STUDIES ON THE EFFECT OF SOLVENT ON THE THERMODYNAMICS AND KINETIC REACTIVITY OF GENETIC VARIANTS OF HUMAN ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE WITH G6P.

BY

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A B S T R A C T

The characterization of a new variant G6PD, "Mould", has been evaluated in terms of the kinetics and thermodynamics of the binding of G6P to the new enzyme. This enzyme in comparison with G6PD B, is a new variant associated with a slow electrophoretic mobility and a slightly higher red cell G6PD activity. The variation of the kinetic and thermodynamic parameters with pH are however similar suggesting that the structural locus is not part of the binding site for G6P, but away from it.

Comparison of the properties of the Mould enzyme with other known G6PD variants found in West Africa has also established the fact that the Mould enzyme is a new sporadic variant in the region.

Negative cooperativity was observed for the binding of G6P to the B and Mould G6PDs. Previous kinetic data on G6P binding had given normal Michealian kinetics due to the concentration range of G6P utilized. Km_2^{G6P}, the Km for the high affinity state of the enzyme was found to be similar to the previously determined Km^{G6P}, implying that kinetic measurements had previously been determined at concentrations where binding will be only at the high affinity site for G6P binding on the enzyme. Since G6PD dissociates to the inactive monomer at high G6P concentrations, the observed negative cooperativity was therefore associated with the probable mechanism by which dissociation of the enzyme to the inactive form is prevented by the enzyme changing to a conformation with a lower

affinity for G6P.

The thermodynamic and kinetic functions of the G6P binding reactions have also been determined for G6PD B in water-glycerol mixtures, water, and D_2O. In the presence of glycerol, the observed sigmoid kinetics was abolished. This behaviour is probably due to the deformation of one of the G6P binding sites, due to pertubations of protein hydration in the presence of glycerol. Log $V_{max.}$ is a linear function of dielectric constant and surface tension while V_{max} is a linear function of viscosity. These correlations show a strong dependence of V_{max} on the properties of the bulk solvent.

Motive type compensation has been observed, implicating the existence of "linkage process" in G6PD reactions.

For the experiments in water and D_2O, anomalous behaviour was observed for the kinetic and thermodynamic functions of the enzyme at the temperature of maximum density for water ($4.0^{\circ}C$) and D_2O ($11.0^{\circ}C$) Correllaion of $V_{max.}$ and K_m values of the enzyme with temperature, and therefore mass composition of the solvents showed that these parameters are dependent on the mass composition. Linear dependence of $V_{max.}$ on viscosity of water was observed until at $4.0^{\circ}C$, where there was a discontinuity. Km and V_{max} were also strongly dependent on the internal pressure of the two solvents.

All these observations do therefore suggest that the catalytic properties of the G6PD enzyme are dependent on the intrinsic properties of the solvent in which it functions, implying that the solvent plays an important role in the catalysis of the enzyme.

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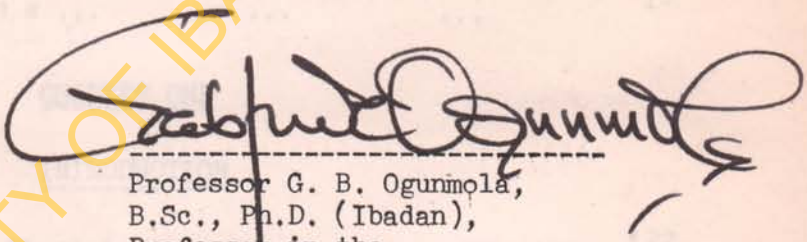
DEDICATION

This thesis is dedicated to the memory of my dead mother, Mrs. J. U. Abugo who toiled hard to bring me up, but did not live long enough to enjoy the fruits of her labour, and my dead cousin, Mr. M. Unufe who lost his life prematurely. May their souls rest in peace.

C E R T I F I C A T I O N

I certify that this work was carried out by Mr. Omoefe O. Abugo in the Department of Chemistry, University of Ibadan between October 1981 and September, 1984.



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A B B R E V I A T I O N S

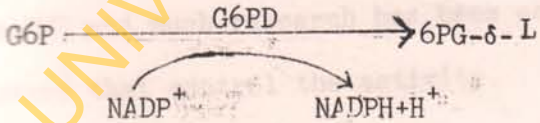
G6PD	:	Glucose-6-Phosphate Dehydrogenase
$NADP^+$	:	Nicotinamide Adenine Dinucleotide Phosphate
G6P	:	Glucose-6-Phosphate
GPG-δ-L	:	6 Phosphoglucono-δ-Lactone
G6G	:	6 Phosphogluconate
NAD	:	Nicotinamide Adenine Dinucleotide
NADPH	:	Reduced Nictotinamde Adenine Dinucleotide Phosphate
HMP	:	Hexose-Mono-Phosphate
GSH	:	Reduced Glutathione
GSSG	:	Oxidised Glutathione
TRIS	:	TRIS (2-hydroxy methyl) methylamine, $NH_2.C(CH_2.OH)_3$
EDTA	:	Ethylenediamine Tetra-Acetic acid
PAGE	:	Polyacrylamide Gel Electrophoresis
SDS	:	Sodium Dodecyl Sulphate
TEMED	:	N, N, N^1, N^1 - Tetramethyl ethylenediamine
D_2O	:	Deuterium Oxide
CM	:	Carboxy methyl
DEAE	:	Diethyl aminoethyl

ADP	:	Adenosine diphosphate
LSFITW	:	A least square curve fit program which fits to the equation: Y = A(0)+A(1)*X+A(2)*X2+.....A(M1)* X (M1).
WHO	:	World Health Organization
MWC	:	Monod - Wyman - Changeux (model)
KNF	:	Koshland - Nemethy - Filmer (model)
SDW	:	Somogyi - Damjanovich - Welch (model)
L.A.(or l.a)	:	Low Affinity
H.A. (or h.a.)	:	High Affinity

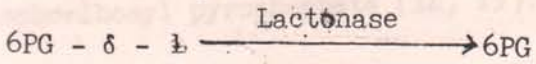
CHAPTER ONE

INTRODUCTION

1.1.1. Glucose-6-phosphate dehydrogenase (D-Glucose-6-phosphate NADP oxidoreductase, EC(1.1.1.49)) was first discovered by Warburg and Christain (1). It was named Zwischenferment (zwischen means between) because Warburg considered it to have an intermediary function in the oxidation of glucose-6-phosphate by methylene blue in lysed erythrocytes. Glucose-6-phosphate dehydrogenase, G6PD, catalyzes the oxidation of glucose-6-phosphate, G6P, to 6-phosphogluconate, 6PG, although the reaction actually takes place in two steps (2, 3). In the first of these steps, G6P is oxidized to its corresponding lactone, 6-phosphoglucono-δ-lactone (6PG-δ-L) under the influence of G6PD. $NADP^+$ is an obligatory cofactor (4, 5); two electrons are directly transferred to $NADP^+$ (6) and NADPH is evolved as a second product of the reaction :



In the second step, 6PG is produced by the enzymatic hydrolysis of the lactone which is very unstable in an essentially irreversible reaction:



The first partial purification of the enzyme, from brewers yeast, was reported in 1936 (7) but another 25 years elapsed before the first successful isolation of G6PD in homogenous form, also from brewers yeast (8). In the meantime the enzyme had been found in a wide variety of animals, plants and micro-organisms (9, 10). In the last two decades, G6PD has been isolated in highly purified and homogenous forms from several mammalian and microbial sources and a considerable body of information has accumulated on structural, catalytic and regulatory features of these enzymes.

