements, experimental ideas, materials, expert supervision and constructive analysis of the work. I remain ever grateful Sir. Also I am indebted to Dr. A.I. Ukoha for co-supervising this work, especially in the electrophoretic aspect and also for his sincere help and advice. I am sincerely grateful to Dr. (Mrs.) Ogbonna and her husband for their assistance towards the graphical drawings.

I also thank all the members of staff especially Professor Rahman, Dr. Abalaka, Dr. Ogbadu and Dr. Oyeleke for various helps and kindness.

My sincere thanks go to my millions of friends, class-mates, well-wishers and undergraduate students too numerous to be mentioned by names here. Prominent among them are: Oniye, Adeniran, Akinsanya, Moneke, Oduche, Nzelibe, Nwoke, Ukpabi, Sani, Ibrahim, Miss Onyike,

Miss Doris Dabor, Miss Helen Obasuyi, Emma Oti, Lamidi, Junji Coker, Tunde Ishola, Awoniyi Kimbo, Oladokun Simeon, Okoduwa Leo, Obasa Sola, Gare Joe, Gani (Yah-alhaji), Fatai, Bola (on-the-tarmac), Moses, Labe (Big shot), Biggest Ben, Victor and Abodunde for encouraging or helping me when the going was really rugged.

I remain ever grateful to my Papa (Chief Ationu, senior), my mother (to whom this book is dedicated to, because she's the best Mum of the decade); Sisters: Stella, Glory and Jane; brothers: Isreal and Henry; Uncle: Dr. U.K. Agwu and his family for their continued supports, prayers, undying love, encouragements and endurance. May God bless you all. I am also highly grateful to the Federal government for awarding me scholarships for both the B.Sc. and M.Sc. degrees.

Finally, I am deeply and forever grateful to Abosede for her undying love, endurance, encouragement, advice and help throughout the period. I also thank all her friends and relatives for their sincere interest and kindness.
May God bless you all.

Miss Doris Dabor, Miss Helen Obasuyi, Emma Oti, Lamidi, Junji Coker, Tunde Ishola, Awoniyi Kimbo, Oladokun Simeon, Okoduwa Leo, Obasa Sola, Gare Joe, Gani (Yah-alhaji), Fatai, Bola (on-the-tarmac), Moses, Labe (Big shot), Biggest Ben, Victor and Abodunde for encouraging or helping me when the going was really rugged.

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ABSTRACT

Glucose-6-phosphate dehydrogenase D-glucose-6-phosphate: nicotinamide adenine dinucleotide phosphate oxidoreductase (E.C. 1.1.1.49) (G6PD) has been purified from white yam tuber (Dioscorea rotundata) by procedures involving ammonium sulphate fractionation, ion-exchange chromatography, sephadex gel chromatography.

G6PD was stable throughout the purification stages and showed two sharp protein bands which were active upon activity staining on polyacrilamide gels.

The molecular weight of the yam tuber G6PD determined by gel filteration was 163,000 $\pm$ 28,000; and the deactivation energy, Ea, determined from Arrhenius plots was 6.45 Kcal $mole^{-1}$.

The yam tuber G6PD showed two pH maxima at acidic region (pH 5.0) and at basic region (pH 8.2) when assayed at 29 $\pm$ 2^{o}C. Groups associated with enzyme catalysis and binding of substrate have pK_a valuew of 5.1, 8.9, 6.08 and 9.6.

The half-life of the enzyme activity was found to be 100 days when stored in the deep freezer, 20 days in the refrigerator and 7 days at ambient temperature.

The initial velocity measurements of the enzyme using G6P and $NADP^+$ indicated a sequential mechanism.

The Km values obtained for G6P, $NADP^+$, NAD^+, glucosamine-6-phosphate and fructose-6-phosphate were 0.8mM, 0.46mM, 1.66mM, 2.0mM and 5.5mM, respectively.

The product inhibition studies with NADPH as product inhibitor showed noncompetitive inhibition with respect to G6P and competitive inhibition with respect to $NADP^+$. The inhibition constants, Ki, obtained were 1×10^{-3}mM and 6.0×10^{-3}mM with $NADP^+$ and G6P respectively.

Alternate substrate inhibition studies using glucosamine-6-phosphate and NAD^+ as substrate analogues for G6P and $NADP^+$, respectively showed that yam tuber G6PD catalysis followed rapid equilibrium random mechanism.

ATP inhibition on the enzyme was competitive with respect to G6P.

INTRODUCTION

Yams (Dioscorea species) are very important food crops in many tropical countries where they are cultivated, and represent a source of energy and revenue for the people of Nigeria and some other tropical countries.

Not only that, yams can be used in preparing a lot of African foods - it could be boiled for food, fried, used in thickening soup, dried and ground to flour (elubo) which is used in making "amala" it could be used in making yam porridge or pounded to be used as "utara ji" or "yam fufu".

It is also worth mentioning that yams can now be processed for glucose, starch and alcohol, while the wild varieties with high sapogenin content are being used as sources of precursors of synthetic sex hormones and cortisone (Onwueme, 1978).

Since it has been established as a source of calorie and revenue for the millions of people of West Africa and other tropical countries (Onwueme, 1978), it will be important to study yams, especially the common one (*Dioscorea rotundata*) in great details.

Unfortunately, it is a common observation that white yam (Dioscorea rotundata) being used as food cannot be stored long after harvest before going bad even when no spoilage organism is involved. This therefore represents a source of great loss in terms of calorie and revenue to both farmers and consumers. Since yams are cultivated and harvested over a given season in the year, some must therefore be stored for food during the cultivation season so as to meet the demand by consumers, but this has proved difficult because of spoilage on storage due to rot, microbial infections, transportation injuries, sprouting and insect attacks.

The effects of these can be devastating if left unchecked since it has been estimated (Coursey, 1965) that over 5 million tons of yams are lost annually during storage and preservation.

Also yam cannot be stored at low temperatures either, because of chilling injury - this senescence in yam results during storage at low temperature (Coursey, 1967).

The yam as a living organ is subject to both physiological and biochemical changes during storage. The work of Ugochukwu et. al. (1977) has shown that certain enzymes, especially glucose-6-phosphate dehydrogenase (G6PD), involved in carbohydrate metabolism have high activities as yams grow older during storage. This observation was later confirmed by the work of Ikediobi and Oti (1981).

Work by Ikediobi and Oti (1981) has also demonstrated without any doubt, relatively high level of G6PD in new and stored yam tubers. Their work has thus suggested that the hexose monophsphate pathway, HMP, might be an important pathway for carbohydrate metabolism in the tuber; and it has already been established that G6PD catalyzes the rate - limiting step of this pathway (Levy, 1979).

Their work (Ikediobi and Oti, 1981) showed further that lipid biosynthesis may be important in the sprouting incident. Already it is known that NADPH is one of the products of G6PD reaction in the HMP and is used for the reductive biosynthesis of lipids (Levy, 1979).

So it will be very interesting to study this enzyme,

G6PD, *in vitro* as a step in trying to understand its *in vivo* function in the yam tuber. Currently, there is research at Tropical Products Institute, London (Ikediobi, personal communications) aimed at using G6PD as a marker of onset of chilling injury in yam tubers stored at low temperatures.

If this is so, it will be of great importance to know in what respects the properties of the yam tuber G6PD differ from those of G6PDs from other sources.

Such knowledge about the properties of the enzyme might prove useful in estimating the relative contribution of HMP to carbohydrate metabolism in the yam tuber.

Extensive literature search has yielded little information about the biochemistry of the yam tuber. The successful storage of yams under tropical conditions must depend heavily on a complete understanding of the basic physiology and biochemistry of the yam tuber.

So a lot of basic research must be done before we can actually begin to understand why the yam tubers cannot be stored for long before going bad.

From the foregoing , it is therefore important

that G6PD be studied *in vitro* as a first step in trying to understand its *in vivo* importance in the yam tuber.

So the purpose of the present research is to isolate, purify, characterize G6PD from white yam tubers (*D. retundata*). The properties of the purified enzyme are studied. The enzyme is also subjected to various kinetic analysis in order to obtain basic information about its kinetic features.

Such information might prove very useful in the storage of white yam tuber under tropical conditions.

Finally, our knowledge of the structure, mechanism of action and mechanism of regulation of G6PDs is still at a relatively primitive level (Levy, 1979)—there is no information available at present on the amino acid sequence or X-ray crystallography of any G6PD; the secondary structure of almost all G6PDs has not been well elucidated (Levy, 1979); and so also is the fact that little work has been published on the identification of essential amino acids.

So our research might prove useful in providing some information in regard to G6PDs in general.

LITERATURE REVIEW

Historical background

G6PD is the first enzyme of Pentose phosphate pathway (PPP) and is solely responsible for the oxidation of D-glucose-6-phosphate (G6P) to D- glucono-8-lactone-6-phosphate in the presence of $NADP^+$ or (NAD^+).

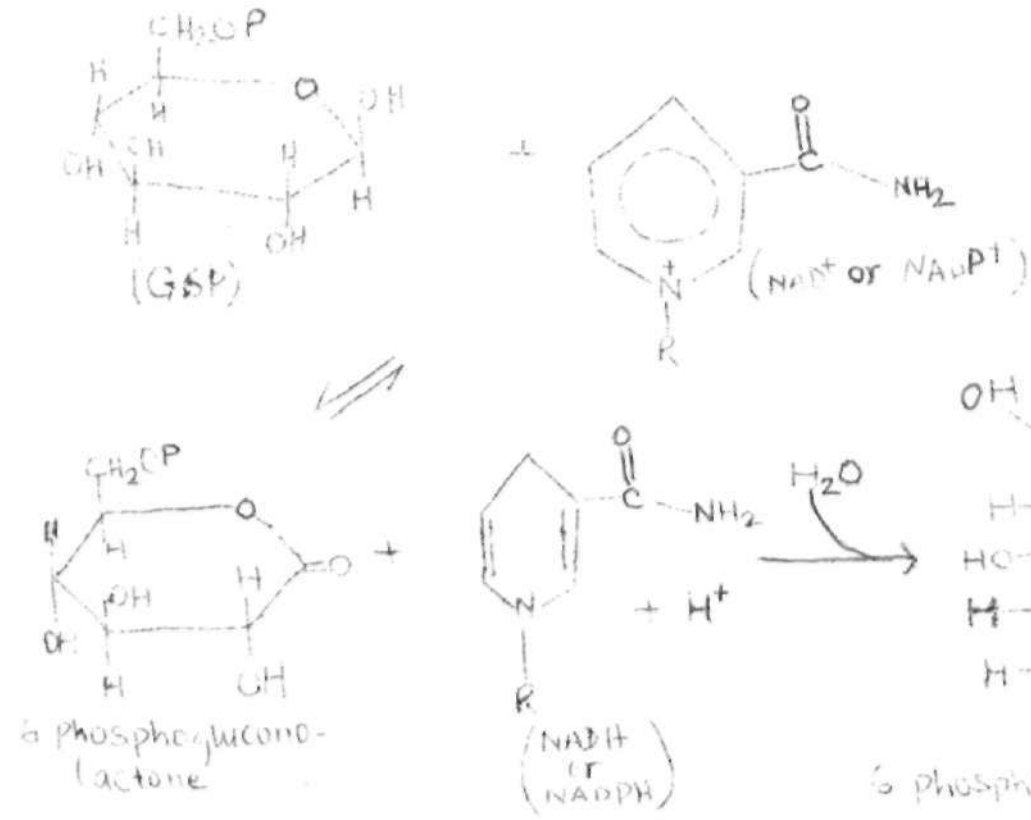
The enzyme was first discovered by Warburg and Christian in 1931 from horse erythrocytes and also in mammalian erythrocytes (Warburg and Christian, 1931). In 1932, they discovered the enzyme in brewer's yeast and a year later isolated it from lysed erythrocytes (Levy, 1979).

The first partial purification of the enzyme was performed in yeast by Negelein and Gerischer in 1936 (Levy, 1979). After this, subsequent isolations have been made of the enzyme from a vast number of sources and a wealth of literature has accumulated about the enzyme since 1936.

The Commission on enzymes assigned G6PDs a general nomenclature (D-glucose-6-phosphate; nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49).

Reaction catalyzed by G6PD.

G6PD is responsible for the oxidation of D- glucopyranose 6-phosphate to 6-phosphogluconolactone according to the scheme below:



From the scheme, R represents the adenosine-diphosphate -ribose or 2' phosphoadenosine-diphosphate - ribose moiety of NAD^+ and $NADP^+$, respectively.

This reaction involves the transfer of hydrogen

atom from G6P to NAD(P); (Levy, 1979). The D-glucono-δ-lactone-6-phosphate produced is hydrolysed to 6-phosphogluconate.

Isolation and Purification of G6PD

The enzyme, G6PD, has been isolated from a wide variety of plant and animal tissues. In animal tissues, G6PDs are cytoplasmic enzymes (that is, soluble). In green plants, it is known that G6PDs occur in both the cytoplasm and chloroplasts (Anderson et. al., (1974); Schnarrenberger et al, 1973).

The most difficult property of some of the G6PDs during isolation is their lability. They easily lose activity during purification (Levy, 1979), and this has been attributed to certain components present in the enzyme source. G6PDs from animal sources can be easily stabilized by $NADP^+$ (Carson et al, 1959; Marks, et. al. (1959). This has been employed in almost all isolations of G6PD from mammalian tissues, however G6PD from Penicillium duponti, which is very unstable was stabilized by G6P instead of $NADP^+$ (Malcolm and Shepherd, 1972). Glycerol has also

been used as a stabilizing factor during the isolation of the enzyme from the lactating rat mammary gland (Levy, 1963; Nevaldine et al, 1964). De Flora et al (Levy, 1979) stated that the erythrocyte G6PD cannot be stabilized by glycerol, but no data are given.

The most spectacular of these isolations and purifications of the enzyme was done by Yoshida from human erythrocytes (Yoshida, 1966). He got a 63,000 fold purification with almost 50% yield by utilizing over 20 purification steps! Subsequently, Yoshida's method was simplified by other investigators.

Recently, with the introduction of affinity chromatography, two procedures were published that are exceedingly simple, rapid and give high yield of purified enzyme (De Flora et al, 1975). This method of purification of G6PD involved the coupling of N^6 (aminohexyl)- adenosine 2' 5' - biphosphate to BrCN-activated agarose. The three steps involved were essentially chromatography on DEAE-sephadex, chromatography on Phosphocellulose and affinity

chromatography on the above ligand-matrix complex (De Flora et al, 1975).

However, apart from the sweet potato G6PD isolated and purified in 1970 (Muto and Uritani, 1970), the only other major plant tuber from which the enzyme has been isolated and purified till date is white yam tuber although it has been purified from various plant sources, but not from any yam cultivar.

Amino Acid Composition.

Published data on amino acid composition of G6PDs (Levy, 1979) has shown a wide range of variability and the most prominent being cysteine content from zero in L. mesenteriodes enzyme to 32 in N. crassa G6PD. There has been no evidence for disulphide bonds in G6PDs even in human erythrocyte G6PD (Yoshida, 1973a). The generally low isoelectric points for these enzymes have been explained by the fact that (ASX + GlX) exceeds (Lys + His + Arg) (Levy, 1979).

Non-amino Acid Components.

The presence of zinc ions (Zn^{++}) and other metal

ions in yeast G6PD were reported by Vallee et al. (1956). Work done by Yue et al, (1969) have demonstrated that S. carlsbergensis G6PD contained no significant amount of any metal ions, Zn^{++} inclusive. Quantitative analysis of bovine adrenal G6PD has revealed significant levels of Ca^{++}, Mg^{++} and Cu^{++} in this enzyme (Singh and Squire, 1975). The human erythrocyte G6PD has been subjected to analysis, and it was found that chelating agents had no effect on the enzyme - thus suggesting it was not a metallo-enzyme (Warburg and Christian, 1931; Levy, 1979). The only non-amino acid component found inG6PDs is probably tightly bound $NADP^+$ (Levy, 1979).

Secondary Structure.

Very few studies have been done on the secondary structure of G6PDs. Jirgensons evaluated the moffit constant, b_o, and cotton effects for G6PD from S. cerevisiae (Levy, 1979). The values for b_o and the magnitude of the positive cotton effect were low- thus suggesting a relatively low degree of helical structure in this enzyme.

Subunit nature and Quaternary structure.

There has been no indication of non identical subunits in most of the G6PDs. The nonidentical subunits reported for human erythrocyte G6PD have been attributed to the use of impure enzyme (Chung and Langdon, 1963). However, hybridization studies suggested that the human erythrocyte G6PD and rat erythrocyte enzyme, each has identical subunits (Beutler and Collins, 1965). It was also suggested by Scott and Brady (1973) that N. crassa G6PD contains at least three nonidentical subunits, however, the numerous reports of multiple bands in gel electrophoresis and isoelectric focusing of G6PDs have disproved the suggestion of Scott and Brady (Levy, 1979).

The minimum quaternary structure for catalysis in G6PDs is the dimer. There is no evidence of catalytically active monomeric G6PD (Levy, 1979). G6PD from human erythrocyte is catalytically active as a dimer (Yoshida and Hoagland, 1970) at pH 6.0 and pH 8.0, and this suggests that the enzyme is a dimer in vivo.

It is known that many G6PDs aggregate to form tetramers or large oligomers. Tetrameric forms have been described for G6PDs from C. utilis (Chilla et al, 1973; Domagk et al, 1973), N. crassa (Scott, 1971), Mouse liver (Hizi and Yagil, 1974), rat liver (Holten, 1972; Matsuda and Yugari, 1967), human erythrocytes (Bonsignore et al, 1971; Cohen and Rosemeyer, 1969; Yoshida, 1973a) and sweet potato (Muto and Uritani, 1972). The factors responsible for the interconversion of dimers and tetramers have been examined properly in human erythrocyte G6PD.

Temperature, enzyme concentration and the concentration of $NADP^+$ or NADPH are implicated in this interconversion (Chung and Langdon, 1963; Levy, 1979).

Cohen and Rosemeyer (1969a; 1969b) have demonstrated that the human erythrocyte G6PD exist as an equilibrium mixture of dimers and tetramers, profoundly influenced by pH, ionic strength as well as by divalent cations (Levy, 1979; Beitner and Naor, 1972; Kanji et al, 1976).

Just as the dimer of G6PD can form tetramers, so also could it dissociate into monomers. This dissociation requires incubation in a strong detergent such as sodium dodecyl sulphate (SDS), Urea, or guanidine. The dissociated subunits are generally inactive. Some G6PDs are however difficult to dissociate (Singh and Squire, 1974; Shrere and Levy, 1977). Some G6PDs can be dissociated into monomers by the removal of $NADP^+$ (Rattazi, 1968; Levy, 1979). The forces responsible for maintaining the tetrameric structure of G6PDs include hydrophobic interactions, hydrogen bonding and stability offered by $NADP^+$ (Levy, 1979).

Molecular Weight.

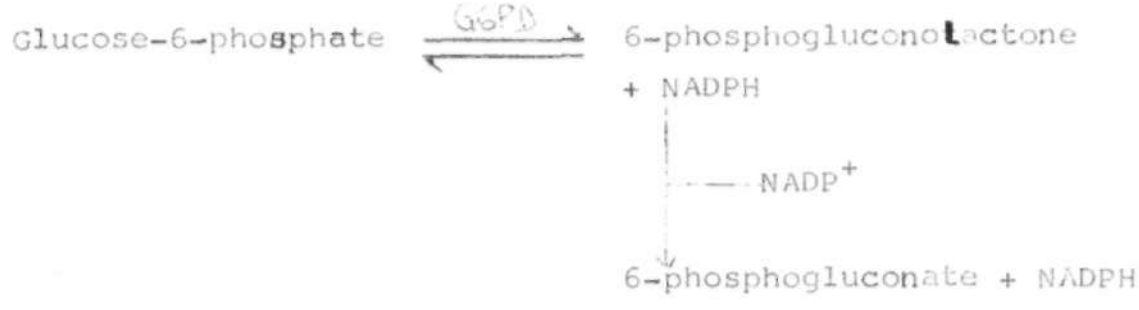
The molecular weight of purified or homogenuous G6PDs differ according to sources of isolation. Most of the reports of the molecular weights of G6PDs were based on reports obtained from gel filtration, disc gel electrophoresis, sedimentation velocity and sedimentation equilibrium techniques (Levy, 1979). The following molecular weights were determined on crude or partially purified G6PDs:

Rat erythrocyte 131,000 (Rattazi, 1968); Drosophila 130,000 and 150,000 (Levy, 1979); Spinach leaves 105,000 (Schnarrenberger et al., 1973); Tobacco (suspension culture) 91,000 and 115,000 (Hoover et al., 1977); sweet potato 110,000 (Muto and Uritani, 1970); Penicillium duponti 113,000 - 126,000 (Malcolm and Shepherd, 1972); Bacillus subtilis spores 240,000 (Levy, 1979); Pseudomonas fluorescens (NADP - preferring enzyme 265,000 (Lessmann et. al., 1975); Rhodopseudomonas spheroides 180,000 - 200,000 (Levy, 1979) mammary gland (in the presence of $NADP^+$) 241,000 (Levy, 1979); human erythrocyte (with $NADP^+$) 240,000 (Levy, 1979); human erythrocyte (without $NADP^+$) 123,000 - 180,000 (Levy, 1979); adrenal gland (in the presence of $NADP^+$) 236,000 (Levy, 1979).

G6PD CATALYSIS

Thermodynamics of the reaction

The reaction catalyzed by G6PD is represented schematically as:



The above reaction catalyzed by G6PD is thermodynamically reversible (Horecker and Smyrniotis 1953), but this is made irreversible by the fast hydrolysis of D-gluconc-δ-lactone-6-phosphate, which is the actual product of glucose-6-phosphate oxidation (Levy, 1979). The hydrolysis of this lactone is known to be dependent on pH ; at pH 7.4, the half-life is about 1.5 minutes (Horecker and Smyrniotis, 1953) and at pH 6.4 and 28°C, the lactone has a half-life of 24 minutes (Glaser and Brown, 1955). Added to this rapid hydrolysis, a wide range of lactonase (Brodie and Lipmann, 1955) prevents the reversibility of the G6PD catalyzed reaction under physiological conditions.

G6PD is known to utilise the open-chair form or the pyranose ring form of the substrate (Salas et. al, 1965; Levy, 1979; Muto and Uritani, 1972;

Malaisse et. al, 1976). The anomeric specificity is essential and β-D-glucopyranose-6-phosphate is the form utilized by the enzyme (Levy, 1979).

Substrate specificity

In all G6PDs, D-glucose-6-phosphate (which is the natural substrate is known to give the highest V_{max} and lowest K_m; although some G6PDs can equally oxidize analogs of the substrate (Grove et. al, 1976; Adams et. al, 1976; Bessel and Thomas, 1973; Egyud and Whelan, 1974; Kirkman, 1959; Metzger et. al., 1972; Salas et. al., 1965). The investigation involved in these tests in substrate specificities concluded that the apparent affinity of the analog for G6PD is lower than the natural substrate because of the structural change which might have caused slight geometric change (Levy, 1979). Among substrates used were 2-deoxyglucose-6-phosphate, 3-deoxyglucose-6-phosphate, galactose-6-phosphate, glucosamine-6-phosphate and glucose-6-sulphate to mention but few. These substrates were utilized at relatively reduced rates and with low affinities in comparison with the natural substrate.

(Bonsignore and De Flora, 1972).

Coenzyme Specificity

G6PD from L. mesenteroides isolated by De Moss et. al (Levy, 1979) is known to react with both $NADP^+$ and NAD^+. Initially, studies with G6PDs from S. carlsbergensis and mammalian tissues showed these enzymes to be specific for $NADP^+$ and could not use NAD^+. It has been shown that some other G6PDs could utilise high concentrations of NAD^+ (Levy, 1961). On the basis of these studies, five classes of G6PDs can be defined:

(i) NADP-specific G6PDs, which do not react with NAD^+;

(ii) NADP-preferring G6PDs, which can react with NAD^+ under forcing conditions, but do not appear to utilise it physiologically;

(iii) G6PDs with dual-nucleotide specificity which utilise both activities equally in vivo;

(iv) NAD-preferring G6PDs which can also react with $NADP^+$ but under physiological conditions only with NAD^+;

(v) NAD-specific G6PDs which can only react with NAD^+. There is a vast literature about coenzyme specifities of G6PD (Levy, 1961, 1979; Turnail and Schlegel, 1972; Vander Wyk and Lessie, 1974; Banziman and Mazover, 1973).

Requirement for divalent cations

Majority of G6PDs are activated by mg^{++} although it is not an absolute requirement for catalytic activity (Glaser and Brown, 1955; Kornberg, 1950). Some other G6PDs show absolute requirement for this divalent cation. An example is G6PD from pea chloroplast (Yoshida, 1966). The reported effects of mg^{++} on C. utilis G6PD include stimulation of catalytic activity, increase of the protective effect of glucose-6-phosphate against various reagents that alter catalytically essential amino acids (Domschke et. al, 1970), promotion of aggregation (Domagk et. al, 1973; Chilla and Domagk, 1973); and alteration of the moffit constant of the enzyme, bo, upon binding (Domschke et. al, 1970). In addition to mg^{++}, other divalent or monovalent

cations have been shown to activate G6PDs from several sources (Glaser and Brown, 1955; Glock and Mclean (1953; Jagannathan et. al, 1956).

Kinetic Mechanisms

The use of steady-state kinetic techniques to determine the kinetic mechanism of any enzyme with two or more substrates entails the determination of the initial velocity pattern in both directions, and the patterns of inhibition by all the products (Cleland, 1963). For G6PDs, it has been difficult to study the enzyme-catalyzed reaction in the reverse direction or to make full use of the product inhibitions because of the lability of the gluconō-d-lactone-6-phosphate at pH's above 6.5 (Glaser and Brown, 1955; Levy, 1979) and of NADPH under acid conditions (Levy, 1979).

All G6PDs proceed by the sequential (Cleland, 1963) or ternary complex (Levy, 1979) mechanisms but in some there is an ordered addition of substrates and release of products, while in others it is random (Levy, 1979). One clear-cut evidence for a random mechanism was obtained by Malcolm and Shepherd (1972)

with G6PDs from P. duponti and P. notatum. In these enzymes, NADPH inhibits competitively with respect to both $NADP^+$ and glucose-6-phosphate (Levy, 1979).

A steady state kinetic analysis of the NAD - and NADP-linked reactions catalyzed by L. mesenteroides G6PD was conducted by Olive et. al, (1971). They found out that the initial velocity measurements demonstrated sequential mechanisms with both coenzymes, but the kinetics of inhibition by the reduced coenzyme product differed for the two reactions. NADPH inhibited the NADP-linked reaction competitively with respect to $NADP^+$ and non-competitively with respect to glucose-6-phosphate, but in the NAD-linked reaction, NADP was a non-competitive inhibitor with respect to both glucose-6-phosphate and NAD^+. The data were interpreted as demonstrating an ordered sequential mechanism (ordered bibi) (Clevand, 1963) for the NADP-linked reaction and an ordered sequential mechanism with enzyme isomerization (iso ordered bibi) (Cleland, 1963).

The use of alternate substrates was utilized by

Kosow (1974) to establish that human platelet G6PD (a partially purified extract) followed an ordered sequential mechanism in which $NADP^+$ binds first and NADPH is released last. Studies with pig liver G6PD (Levy, 1979) using glucosamine-6-phosphate as dead end inhibitor, led to the conclusion that this enzyme used an ordered sequential mechanism with $NADP^+$ binding first and NADPH released last.

Several G6PDs have been reported showing non-Michaelian (Sigmoid) Kinetics. G6PDs from the following sources show such kinetics when glucose-6-phosphate was varied: P. multivorans (Lessie and Vander Wyk, 1972); P. fluorescens (Vander Wyk and Lessie, 1974); P. aeruginosa (Lessie and Neidhardt, 1967); Hydrogenomonas eutropha H-16 (Black-Kolb and Schlegel, 1968); Azotobacter beiyerickii (Senior and Davies, 1971); Paracoccus denitrificans (Levy, 1979); R. spheroides (Marks et. al, 1961); sweet potato (Mute and Uritani, 1970); Ancystis nidulans (Grossman and McGowan, 1975). Non-Michaelian kinetics were seen when $NADP^+$ was varied in the

G6PD from tobacco suspension (Hoover et. al, 1977).

Kinetic Constants

The kinetic constants of most G6PDs have been obtained through kinetic studies, hence information on kinetic constants has been obtained from double -reciprocal plots (Levy, 1979). The apparent K_m's are identical with true K_m's for one or both substrates for some G6PDs(Anderson et. al, 1974; Opheim and Bernlohr, 1973; Levy, (1979).

It is known that these kinetic constants are influenced by other reaction conditions particularly pH and ionic strength. The proper interpretation of kinetic constant data entails relating in vitro experiments to in vivo conditions (Levy, 1979). The ratio NADPH/$NADP^+$ is quite high in many cells and tissues, and, also the Ki for NADPH exhibited by most G6PDs leads to the almost total inhibition of G6PD in cells and tissues (Levy, 1979). This fact is of great importance when we consider regulation of the enzyme.

Table I summarizes the kinetic constants for some G6PDs.

Enzyme source	K_m G6PD (mM)	K_m $NADP^+$ (μM)	K_m NAD^+ (mM)	Ki NADPH (μM)	Ki $NADP^+$ (μM)
A. Subocydans	0.056	120	6.2	-	-
P. fluorescens	(2.7), (2.33)	360	0.15	-	-
A. beijerickii	-	192	8.0		
V. alginolyticus	1.58	25.3	-	22	39.2
H. eutropha H-16	1.0	150	0.9	-	-
R. spheroids	0.4	130	-	-	-
A. aerogenes	0.76	36	-	-	-
L. mesenteroids	(0.081) (0.053)	5.7	0.106	37.6	5.0
L. lactis	0.2,0.19	14	0.11	-	-
B. subtilis	0.075	6.7	-	-	-
S. carlsbergensis	0.015	2.5	-	0.07	2.5
Tobacco suspension	0.22	50	-	-	-
Sweet potato root	-	29.7	10.0	-	-
Mouse liver	(6.8), (200)	(5.5), (450)	-	-	-
Bovine adrenal	0.19	3.8	2.36	-	-
Human erythrocyte	0.07	2.0-4.4	4.0	9.0	-
B. licheniforms	0.2	5.0	3.0	10.0	-

Table I showing kinetic constants for some G6PDs modified after Levy (1979).

Effects of pH and ionic strength

Kuby and Roy (1976) analysed the effects of pH and temperature at constant ionic strength on Vmax and the Kms of glucose-6-phosphate and $NADP^+$, and were able to describe the events that govern the kinetic mechanism of G6PD from S. carlsbergensis. They observed that the pH titrations involved two groups on the enzyme, with values of pK_a of about 5.7 and 9.2 at 25°C, and with standard enthalpies of ionization of approximately 5 and 11 Kcal/mole respectively, which involve the response of Vmax to pH. So, from these parameters Kuby and Roy (1976) concluded that a histidine (in the acid range) and a lysine (in the basic range) are involved in catalysis.

Olive et. al. (1971) showed that Vmax was unaltered between pH 6.5 and 9.9 in their investigation of the effects of pH on the kinetic parameters of the NAD-linked reaction catalysed by G6PD from L. mesenteroides; hence, no conclusion could be drawn on the nature of the enzyme groups that

participated in catalysis. Soldin and Balinsky (Levy, 1979) studied the pH effect on kinetic parameter of G6PD from human erythrocyte (Bantu blood). Their experiments suggested that a group with pK_a of 6.6, probably histidine, was involved in catalysis and that a cysteine residue was also involved in binding both glucose-6-phosphate and $NADP^+$. Kahn et. al (Levy, 1979) and Babalola et.al. (1976) also concluded that a histidine and a cysteine were important for catalysis. Other reports on effects of ionic strength on G6PDs are vast and varied (Anderson et. al., 1968; Glaster and Brown, 1955; Levy, 1979; Rutter, 1957).

Enzyme Stability

Animal tissue G6PDs are very sensitive to assay conditions (Yoshida, 1966) and they undergo rapid reversible partial inactivation upon dilution (Kirkman and Hendrickson, 1962). Yoshida has found that this partial inactivation did not depend on the presence or absence of $NADP^+$ or bovine serum albumin, or on the pH, but was entirely dependent on the concentration of the enzyme in the assay

solution (Levy, 1979).

Regulatory Features of G6PD

Extensive investigations on the control of the hexose monophosphate shunt and other metabolic pathways in which G6PD takes part have been done; and it is known that G6PD catalyzes the rate - limiting reactions in these pathways under most physiological conditions; and regulation is only affected by control of G6PD activity and concentration (Levy, 1979).

Eggleston and Krebs (1974) emphasized two features of the G6PD catalyzed reactions that must be considered in seeking to understand how G6PD activity is regulated. The first is its effective thermodynamic irreversibility which should cause the reaction to go to completion and the inhibition by NADPH. This inhibition which has been observed for all G6PDs is competitive with $NADP^+$ (Levy, 1979). NADPH inhibition is very important in establishing the rate of the G6PD - catalyzed reaction and also to account for almost total inhibition of G6PDs in vivo in rat liver (Eggleston and Krebs, 1974;

Levy, 1979) and normal erythrocytes (Yoshida, 1973).

G6PD concentration is also important in its regulation (Levy, 1979). Enzyme concentrations are generally much higher *in vivo* than under assay conditions, a fact that can influence kinetic and regulatory behaviour dramatically (Shrere 1967; 1970). Yoshida (1966) has shown that human erythrocyte G6PD activity is inactivated approximately 75% on dilution.

Coenzymes concentrations and ratios also serve to regulate the activity of G6PD. Yoshida (1973) has proposed that high concentration ratio of NADPH to $NADP^+$ in human erythrocytes plays a dominant role in regulating G6PD *in vivo*. The NADPH/$NADP^+$ ratio has also been considered to influence erythrocyte G6PD by way of structural changes than its activity (Levy, 1979).

In photosynthesizing plants, the activity of G6PDs is strongly influenced by the NADPH/$NADP^+$ ratio (Lendzian and Bassham, 1975). They also

pointed out (Lendzian and Bassham, 1975) that in the chloroplast, where the Calvin cycle functions during photosynthesis and the hexose monophosphate shunt functions under cell respiration, control mechanisms must operate to prevent wasteful cycling. These investigators found that the NADPH/$NADP^+$ ratio operates within the chloroplast to control the key reactions of these two pathways in an inverse way. The rate - limiting enzymes of the Calvin cycle are stimulated by a high NADPH/$NADP^+$ ratio and G6PD is inhibited (Lendzian and Bassham, 1975.

ATP and other nucleotides have a general regulatory significance for G6PDs. Inhibitory effects of ATP on numerous G6PDs have been reported (Alberty, 1968; Velose et. al., 1973). Only a few of these effects have been shown to be significant under physiological conditions, where ATP exists to a considerable and variable extent chelated with mg^{++} (Alberty, 1968; Velose et.al. 1973). It has been observed that ATP is a much poorer inhibitor of G6PDs when assayed in the presence of mg^{++} than in its absence (Levy, 1979). Further studies with ATP on the enzyme from human erythrocyte have shown that ATP inhibits competitively with respect to

glucose-6-phosphate and noncompetitively with respect to $NADP^+$ with Ki of 0.08mM (Yoshida, 1973.

Among the higher plant G6PDs, the enzyme from mung beans was shown to be inhibited by ATP and ADP (Levy, 1979). The sweet potato G6PD was found to be inhibited by ATP competitively with respect to glucose-6-phosphate, with Ki of 2.1mM at pH 7.0; at higher pH however, the Ki for ATP was increased (Levy, 1979). It has also been shown that ADP and AMP were found to be inhibitors of G6PD by a mechanism fully competitive towards glucose-6-phosphate, although this was to a reduced extent and the inhibition showed sigmoidal kinetics (Bonsignore and De Flora, 1972)

Coenzyme A and acetyl-CoA are known to inhibit G6PD from L. mesenteroides. This was first reported by Levy and Ishaque (Levy, 1979) and was later confirmed by Coe and Hsu (1973). Acetyl CoA and CoA have also been found to inhibit G6PDs from P. fluorescens and mung beans (Levy, 1979).

Quite a number of metabolic intermediates have been shown to inhibit various G6PDs. Glyceraldehyde -3-phosphate and erythrose-4-phosphate were shown to inhibit G6PD from C. utilis (Chilla et. al., 1973; Domagk et. al., 1973). Both compounds are ofcourse intermediates of the HMP shunt. Interesting enough, no regulatory significance was found for these inhibitions (Chilla et. al., 1973). Ribulose-1,5-di-phosphate (RuDP) has also been found to inhibit G6PD from spinach leaves (Lendzian and Bassham, 1975)and Aphanocapsa 6309 (Levy, 1979). The inhibitions of RuDP on the spinach G6PD have been shown to be of regulatory significance (Lendzian and Bassham, 1975).

It has also been reported by several Investigators that long-chain acyl CoA or long chain fatty acids have inhibitory effects on G6PDs and other enzymes (Malcom and Shepherd, 1972). Since these compounds have a somewhat detergent-like effect on proteins, they are therefore considered

to be non-specific effectors without regulatory significance (Levy, 1979). These compounds may represent significant control mechanism for G6PD as stated by Levy (1979).

Mckerns et. al., (Levy, 1979) and Marks and Banks (1960), have shown that some G6PDs are inhibited by steroids. This inhibition was found to (i) affect only mammalian G6PDs (Bonsignore and De Flora, 1972); (ii) occur with only certain steroids with specific structural characteristics (Levy, 1979); (iii) require concentration of most effective steroids in the micromolar range (Levy, 1979); and (iv) be uncompetitive with respect to both $NADP^+$ and glucose-6-phosphate. However, the physiological significance of the inhibition of G6PDs by steroids is still not clear (Levy, 1979; Bonsignore and De Flora, 1972). G6PDs from the following sources have been shown to be inhibited by steroids: erythrocytes from normal individuals (Marks and Banks, 1960) and erythrocytes from G6PD- deficient individuals (Levy, 1979); human

liver and adipose tissue (Marks and Banks, (1960), testis, brain and placenta (Levy, 1979).

The Pentose Phosphate Pathway.

The pentose phosphate pathway (PPP) occurs in both animals and plants tissues (Horecker, 1968). It involves mechanisms which lead to the inter-conversion of sugars and is of primary importance as a source of ribose and deoxyribose for nucleic acid and nucleotide coenzymes; and it appears to have a significant role as a source of reducing power or metabolic hydrogen (Horecker, 1968). The PPP is related to Embden-Meyerhof pathway in some aspects.

The two pathways are interconnected at glucose-6-phosphate, fructose-6-phosphate and glyceraldehyde-3-phosphate levels (Horecker, 1968). Interesting enough, all the individual enzymes and intermediates have been studied in great details and there is a wealth of literature on the enzymes involved. A complete knowledge of the PPP entails knowing the individual enzymes involved and the reactions they catalyze.

Importance of hexose monophosphate pathway (HMP)

A major requirement of the cell which is fulfilled by the HMP is the production of NADPH which is used by cells for many synthetic processes. NADPH generated by HMP provides almost half of the reducing power for fatty acid synthesis (Horecker, 1968).

NADPH and NADH are generated for the biosynthesis of fatty acids, folic acids, purine biosynthesis and steroids (Horecker, 1968).

The HMP ensures the supply of hexose, pentose, erythrose and triose sugars for important synthesis going on in the cell. Ribose-5-phosphate, for example, is important in the synthesis of RNA, DNA and nucleotide coenzymes (Stryer, 1974).

Also in plants, the carbon dioxide (CO_2) generated in HMP might be utilised in the synthesis of hexose during photosynthesis by green plants (Horecker, 1968).

Of particular importance is the role of HMP in

the maintainance of reduced sulfyhydryl compounds of the cell, since NADPH is the primary reductant of glutathione, and this in turn has been correlated with the increase in red cell fragility in individuals with congenital G6PD deficiency (Keller, 1971).

The use of glucose by the corneal epithelium appears to be controlled by the oxidative steps of HMP since most of the CO_2 produced by this tissue is from carbon atom number one of glucose (Horecker, 1968). In corneal and nerve tissues, however, and possibly in the erythrocyte another mechanism is obtained for the oxidation of NADPH. This is the polyol dehydrogenase which uses NADPH for reduction of aldoses such as glucose, galactose and xylose which are abundant in the lens and nerve tissues (Horecker, 1968).

These aldoses are reduced in the lens to form the corresponding polyols, sorbitol, dulcitol or xylitol. Polyols, especially sorbitol accumulate in the lens of diabetic animals. So cataract formation has been associated with increased

polyol content of lens, but whether this is a direct effect on osmotic equilibrium or a consequence of the removal of the NADPH required to maintain protein sulfyhydryl groups remains to be established (Horecker, 1968).

METABOLIC AND PHYSIOLOGICAL ROLE OF G6PD IN RED CELLS

The red cell depends on glycolytic pathway for its energy production, but glycolytic energy scheme is far less efficient source of energy (Keller, 1971). An alternative oxidative pathway for glucose is present in the red cell - and this is the HMP. The most important function of HMP to the matured red cell is production of NADPH which is the only source of NADPH available to the erythrocytes (Keller, 1971).

1. Methemoglobin Reduction

The hemoglobin in normal red cells is always changing from active ferrohemoglobin to the less active ferric state called methemo-

globin. Methemoglobin cannot transport oxygen (O_2) so is non-functional as a carrier of O_2. The red cell in order to prevent this has two enzyme systems - one associated with NADPH which is NADPH-dependent, and the other which is NADH-dependent. The enzyme that is NADPH-dependent is called NADPH-dependent methemoglobin reductase (Keller, 1971). The hereditary deficiency of the NADPH-dependent reductase is associated with methemoglobinemia (Keller, 1971).

2. Maintainance of reduced glutathione

The basis of drug-induced hemolytic anemia is that there is a deficiency in G6PD in red cells and this leads to reduced amount of NADPH present, which in turn entails lowered concentration of reduced glutathione, a tripeptide that contains a sulfyhydryl group (Keller, 1971). Reduced glutathione keeps the cysteine residues in glutathione in the reduced form and this reduced form is regenerated from oxidized glutathione by reaction with NADPH (Stryer, 1974). Reduced glutathione appears to be essential for

the maintainance of normal red cell structures (Horecker, 1968). Cells with lower level of reduced glutathione are more exposed to hemolysis for unknown reasons (Horecker, 1968).

3. Reduction of Oxidant compounds and drugs

Oxidant compounds tend to injure the erythrocyte by their ability to act as electron carriers by taking electrons from cellular constituents and passing them to oxygen (Keller, 1971). By doing this, these compounds shield the dangerous charge of oxygen they carry. So NADPH plays a vital role in protecting the erythrocytes directly by detoxifying these compounds (Keller, 1971).

4. Lipid Synthesis

About 90% of erythrocyte lipid is located in the cell membrane (Keller, 1971). The integrity of the membrane depends on adequate original synthesis of membrane lipids during the cell's life. It has been controversial as to whether the observed increase in G6PD is

necessary for the accompanying increased rates of fatty acid synthesis or whether it is merely a reflection of the greater fat synthesis (Freedland and Briggs, 1977). Red cell fatty acid synthesis requires NADPH which is used for the reductive steps; and the source of NADPH is via HMP which is dependent on the presence of G6PD (Freedland and Briggs, 1977). In G6PD deficient red cells, there is decreased membrane lipids found in the cell membranes (Keller, 1971).

5. Pathophysiology of G6PD Deficiency

Since the presence of G6PD in HMP is required as a source of NADPH, a deficiency in G6PD is likely to affect the concentration of reduced glutathione which in turn will affect red cells since they now become susceptible to hemolysis (Keller, 1971). Drugs such as pamaquine, primaquine and furadantin may distort the surface of red cells in the absence of reduced glutathione - thus making them more

liable to destruction and removal by the spleen (Stryer, 1974). This effect leads to drug induced hemolytic anemia. The practical importance of detecting G6PD deficiency has prompted the formulation of techniques for assaying the enzyme (Levy, 1979).

6. Inheritance of G6PD deficiency

G6PD deficiency is not a rare disease. It is an inherited sex-linked trait and it has been established that female heterozygotes have two populations of red cells: One has normal enzymatic activity, whereas the other is deficient in G6PD (Levy, 1979). It has also been observed that there is a high gene frequency of G6PD deficiency in Black Americans (Keller, 1971); and this high frequency is thought to be advantageous under certain environmental conditions (Keller, 1971).

Infact, it has been established that G6PD deficiency in red cells appears to protect an individual from falciparum malaria because the

parasites need the HMP and reduced glutathione for optimal growth (Stryer, 1974). Hence G6PD and sickle-cell trait have similar mechanisms of protection against malaria. This reason therefore accounts for the high gene frequencies of G6PD in malaria-infested regions of the world (Stryer, 1974).

Many of the varying aspects of G6PD deficiency have been thoroughly studied and integrated (Keller, 1971). There are several advances being made towards the elucidation of mechanisms, both biochemical and genetic in G6PD deficiency. These will no doubt facilitate a better and final understanding of still other hereditary diseases (Levy, 1979).

7. Genetic Variants

A large number of mutants have been identified in human erythrocytes (Levy, 1979). Yoshida (1967) has shown that two variant G6PDs, the Negro variant A and the Hetoen variant, which is associated with enzyme overproduction,

differ from the normal variant B by single amino acid substitution. These amino acid substitutions have a relatively minor effect on the catalytic properties of the enzyme (Levy, 1979).

8. G6PD and aging of erythrocytes

It has been very uncertain whether aging of erythrocytes could be attributed to G6PD level in the cell so an additional reason for the current interest in the structural and regulatory properties of G6PD is the striking correlation between decrease of erythrocyte G6PD activity and aging of the cell (De Flora et. al., 1972).

METABOLIC AND PHYSIOLOGICAL ROLE OF G6PD IN PLANTS

1. Lipid Synthesis

The biosynthesis of lipids in general entails the presence and participation of NADPH for the reductive steps (Freedland and Briggs, 1977). The availability of NADPH could be as a result of G6PD present as one of the enzymes in

HMP. Thus in plant tissues, a deficiency in G6PD in the cell might result in a decrease in the amount of lipid. The work by Ikediobi and Oti (1981) suggests that the high activity of G6PD in white yam tuber is responsible for the high lipid biosynthesis in this tuber, and might also be related to onset of sprouting. It therefore appears that G6PD is actively involved in lipogenesis in the tuber. Their work also suggests that the HMP might be important in carbohydrate metabolism in the tuber because of the high level of G6PD observed. This idea has been hinted by Ugochukwu et. al. (1977) in their studies of enzyme activities in aging white yam tubers.

2. G6PD and aging of yam tubers.

The relatively high activity of G6PD in aged yam tubers tends to suggest that the contribution of the HMP to carbohydrate metabolism increases with age (Ugochukwu et. al., 1977). It may be possible to relate G6PD activity in yam tubers to the age of the tuber.

MATERIALS AND METHODS

Materials:

Thirty-one different yam cultivars of the Dioscorea species were purchased from different parts of Nigeria. The table below depicts the number of yams for each cultivar.

Table 2: Number of yams for each cultivar.

CULTIVARS	NUMBER OF YAMS
D. rotundata	24
D. bulbifera	1
D. dumentorum	2
D. alata	3
D. cayenesis	1

Chemicals.

Marker proteins - Bovine serum albumin, Lactate dehydrogenase, alcohol dehydrogenase were obtained from Sigma Company St. Louis U.S.A.; egg albumin, hemoglobin and cytochrome C were purchased from BDH chemicals Poole England. Nicotinamide adenine dinucleotide (NAD^+), magnesium chloride ($MgCl_2$),

nicotinamide adenine dinucleotide phosphate (NADP) [disodium salt], nicotinamide adenine dinucleotide phosphate reduced (NADPH) [sodium salt], glucose-6-phosphate (G6P) [disodium salt; specific rotation $[\alpha]_D^{20^\circ}$], Tris- (hydroxymethyl)- methylamine [2-amino-2- (hydroxymethyl) propane-1,3-diol, (tris)] were purchased from BDH chemicals Poole England.

Blue dextran 2000, glucosamine-6-phosphate, fructose-6-phosphate (F6P), sephadex G-150 and DEAE sephadex A_{50} were obtained from Pharmacia fine chemicals U psala Sweden.

Acrylamide N N methyl-bisacrylamide (BIS), ammonium persulfate, N,N,N^1, N^1, - tetramethylene diamine (TEMED), bromophenol blue, coomasie brilliant blue were obtained from Shandon Products Ltd. Chesire England.

Other chemicals were either products purchased fro BDH chemicals Poole England, or Sigma chemical Co. St. Louis U.S.A., or Hopkins and Williams (Chadwell heath Essex England) or koch-light

laboratories Ltd. Colnbrook Bucks England. All reagents used were of analytical or pure grade.

Equipment.

Chemical balance-Mettle type H6T No. 215552

pH meter - Corning model 7

High speed centrifuge-Beckmann model J-21

Beckann double beam DB-GT grating spectrophotometer

DC power suppler for electrophoresis: BIO-Rad laboratories model 500 power supply

Ultrorac LKB 7000 fraction collector LKB Produkter Bromma Sweden

Glass columns from Wright Scientific Ltd. England

LKB peristaltic pump type 4912A H. Jurgens and Co. Bromma Sweden.

PURIFICATION OF G-6-PD

Preparation of acetone powder

White yam tuber (D. rotundata) weighing 125 gm was peeled and cut into small cubes and soaked in excess chilled acetone. This was then homogenized in a Potter homogenizer to a fine slurry. The slurry was squeezed to dryness by using double layers of

cheese cloth. The resulting whitish hard cake was homogenized again in excess fresh chilled acetone. The resulting white slurry was again squeezed to dryness by the use of double layers of cheese cloth. The whitish hard cake was spread inside the deep freezer to dry overnight. The dried powder was collected in a conical flask and stored in the freezer for use.