Interests in G6PDs have arisen from several perspectives. In animal tissues and many plants and micro-organisms, G6PD is the initiating and primary enzyme of the hexose monophosphate shunt (HMP), which serves to generate NADPH and under some circumstances, pentose phosphates (11, 12, 13, 10). Although the principal role of the HMP is to generate NADPH, the precise role of this pathway of G6P oxidation varies among different tissues and under various metabolic conditions. In some animal tissues under certain circumstances a major fraction of glucose is metabolized by way of the HMP and much research has been concerned with elucidating those factors that control the activity.

The principal function of the hexose monophosphate shunt in human erythrocytes is the generation of NADPH and limited amounts of 5-phosphoribosyl pyrophophate (14, 15).

NADPH is used for several functions, the most important being as the co-enzyme for glutathione reductase that maintains glutathione in its reduced form. Reduced glutathione is in turn essential for the maintenance of viable erythrocytes (14, 15). Under physiological conditions G6PD catalyzes the rate limiting reaction in the hexose monophosphate shunt in human erythrocytes (13). Intense interest in human erythrocyte G6PD was aroused in the mid-1950s by the discoveries that G6PD deficiency is associated with primaquine-sensitive hemolytic anemia (16) and that the structural gene for human G6PD is located on the X - chromosomes (17). It soon became apparent that a wide variety of clinical conditions are associated with erythrocyte G6PD deficiency or with genetically altered G6PDs (14, 15, 18-22). Over 140 variants of human erythrocyte G6PD have been found (21) and it is estimated that over 100 million people in the world have some form of erythrocyte G6PD deficiency (15). Since human G6PDs (23), as well as G6PDs in other animals (24, 25) are coded for by a gene on the X-chromosome it is curious that the erythrocyte G6PD deficiency seen in some variants is not accompanied by a similar deficiency in the G6PD in other cells of the same individual (26) and that no satisfactory animal model has been found for human erythrocyte G6PD deficiency.

1.2.1. METABOLIC AND PHYSIOLOGIC ROLE OF ERYTHROCYTE G6PD

The G6PD enzyme is metabolically important particularly to the erythrocyte. Despite its non-nucleated state, the mature human red cell continues to be an actively metabolizing cell throughout its period of circulation in the blood. Normally, the mature red cell relies primarily on the anaerobic glycolytic (Embden-Meyerhof) pathway for its energy production. However, an alternative and oxidative pathway for glucose, in the form of the HMP, is available to the red cell. As previously stated, G6PD is the initiating and primary enzyme in this alternate pathway. To the mature red cell, the most important function of the HMP is the production of reducing power in the form of NADPH. Indeed, this pathway represents the only source of NADPH available to the erythrocytes (27). NADH is produced during anaerobic glycolysis, but unlike NADPH, is oxidized rather rapidly by molecular oxygen (28). The NADPH remains available to the cell for reductive reactions and other crucial functions, mainly having to do with the protection of cell components against oxidation. The red cell is constantly exposed to some degree of oxidative injury from a variety of sources, including the very oxygen it is destined to transport (29). To counteract this threatened oxidative injury, the erythrocyte is endowed with several specific enzymatic systems each of which requires NADPH as a cofactor. These protective systems are primarily concerned with methemoglobin reduction, the maintenance of reduced glutathione, the reduction of oxidant

compounds and drugs, and possibly the synthesis of lipids.

In the normal red blood cells, the hemoglobin is constantly shifting from the functional, reduced state (ferrohemoglobin) to the oxidized (ferric) state, more often called methemoglobin. Methemoglobin does not combine with oxygen and is non-functional as an oxygen transporting pigment. To counteract this oxidation of hemoglobin and maintain its functional state, the erythrocyte carries two specific enzyme systems, one NADPH dependent, the other NADH dependent (30).

$$MetHb^{3+} + NADPH \xrightarrow{\text{NADPH-MetHb Reductase}} Hb^{2+} + NADP$$

$$MetHb^{3+} + NADH \xrightarrow{\text{NADH-MetHb Reductase}} Hb^{2+} + NAD$$

Although the later system is considered to be the more important under physiologic conditions (30, 31), the NADPH-dependent methemoglobin reductase must assume major importance under certain abnormal circumstances (32, 33).

Glutathione is a tripeptide of complex functions that is found in the blood almost entirely within the red cells (32) and accounts for over 95 percent of the reduced, non-protein, sulfhydryl compounds in the erythrocyte (28). Glutathione is maintained in its functional reduced state (GSH) in the normal erythrocyte by a specific enzyme, glutathione reductase, whose preferred cofactor is NADPH (34).

$$GSSG + 2NADPH \xrightarrow{\text{GSSG Reductase}} 2GSH + 2NADP$$

The reaction appears to be irreversible under physiologic conditions. GSSG reductase is ubiquitous throughout the biologic world (35) emphasizing the important protective role of GSH in the maintenance of living cells. Instances of drug-sensitivity have been attributed to a primary deficiency of glutathione reductase itself (36), or to an inability to synthesize glutathione (37). GSH appears necessary for the stability of many cell proteins, including several enzymes (38), coenzymes (39), and hemoglobin (39). The GSH may well fulfil this protective function by being more readily oxidized than are vital cellular constituents (35) or at least by promptly reversing the earliest oxidative changes in them (40). In doing so, the GSH becomes reoxidized to its disulfide form, GSSG. The preferential oxidation of GSH protects the integrity of the red cell while at the same time offering minimal risk to it, since the cell can again reduce the oxidized glutathione and thus regenerate the protective compound.

GSH of red cells is also coupled to an enzyme, glutathione peroxidase, that specifically reacts with the potent oxidizing agent H_2O_2 to convert it to water, while the GSH is reoxidized in the reaction (41).