Preparation of crude enzyme from acetone powder

Unless otherwise stated, all experiments were performed in the cold. Forty grams of the dried acetone powder were soaked in 120 ml of cold 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercapto-ethanol. The resulting slurry was stirred continuously with a glass rod for two hours, after which it was then centrifuged at 2,500g for 15 minutes at $5^{o}C$. Then 120 ml of the supernatant was collected as source of crude enzyme.

Ammonium sulphate $(NH_4)_2SO_4$ fractionation.

Initial experiments were performed using 20 ml of crude enzyme to determine the percentage of solid

ammonium sulfate that was required to produce high enzyme activity. From these initial experiments it was found that the enzyme had very high activity between 45% to 75% $(NH_4)_2SO_4$ saturation.

The remaining 100 ml of crude enzyme was then brought to 45% $(NH_4)_2SO_4$ saturation by the addition of 27.7gm of solid $(NH_4)_2SO_4$ crystals. The resulting solution was stirred until all the crystals dissolved and there was precipitation. This was then centrifuged at 2,517.75g for 15 minutes. The supernatant (100 ml) was collected and brought to 75% saturation by adding 21.0 gm of $(NH_4)_2SO_4$ crystals. The solution was stirred and allowed to stand until all the crystals dissolved and there was precipitation. It was again centrifuged at 2,517.75g for 15 minutes; then 25ml of 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol was used in dissolving the $(NH_4)_2SO_4$ precipitated enzyme.

Dialysis

The $(NH_4)_2SO_4$ precipitated enzyme was then dialyzed against 0.055M Tris-Hcl buffer of pH 7.6

containing 0.2% B-mercaptoethanol. The buffer which was stirred constantly by a magnetic stirrer, was changed after every 30 minutes.

Ion-exchange chromatography on DEAE-sephadex

DEAE-sephadex A_{50} (5 gm) was weighed and swollen in 0.055M Tris-Hcl buffer of pH 7.6 for two days. The resulting slurry was packed in a glass column (3.0 x 21cm) and was equilibrated overnight by constant washing with 0.055M Tris-Hcl buffer of pH 7.6, until the effluent recorded a pH of 7.6.

Fifteen milliliters of the dialyzed $(NH_4)_2SO_4$ precipitated enzyme were then applied on the ion-exchange bed and allowed to drain into the column. Thereafter it was eluted with a linear gradient between 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol to 1M Nacl in the same buffer. The flow rate was maintained at 1ml/3minutes. Fractions (5ml) were collected and assayed for enzyme activity and the protein profile was followed by reading absorbance at 280 nm for each fraction. Fractions containing significantly high enzyme activity

were pooled and concentrated to a small volume by using either $(NH_4)_2SO_4$ precipitation (above 90% saturation) or dry sephadex G-150. In the case of $(NH_4)_2SO_4$ precipitated enzyme, it was centrifuged at 2,517.75g for 15 minutes and the resulting precipitate was dissolved in 15ml of 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol.

Gel filtration chromatography

Sephadex G-150 (5gm) was swollen in distilled water for 3 days after which it was packed in a glass column (2.6 x 40 cm) and equilibrated with 0.055M Tris-Hcl buffer of pH 7.6 until the effluent recorded a pH of 7.6.

A five milliliter portion of the enzyme from the ion-exchange step was applied on the sephadex bed and allowed to drain completely before being eluted with 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol. The flow rate was maintained at 1.75ml per minute. Fractions (5ml) were collected and assayed for enzyme activity and protein profile was followed by taking absorbance readings at 280 nm.

Fractions containing significantly high enzyme activity were pooled and concentrated against sephadex G-150 to a small volume. This concentrated extract (5ml) was the source of the pure enzyme.

Determination of G6PD levels in selected yam cultivars

Twenty grams of each of 24 cultivars of D. rotundata, one cultivar of D. bulbifera, two cultivars of D. dumentorum, 3 cultivars of D. alata and one cultivar of D. cayenesis were peeled into separate 60ml 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol. Each was homogenized in a potter homogenizer and the slurry was then squeezed through cheese cloth. The supernatant resulting from each slurry was used as a source of crude enzyme. The activity of G6PD in each supernatant was determined as described below.

Assay of G6PD activity

G-6-PD activity was measured spectrophotometrically by the initial rate of reduction of $NADP^+$ at 29 $\pm$ 2°C. The increase in absorbance at 340nm was recorded for 2 minutes using a Beckmann double beam grating spectrophotometer. The assay method in a cuvette (1 cm

light path) contained the following: NADP (0.2mM, 0.3ml); G6P (6mM, 0.3ml); $MgCl_2$ (M, 0.3ml); Tris-Hcl buffer (0.055M pH 7.6, 2.05ml) and enzyme solution (0.05ml) to give a total volume of 3ml. Reaction was always initiated by the addition of enzyme last. A reference cuvette (1cm light path) contained all the above mentioned materials with the exception of the enzyme. The enzyme activity was expressed as the increase in absorbance at 340nm (A_{340}) per minute per ml of enzyme; and one unit of enzyme activity is defined as the amount of enzyme that will reduce one micromole of NADP per minute at 29 $\pm$ 2°C.

Determination of protein

The protein concentration was determined by the method of Lowry et. al. as modified by Plummer (1971) using bovine serum albumin as standard.

Disc gel electrophoresis

Electrophoresis was carried out by a modification of the method of Davies (1964); 7.5% gels were run in Tris-glycine buffer at pH 8.4. The enzyme solution was mixed with an equal volume of 0.01M Tris-glycine

buffer of pH 8.2 containing 2M sucrose, and this was layered on top of the stacking gel. A current of 2mA per gel was used until the tracking dye entered into the running gel whereby the current was changed to 5mA per gel. This was applied until the tracking dye was about 3mm from the bottom of the gel hence the electrophoresis was terminated. The gels were put into 12% trichloroacetic acid for 30 minutes (to fix the proteins) after which they were rinsed and transferred into Coomasie blue stain for one hour. They were rinsed with 7% acetic acid and stored in same acetic acid for observation.

Staining of polyacrylamide gels for G-6-PD activity.

The procedure of electrophoresis described above was carried out but the gels were stained for enzyme activity by the method of Harry and Hopkinson (1976). The staining reaction mixture contained the following: 25ml of 0.2M Tris-Hcl buffer of pH 8.0; 5ml of 0.2M $MgCl_2$, NADP (5mg in 1ml of H_2O), methyl thiazolyl tetrazelium (MTT) (7.5mg in 1.5ml of H_2O).

Phenazine methosulphate or PMS (5mg in 1ml of H_2O), and 0.3ml of 0.6mM G6P. All staining reactions were carried out in the dark since MTT is affected by light.

Molecular weight determination

A column (2.6 x 40cm) of sephadex G-150 was equilibrated with 0.055M Tris buffer of pH 7.6 until the effluent recorded a pH of 7.6. A solution of Dextran blue 2000 was made by dissolving 2mg of Dextran blue in 10ml 0.055M Tris-Hcl buffer of pH 7.6. Five milliliters of this solution was applied on the sephadex bed and allowed to drain completely before it was eluted with 0.055M Tris-Hcl buffer of pH 7.6 containing 0.2% B-mercaptoethanol. The flow rate was maintained at 1ml per 2 minutes. Fractions (5ml) were collected until the whole dextran blue was eluted. The void volume, Vo, of blue dextran was 75ml.

Two milligrams of each of the following marker proteins cytochrome C (Mw 12,500); egg albumin (Mw 45,000); Lactate dehydrogenase (Mw 146,000);

bovine serum albumin (67,000); hemoglobin (MW 65,000) and alcohol dehydrogenase (MW 80,000) were dissolved in 10ml of 0.055M Tris-Hcl buffer of pH 7.6. One milliliter of each marker protein solution was removed and mixed to give a final volume 5ml of combined marker protein solution, and this was applied on the sephadex G-150 bed. Then it was eluted with 0.055M Tris-Hcl buffer and 5ml fractions were collected. Four milliliters of the purified enzyme preparation was then applied to the same column after the elution patterns of the marker proteins had been determined by measurement of absorbance at 280 nm with Beckmann spectrophotometer. The elution pattern of the pure enzyme was determined by assaying each fraction for enzyme activity spectrophotometrically at 340nm as described before. The elution volumes of both marker proteins and the pure enzyme were determined.

Determination of pH profile of G-6-PD

The activity of the purified enzyme was determined as a function of pH using 6mM G6P as substrate and 0.2mM NADP as the coenzyme. The assay procedure was

modified in terms of pH of buffers. The following buffers were used in the assay of enzyme activity:

0.1M citrate buffer for the pH range of 3-6; 0.1M Tris-Hcl buffer for the pH range of 7-9; and 0.1M glycine-NaOH buffer for pH range of 9-10.

Effect of pH on K_m and V_{max} of purified G-6-PD

The following buffers were used in the assay of enzyme activity: 0.1M citrate buffer for pH range of 3-6, 0.1M Tris-Hcl buffer for pH range of 7-9 and 0.1M glycine-NaOH buffer for pH range of 9-10. The enzyme activity was recorded spectrophotometrically at 340nm and at varying concentrations of G6P. The assay procedure was essentially the same except for the change in buffer pH. The Line-weaver Burk plots of the results at the different pH values were drawn to obtain values for K_m and V_{max}. Values of log V_{max}, K_m and V_{max}/K_m were found for each corresponding pH. These values were then plotted against pH using the method of Dixon and Webb (1964).

Stability of G6PD at different temperatures

A solution (0.2ml) of the purified enzyme was

measured into 8 different tubes which were sealed with aluminium foil. Each tube was incubated in a waterbath for 20 minutes at the following temperatures: 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 100°C. The heated enzyme solution was then assayed for activity at 29 ± 2°C after cooling down to room temperature. The deactivation energy, ΔE, was calculated from the slope of plot log. initial velocity versus reciprocal of temperature (in °K).

Stability of G-6-PD in storage

A solution (0.2ml) of the purified enzyme was put into 24 tubes sealed with aluminium foil. Eight of these tubes were left at the laboratory temperature of 27°C; another 8 of the tubes were left at 4°C and the last 8 tubes were left in the frozen state (deep freezer) for 16 days. On every other day, 0.05ml of the enzyme was removed from a tube under the various listed conditions and assayed for activity as described previously.

Substrate specificity

Six millimolars of fructose-6-phosphate (F6P),

6mM glucosamine-6-phosphate and 0.2mM NAD^+ were each used as substrate for the purified enzyme. The assay procedure used was essentially those described previously except the change in substrate. Line-weaver Burk plots of the results were constructed to obtain the K_m values.

KINETIC STUDIES

Initial velocity studies:

1. The pure enzyme was assayed with various concentrations (100uM, 80uM, and 60uM) of G6P. The assay solution contained the following: $MgCl_2$ (1M, 0.3ml), enzyme solution (0.05ml), 0.3ml of G6P at different levels mentioned above, 0.3ml of 0.2mM NADP as varying substrate, and various volumes of 0.055M Tris-Hcl buffer of pH 7.6 in a total volume of 3ml. The reaction was initiated by adding the enzyme last and was followed spectrophotometrically at 340nm for over 2 minutes at 29 $\pm$ 2°C. A reference cuvette (1cm light path) containing all the

the above mixture with the exception of 0.2mM NADP was used.

2. The pure enzyme was assayed under various concentrations (0.2mM, 0.4mM, 0.6mM and 0.8mM) of NADP. The assay procedure was essentially the same as the previous one described above except that NADP was the changing fixed substrate and 6mM G6P was the varying substrate. A reference cuvette containing all mixtures except 6mM G6P was used.

3. Various concentrations of NAD^+ (0.1mM, 0.2mM, 0.4mM and 0.6mM) were used to assay the pure enzyme. The assay procedure was essentially that described previously except that G6P was the varying substrate and NAD^+ the changing fixed substrate.

Product Inhibition Studies

Experimental protocol involves holding one substrate constant at subsaturating levels and varying the other substrate in the region of its K_m at different fixed concentrations of one of the products. This procedure is repeated so that the fixed substrate in the

first experiment is varied and the other substrate is fixed at subsaturating levels. The same procedure is then extended to the other product (Ikediobi personal communication). NADPH was prepared in the following concentrations:
10^{-4}mM; 10^{-5}mM; and 10^{-6}mM. Initial velocity experiment was carried out in the absence of the inhibitor (NADPH). Six millimolars G6P was used as the varying substrate while 0.2mM NADP was held constant at subsaturating levels, and the NADPH was used at various levels at fixed volume. The reference cuvette contained every reaction mixture with the exception of 6mM G6P. Assays were carried out in duplicate.

The assay procedure was then changed. 0.2mM NADP was made the varying substrate while 6mM G6P was held constant at subsaturating levels and NADPH was used at various levels at fixed volume. The assay procedure was similar to the one described above.

Inhibition studies using substrate analogue, Glucosamine-6-phosphate.

Different levels of glucosamine-6-phosphate (1mM, 2mM, 4mM and 8mM) were prepared. Initially 6mM G6P was used as the varying substrate and 0.2mM NADP was nonvaried substrate held at subsaturating level around its K_m value. The above was repeated using 6mM G6P as the nonvaried substrate and 0.2mM NADP as the varying substrate.

Competitive substrate Inhibition Studies using substrate analogue, NAD^+.

Different levels of NAD (0.2mM, 0.4mM and 0.6mM) were prepared and 6mM G6P was used as the varying substrate and 0.2mM NADP was nonvaried substrate. This was repeated using 6mM G6P as nonvaried substrate and 0.2mM NADP as varied substrate.

The condition of competitive substrate inhibition studies using substrate analogues entails that a dead end competitive inhibitor be available for each substrate (Ikediobi personal communication). For each of the competitive substrate inhibition study, initial velocity experiment was carried out in the absence of inhibitor.

Inhibition by ATP

The following concentrations of ATP were prepared - 1mM, 2mM and 3mM. The inhibition by ATP was carried out first in its absence in the reaction mixture and then at different constant levels as indicated above. In each case, 6mM G6P was varied and 0.2mM NADP was the non-varied coenzyme. A reference cuvette containing all reaction mixtures except 6mM G6P was used. The assay procedure was similar to those already described.

RESULTS

Isolation and purification of G6PD

G6PD was found to be stable throughout the purification steps. From the initial $(NH_4)_2SO_4$ fractionation experiment (Fig. 1) carried out on the crude G6PD extract, it can be observed that the enzyme protein was precipitated between 45%-75% $(NH_4)_2SO_4$ saturation. The 45%-75% $(NH_4)_2SO_4$ cut did increase the purification of the enzyme by approximately 16 times over the crude extract as shown on Table 3.

Figure 2 shows the elution pattern obtained for G6PD after ion-exchange chromatography using DEAE-sephadex A_{50}. A single protein peak and single enzyme activity peak are observed. This purification step also increased the fold purification of the enzyme by 28 times over the crude (table 3).

FIG. 1: Ammonium sulphate fractionation of crude G6PD.

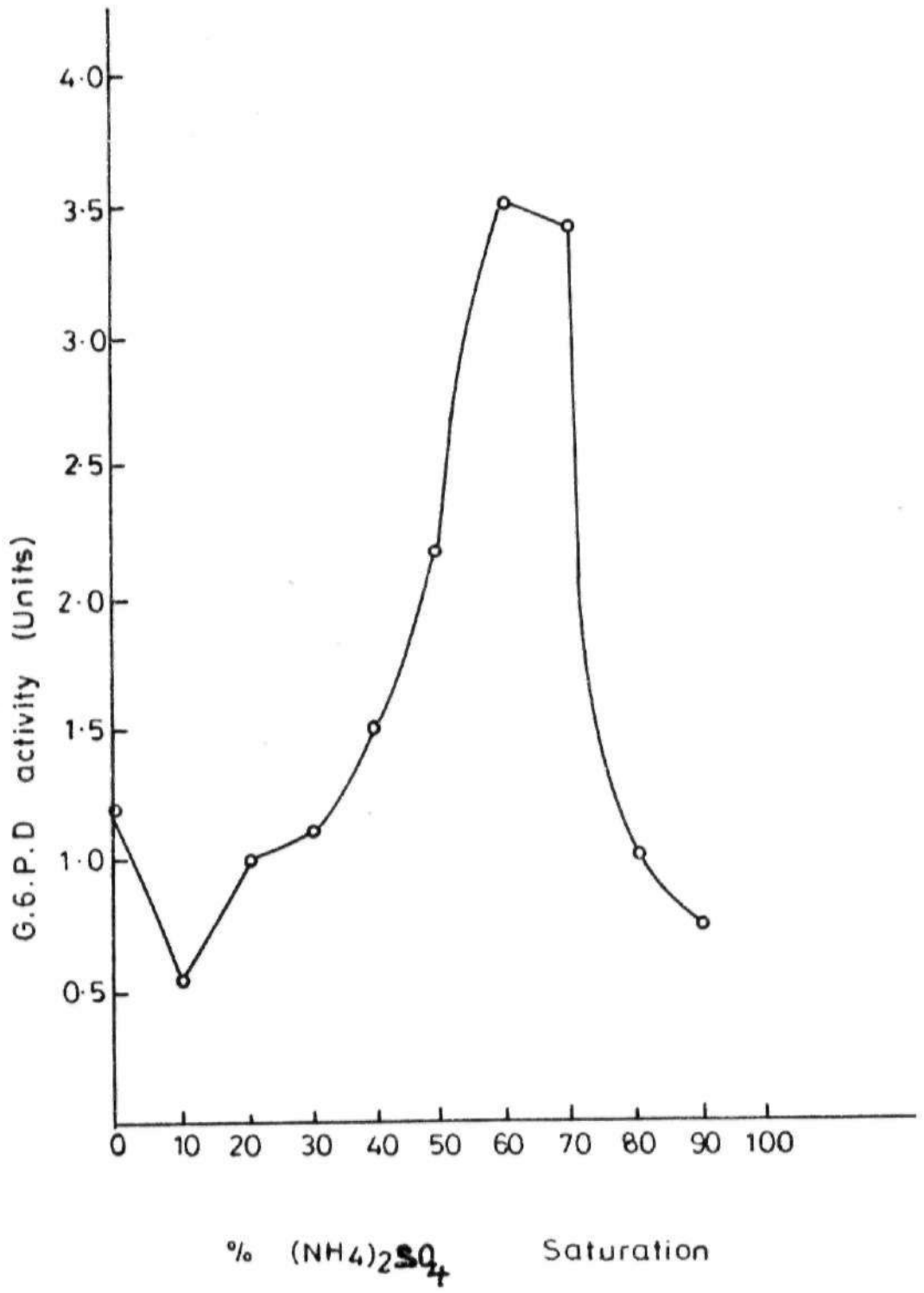
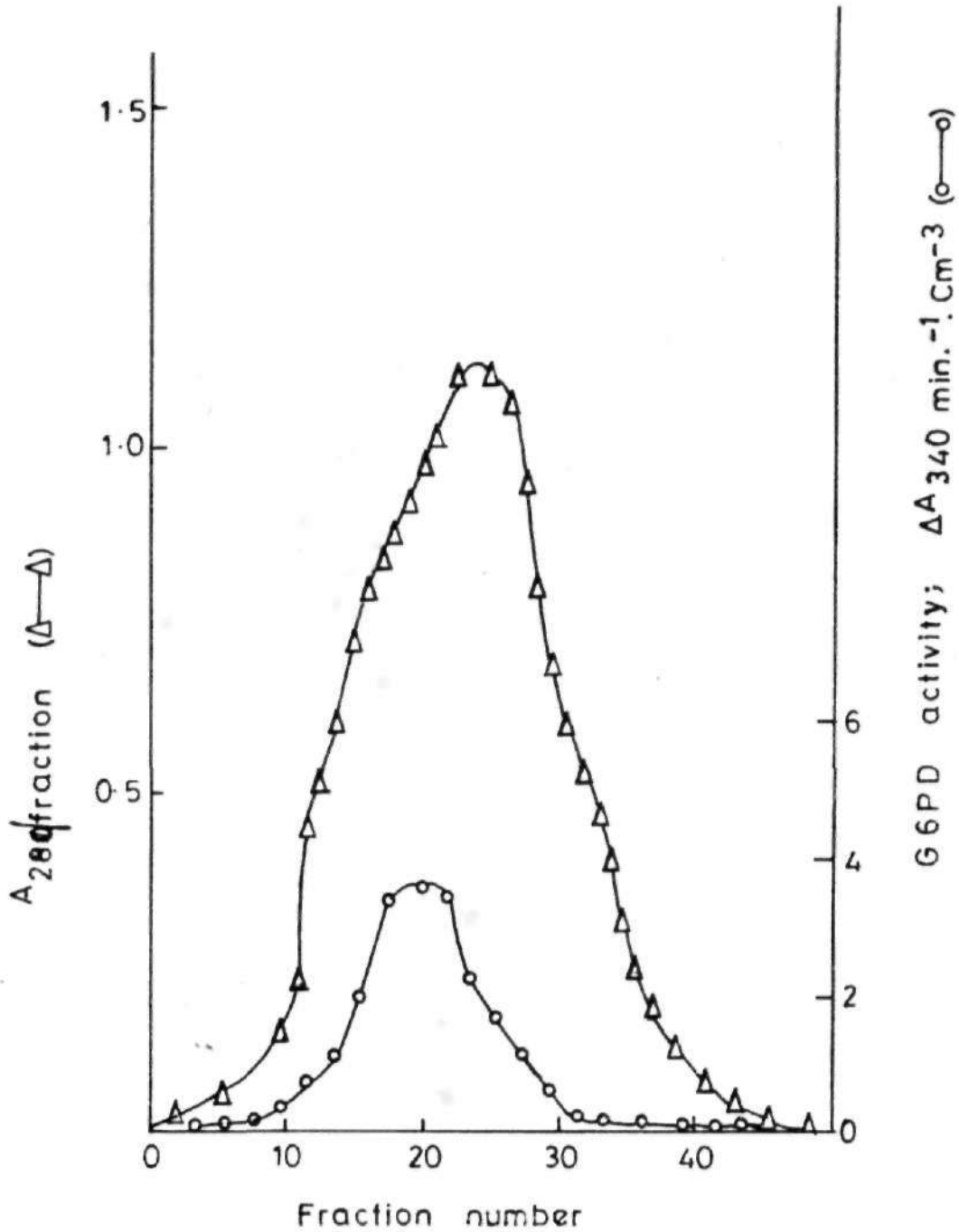

G.6.P.D activity (Units)
4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0
10
20
30
40
50
60
70
80
90
100
% (NH4)2SO4 Saturation

Table 3. - Purification table of Yam tuber G6PD

Purification step	Volume (ml)	Protein (mg ml^{-1})	Total Protein (mg)	Activity (Unit ml^{-1})	Total activity (units)	Specific activity (Units mg^{-1}	Fold Purification	Yield %
Crude	100	1.92	192	2.66	266	1.385	-	100.00
Ammonium sulfate	25	0.09	2.25	1.96	49	21.77	15.71	18.42
Ion-exchange	15	0.05	0.75	1.91	28.65	38.20	27.58	10.77
Sephadex G-150	2.5	0.011	0.028	1.98	4.95	180.0	129.96	1.86


1·5
1·0
0·5
A_{280} fraction (Δ—Δ)
0
10
20
30
40
Fraction number
6
4
2
0
G6PD activity; ΔA_{340} min.$^{-1}$. Cm^{-3} (o—o)

after

The elution pattern / gel filtration on sephadex G-150 for G6PD (Fig. 3) shows one protein peak and one enzyme activity peak. This gel filtration resulted in over 100 -fold purification of the enzyme (table 3).

G6PD levels in different yam cultivars

The G6PD levels in different yam cultivars were measured spectrophotometrically and expressed as specific activities. The result shows that different Dioscorea cultivars have different levels of activities of G-6PD and also different protein contents (table 4). The highest activity of G6PD is obtained for D. rotundata cultivar locally called 'alo' from Ondo; while the lowest activity was obtained for D. rotundata cultivar locally called 'buki' from Kaduna.

Table 4 - G6PD levels in selected Yam cultivars from parts of Nigeria.

Cultivars	local name	Area harvested	Specific activity (Units/mg protein)
D. rotundata	amaran	Zaria	77.00
D. rotundata	chikakwando	Zaria	29.40

Table 4 Contd.

Cultivars	local name	Area harvested	Specific activity (Units/mg protein)
D. rotundata	mampa	Zaria	42.85
D. rotundata	-	Gongola	27.90
D. rotundata	buki	Kaduna	10.52
D. rotundata	amaran	Kaduna	62.00
D. rotundata	omi	Benin	95.00
D. rotundata	emowe	Benin	64.00
D. rotundata	arhebu	Benin	55.00
D. rotundata	okun	Ilorin	23.30
D. rotundata	okunmado	Ilorin	34.90
D. rotundata	ojo	Ilorin	54.00
D. rotundata	ataoja	Ogbomosho	34.00
D. rotundata	Fele	Ogbomosho	20.00
D. rotundata	churu	Ilorin	58.00
D. rotundata	Igangaran	Ogbomosho	18.00
D. rotundata	eleyintu	Ede	38.00
D. rotundata	eleyintu	Ede	40.70
D. rotundata	alo	Ondo	125.20
D. rotundata	pepa	Abuja	32.50
D. rotundata	ashabamu	Okene	29.00

Table 4 contd.