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH Peroxidase}} 2H_2O + GSSG$$

NADPH plays a vital role in protecting the erythrocyte against oxidative injury from any oxidant compounds, whether endogenous or exogenous. These compounds threaten the cell by their ability to act as electron carriers, readily taking on electrons from cellular constituents and passing them to oxygen. Such compounds thus can

bridge the metabolic gap that insulates the red cell from the dangerous charge of oxygen it carries (29). To counteract these agents, NADPH may act directly to detoxify oxidant compounds (29), while GSH, also a reducing agent, is a second mode of protection. However, both mechanisms depend ultimately on the activity of the HMP. This shunt is accordingly stimulated by oxidant compounds which have been shown to accelerate the pathway and also the degree of its recycling, augmenting the production of NADPH (22).

Over 90 percent of the lipid content of an erythrocyte is found in the cell membrane. Therefore, the integrity and viability of the cell must depend to a great extent on the adequate original synthesis of membrane lipids and possibly on their renewal during the cells life. Again, these vital functions, at least in part, depend upon a supply of NADPH since the reduced coenzyme is necessary for certain reductive steps in lipid synthesis (42). In the red cell, lipid synthesis occurs mostly in immature nucleated cells and reticulocytes (43). It may also occur to a slight extent in mature erythrocytes (44), but with doubtful significance. Regardless, the original synthesis of membrane lipids in the nucleated cell should greatly influence that cells subsequent function and survival in the circulation. The vital role of the HMP is indicated by the decreased membrane lipids found in G6PD deficient red cells, even at earlier stages of development. In addition to red cells, G6PD and other enzymes of the HMP are present to an appreciable extent in many other

human tissues, but in varying amounts (45). The enzyme shows highest activity in those tissues with a strong potential for lipogenesis such as lactating breast, adrenal cortex and adipose tissue. This distribution of shunt enzyme points up the importance of the pathway in lipid synthesis.

It must be re-emphasized that the only source of NADPH available to the mature red cell is the HMP, and these several protective mechanisms are thus intimately reliant upon the activity of G6PD.

1.3.1. S P E C I F I C I T Y :

D-Glucose-6-phosphate, is the natural substrate for G6PD. It gives the highest value of maximum velocity and lowest K_m for all the G6PDs tested. Most G6PDs can oxidize compounds with minor changes in the substrate structure, but always with less efficiency. It has been shown by a couple of authors working on G6PDs from different sources that the β - D - glucopyranose - 6 - phosphate is the form of G6P utilized by the enzymes (46-49) as is indicated in fig. 1.1.

The C-1 carbon atom is the anomeric carbon atom. Changes in the substrate structure at the anomeric carbon atom cause loss of activity as a substrate. A typical example is when glucose-1-phosphate is used as the substrate for some G6PDs (50-52). There appears to be some degree of tolerance with respect to the -OH group at C-2: 2-deoxyglucose-6-phosphate can serve as a weak substrate for many G6PDs (53, 54). The few attempts to test compounds altered at C-3

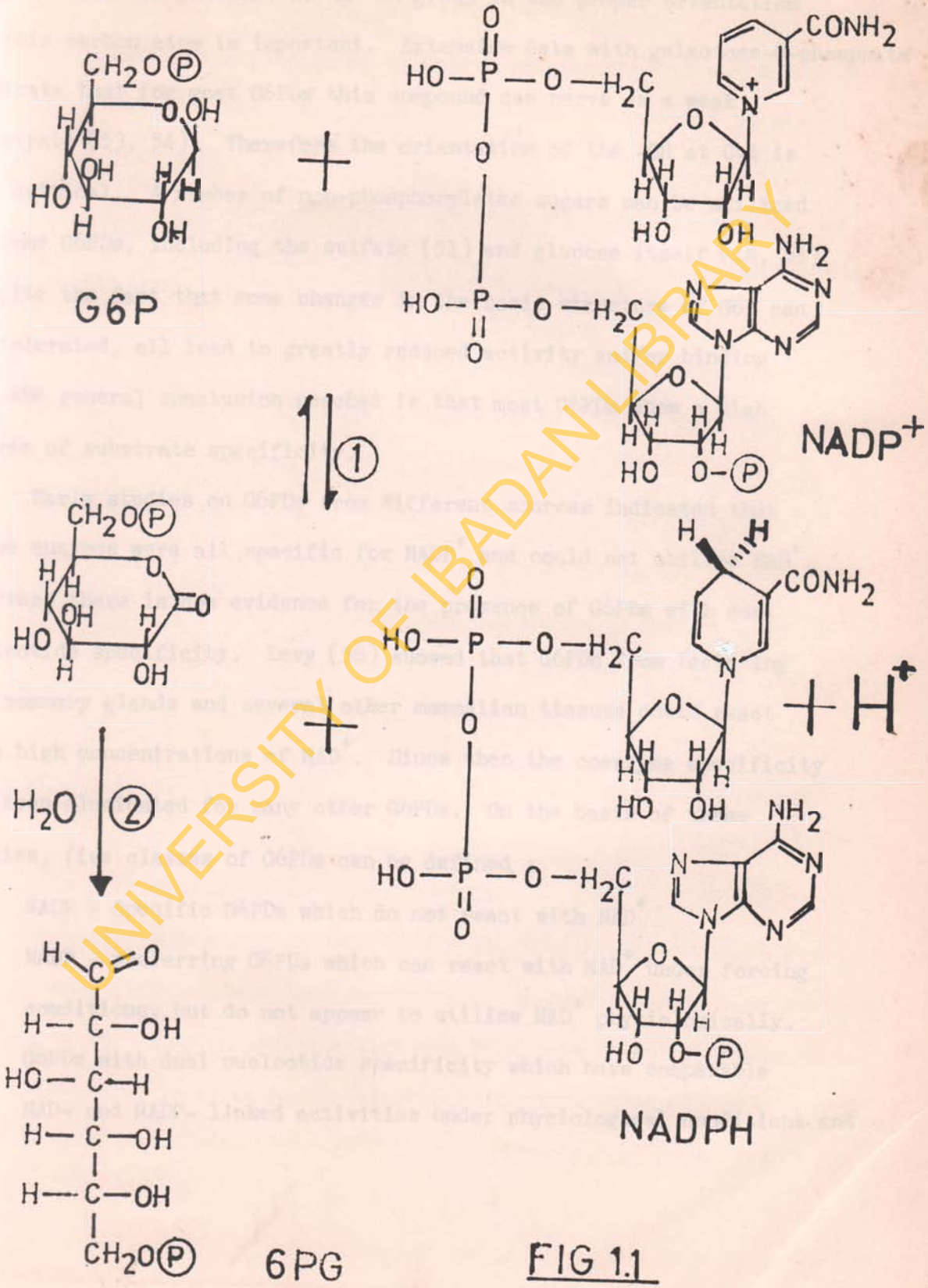


FIG 1.1

suggest that the presence of an -OH group in the proper orientation on this carbon atom is important. Extensive data with galactose-6-phosphate indicate that for most G6PDs this compound can serve as a weak substrate (53, 54). Therefore the orientation of the -OH at C-4 is not critical. A number of non-phosphorylated sugars can be utilized by some G6PDs, including the sulfate (51) and glucose itself (48, 55). Despite the fact that some changes in the basic structure of G6P can be tolerated, all lead to greatly reduced activity and/or binding and the general conclusion reached is that most G6PDs show a high degree of substrate specificity.