Cultivars	local name	Area harvested	Specific activity (Units/mg protein).
D. rotundata	ekpe	Akokwa	54.00
D. rotundata	oeha	Akokwa	62.00
D. rotundata	obiaturugo	Akokwa	22.00
D. bulbifera	-	Gongola	65.85
D. dumentorum	-	Gongola	64.28
D. dumentorum	una	Akokwa	44.50
D. alata	sakata	Zaria	56.49
D. alata	ewuru	Ede	29.00
D. alata	mbila	Akokwa	80.00
D. cayenesis	ikpen	Benin	61.00

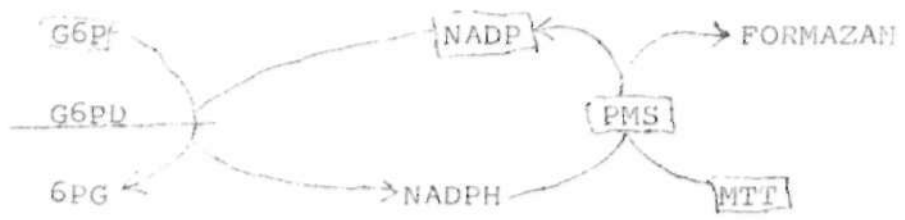
Assay of G6PD activity.

There was no noticeable difference in activity when G6PD was assayed in the presence of 6-phosphogluconate (6PG) with G6P, and when it was assayed with only G6P. So this resulted to the use of G6P only in all the assay methods because of the absence of effect of 6PG-dehydrogenase activity. Under this condition, the initial rate of $NADP^+$ reduction was

linear for two minutes (Fig. 4). The non linear trace after 2 minutes could be due to rapid depletion of substrate by G6PD or accumulation of product which thus inhibited the reaction.

Disc gel electrophoresis

Staining system for G6PD: The gel containing G6PD was stained for enzyme activity after electrophoresis in the reaction mixture described below. This reaction mixture contained 0.055M Tris-Hcl buffer pH 8.0, 6 mM G6P (in excess), 0.2M $MgCl_2$ (5mg in 1 ml H_2O), NADP (5mg in 1ml H_2O), MTT (7.5mg in 1.5ml H_2O) and PMS (5mg in 1ml H_2O). The reaction in the system is schematically represented thus:



Mechanism of reaction: The electron transfer dyes such as methyl thiazolyl tetrazolium (MTT) which is an acceptor for dehydrogenase reaction is a widely used staining reagent for the detection of enzyme after electrophoresis.

In the reaction, MTT is reduced by electron donors to form a dark blue purple insoluble formazan. The reaction proceeds rapidly in the presence of phenazine methosulfate (PMS) which acts as an intermediate catalyst, and these two reagents, each at a concentration of about 0.1mg/ml are standard ingredients in stains for the detection of reactions which lead to the generation of the reduced forms of the coenzymes NAD^+ and $NADP^+$.

Figure 5 shows the disc gel electrophoresis patterns of the purified G6PD. Two protein bands were observed for the enzyme. These protein bands coincided with a diffuse enzyme activity band when G6PD was stained for activity.

Molecular Weight determination

The elution pattern of the purified G6PD showed a single peak for enzyme activity. The molecular weight of D. rotundata G6PD estimated 3 times in sephadex G-150 chromatography using well characterized proteins as markers

yielded a value of 163,000 ± 28,000 (Fig. 6 and 7).

Effect of pH on enzyme activity

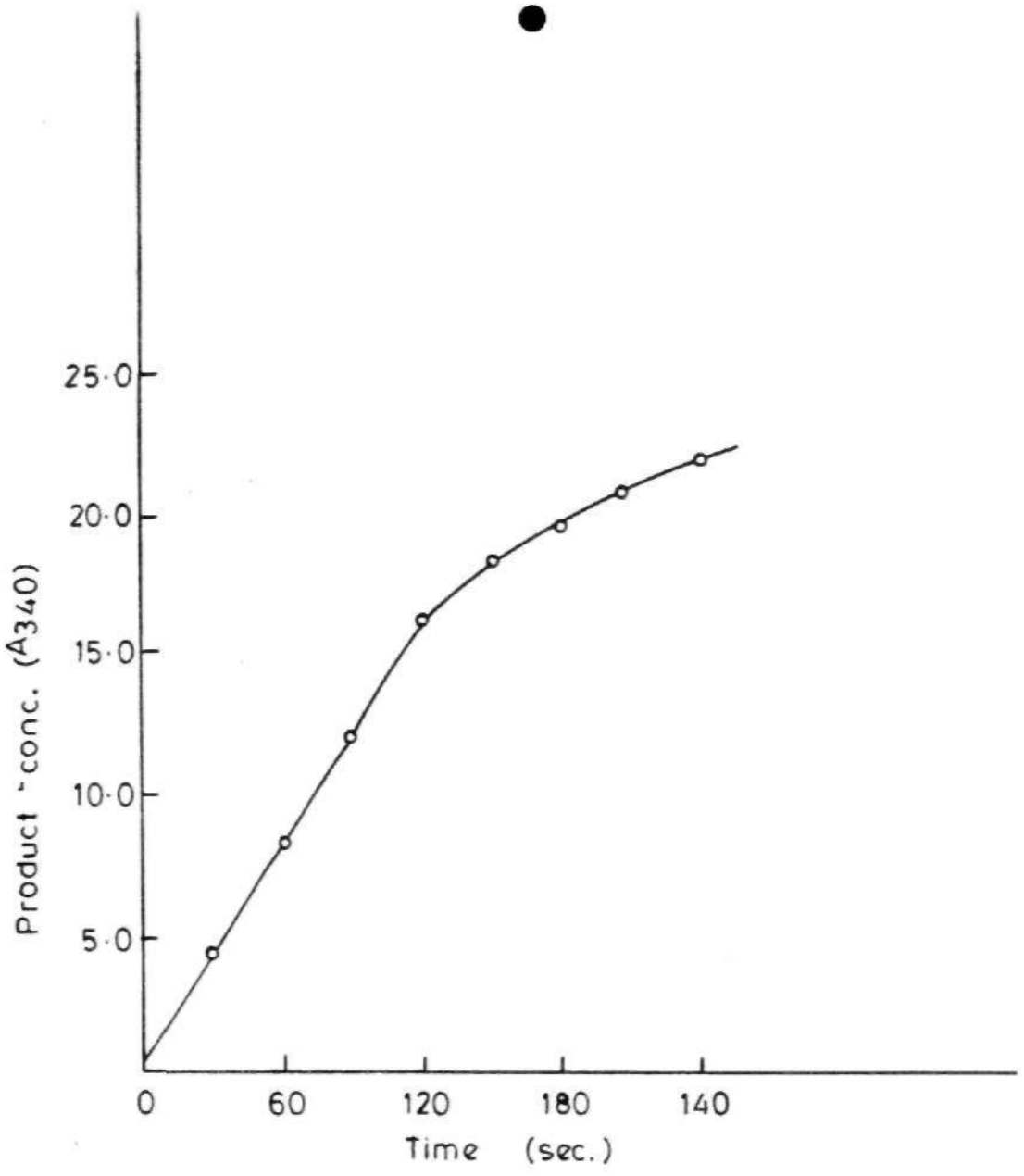
The activity of the purified G6PD determined as a function of pH using G6P as substrate and $NADP^+$ as the coenzyme shows that the enzyme has two pH optima. The first one is observed at the acidic range (pH 5.0) while the other is observed at the basic range (pH 8.2) as shown in Fig. 8.

The effect of pH on K_m and V_{max} of the purified G6PD was carried out by determining the apparent K_m and V_{max} values at each pH value using G6P as substrate and $NADP^+$ as coenzyme. Log V_{max}/K_m against pH (Fig. 9.) shows two peaks- one at pH 5.1 and the other at pH 8.8. Also, two peaks are obtained when Log V_{max} is plotted against pH - one at pH 6.0 and the other at pH 9.6.

Temperature stability of G6PD

The Arrhenus plot (Fig. 10) of log velocity against reciprocal of temperature gave the energy of deactivation value, Ea, of 6.45Kcal $mole^{-1}$. This is the deactivation energy value of G6PD since it was the enzyme and not the reaction mixture that was heated at various temperatures.

FIG. 4: Progress curve for G6PD reaction.


25·0
20·0
15·0
10·0
5·0
Product - conc. (A_{340})
0
60
120
180
140
Time (sec.)

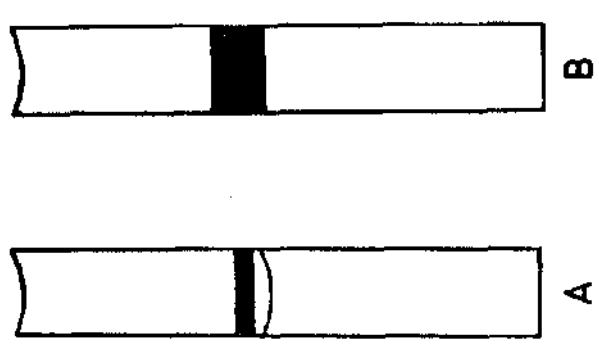

B
A

FIG. 5: Electrophoretic patterns of purified G6PD. (A) Protein stain; (B) activity stain for G6PD.

FIG. 2: Elution pattern after ion-exchange chromatography of $(NH_4)_2SO_4$ precipitated enzyme on a DEAE-sephadex Column. (△—△) absorbance at 280nm; (●—●) G6PD activity (ΔA_{340}/minute/ml).

FIG. 3: Elution pattern of enzyme protein chromatography step on a sephadex G-150 column. (-△-△-) absorbance at 280nm; (-○-○-) G6PD activity (ΔA_{340}/minute/ml).

FIG. 6: Elution pattern on sephadex G-150 of purified G6PD (-△-△-△-) and standard proteins (○—○—○—).

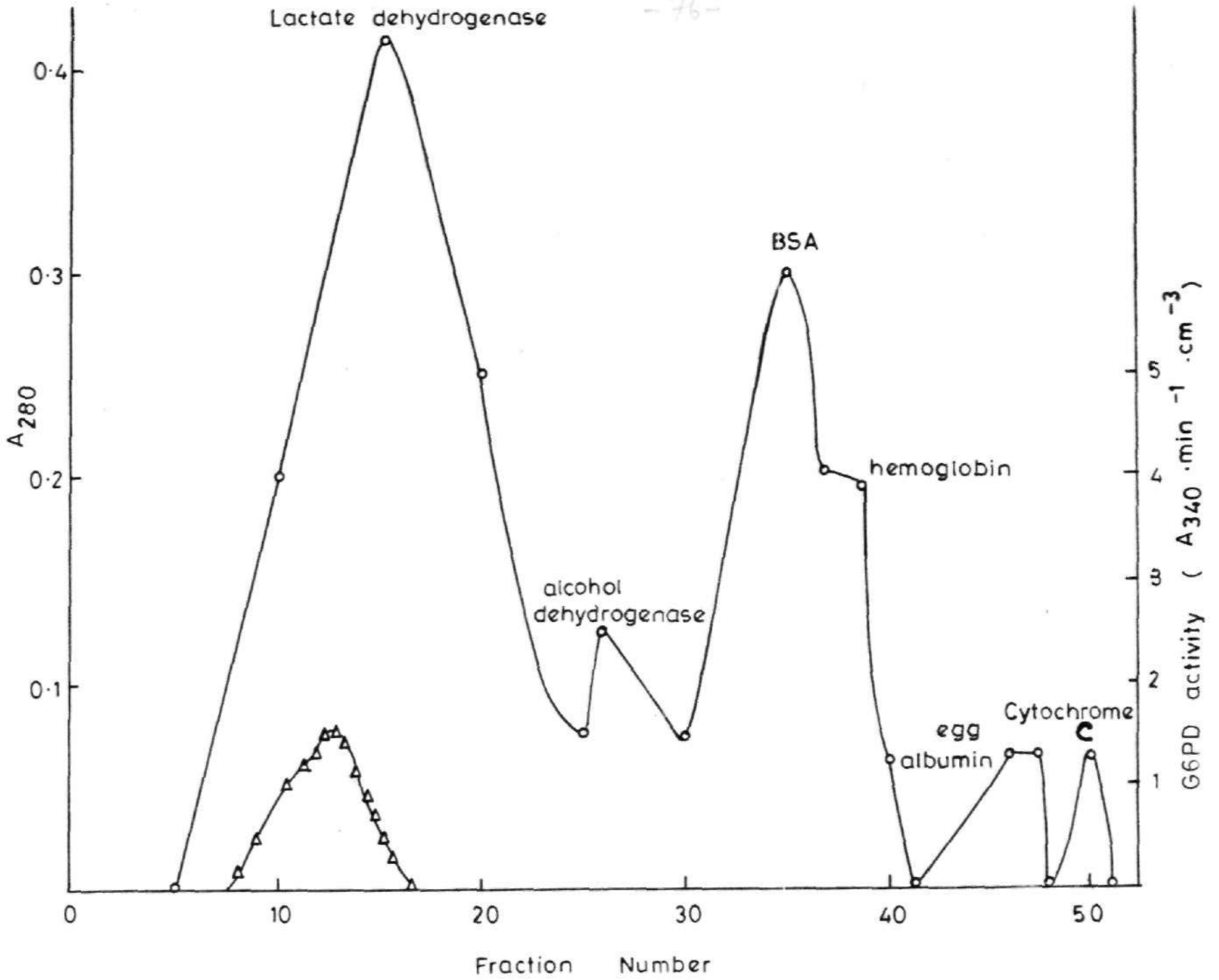

Lactate dehydrogenase
BSA
hemoglobin
alcohol dehydrogenase
egg albumin
Cytochrome c
A280
0·4
0·3
0·2
0·1
0
10
20
30
40
50
Fraction Number
G6PD activity (A340 ·min -1 ·cm -3)
5
4
3
2
1

FIG. 7: Plot of elution Volume (mls) against logarithm of molecular weights of standard proteins for the estimation of molecular weight of G6PD.

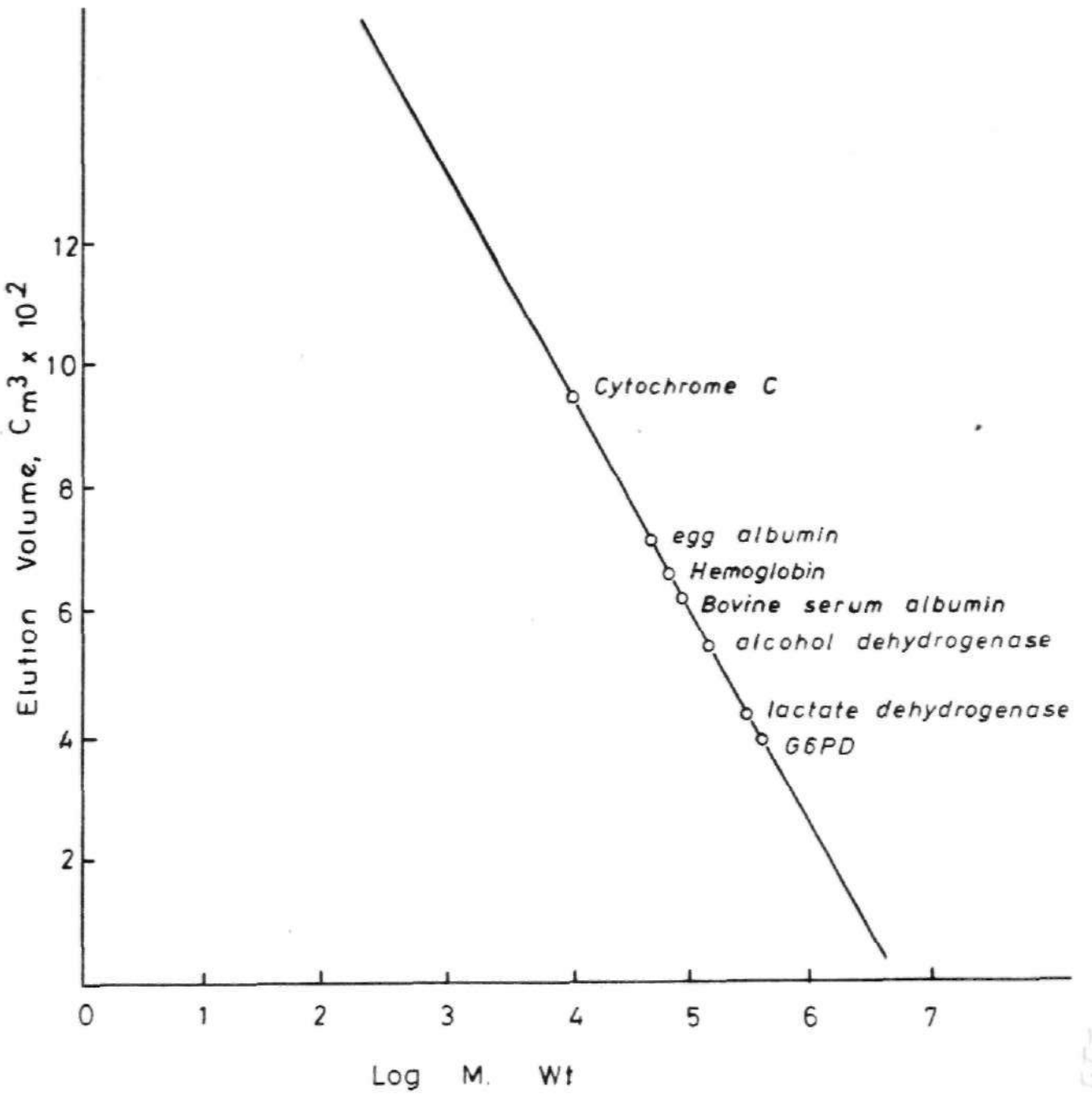

Elution Volume, $C_m^3 \times 10^{-2}$
12
10
8
6
4
2
0
1
2
3
4
5
6
7
Log M. Wt
Cytochrome C
egg albumin
Hemoglobin
Bovine serum albumin
alcohol dehydrogenase
lactate dehydrogenase
G6PD

FIG. 8: Effect of pH on activity of purified G6PD.

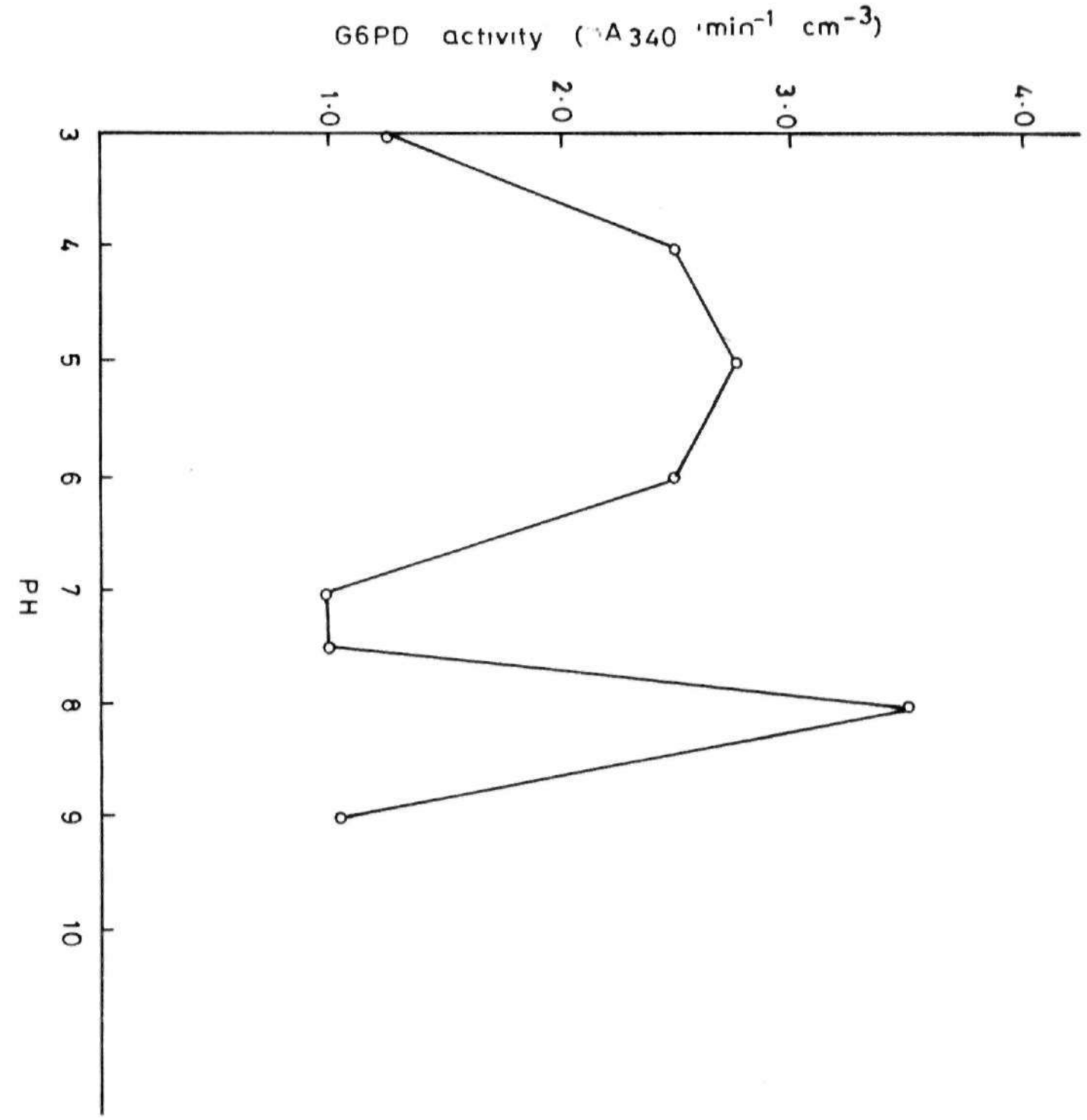

G6PD activity (ΔA340 min-1 cm-3)
1·0
2·0
3·0
4·0
PH
3
4
5
6
7
8
9
10

FIG. 9: Effect of pH on Log V_{max}/K_m.