Early studies on G6PDs from different sources indicated that these enzymes were all specific for $NADP^+$ and could not utilize NAD^+. However, there is now evidence for the presence of G6PDs with dual nucleotide specificity. Levy (56) showed that G6PDs from lactating rat mammary glands and several other mammalian tissues could react with high concentrations of NAD^+. Since then the coenzyme specificity has been elucidated for many other G6PDs. On the basis of these studies, five classes of G6PDs can be defined :

1. NADP - Specific G6PDs which do not react with NAD^+
2. NADP - preferring G6PDs which can react with NAD^+ under forcing conditions, but do not appear to utilize NAD^+ physiologically.
3. G6PDs with dual nucleotide specificity which have comparable NAD- and NADP- linked activities under physiological conditions and

probably make use of both activities in vivo.

4. NAD- preferring G6PDs which can react with both coenzymes, but, under physiological conditions, would be expected to react with only NAD^+.

5. NAD- Specific G6PDs which can only react with NAD^+

Most G6PDs are found in the first three classes, only a few examples of G6PDs in the last two groups are known at present.

The reaction catalyzed by G6PD involves direct transfer of the hydrogen on the anomeric carbon atom to the B side of the coenzyme molecule. This was first shown by Stern and Vennesland with the NADP-specific G6PD from S. Carlsbergensis (6). The absolute configuration of the hydrogens at the prochiral centre in the nicotinamide ring of NADH and NADPH was elucidated by Cornforth et al (57), thus it is possible to specify that the hydrogen is transferred from G6P to the si face at C-4 of the nicotinamide ring of $NADP^+$. This stereospecificity is identical for all G6PDs in which this has been tested.

1.4.1. PRIMARY AND SECONDARY STRUCTURE

A recent review by Levy (58) gives the amino acid composition of some G6PDs. There is a considerable variability, the most striking being the range of cysteine contents from zero in the L.mesenteroides enzyme (59) to 32 in N. Crassa G6PD (60, 61). There is also a wide range of methionine contents. In all the G6PDs whose amino acid composition

has been determined, the amount of (Aspartic acid + Glutamic acid) always exceed (Lysine + Histidine + Arginine). This is consistent with the general low isoelectric points for these enzymes.

The N- and C- terminal amino acids for some G6PDs has been determined. The G6PD from C. Utilis appears to consist of two different subunits, both of which have C-terminal glycine residues, but one of which has an N-terminal glycine and the other an N-terminal alanine (62). Chung and Langdon (63) tentatively concluded that human erythrocyte G6PD contains two different subunits, since they found both tyrosine and alanine as N-terminal amino acids. However, their conclusion was premature because their enzyme was not homogenous. Subsequently both Yoshida (64) and Rattazzi (65) presented evidence to indicate that the N-terminal amino acid was tyrosine. Finally Yoshida showed by Edman degradation that this enzyme contains no unblocked N-terminal amino acid (66). The earlier identification of tyrosine was the result of a misinterpretation. The amino terminus is blocked and it was identified as pyroglutamic acid (66). It however, seems possible that the pyroglutamic acid is generated by proteolytic or other modification during the enzyme isolation, although this has not been proved (66). The C-terminal amino acid in human erythrocyte G6PD types A and B (64) as in the C. Utilis G6PDs (62) is glycine. Kahn et al also found that human leukocyte G6PD contains a C-terminal lysine (67, 68). This was true of three different forms of G6PD corresponding to three different stages of post translational modification.

The structural difference between erythrocyte G6PDs A, B and Hektoen variant associated with fourfold increased enzyme activity was elucidated by peptide mapping of their tryptic and chymotryptic peptides by Yoshida (69, 70). A single amino acid substitution from asparagine in the B enzyme to aspartic acid in the A variant, and from histine in the B enzyme to tyrosine in the Hektoen variant was found.

The subunits of most G6PDs are identical. From peptide mapping studies on tryptic digests, Singh and Squire concluded that bovine adrenal G6PD consists of identical subunits (71). Hybridization studies also suggested that the human and rat erythrocyte G6PDs each consist of identical subunits (72). Yoshida later confirmed this for the human enzyme, variants A and B (64, 69). The subunit (monomer) molecular weights of microbial G6PDs are 50,000 - 60,000 while those of mammalian G6PDs are 58,000 - 67,000 (76 - 78). Amongst the microbial G6PDs, those from C. Utilis and S. Cerevisiae are exceptions. Subunit molecular weights of C. Utilis has been given to be about 14,000 in some instances (62), while that of S.Cerevisiae has been given as 22,000 (79). It has however being speculated that proteolytic degradation during isolation might be responsible for such low subunit molecular weights.

Very few studies have been conducted on the secondary structure of G6PDs. Jirgensons working on a number of proteins including G6PD from S. Cerevisiae (80) obtained values for the Moffit constant,

bo, and the positive cotton effect, that were relatively low, indicating a relatively low degree of helical structure. Optical rotatory dispersion results for two G6PDs from C. Utilis were also interpreted as indicating low helical contents in these two enzymes (81). In contrast to these results, circular dichroism studies on rat liver G6PD (obtained from Holtzmann strain rats) indicated that these enzymes have a large helical content (82).

1.4.2. TERTIARY AND QUATERNARY STRUCTURE

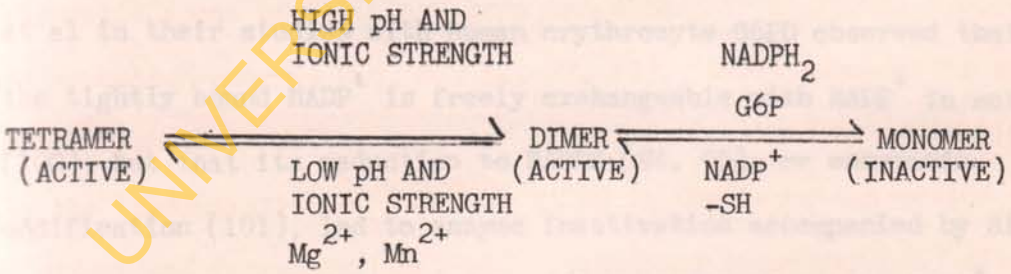
Structural studies conducted on G6PDs indicate two fundamental molecular forms, an inactive monomer and an active dimer (2, 83). Most G6PDs are in the dimeric form at the optimum pH (8-9) (84), the minimum structure that is catalytically active.