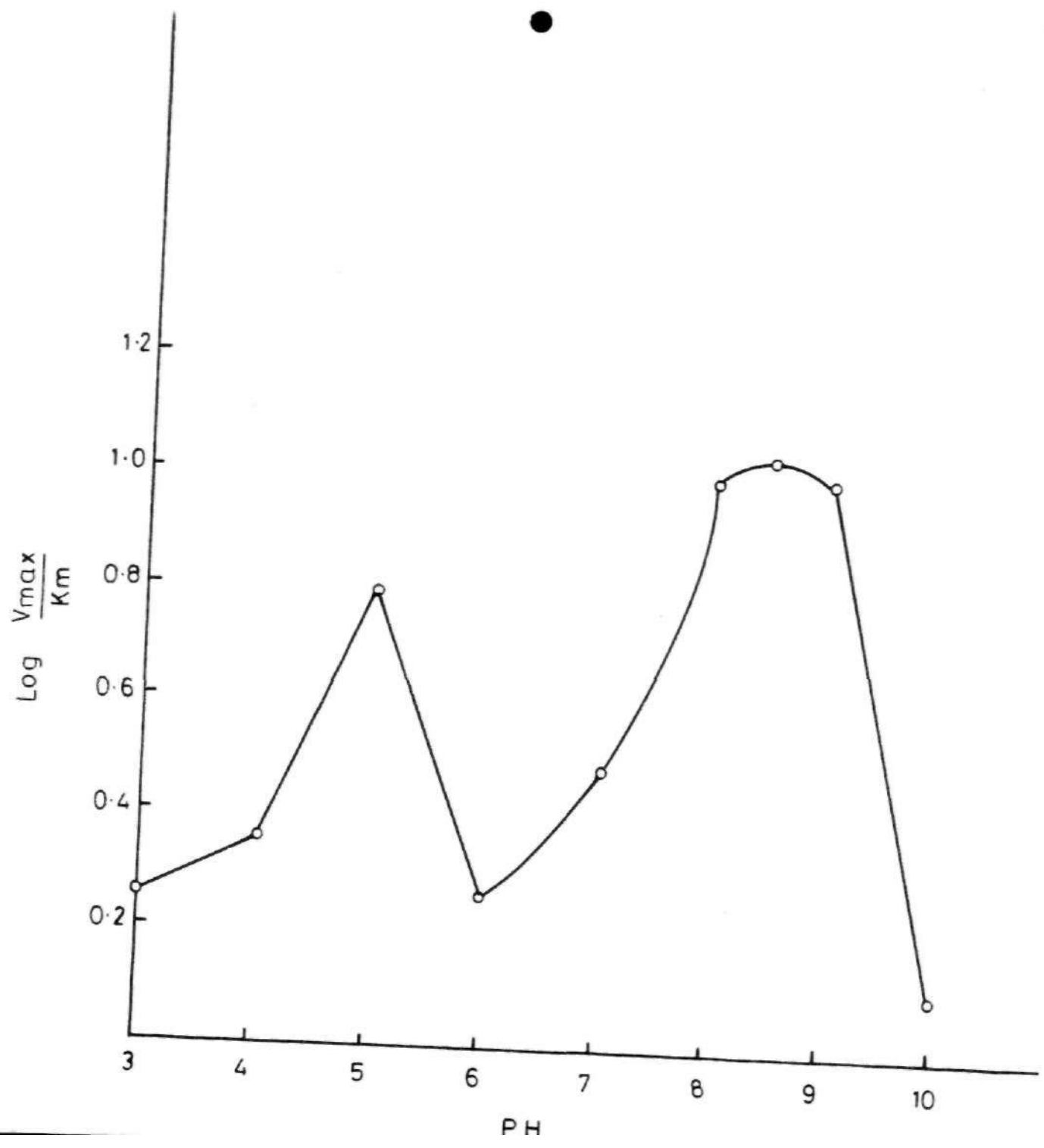
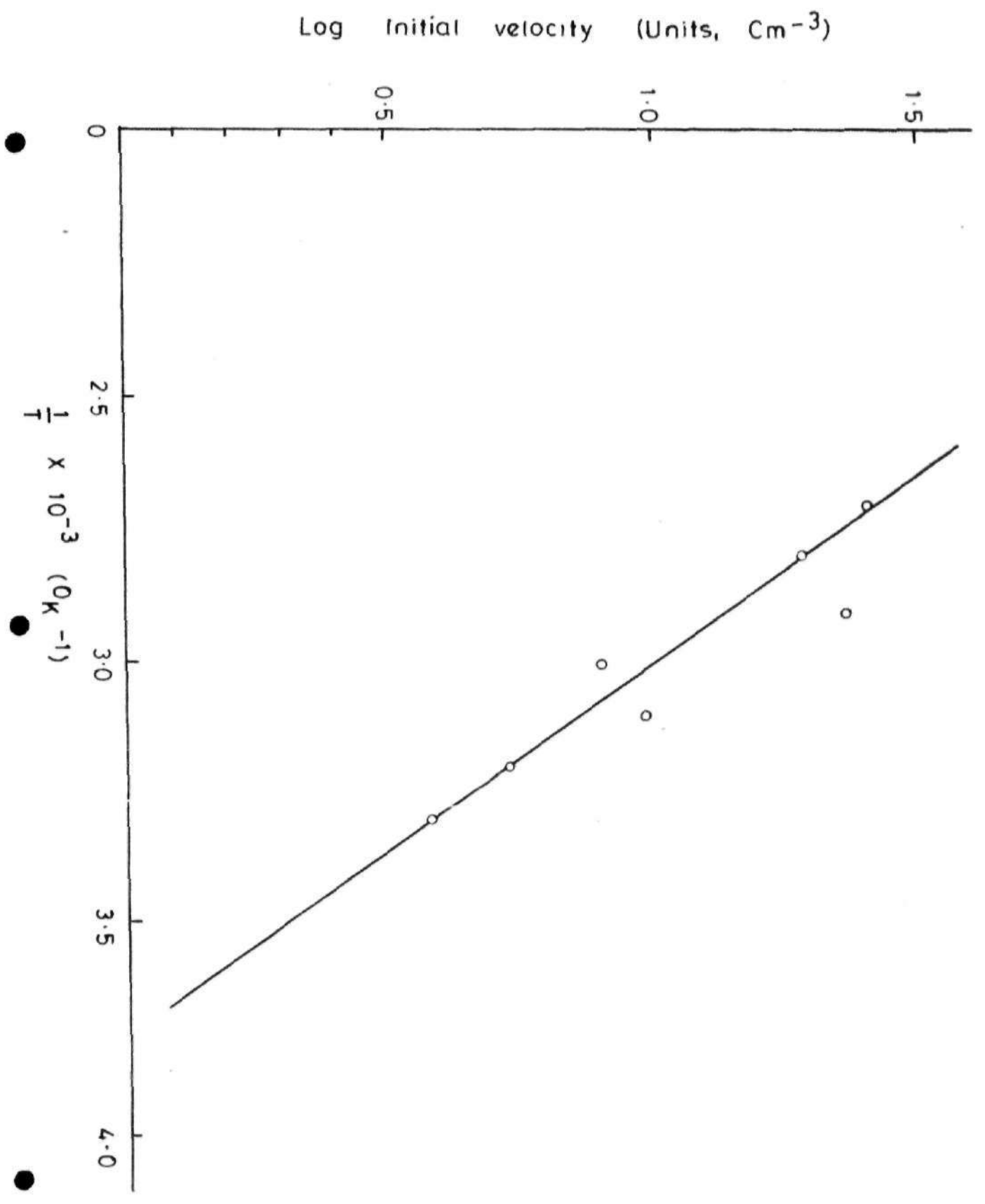

Log Vmax/Km
1·2
1·0
0·8
0·6
0·4
0·2
3
4
5
6
7
8
9
10
PH

FIG. 10: Arrhenius plot of logarithm of initial velocity against $1/T\ ^{o}K$. Slope of curve = $E/2.303R$; E = Energy of activation; R = Gas constant ($1.986\ Cal\ deg^{-'}\ mole^{-'}$).


Log Initial velocity (Units, Cm^{-3})
0
0·5
1·0
1·5
2·5
3·0
3·5
4·0
$\frac{1}{T} \times 10^{-3}$ ($^0K^{-1}$)

Effect of storage on activity

The profile of stability of G6PD on storage for 16 days at 28°C, 4°C and in the frozen state are shown in fig. 11. The plot of relative activity (expressed as % activity of enzyme versus days shows that G6PD is not very stable at 28°C. It is more stable on storage in the refrigerator (4°C) and in deep-frezer (Fig. 11).

Half-life of G6PD at different storage environments

It is known that loss of enzyme activity frequently follows an exponential decay curve that becomes a straight line when log of activity is plotted against time. The relationship log Eo/E = $K_D t/2.303$ is known to hold for such enzymes, where Eo, is specific activity of enzyme at zero time; E, specific activity of enzyme at any time; K_D, rate constant and t, time. When logEo/E was plotted against t, a straight line passing through the origin was obtained whose slope is $K_D/2.303$. For a first order reaction the half-life is given by $t_{½} = 0.693/K_D$.

The half-life of G6PD was then calculated for each storage environment - at 28°C, 4°C and for the frozen state. The specific activity of the enzyme initially was

taken as Eo, while the specific activity of the enzyme on subsequent days of storage was regarded as E. So a plot of logEo/E against t in hours was constructed and $K_D/2.303$ was determined from the slopes of the plots.

The half-life of G6PD thus calculated was found to be 7 days at 28°C, 20 days at 4°C and 100 days in the frozen state (Fig. 12 and 13).

Substrate specificity

NAD^+ was reduced by G6PD as a possible substrate. Also, F6P and glucosamine-6-phosphate ($G-NH_2-6-P$) were oxidized by the enzyme as possible substrates too. The apparent K_m values of 1,66mM was obtained for NAD^+; 5.5mM and 2.0mM for F6P and $G-NH_2-6-P$ respectively (Fig. 14,15, and 16). The kinetic constants obtained are shown in table 5.

Table 5 - Kinetic constants for Yam tuber G6PD substrates.

Substrate	Apparent K_m (mM).
G6P	0.76
$NADP^+$	0.5
NAD^+	1.66
GNH_2-6-P	2.0
F-6-P	5.50

Inhibitors

The inhibition patterns of ATP using G6P as varied substrate and $NADP^+$ as nonvaried substrate at different constant levels of ATP shows a competitive inhibition pattern (Fig. 29).

Initial velocity studies

The results of the initial velocity experiments performed at pH 7.6 are illustrated in Fig. 17 and 18. The intersecting patterns observed are consistent with a sequential mechanism for the white yam tuber G6PD. The replots for slopes and intercepts from these patterns were linear and yielded the following Michaelis constants: 0.8mM for G6P and 0.42mM for $NADP^+$ (fig. 19 and 20).

Product inhibition studies.

The inhibition of yam tuber G6PD by NADPH was examined by measuring initial velocities at different concentrations of one substrate in the presence of NADPH and at a constant non-saturating concentration of the other substrate.

The inhibition patterns observed were:

(i) competitive with respect to $NADP^+$ (Fig. 21)

(ii) Noncompetitive with respect to G6P (Fig. 22)

Secondary plots of these results (Fig. 23 and 24) gave the following inhibition constants for NADPH: 1×10^{-3} mM when $NADP^+$ was varied substrate and 6×10^{-3} mM when G6P was the varied substrate.

Alternate substrate inhibition studies

$G\text{-}NH_2\text{-}6\text{-}P$ was used as the alternate substrate to G6P and NAD^+ used as that for $NADP^+$. In order to employ this method of alternate substrate inhibition studies, it was necessary to determine the apparent Michaelis constants for the alternate substrates. When G6P was the varied substrate and $NADP^+$ nonvaried at different constant levels of $G\text{-}NH_2\text{-}6\text{-}P$, the inhibition pattern was found to be competitive (Fig. 25). When $NADP^+$ was the varied substrate and G6P nonvaried substrate at different constant levels of $G\text{-}NH_2\text{-}6\text{-}P$, the inhibition pattern was again found to be competitive (Fig. 26).

When G6P was the varied substrate and $NADP^+$ the nonvaried at different constant levels of NAD^+, the inhibition pattern was found to be noncompetitive.

Also, when $NADP^+$ was the varied substrate and G6P the nonvaried at different constant levels of NAD^+, the inhibition pattern was found to be competitive (Fig. 27 and 28).

11 APR 1983

FIG. 11: Stability of G6PD on storage in the deep freezer (—○—○—); refrigerator (—△—△—); and room temperature (—●—●—).

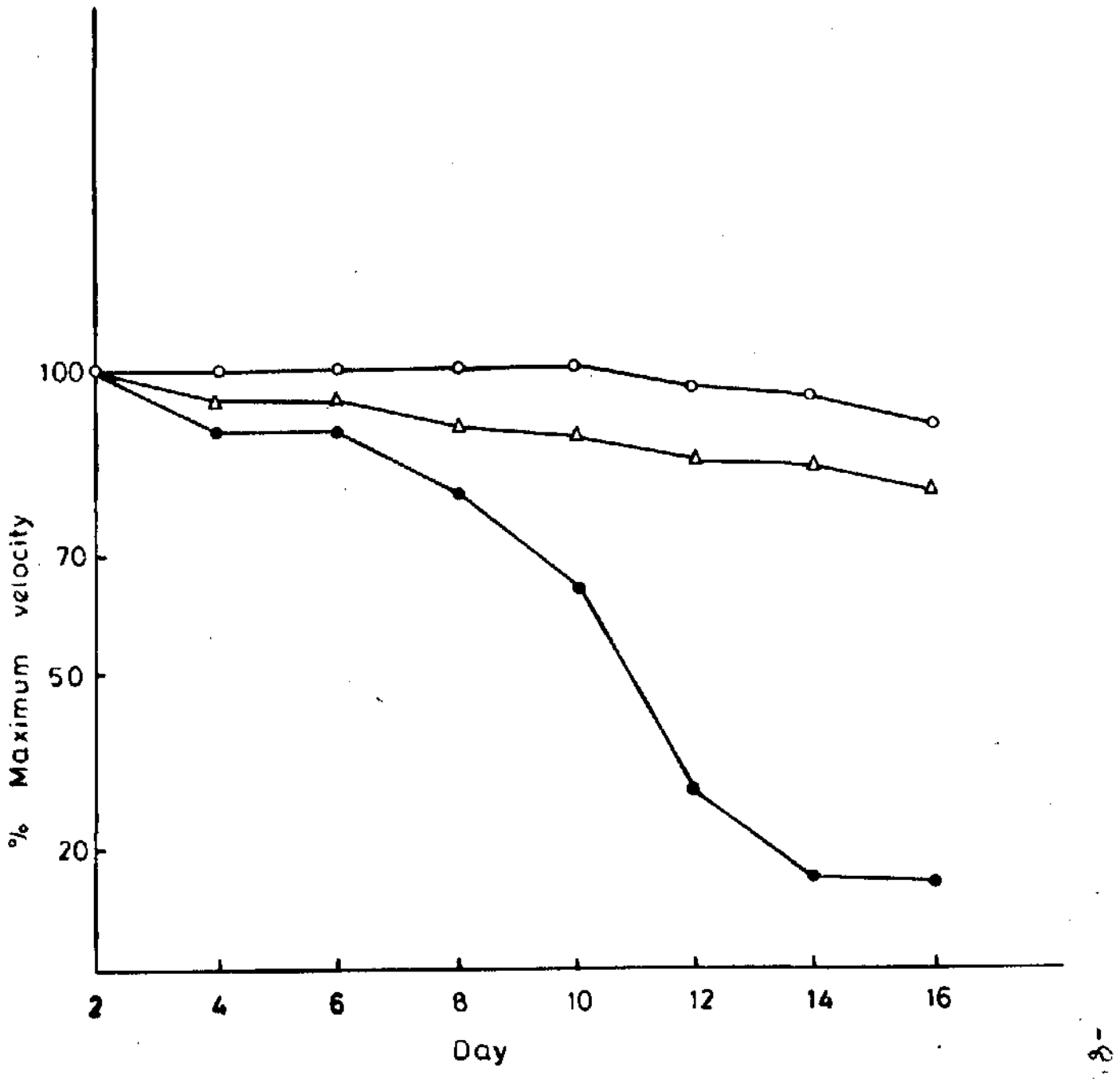

100
70
50
20
% Maximum velocity
2
4
6
8
10
12
14
16
Day

FIG. 12: Plot of logarithm Eo/E against time in hours to determine the half-life of G6PD at room temperature.

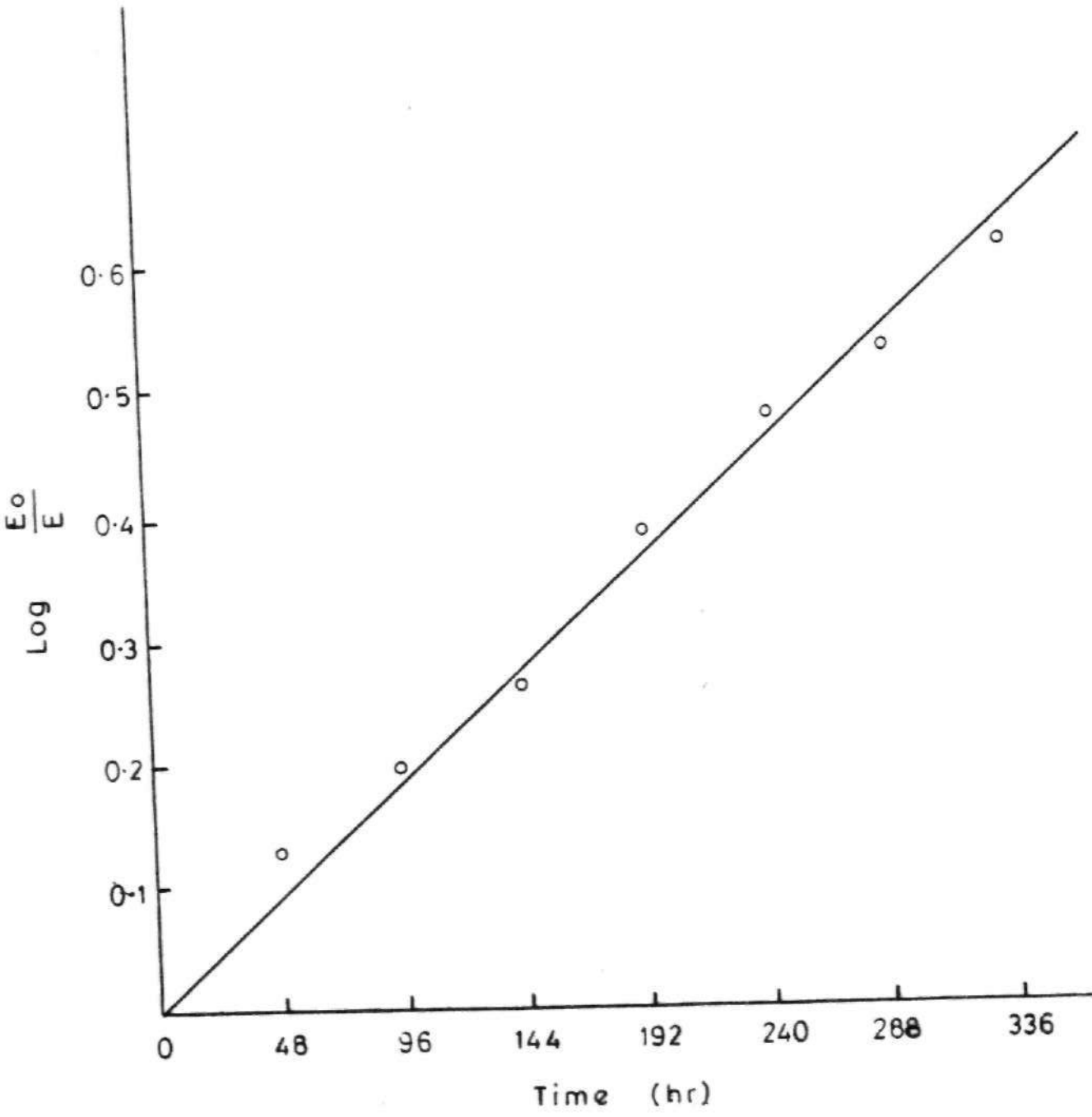

Log E°/E
0·6
0·5
0·4
0·3
0·2
0·1
0
48
96
144
192
240
288
336
Time (hr)

FIG. 13: Plot of logarithm Eo/E against time in hours to determine the half-life of G6PD in the refrigerator.

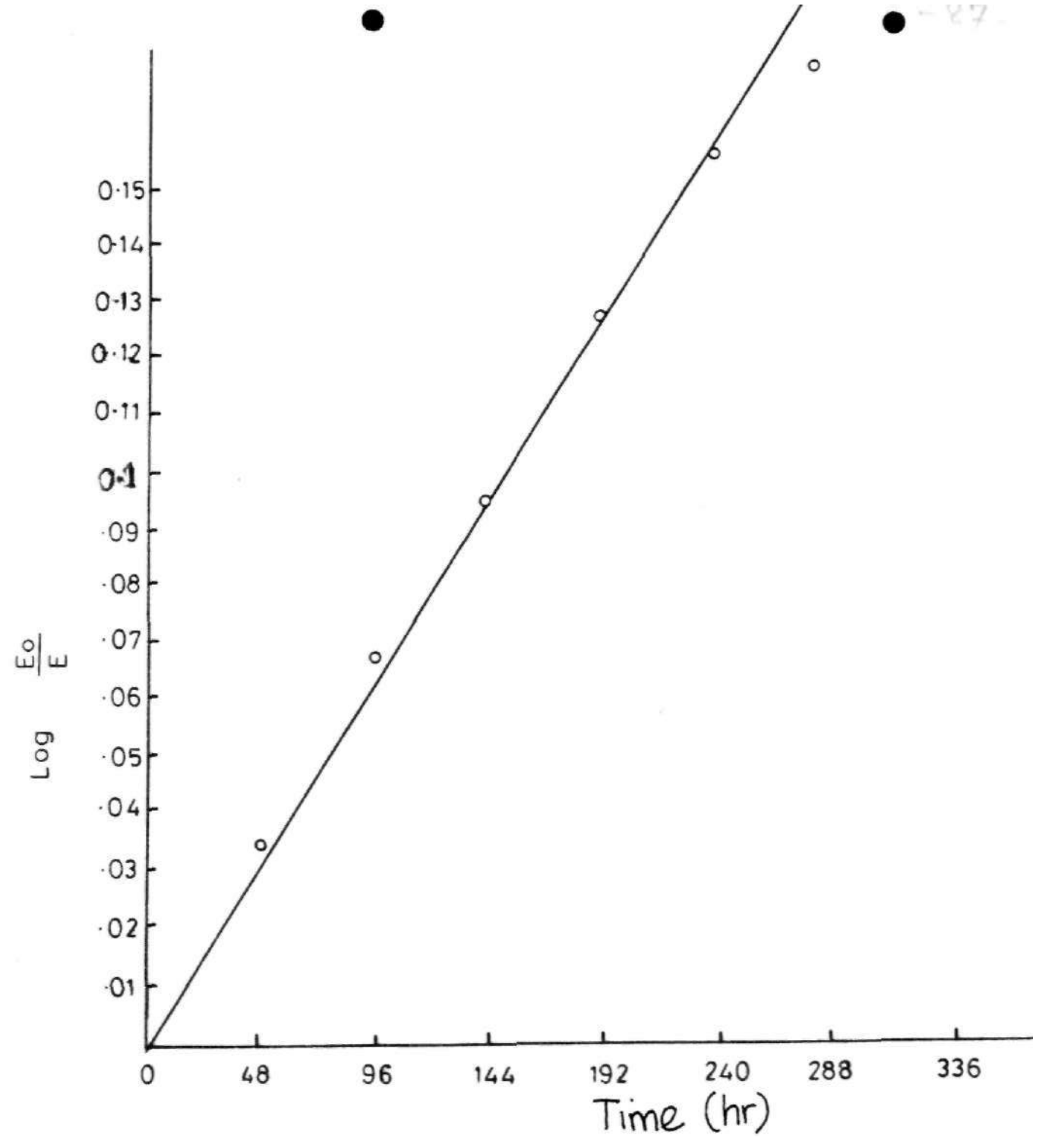

0·15
0·14
0·13
0·12
0·11
0·1
·09
·08
·07
·06
·05
·04
·03
·02
·01
Log Eo/E
0
48
96
144
192
240
288
336
Time (hr)

FIG. 14: Line-weaver Burk plot of 1/V against 1/ $[NAD^+]$ to determine the K_m of G6PD for NAD.

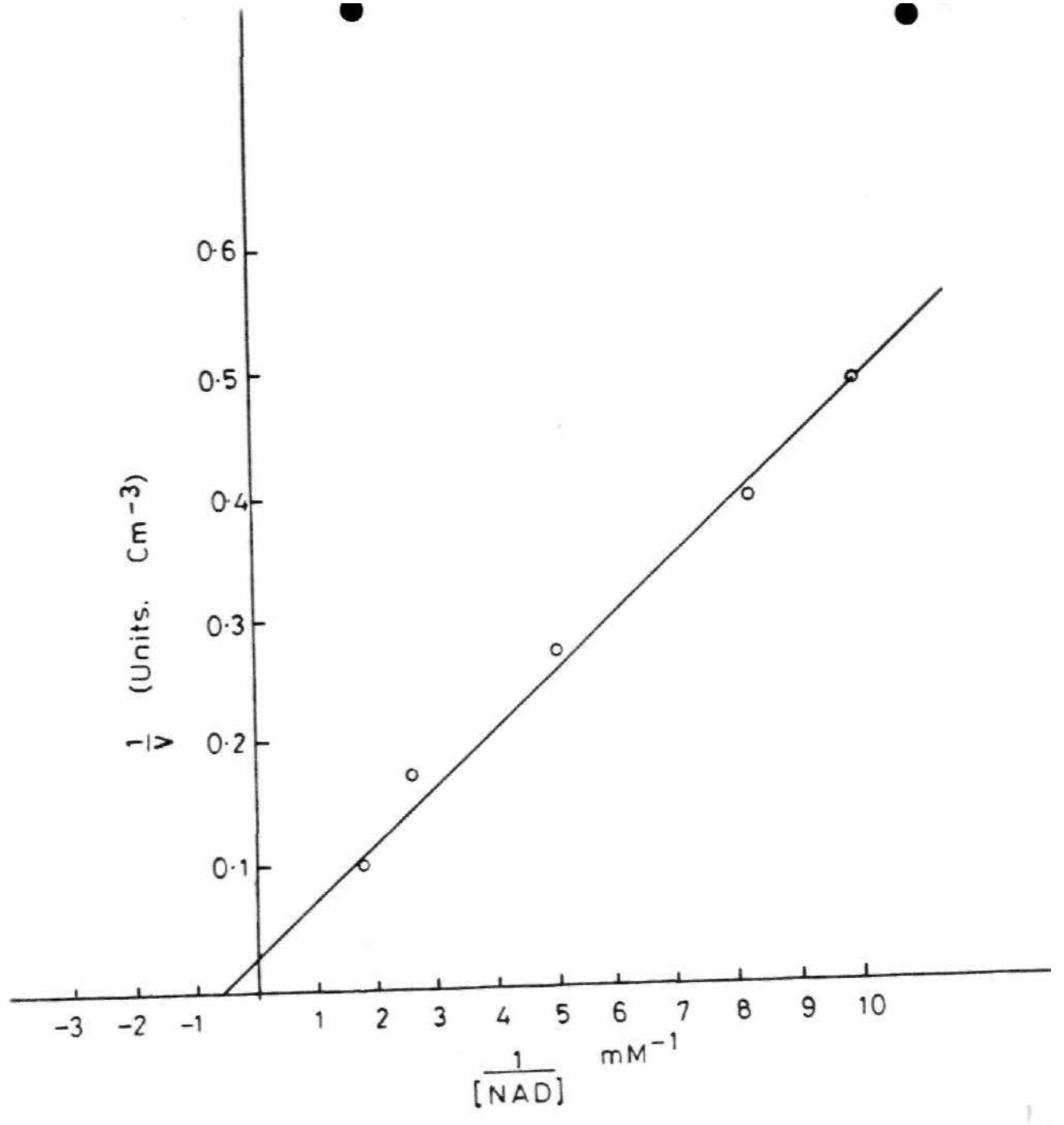

0·6
0·5
0·4
0·3
0·2
0·1
$\frac{1}{V}$ (Units. Cm^{-3})
-3 -2 -1
1 2 3 4 5 6 7 8 9 10
$\frac{1}{[NAD]}$ mM^{-1}

FIG. 15: Line-weaver Burk plot 1/V against 1/[G-NH_2-6-P] to determine the K_m of G6PD for glucosamine-6-phosphate.