Although the minimum quaternary structure necessary for catalysis in G6PDs is the dimer many G6PDs aggregate to form tetramers. Tetrameric forms of G6PD have been described for microbial (75, 85, 86) and mammalian sources (66, 83, 84, 87, 88). Early experiments with human erythrocyte G6PD implicated temperature, enzyme concentration, and the concentration of $NADP^+$ or NADPH in the inter conversion of dimers and tetramers (89 - 91). Cohen and Rosemeyer (84) also working on erythrocyte G6PD showed that the enzyme has two discrete polymeric forms corresponding to tetramers and dimers, the equilibrium of which is closely related to the pH and ionic strength of the solvent, and to some other enviromental parameters. High pH and ionic strength favour formation of dimer and lower pH and ionic strength

promote tetramer formation. These results were confirmed by Bonsignore et al (92), who also found that Mg^{2+} and other divalent cations, at moderate concentration promote tetramer formation.

A second equilibrium exists between dimers and monomers. A major regulatory factor of this dissociation system is the apoenzyme - bound, NADP (90, 91, 93). Thus reduction of the tightly bound $NADP^{+}$ results in the dissociation of the dimeric enzyme to monomers (87, 94, 95). Conversely reincubation of the individual monomers in the presence of NADP and sulfhydryl reagents is followed by reassembly to the dimeric form (52, 57) G6PD dissociation to monomers can also be effected by incubation in sodium dodecyl sulfate (SDS), urea, or guanidinium chloride.

The general features of both equilibria may be summarized as follows :



The two sets of equilibrium i.e. between tetramers and dimers, and between dimers and monomers, appear to be closely interdependent on an equilibrium basis. These facts support the view that the catalytic operations of erythrocyte G6PD in vitro involve a continuous interconversion of multiple molecular forms arising from

three association - dissociation systems (96 - 98).

From studies on factors involved in the aggregation and dissociation of G6PDs some ideas have emerged concerning those forces involved in interactions between subunits. As is true in some other proteins consisting of four subunits (99), tetrameric G6PDs appear to exist as "dimers of dimers," with two planes of dissociation across which different kinds of forces predominate. From their studies of the effects of ionic strength and pH on the structure of human erythrocyte G6PD, Cohen and Rosemeyer concluded that ionic bonds were of primary importance in stabilizing the interaction between dimers within the tetramer, whereas predominantly hydrophobic forces served to stabilize the interaction between the subunits within the dimer (84).

The precise role of $NADP^+$ in the quaternary structure of human erythrocyte has been elucidated by a number of authors. Bonsignore et al in their studies with human erythrocyte G6PD observed that the tightly bound $NADP^+$ is freely exchangeable with $NADP^+$ in solution (100), but that its reduction to NADPH (94, 95), or enzymatic modification (101), led to enzyme inactivation accompanied by dissociation to monomers. They referred to the tightly bound $NADP^+$ as "structural $NADP^+$" (100), and claimed that the human erythrocyte G6PD was unique in possessing such structural $NADP^+$. Although Yoshida originally claimed that the removal of $NADP^+$ caused dissociation from tetramer to dimer (52), he subsequently stated that the dissociation

is to monomers (102). Thus there is a general agreement that removal of (structural) $NADP^+$ leads to dissociation of the enzyme to its subunits (94, 101, 102). This therefore means that $NADP^+$ must play a specific role in the quaternary structure of human erythrocyte G6PD by stabilizing the interaction between subunits in the dimer.

There is disagreement on the stoichiometry of $NADP^+$ binding to human erythrocyte G6PD. Some investigators have reported one $NADP^+$ per dimer (84, 93, 103), or one NADPH, but never both (103). Others have shown two classes of NADP(H) binding sites; with different affinities for the coenzyme: four sites per tetramer binding four molecules of $NADP^+$ very tightly and four additional sites binding either four more $NADP^+$ molecules or two NADPH molecules (104, 105). These investigators thus propose distinct structural and catalytic sites for $NADP^+$, a view that is strenghtened by the kinetic studies of Luzzatto and Afolayan (106, 107).

Based on electron microscopy studies of erythrocyte G6PD B variant, Wrigley et al (83) suggested that the subunits of the enzyme were arranged to give a tetrahedral structure, with D_2 sysmmetry for the tetramer. These studies were used to examine the interconversion of monomers, dimers and tetramers and to elucidate the shape and dimensions of the different forms of this enzyme. The monomers were found to be of approximately cylindrical shape; with dimensions of $68A^\circ$ by $34A^\circ$. The shape of the monomers is modified in the dimer, which is V-shaped and involves isologous association between the subunits.

The molecular weight of most G6PD dimers is in the range of

100,000 to 120,000, while that of tetramers is about 200,000 to 250,000. Tetramer molecular weights of 280,000, 284,000 and 350,000 have been obtained however for G6PDs from rat liver (108) bovine adrenal (77) and B. Subtilis (74) respectively. There is the possibility that these are higher oligomers other than tetramers.

1.5.1. PURIFICATION

Glucose-6-phosphate dehydrogenase has been isolated from a large number of animal, plant and microbial sources (8, 52, 76, 77, 78, 109), and has been purified to varying degrees from yeast (8, 110), mammary gland (56, 88), adrenal gland (76), liver (78, 109), human erythrocyte (52, 63, 111, 112) and other sources. A recent review by Levy (58) has however shown that there have been some imprecise designation of enzyme sources, especially for enzymes isolated from yeast.

One of the problems encountered in isolating some G6PDs is their lability, while others appear to be quite stable. In general, G6PDs from human and fungal sources appear to be more labile than those from bacterial sources.

Because of the existence of many genetic variants of G6PD in man, those from erythrocytes have been of particular interest in the studies of human biochemical genetics. This has thus led to the attempts, by many workers to purify the enzyme to a very high state of purity so as to elucidate more accurately the structure, physico-chemical and other properties of the enzyme.

Chung et al (63) purified human erythrocyte G6PD to a specific activity of up to 113 enzyme units per milligram of protein. This preparation had a purity of about 80 percent by the criteria of ultracentrifugal and electrophoretic measurements. Yoshida (52) however reported that the enzyme contained impurities of smaller molecular weights and therefore said that their preparation was about 65 percent pure, since it was presumed that they measured the enzyme activity in partially inactive form. Yoshida (52), using DEAE-cellulose, calcium phosphate gel, CM-cellulose, DEAE Sephadex and CM-Sephadex chromatographic columns, purified G6PD to a specific activity of 750 enzyme units per milligram, the highest till date. When the enzyme preparation was diluted to about one milligram per millilitre or less, the specific activity went down to about 170 to 180 units per milligram. In this preparation, care was taken to avoid inactivation of the enzyme by proteolytic enzymes, e.g. plasmin, at the early stages of purification by the addition of ε-amino-N-caproic acid and/or diisopropylfluorophosphate so as to improve the yield. The purification process was however time consuming requiring as many as twenty steps and also large quantities of blood.

When abnormal and unstable enzyme variants or small amounts of blood are to be purified, Yoshidas procedure is not feasible, thus subsequent modifications were aimed at simplifying it. The same remarks can be made about the methods established by Cohen et al (111), Chung et al (63) and Bonsignore et al (112). Considering the lengthy

methods of purification, Rattazzi (65), came up with a shorter and simpler one, also involving smaller volumes of blood. He obtained an overall yield of 60 percent with specific activity of 190 units per milligram. The procedure consisted of three steps, but the final specific elution step by the substrate G6P yielded a highly labile enzyme species due to the catalytic reduction of the apoenzyme bound $NADP^+$ which stabilizes the quarternary structure of the G6PD. The preparation of Cohen et al (111), although leading to a simplification of the Yoshida procedure still involved some steps which according to Bonsignore et al (112) are not easily reproducible from one preparation to another. Bonsignore and coworkers (112), thus described a method for purifying G6PD from human erythrocytes which represent a modification of two procedures developed by Cohen et al (111) and Rattazzi (65). They claimed to obtain stable and homogenous enzyme preparations by this method. Here the specific elution from the final column was by $NADP^+$ which has been shown by many workers to have a lower K_m (and therefore a higher affinity for the enzyme) than the substrate glucose-6-phosphate (G6P). The preparation of Kahn et al (113) was eluted specifically by high concentrations of the coenzyme $NADP^+$, which explains the stability of the enzyme prepared by these workers. The total yield here was between 80 - 90%. The higher yield, the simplicity of the method and above all, the stability of the enzyme represented a major advantage of the method of Kahn et al over that described by Rattazzi and the other workers described above.

All the purification procedures described so far are time consuming. This consideration prompted DeFlora et al (114) to develop a new method of purification based on two sequential steps of affinity chromatography. This is a type of adsorption chromatography in which the bed material has biological affinity for the substance to be isolated. The basic principle of affinity chromatography is to immobilize one of the components of the interacting system (e.g. ligand) to an insoluble porous support in most cases through a spacer arm which can then be used selectively to adsorb, in a chromatographic procedure that component (e.g. enzyme) of the bathing medium with which it can selectively interact. Desorption and elution are subsequently carried out by changing the experimental conditions (e.g. introduction of a competing soluble ligand with appreciable affinity for the enzyme or by inducing conformational changes which decrease the enzyme affinity for the immoblized ligand) which results in the dissociation of the enzyme - immoblized ligand complex after unbound substances have been washed away. This DeFlora's method can be applied in the purification of G6PD from single donors and can therefore be conveniently employed for the study of structural and functional modifications in genetic variants of this enzyme. It was also a substantial improvement over the lengthy techniques of purification of G6PD. DeFlora et al (114) however experienced some difficulties, especially poor reproducibility of the affinity chromatographic step due to marked modifications in $NADP^+$ structure occuring during the carbodiimide directed coupling of the

dinucleotide to the matrix. Hence a more convenient affinity gel, N^6 - (6-aminohexyl) adenosine 2, 5 -biphosphate was synthesized (115), and coupled to Cyanogen bromide activated agarose. With this the variability in recovery of G6PD was overcome, and a good yield was obtained in two days.

Attempts by DeFlora and workers (115) to further simplify the procedure by omitting the phosphocellulose step was unsuccessful. Preparations of the enzyme obtained without this step were found to be contaminated with substantial amounts of another protein later on designated as FX. Morelli and DeFlora (116) however eliminated this protein by the inclusion of the CM-Sephadex step after passage through the affinity column. An enzyme preparation with a specific activity of 174 units per milligram, and which was homogenous by electrophoretic criteria was obtained.

Craney and Goffredo (117) claimed that due to the lability of the enzyme, the isolation procedure should be as rapid as possible, containing very few chromatographic steps. Thus the number of chromatographic steps required by the procedure of DeFlora and coworkers led them to reexamine the use of $NADP^+$ as an affinity ligand in the purification of G6PD. Adaptations of standard affinity chromatography procedures (118, 119) led to the synthesis of an affinity chromatography matrix which binds erythrocyte G6PD. Selective elution of the enzyme from the affinity matrix followed by chromatography on Bio-Gel P-150 column yielded a highly purified enzyme preparation which was homogenous on the criteria of polyacrylamide disc gel electrophoresis.

The specific activity of the preparation was 173 units per milligram, with a yield of 66%. The whole process was completed in twenty five hours.

With these purification methods, which can give very pure enzymes using small quantity of blood, the previous limitations of physico-chemical and molecular studies is now averted. These studies have always been limited by unavailability of purified enzyme. A lot of discrepancies both in kinetic and molecular properties of the different variants of the enzyme had been due in part to various workers using enzymes of varying purities. Thus presently we can get most variants of G6PD in very pure forms for physico-chemical and comparative studies.

1.6.1. GENETIC VARIANTS OF G6PD

Over the past few decades, defects and variants of human G6PD have attracted attention both as causes of various hemolytic disorders and as useful genetic markers. Deficiencies in the activity of human red cell G6PD are found in many countries and in over one hundred million persons, some of whom may suffer, as a result from hemolytic anemias induced by certain types of drugs and other agents, including important antimalarial compounds. The number of G6PD variants already identified is second only to hemoglobins (120) in terms of genetic variability of a human protein. More than 140 types of G6PD which are distinginshable by electrophoretic mobility, or by enzymatic characteristics, or by both methods have been reported (18, 20). Different types of the enzyme have been found among populations from diverse areas as Sardinia, Northern Europe, Africa,

North America, China, New Guinea and other places (22, 121 - 123).