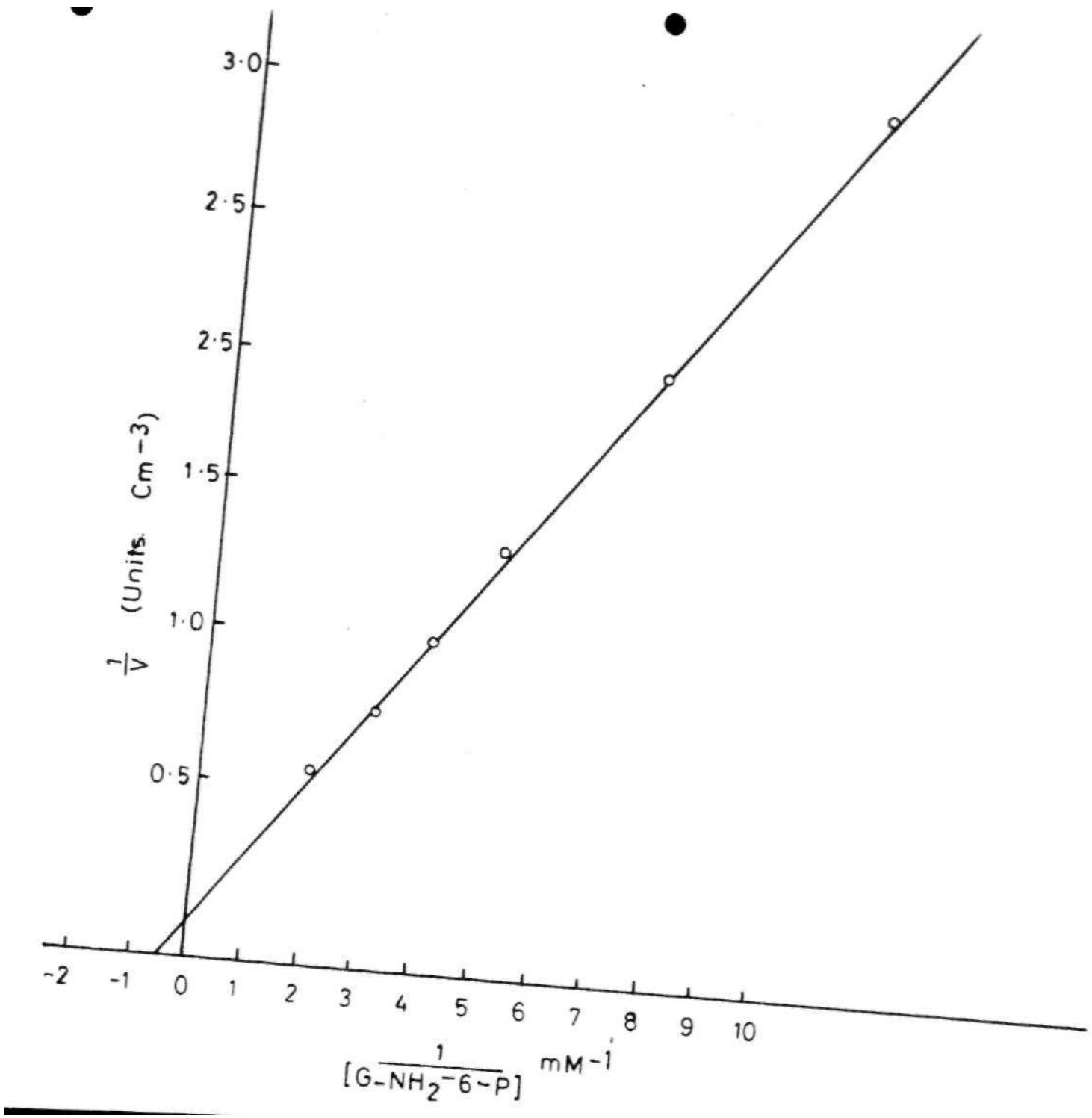

3·0
2·5
2·5
1·5
1·0
0·5
$\frac{1}{V}$ (Units. Cm⁻³)
-2
-1
0
1
2
3
4
5
6
7
8
9
10
$\frac{1}{[G\text{-}NH_2\text{-}6\text{-}P]}$ mM⁻¹

FIG. 16: Line-weaver Burk plot 1/V against 1/[F6P] to determine the K_m of G6PD for F6P.

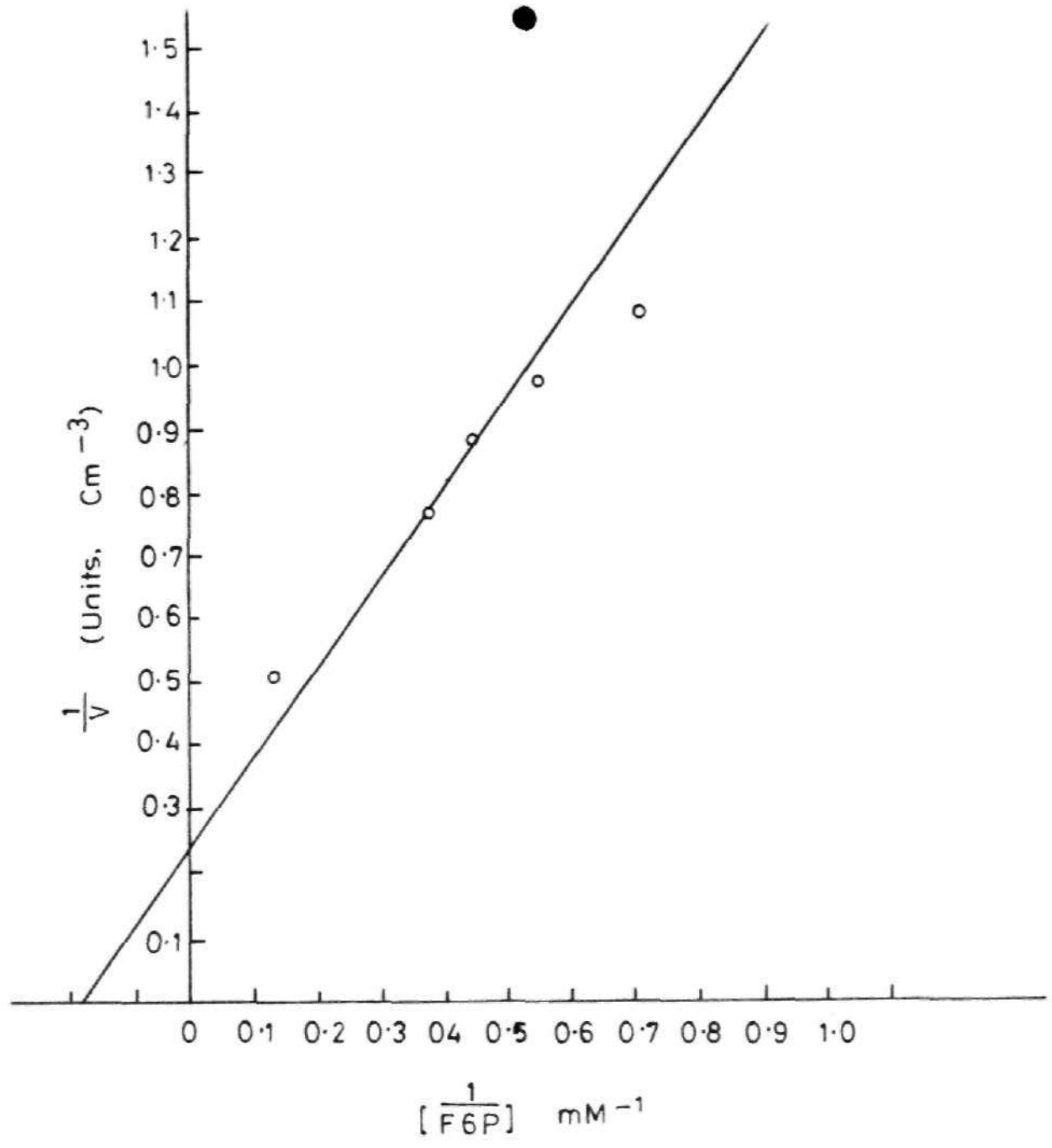

1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·1
$\frac{1}{V}$ (Units, Cm^{-3})
0
0·1
0·2
0·3
04
0·5
0·6
0·7
0·8
0·9
1·0
$[\frac{1}{F6P}]$ mM^{-1}

FIG. 17: Initial velocity plot of 1/V against 1/ [G6P] at various concentrations of $NADP^+$: (—o—o—) 0.8mM $NADP^+$: (—Δ—Δ—) 0.6mM; (—•—•—) 0.4mM $NADP^+$; and (—□—□—) 0.2mM $NADP^+$.

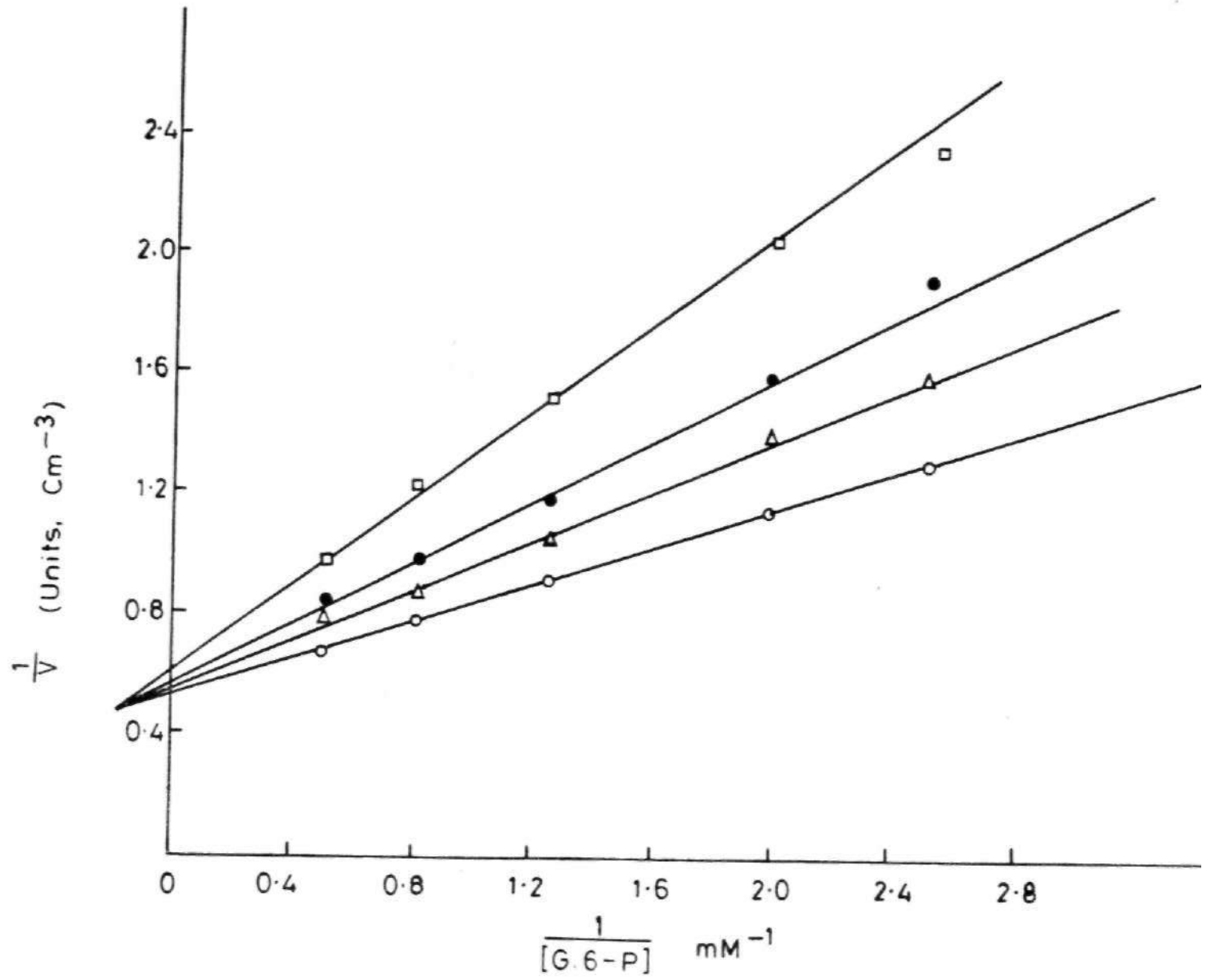

2·4
2·0
1·6
1·2
0·8
0·4
0
0·4
0·8
1·2
1·6
2·0
2·4
2·8
$\frac{1}{V}$ (Units, Cm^{-3})
$\frac{1}{[G.6-P]}$ mM^{-1}

FIG. 18: Initial velocity plot of $1/V$ against $1/[NADP^+]$ at various concentrations of G6P: (—○—○—○—) 100mM G6P; (—△—△—△—) 80mM G6P; (—●—●—●—) 60mM G6P.

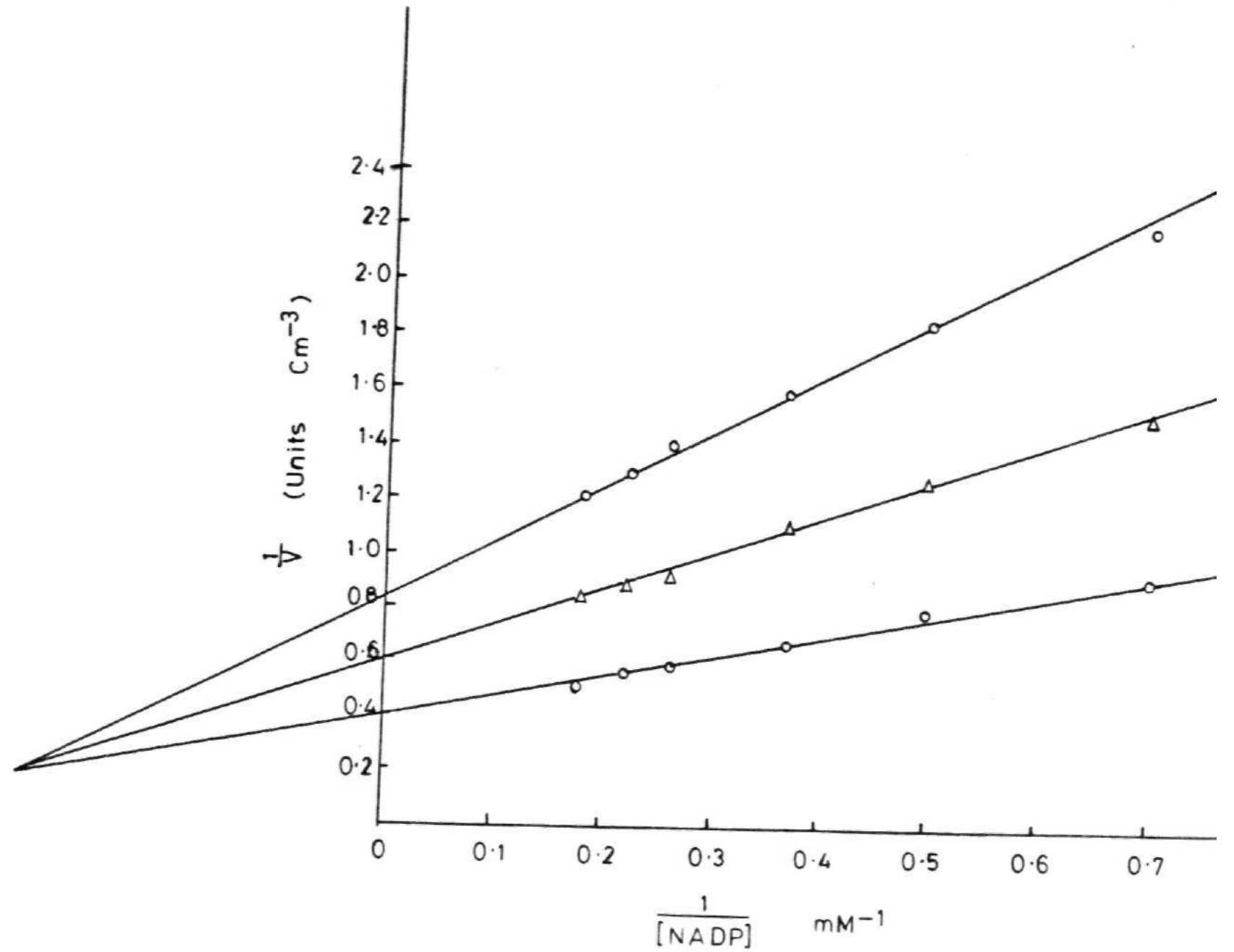

2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
(Units Cm⁻³)
1/[NADP] mM⁻¹

FIG. 19 and 20): Secondary plots of intercepts against $1/[NADP^+]$ and $1/[G6P]$ to determine K_m for $NADP^+$ and G6P.

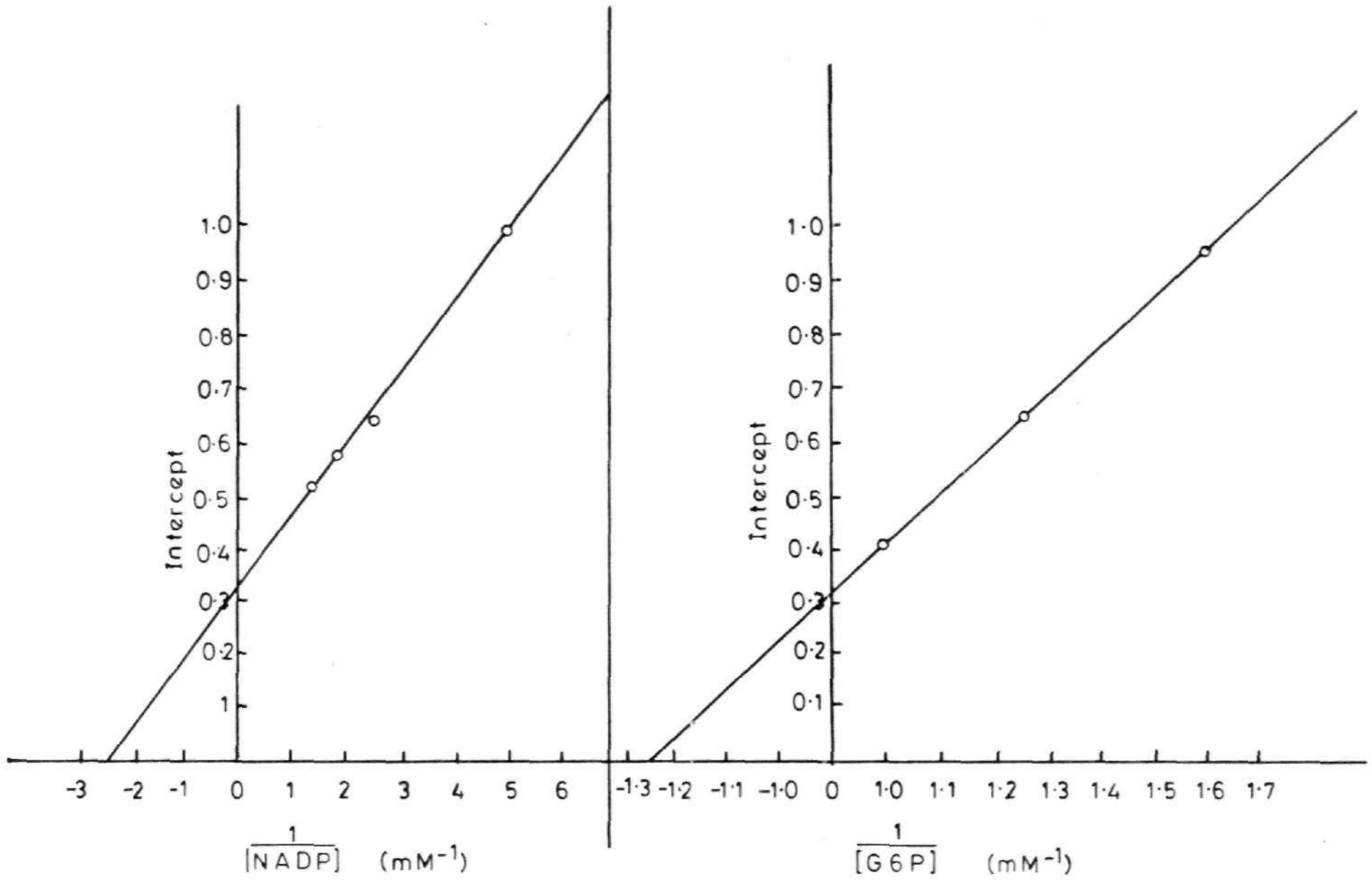

1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Intercept
-3 -2 -1 0 1 2 3 4 5 6
1/[NADP] (mM⁻¹)
-1·3 -1·2 -1·1 -1·0 0 1·0 1·1 1·2 1·3 1·4 1·5 1·6 1·7
1/[G6P] (mM⁻¹)

FIG. 21: Product inhibition by NADPH using G6P as varying substrate at constant level of $NADP^+$. (-○-○-) NO NADPH; (△-△-△) 10^{-6}mM NADPH; (●-●-●) 10^{-5}mM NADPH; and (□-□-□) 10^{-4}mM NADPH.

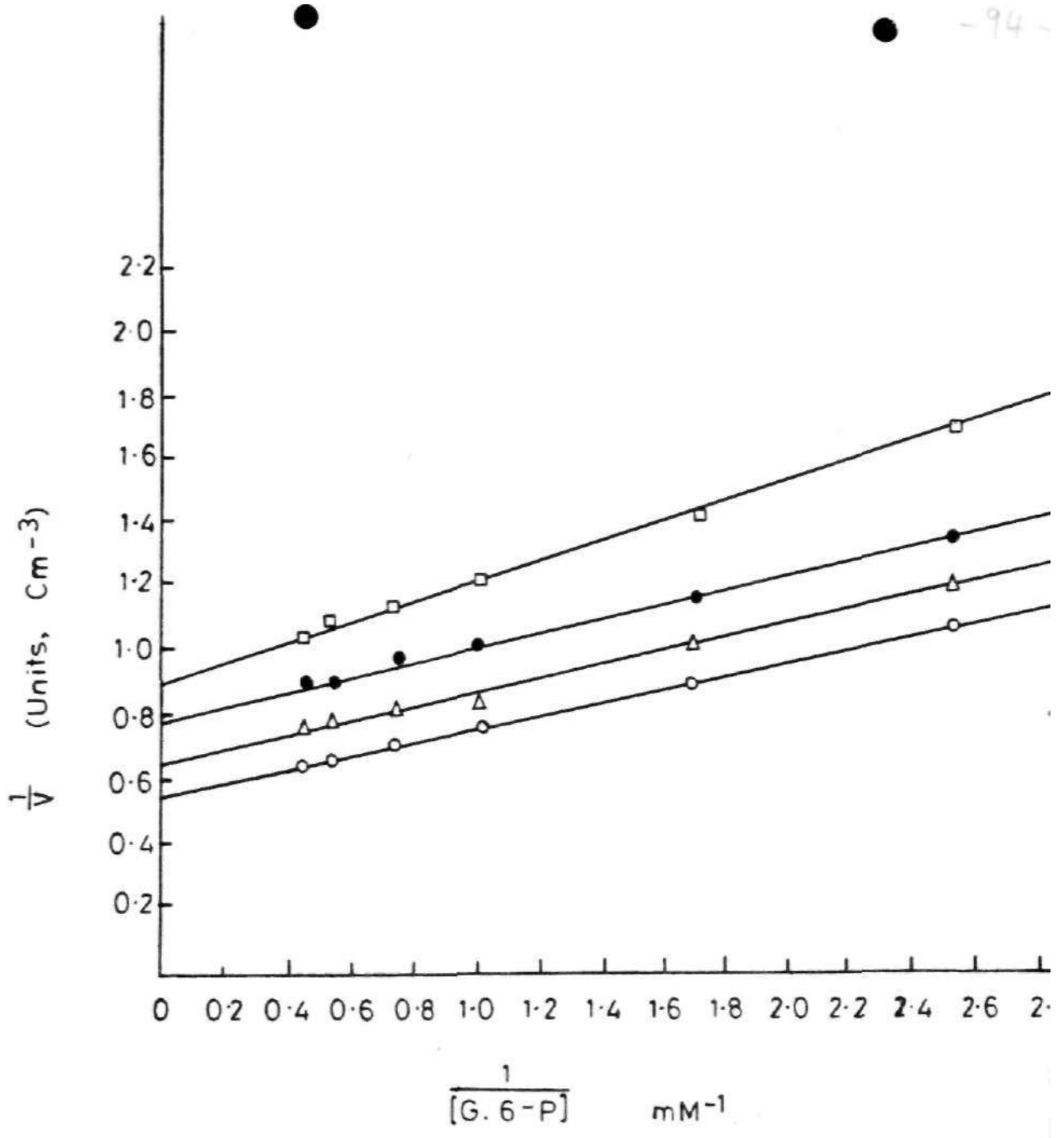

2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
$\frac{1}{V}$ (Units, Cm^{-3})
0 0·2 0·4 0·6 0·8 1·0 1·2 1·4 1·6 1·8 2·0 2·2 2·4 2·6
$\frac{1}{[G.6-P]}$ mM^{-1}

FIG. 22: Product inhibition by NADPH using $NADP^+$ as varied substrate at constant level of G6P. (-○-○-○-) NO NADPH; (-△-△-△-) 10^{-6}mM; (-●-●-●-) 10^{-5}mM NADPH; and (-□-□-□-) 10^{-4}mM NADPH.