The genetic determinant of G6PD is located on the X-chromosome in man (23). Therefore, males (XY) are always hemizygotes and females (XX) are homozygotes or heterozygotes. G6PD deficiency is inherited as an X linked trait. Males who carry the gene show full expression, and male to male transmission is not observed. In each cell of the female, only one of the two G6PD genes is active. The tissues and the blood cells of heterozygous females therefore represent a mosaic of normal and variant cells. The proportion of normal to variant cells may vary greatly.

G6PD variants can be classified in various ways. Accoring to the prevalence in particular populations, a G6PD type can be classified as :

(a) Sporadic when only single or very few cases have been encountered.

(b) Polymorphic when its frequency in at least one population is above one percent.

According to clinical manifestations, G6PD variants can be divided into three groups namely :

(a) "Normal" or basically non-deficient, when they are not associated with any known clinical implications e.g. G6PD A and B.

(b) Common or uncommon deficient variants associated only with acute hemolytic anemia (AHA). This group however require the administration of exogenous agents such as drugs, foods, infections or fava

beans for hemolysis to occur. These variants have lower K_m for $NADP^+$ and higher K_i for NADPH than for the normal variant.

(c) Variants associated with chronic nonspherocytic hemolytic disease (CNSHD) even in the absence of exogenous agents. These variant have very low enzyme activities and have higher K_m values for $NADP^+$ and lower K_i values for NADPH than the normal B type. Typical examples are G6PD Alhambra (124). Chicago (125). Tripler (126) and Albuquerque (127).

These enzymes can also be classified according to the level of the G6PD in the red blood cell namely:

(a) Deficient when associated with more or less severe enzyme deficiency.

(b) Non-defieient when associated with no deficiency at all,

G6PD variants were formerly identified basically on the basis of electrophoretic mobility in starch gel (128, 129). This created a lot of confusion until in 1966 when a study group convened by the World Health Organization (W.H.O.) in Geneva recommended the following (130): That the most common and ubiquitous variant of G6PD be called B, and should be used as reference with which all other variants can be compared, and for convenience be called the "normal" type. Its red blood cell activity and electrophoretic mobility was hypothetically assumed to be 100 percent. The variant with normal activity and electrophoretically faster than B (about 110 percent mobility of B) which occurs in polymorphic frequency in negro (African and American) populations, representing about 20 percent (131) of the

population of people of African ancestry, should be called A. The variant commonly found among the same people representing about 10 to 15 percent (131) of their population, which has the same electrophoretic mobility like A but is associated with enzyme deficiency, should be called A^-.

G6PD B as earlier noted occurs in polymorphic frequency in many populations. It is the only G6PD phenotype observed thus far amongst Caucasians and is found in about 80 percent negroes. The A^- type enzyme has been found to be associated with a lot of drug and food induced acute hemolytic anemia (131). Some other common G6PDs are the G6PD Baltimore-Austin (132), Ibadan-Austin (132), which are electrophoretically abnormal, but display normal or near normal enzyme activity. G6PD Barbierri (133) and Seattle (134) have clearly diminished enzyme activity, but do not have any known clinical disorder. Just as the common deficient variant amongst the negroes is the A^-, the common mutant amongst the Meditterenean population is the G6PD Meditterenean (135). It has a normal electrophoretic mobility with a red blood cell activity of 8 to 20 percent. It is found amongst Greeks, Asiatic and Sephardic Jews, Sardinians and N.W. Indians. It is associated with drug induced acute hemolytic anemia, neonatal hyperbilirubinaemia and neonatal jaundice. The G6PD Canton (136) is another common G6PD deficient enzyme found amongst Cantonese in South China. These people are

subject to drug induced acute hemolytic anemia. Neonatal hyperbilirubinaemia, leading to kernicterus, occurs in G6PD, deficient Mediterranean, Chinese and Negro infants after exposure of the child or mother to certain drugs or naphthalene, and at times even in the absence of exposure to such agents. Just as some G6PD variants associated with severe enzyme deficiency like G6PD Barbieri (133) and Markham have no hemolytic problems, other variants with less severe enzyme deficiency such as G6PD Alhambra and Tripler (124, 126) are associated with chronic hemolytic disease even in the absence of exogenous agents.

Apart from the normal B, A and A⁻ G6PD types commonly found in West Africa, other sporadic G6PD variants have been identified. G6PD Dakar and Mali (137), highly deficient enzyme types with normal mobilities have been identified in Senegal and Mali respectively. G6PD Gambia (138), and G6PD Takoma from Eastern Senegal (139), slow moving and non-deficient variants have also been identified. From the Western part of Nigeria, a lot of G6PD variants with or without clinical disorders have been identified. Some of these are G6PD Lanlate, Ekiti, Mokola and Abeokuta (140). G6PD Ijebu-Ode and Ita-Bale, slow and non-deficient variants with no associated clinical implications have been worked on extensively by Luzzatto and Afolayan (141). Recently a new G6PD type has been found in a 69 year old Nigerian

woman diagnonized for polycythemia rubra vera (PRV) (142). It is slow moving with a higher red blood cell G6PD activity than the normal enzyme. It is tentatively called the Mould G6PD. This work involves the evaluation of its kinetic characteristics.

Most genetic variants of G6PD presumably have arisen through point mutations causing single amino acid replacements. Yoshida has shown that two variant G6PDs, the common Negro variant A and the Hektoen variant, which is associated with enzyme overproduction, differ from the normal B variant by single amino acid substitutions: in A an aspartic acid residue replaces an asparagine (69) and in Hektoen a tyrosine replaces a histidine (70). The finding of a "variant" G6PD does not however automatically mean that it must have a genetic basis. Acquired modifications are also possible.

Although strikingly different incidence of G6PD deficiency occurs within some geographical areas, its prevalence in tropical and semi-tropical regions is apparent from studies from different parts of the world (143). A rough correlation seems to exist between G6PD deficiency and the presence of Hemoglobin S (HbS) (144). The distribution of the two has been related to the distribution of high incidence areas of falciparum malaria (144, 145). The heterozygous deficient females, but not the hemizygous deficient males appear to be relatively resistant against Plasmodium falciparum malaria (145). The variant A^- conferred a distinct advantage to heterozygous females with respect to infection by

Plasmodium falciparum (146). Thus the plasmodium prefers red blood cells that have sufficiently high level of G6PD, whether by virture of their genetic structure or because of their young age as the parasites tend to invade the youngest cells in G6PD deficient female subjects (144). The high incidence of this inborn error (G6PD deficiency) of metabolism and the presence of HbS have been attributed to their protective effect against Plasmodium falciparum malaria on the basis of their geographical distribution and the demonstration of decreased parasite concentration in enzyme deficient cells (144). The occurence of falciparum malaria has been related to the presence of reduced glutathione, which is very small and highly unstable in the erythrocytes of sensitive individuals (143).