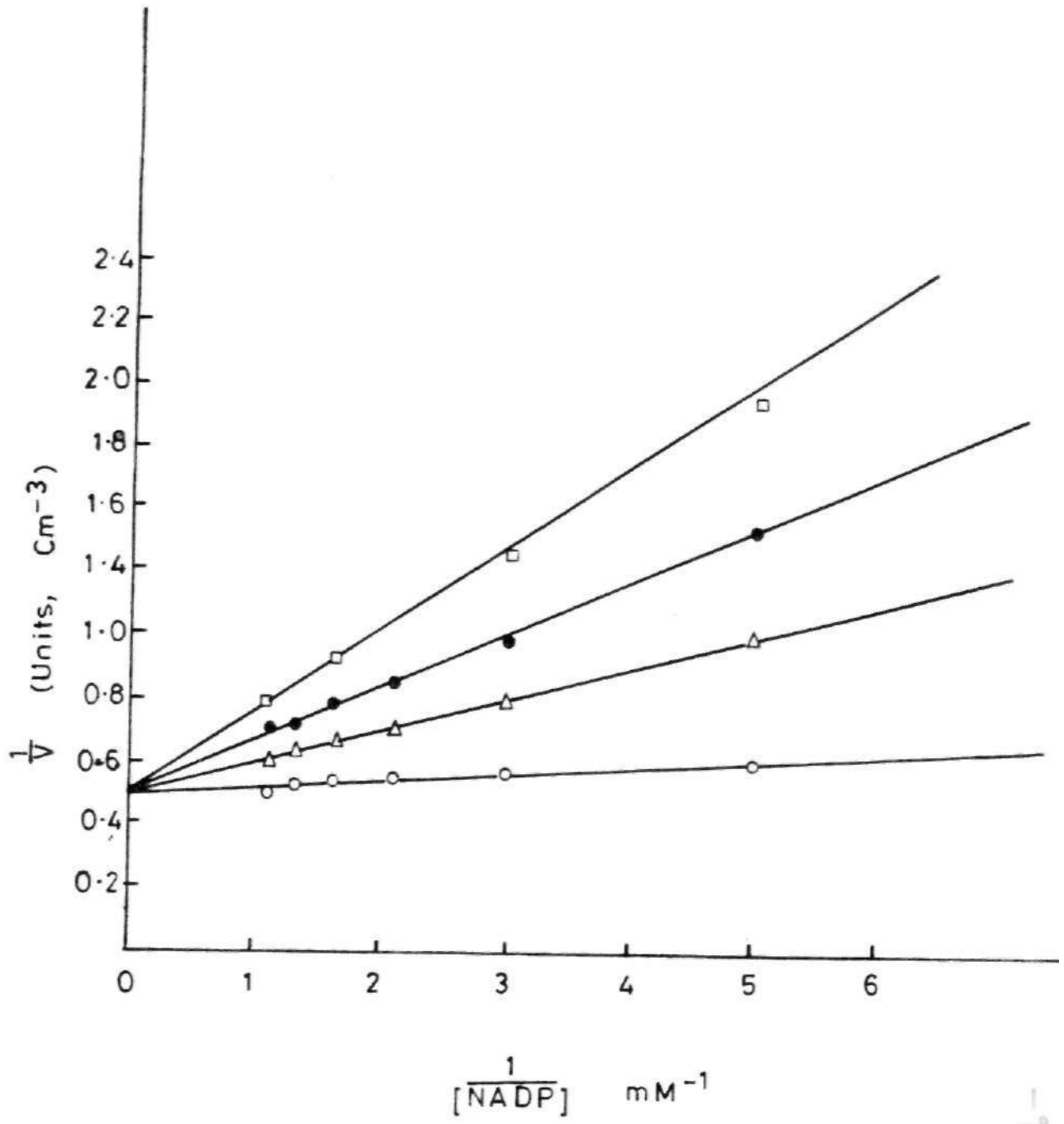

2·4
2·2
2·0
1·8
1·6
1·4
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
6
$\frac{1}{V}$ (Units, Cm^{-3})
$\frac{1}{[NADP]}$ mM^{-1}

FIG. 23 and 24: Secondary plots of slope and intercept versus NADPH concentrations.

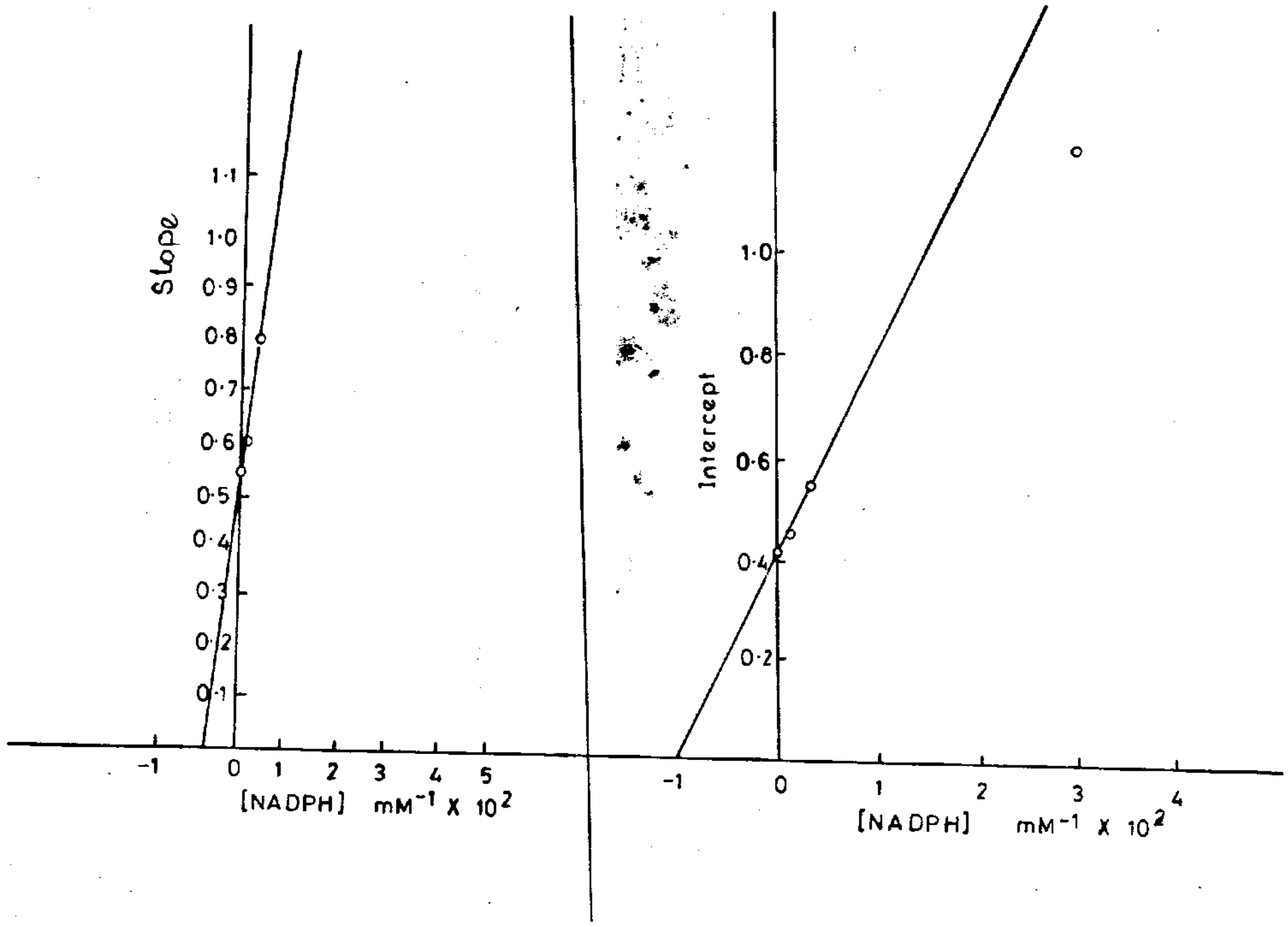

Slope
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
-1
0
1
2
3
4
5
[NADPH] mM⁻¹ X 10²
Intercept
1·0
0·8
0·6
0·4
0·2
-1
0
1
2
3
4
[NADPH] mM⁻¹ X 10²

FIG. 25: Alternate substrate inhibition by $G\text{-}NH_2\text{-}6\text{-}P$ using G6P as varied substrate (○) NO $G\text{-}NH_2\text{-}6\text{-}P$; (●) 1mM $GNH_2 6P$; (△) 4mM $GNH_2 6P$; and (□) 8mM $GNH_2 6P$.

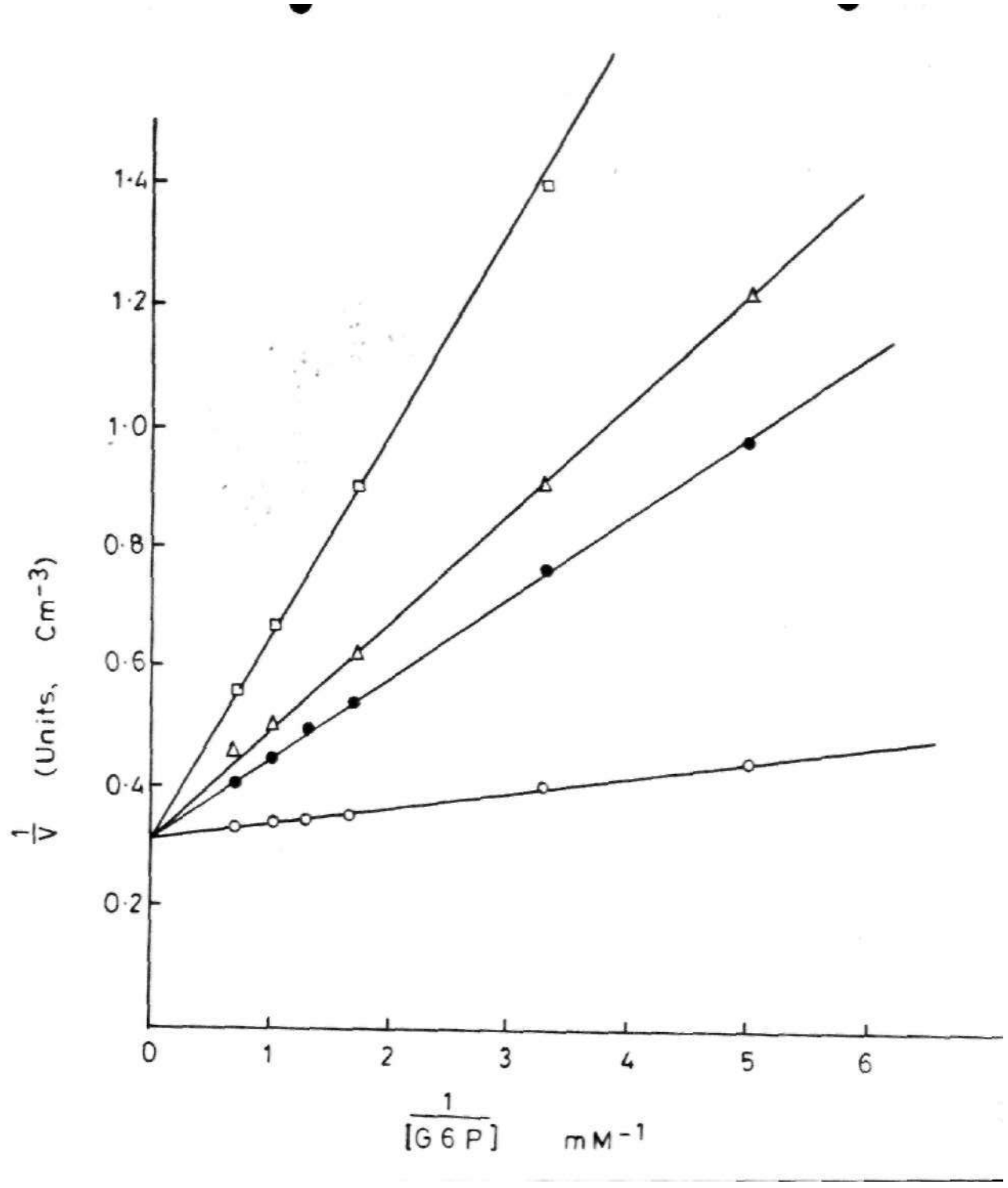

1·4
1·2
1·0
0·8
0·6
0·4
0·2
$\frac{1}{V}$ (Units, Cm^{-3})
0
1
2
3
4
5
6
$\frac{1}{[G6P]}$ mM^{-1}

FIG. 26: Alternate substrate inhibition by $GNH_2 6P$ using $NADP^+$ as varied substrate (-o-o-o-) NO $GNH_2 6P$; (-●-●-●-) 1mM $GNH_2 6P$; (▲-▲-▲-) 4mM $GNH_2 6P$ and (□-□-□-) 8mM $GNH_2 6P$.

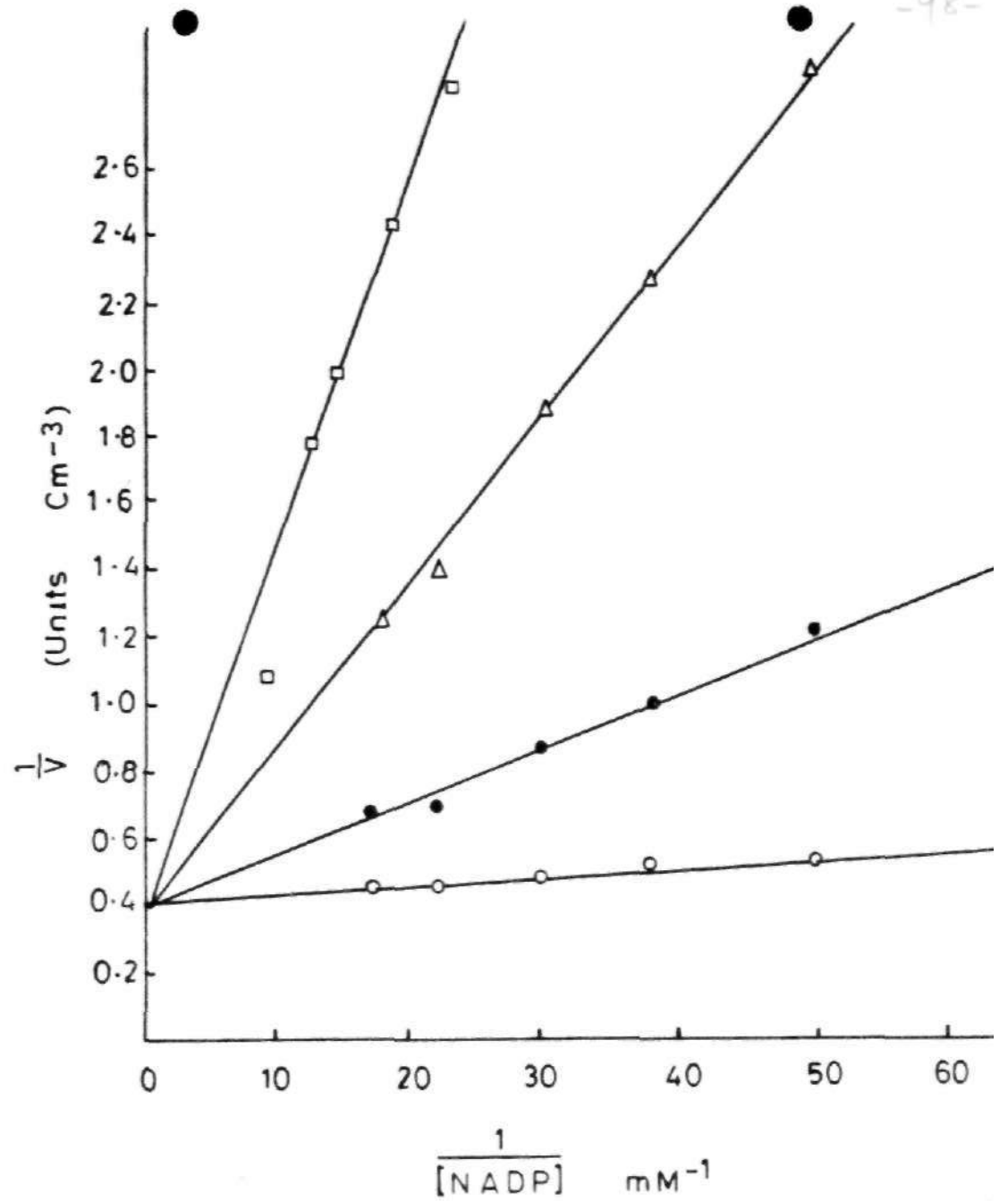

2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
(Units Cm⁻³)
-I>
0
10
20
30
40
50
60
1
[NADP]
mM⁻¹

FIG. 27: Alternate substrate inhibition by NAD^+ using GGp as varied substrate (-o-o-o) NO NAD^+; (-Δ-Δ-Δ) 0.6mM NAD^+; (-●-●-●) 0.4mM NAD^+.

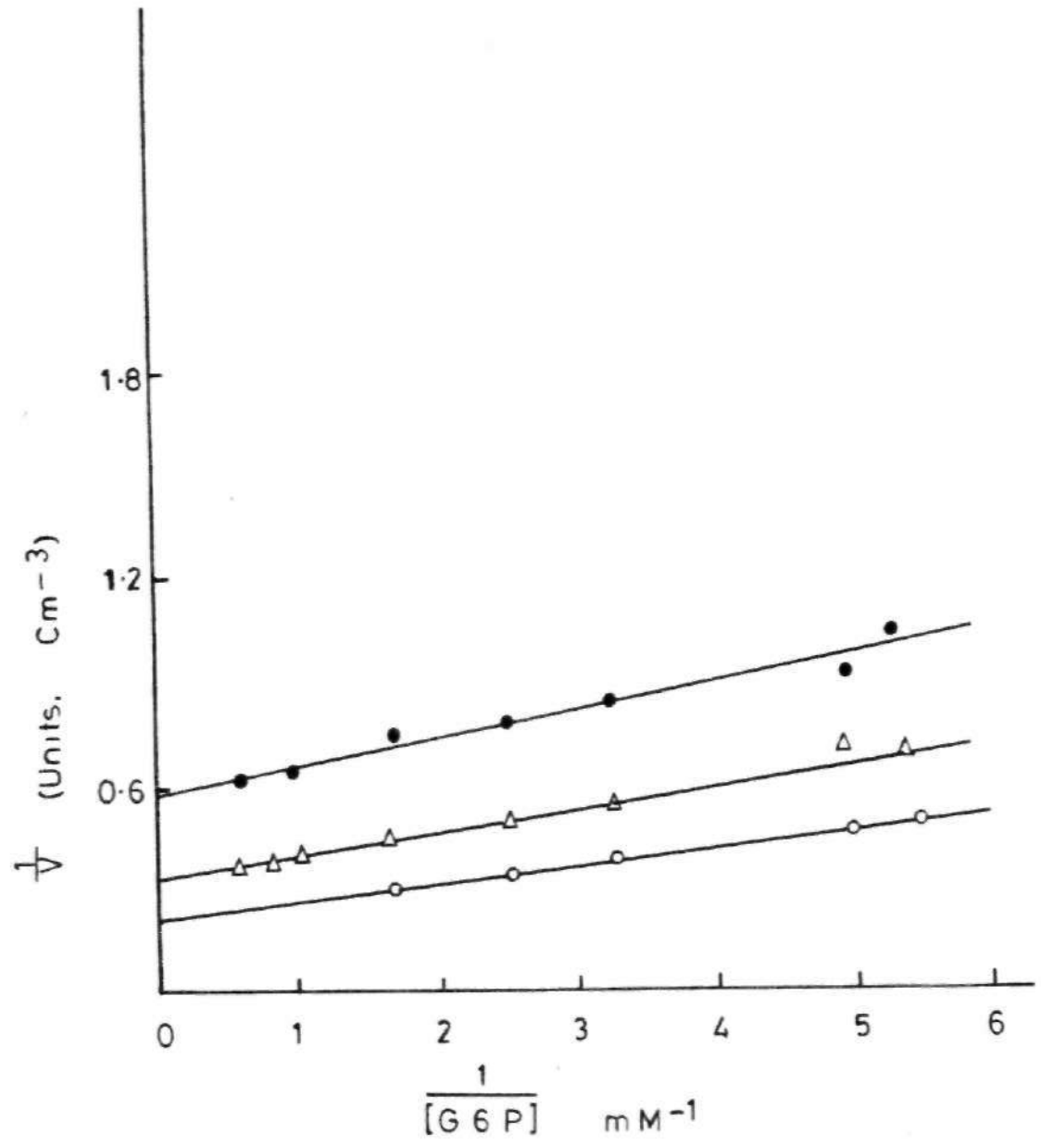
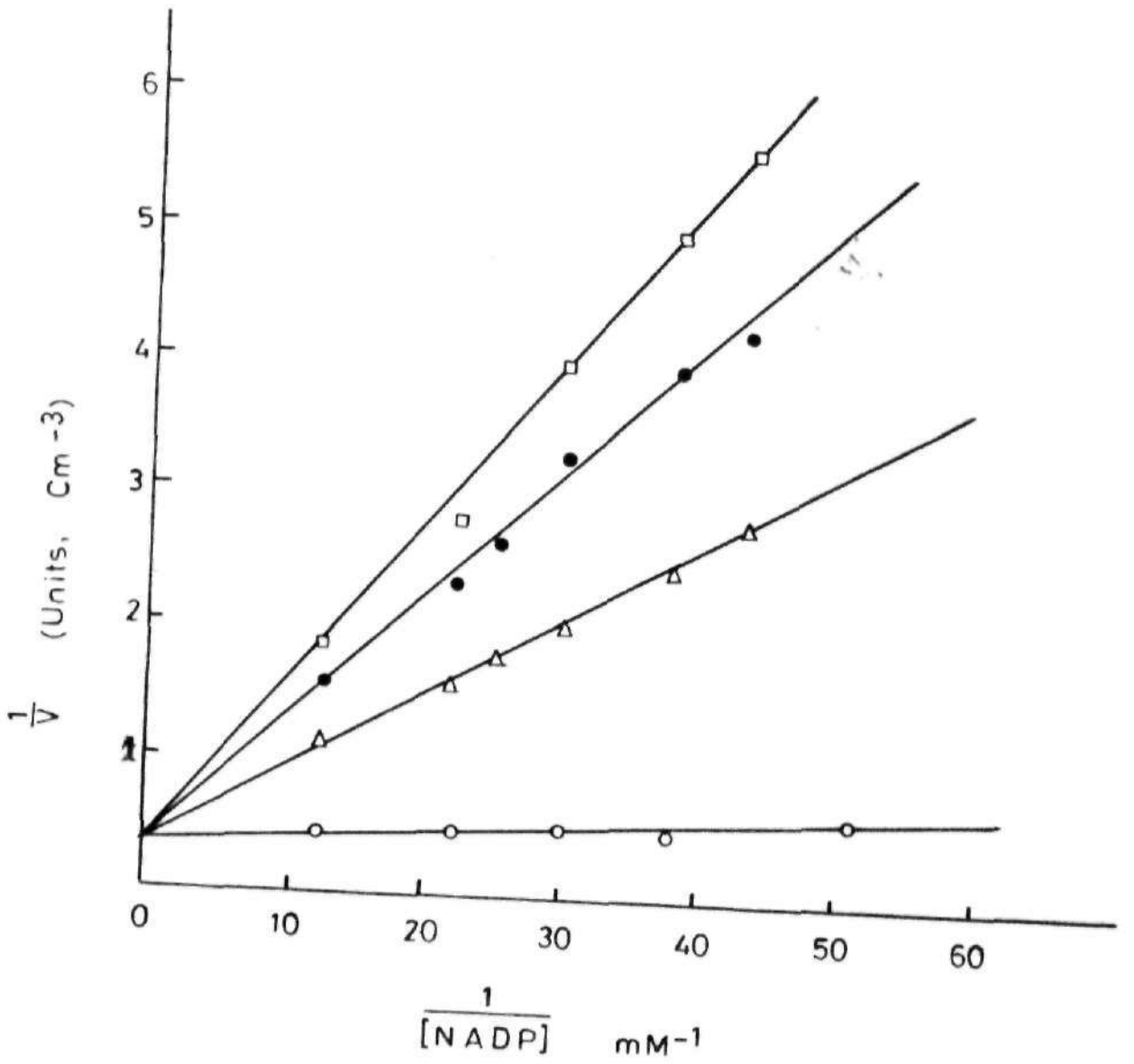

1·8
1·2
0·6
0
1
2
3
4
5
6
1/V (Units, Cm⁻³)
1/[G 6 P] mM⁻¹

FIG. 28: Alternate substrate inhibition by NAD^+ using $NADP^+$; (●—●) NO NAD^+ (Δ—Δ—Δ) 0.2mM NAD^+. (●—●—●) 0.4mM NAD^+ and (□—□—□) 0.6mM NAD^+.


6
5
4
3
2
1
0
10
20
30
40
50
60
1/V (Units, Cm -3)
1/[NADP] mM-1

2 4 4 8 3

In effect, the tables below (Tables 6 and 7) summarise the observed steady-state alternate substrate inhibition patterns.

Table 6 - Observed steady state alternate substrate inhibition pattern.

Alternate substrate	Variable substrate	
	$NADP^+$	G6P
NAD+	CI	NCI
$G\text{-}NH_2\text{-}6\text{-}P$	CI	CI

Table 7 - Observed steady state Inhibition Pattern

Variable substrate	Product Inhibitor	Observed inhibition Pattern
$NADP^+$	NADPH	CI
G6P	NADPH	NCI

CI and NCI denote competitive and noncompetitive inhibition respectively.

DISCUSSION

G6PD has been isolated from a large number of animal, microbial and plant sources. Apart from the sweet potato tuber (Muto and Uritani, 1970), the enzyme has never been isolated from any other main plant tuber. This enzyme occupies a key position in glucose metabolism and the reaction it catalyzes is an important site for control of the pentose phosphate pathway.

In the mammary glands of many mammals, large changes in G6PD activity has been noticed to accompany the onset and cessation of lactation (Levy, 1979). The activity of G6PD, which is not present in the mammary glands of nonpregnant rats rises only slightly during lactation (Levy, 1979). Here the increase in activity of G6PD is correlated with the increased requirement for NADPH in the biosynthesis of milk fat.

In the white yam tuber, a high activity for G6PD in new and stored yam tubers has been reported (Ugochukwu et. al., 1977; Ikediobi and Oti, 1981).

This high activity has been suggested to be connected with sprouting and fatty acid biosynthesis in the white yam tuber (Ikediobi and Oti, 1981).

In the present work, the homogeneity of the white yam tuber G6PD was tested by disc gel electrophoresis at pH 8.3. The enzyme showed two protein bands both of which were active and corresponded with the activity band on the gels. It would appear that the enzyme exists in isozymic form.

The L. mesenteroides G6PD showed a major protein band and two minor ones in the disc gel electrophoresis (Olive et. al., 1971), while that from sweet potato tuber showed one major band with two minor ones in disc gel electrophoresis (Muto and Uritani, 1970). The activity stain in the sweet potato tuber G6PD when matched with the protein bands coincided with just the major protein band (Muto and Uritani, 1970). Our disc gel electrophoresis results were similar to those reported in the literature.

The molecular weight of our yam tuber G6PD determined by gel filtration was found to be 163,000 ± 28,000. This value compares favourably with known values of G6PD dimers from the following sources: rat erythrocyte = 131,000 (Rattazzi, 1968); Drosophila = 130,000 - 150,000 (Levy, 1979); spinach leaves = 105,000 (Levy, 1979); sweet potato tuber = 110,000 (Muto and Uritani, 1970); Penicillium duponti = 113,000 - 126,000 (Levy, 1979); Pseudomonas fluorescens ($NADP^+$ preferring enzyme) = 265,000 (Lessman et. al., 1975) Pseudomonas aeruginosa = 190,000 (Lessie and Neidhart, 1967); Rhodopseudomonas spheroides = 180,000 - 200,000 (Levy, 1979) and mouse liver = 121,000 (Levy, 1979).

During the experiment on temperature stability of the yam tuber G6PD, the enzyme and not the reaction mixture was heated at various temperatures for twenty minutes, ΔE_a value of 6.45 Kcal $mole^{-1}$ got from the Arrhenius plot was looked upon as the energy of deactivation of the enzyme.

The white yam tuber G6PD showed two pH optima-

one at the acidic range (pH 5.0) and the other in the basic range (pH 8.2) at 29 $\pm$ 2°C. Analysis of the effect of pH on V_{max} and K_m of the purified enzyme showed that groups with pK_a values of 5.1 and 8.9 were responsible for enzyme catalysis. Also the plot of log V_{max} against pH shows that groups with pK_a values of 6.08 and 9.6 were responsible for either catalysis or binding of the substrate onto the enzyme. These findings compare with values obtained for G6PD from other sources.

Two groups on L. mesenteroides G6PD are involved in the dissociation of NAD^+ from the binary complex and probably the same groups are concerned with either the binding of NAD^+ to enzyme or with the isomerization step (Olive et. al., 1971). These two groups on the enzyme have pK_a values in the range of 6.7 - 7.0 and 9.0 - 9.5. Olive et. al. (1971) proceeded to interpret these values to mean that the first group on the enzyme is the imidazole of histidine and the second group a sulfhydryl of cysteine, or an ϵ-amino lysine, or a hydroxyl of

tyrosine. Both histidine (Olive et. al., 1971) and tyrosine (Domschke et. al., 1970) have been implicated in the mechanism of action of G6PD from C. utilis. Further experiments will be needed for a definitive identification of groups on L. mesenteroides G6PD.

Studies on S. carlsbergensis G6PD by Ruby et. al. (1976) showed that the pH titrations revealed two groups on the enzyme with pK_a values of 5.7 and 9.2 at 25°C. From their result, they concluded that a histidine (in the acidic range) and a lysine (in the basic range) are responsible for catalysis.

Also pH studies on human erythrocytes G6PD by Babalola et. al. (1976) revealed that a histidine and a cysteine are probably essential for catalysis.

Based on the pK_a data obtained in this work, we may tentatively implicate histidine, tyrosine and cysteine in overall catalysis. More work, of course will be required to confirm these assignments.

Senescence (the deteriorative events which precede the death of a mature cell) in yam might

be induced prematurely by chilling or low temperature injury which occurs around 10°C or lower (Ikediobi and Oti, 1981). It is likely that enzymatic changes are involved in senescence. From the effects of storage on the enzyme, it was observed that yam tuber G6PD had a half-life of 100 days in the deep freezer, 20 days in the refrigerator and 7 days at ambient temperature. It is not certain whether to conclude that yam tuber G6PD might be one of the major factors responsible for chilling injury in yams stored at low temperature. Already, current research at Tropical product Institute, London, indicates that G6PD might be a possible diagnostic enzyme for detection of onset of chilling injury in yam tubers stored at low temperatures (Ikediobi personal communication). The short half-life of 7 days recorded in this work for G6PD at ambient temperature may be a factor in yam tuber deterioration, in storage. Work by Ikediobi and Oti (1981) has also shown that G6PD activity increases during sprouting and falls after at ambient temperature. More work is called for

to actually demonstrate this.

The white yam tuber G6PD shows a wide range of substrate specificities as other G6PDs (Levy, 1979). G6PDs show varying activities on G-6-P with substituted C-1, C-2, C-3 and C-5 (Levy, 1979). G6PDs are also known to utilise nonphosphorylated sugars such as phosphonate analogues, glucose-6-sulphate, glucose, galactose, 2--deoxy-glucose, arabinose and xylose (Levy, 1979). Non-utilization of these sugars might be attributed to experimental conditions. However, it has been shown that some of these sugars are utilized to a very slight extent only.

Also, it has been confirmed that the apparent affinity of the analogue for G6PD is lowered by structural change which might cause loss of interaction between analogue and the enzyme (Adam et. al., 1976).

However, in all cases, D-glucose-6-phosphate, the natural substrate of G6PD, is known to be the one that gives the highest V_{max} and lowest K_m for

all G6PDs tested so far (Levy, 1979).

The white yam tuber G6PD utilises NAD^+, glucosamine-6-phosphate and fructose-6-phosphate in addition to $NADP^+$ and G6P. The K_m values for these analogues are higher than that of the natural substrate.

Initial velocity measurements for the white yam tuber G6PD show intersecting or converging patterns. These clearly indicate a case of sequential mechanism and rule out any case for ping-pong mechanism. This result is consistent with results obtained for other G6PDs and infact, it has been established that all G6PDs (so far isolated) proceed by sequential or ternary complex mechanisms (Levy, 1979). In some G6PDs, there is an ordered addition of substrates while in others it is random. For kinetic mechanisms of enzymes utilising two or more substrates, it is important to elucidate the initial velocity pattern in both directions and also the patterns of inhibition by all the products (Cleland, 1963).

With G6PDs, it has been impossible to elucidate the enzyme - catalyzed reaction in the reverse direction or to utilise all product inhibitions because

of the lability of glucono-δ-lactone-6-phosphate particularly at pHs above 6.5 and of NADPH under acidic conditions (Levy, 1979).

So alternate methods have been used in determining the kinetic mechanisms of G6PDs. There is a vast literature on the methods used in trying to elucidiate the kinetic mechanism of G6PDs (Levy, 1979).

Kuby et. al., (1974) in their investigation of kinetic mechanism for S. carlsbergensis G6PD showed a clear evidence for a rapid equilibrium random mechanism. In their mechanism, NADPH was shown to inhibit competitively with respect to both $NADP^+$ and G6P. Such a result was then interpreted as random mechanism.

Another clear cut evidence for random mechanism was obtained by Malcolm and Shepherd (1972) for G6PDs from P. notatum and P. duponti. For the two G6PDs, the investigators showed that NADPH inhibits competitively with respect to both $NADP^+$ and G6P; however they refused to propose a random mechanism.

For L. mesenteroides G6PD, the initial velocity

measurement shows sequential mechanism (Olive et. al., 1971). The L. mesenteroides G6PD showed dual coenzyme specificities, i.e. it utilised NAD^+ and $NADP^+$. For either coenzyme, the initial measurements show sequential mechanism (Olive et. al., 1971).

The kinetic inhibition by NADPH showed that the reduced coenzyme inhibited the $NADP^+$ linked reaction competitively with respect to $NADP^+$ and non-competitively with respect to G6P; however, for the NAD^+ linked reaction, NADPH inhibits noncompetitively with respect to both G6P and NAD^+. These results were then interpreted as an ordered sequential mechanism or "ordered bi bi" for the $NADP^+$ linked reaction and an ordered sequential mechanism with enzyme isomerization or "iso - ordered bi bi" for the NAD^+ - linked reaction (Olive et. al., 1971).

In L. mesenteroides, evidence exists that G6PD plays a dual role in vivo, leading to the generation of either NADPH or NADH. NADH is responsible for the catabolic reactions by donating hydrogens to the end products of fermentation,

lactate and ethanol; while NADPH is used for the reductive biosynthesis of fatty acids.

Rat mammary G6PD also utilised both NAD^+ and $NADP^+$ as coenzymes. As of now, no known evidence has been advanced for the importance of NAD^+-linked reaction catalized by the mammary enzyme (Levy, 1963); but the $NADP^+$-linked reaction, is closely linked with mammary fatty acid synthesis (Levy, 1963).

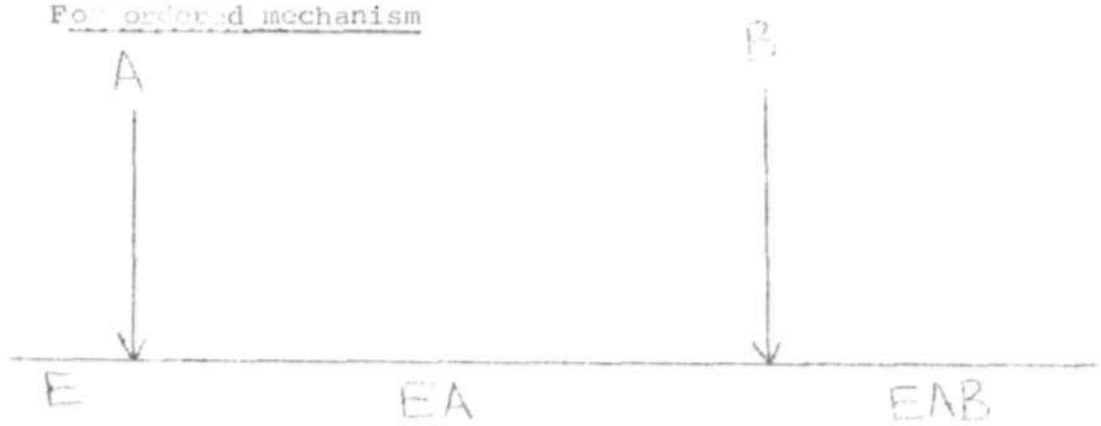
Soldin and Balinsky (Levy, 1979) in their studies on erythrocyte G6PD found that NADPH inhibition was competitive with respect to $NADP^+$ and noncompetitive with respect to G6P. This result is consistent with either a compulsory - order mechanism in which $NADP^+$ bound first, or a rapid equilibrium random mechanism with a dead-end enzyme - G6p -NADPH complex (Levy, 1979).

Data from this work show that NADPH inhibits competitively with respect to $NADP^+$ and noncompetitively with respect to G6P. Such results are infact consistent with an ordered substrate addition

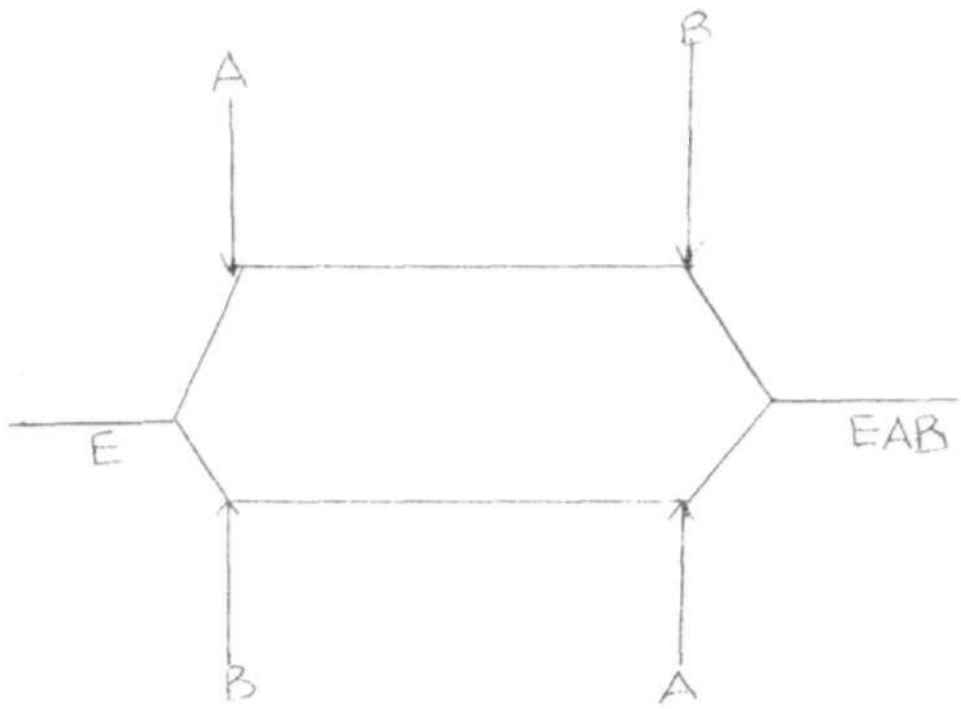
(Cleland, 1963) but further data would be required to substantiate this, since measurements with 6-phosphoglucono-d-lactone are not practicable (Spivey and Katz, 1976; Horecker and Smith, 1953). So product inhibition data alone cannot distinguish between random or ordered additions of substrates and products (Spivey and Katz, 1976).

Spivey and Katz (1976) have suggested that dead-end inhibition studies can reveal the mechanism of substrate addition because a B-substrate analogue will be expected to combine with both the free enzyme, [E], and the enzyme-A, [EA], forms in a random mechanism; but only to the [EA] form in an ordered pathway thus:

For ordered mechanism



and for the random mechanism:



So an uncompetitive inhibition results with a B-substrate analogue and variable substrate in an ordered mechanism (Spivey and Katz, 1976; Ikediobi personal communication).

Spivey and Katz (1976) have therefore summarized the expected kinetic mechanism in Table 7.

Table 7: Expected Kinetic mechanism

Substrate analogue	A		B	
variable substrate	A	B	A	B
Rapid equilibrium-random	CI	CI(NCI)	CI(NCI)	CI
Ordered	CI	NCI	UCI	CI(NCI)

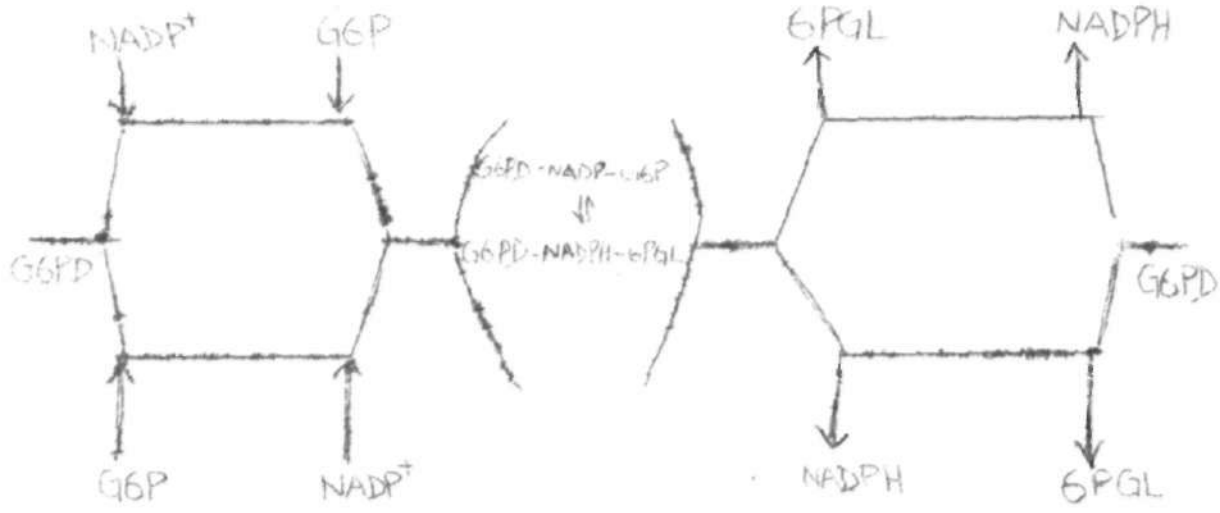
where A and B are substrates and CI, NCI, UCI are competitive, noncompetitive anduncompetitive inhibitions respectiv

Using competitive substrate analogues, our results show that glucosamine-6-phosphate inhibits competitively with respect to both $NADP^+$ and G6P; and NAD^+ (competitive substrate analogue of $NADP^+$) inhibits competitively with respect to $NADP^+$ and noncompetitively with respect to G6P.

These results are therefore interpreted as a rapid equilibrium random mechanism judging from the summary of Spivez and Katz (table 7) above.

In effect therefore, it is very likely that white yam tuber G6PD is governed by a rapid equilibrium random mechanism. If this is the case,

it follows that the kinetic mechanism of the white yam tuber G6PD can be represented schematically as this



where 6PGL represents 6-phosphogluconolactone.

Secondary plots of the initial velocity studies were linear and gave rise to the following Michaelis constants: K_m ($NADP^+$) = 0.46 mM; and K_m (G6P) = 0.8 mM. The K_m value for $NADP^+$ is quite close to that obtained for the mouse liver enzyme 0.45mM (Levy, 1979), but the value is a bit high when compared to those of sweet potato tuber G6PD, K_m ($NADP^+$) = 0.0297mM (Muto and Uritani, 1970); P. fluorescens G6PD, K_m ($NADP^+$) = 0.36mM (Levy, 1979);

and eutropha H-16 G6PD, K_m ($NADP^+$) = 0.15mM (Levy, 1979). In some G6PDs, the K_m values for $NADP^+$ have not been determined - Housefly G6PD (Levy, 1979); human placenta G6PD; Peal leaf G6PD and human platelet G6PD (Levy, 1979).

The K_m value of G6P for our enzyme is very close to some values obtained in a good number of other G6PDs - eutropha H-16, K_m (G6P) = 1.0mM (Levy, 1979); A. aerogens, K_m (G6P) = 0.76mM; R. spheroides, K_m (G6P) = 0.4 mM; P. fluorescens, K_m (G6PD) = 2.7 and 2.33 mM; P. multivorans, K_m (G6P) = 6.0 and 20 mM and H. facilis, K_m (G6P) = 0.36 mM (Levy, 1979).

The effect of adenine nucleotides on yeast G6PD was first established by Bonsignore and De Flora (1972). This inhibition was found to be competitive with respect to G6P. It is also of interest to note that G6PDs from other sources have confirmed this - Yoshida and Lin (1973) have shown that human erythrocytes G6PD was inhibited by ATP. This inhibition was consequently shown to

be competitive with respect to G6P. They also noted that ATP inhibition was very effective in the absence of Mg^{++}.

Pig liver G6PD has also been shown to be inhibited by both ATP and ADP (Kanji, 1976). Ehrlich ascites tumor G6PD was shown to be sensitive to ATP inhibition in the absence of Mg^{++}, but slight inhibition was seen in its presence (Levy, 1979).

In higher plant G6PDs, the enzyme from Mung beans was shown to be inhibited by ATP and ADP but these inhibitions were counteracted by the introduction of Mg^{++} (Levy, 1979). Sweet potato tuber G6PD was inhibited competitively by ATP with respect to G6P (Levy, 1979).

In our experiment, ATP inhibition on white yam tuber G6PD was found to be competitive with respect to G6P . As of now, no regulatory significance can actually be attached to ATP inhibition. However, if this inhibition is assumed to occur *in vivo*, then it will appear as if ATP controls both pathways

of glucose degradation - the glycolytic route at phosphofructo-kinase level (Bonsignore and De Flora, 1972) and the hexose monophosphate shunt by its effect on G6PD.

Evidence has been recently produced to show that ATP can be considered as the most important metabolite regulating glucose metabolism (Bonsignore and De Flora, 1972).

Eggleston and Krebs (1974) have stressed that there are two special features of the G6PD-catalyzed reaction that must be considered when one is seeking to understand how G6PD activity is regulated - firstly, is the effective thermodynamic irreversibility of the reaction it catalyzes and this causes the reaction to go on to completion; secondly, is the fact that NADPH is a potent inhibitor and causes many G6PD - catalyzed reactions to behave like a steady - state system (Eggleston and Krebs, 1974).

In all G6PDs, it is a common knowledge that NADPH inhibition is mainly competitive with $NADP^+$

Since Levy (1979) has put it that the ratio of free NADPH to free $NADP^+$ is very high in mammalian cells, NADPH inhibition has been considered to be of great importance in establishing the rate of the G6PD - catalyzed reaction and to account for inhibition of G6PDs *in vivo* in rat liver.

Activity of G6PDs in photosynthesizing plants is also affected by the NADPH/$NADP^+$ ratio . Lendzian and Bassham (1975) have pointed out that in the chloroplast where the Calvin cycle functions during photosynthesis and the hexose monophosphate shunt functions under conditions of cell respiration, control mechanisms must operate to prevent wasteful cycling. These investigators found that the rate - limiting enzymes of the Calvin cycle are stimulated by a high NADPH/$NADP^+$ ratio and G6PD is inhibited (Lendzian and Bassham, 1975).

Suggestions For Further Work

G6PD being so important an enzyme deserves to be well studied like other enzymes. The importance of G6PD is quite obvious - the *L. mesenteroides* G6PD

is widely used in clinical diagnostic procedures especially in the method of determining serum glucose (Levy, 1979). It is also used to test for phosphoglucose isomerase which is abnormally high in patients with breast or prostatic cancer; it is used in creatine phosphokinase assays for myocardial infarction and cerebrospinal disease and skeletal disease (Levy, 1979).

Since G6PD is that important, will it not be essential to develop purification procedures which are simple, easy, of high purity and yield? Affinity chromatography may provide enzyme preparations of higher specific activities and purity.

Work should be directed at finding out the structure and mechanisms of regulation of the white yam tuber G6PD. It will also be necessary to confirm the proposed identities of the groups responsible for catalysis and binding.

As of now, there is no known information on X-ray diffraction of any G6PD and very little work indeed has been published on the identification of essential amino acids. These then represent worthy challenges which subsequent investigators may wish to tackle.

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