1.6.2. TECHNIQUES FOR THE DETERMINATION OF G6PD VARIANTS

A number of minimal criteria for the identification of new variants was suggested by W.H.O. Standardization Committee (147) due to the high rate of increase of new identified variants. These criteria include :

(1) Red cell G6PD activity

(2) Electrophoretic mobility of enzyme

(3) Michealis constants (Km's) for G6P

(4) Affinity for 2-deoxyglucose-6-phosphate (2dG6P)

(5) Thermal stability.

So many variants have however been described that it has become very difficult to determine whether or not a newly discovered variant is distinct from any other by just these techniques alone. This difficulty has however been partially overcome by the addition of other criteria. These include the use of techniques like elution profile from DEAE and CM Sephadex columns (127, 140), physico-chemical studies, Kinetic and thermodynamic studies (97), peptide mapping (69, 70) and a host of others.

Methods like peptide mapping are not applicable when studying the unusual enzymes often with very weak activity. Yet in these cases the kinetic and thermodynamic studies not only afford a better understanding of the functional abnormality of the enzymatic protein but sometimes allows to suggest a modification of some functional groups.

1.7.1. KINETICS AND THERMODYNAMICS OF SUBSTRATE BINDING

The rate equation describing the steady state kinetics of enzyme catalyzed reactions has the form :

$$v = \frac{dp}{dt} = \frac{f(A,B \ldots..)}{g\ (A,\ B \ldots..)}$$

where f(A, B .....) and g(A, B .....) are linear polynomials involving the concentrations of the reactants. If these equations are integrated in order to follow the time course of the reaction, the expressions are quite complex except in the simplest cases. The rate equations are

thus used in their differential form and the velocity is determined as a function of reactant concentration.

A determination of initial velocity patterns of bireactant reactions usually involves the variation of the concentration of one substrate at different fixed levels of the other one, and in the absence of products. Bireactant mechanisms give one of three patterns. Most sequential mechanisms will give a rate equation of the form (148):

$$v = \frac{V(A)(B)}{K_{ia}K_b + K_a(B) + K_b(A) + (A)(B)} \quad \ldots\ldots \quad 1.1$$

where v is equal to the initial velocity, A and B are substrate concentrations, and V and K's are maximum velocity and Michealis constants respectively. Taking the reciprocal of the plot we have:

$$1/v = K_{ia}K_b/V(A)(B) + K_a/V(A) + K_b/V(B) + 1/V \quad \ldots\ldots \quad 1.2.$$

If the concentration of A is varied, while that of B is kept constant at a saturated level, equation 1.2 will reduce to the

$$1/v = K_a/V(A) + 1/V \quad \ldots\ldots \quad 1.3$$

Taking reciprocals again, we have

$$v = \frac{V(A)}{K_a + (A)} \quad \ldots\ldots \quad 1.4$$

the simple Michealis - Menten equation (149). At very high A, v = V, while at low A, $v = (V/K_a)A$. V and V/K_a (or their reciprocals) are thus the two fundamental kinetic parameters varying independently with the concentrations of other substrates, inhibitors, activators,

or such variables as pH, temperature or ionic strength. The ratio of these two independent parameters, K_a, represents the level of the substrate A giving v = V/2. While it is not an independent constant, it is a very useful one for the biochemist, since it is also a clue to the physiological level of the substrate. A substrate concentration around K_a utilizes most of the catalytic potential of the enzyme, while still maintaining proportional control; at high substrate levels the rate does not vary with substrate concentration, and one has no control. The Michealis constant is not usually identical with the dissociation constant, K_D, of the enzyme substrate complex, but rather it represents the apparent dynamic dissociation constant, under steady state conditions, that is $K_D = (E)(A)/(EA)$ at thermodynamic equilibrium while the Michealis constant equals the same expression under steady state conditions. The Michealis constant thus measures the apparent affinity under steady state conditions, as opposed to equilibrium conditions. Michealis constants may be smaller, larger, or same as dissociation constants, depending on the mechanism and the relative size of certain rate constants. V is the velocity of the reaction when the enzyme is maximally saturated with its substrates. It is directly proportional to the turnover number of the enzyme, K_{cat}, which is the maximum number of substrate molecules converted to products per active site per unit time.

Plots of v against A are hyperbolas and not very informative,

Thus equation 1.4 is best transformed into a linear form, in one of three ways for practical applications :

Lineweaver - Burk (150) : $1/v = K_a/V(A) + 1/V$

Dixon (151) : $(A)/v = K_a/V + (A)/V$

Eadie (152) : $v = -v/K_a(A) + V$

The methods making use of the Lineweaver-Burk and Dixon equations have very little merit, and improvements have been suggested from time to time. Hofstee (153) has pointed out that the reciprocal method of plotting initial rates and substrate concentrations (Lineweaver-Burk's form) could be misleading, and he emphasized the advantages of v versus v/A plots to detect model errors. He emphasized that plots of 1/v versus 1/A tend to obscure experimental inferences that the classical enzyme model is incorrect as well as various kinds of systematic or random errors in the plotted quantities. When a plot of 1/v versus 1/A is made, it is observed that cordinates go to infinity when A tends to zero ($A \rightarrow 0$). Again when a plot of A/v versus A (Dixon's plot) is made, the cordinates tend to infinity when A tends to infinity ($A \rightarrow \infty$). The half maximum point in both cases lie to one side of the curve. Experimental data that may have been obtained with about equal accuracy will be over emphasized at low values of v in the Lineaweaver-Burk plot, and at high values of v in the Dixon plot. The points at the other end of the curve are thus apt to be underestimated or even neglected. This is statistically unsound. Hoftsee further pointed out that none of these objections hold for the

Eadie plot. Here, the curve has a finite and positive intercept with both cordinates. The half maximum point lies in the middle of the curve, and on both sides of this point equal emphasis is laid upon all data.

Wilkinson (154) however questioned Hoftsee's argument. He said that though the Eadie's plots are somewhat more reliable when it comes to fitting parameters, they also squeezed the data about as badly although in a different form.

1.7.2. KINETICS OF $NADP^+$ BINDING TO G6PD

The binding of $NADP^+$ to G6PD at a saturating concentration of G6P has always been thought to conform to the classical Michealis-Menten model. Hence only a single dissociation constant of enzyme-$NADP^+$ complex has always been reported for the G6PD variants. But Luzzatto (107) in 1967 reported that the saturation function of $NADP^+$ for the A variant is sigmoid shaped and hence does not follow the usual Michealis-Menten equation. Luzzatto (107) interpreted his quantitative kinetic data as indicative of the existence of multiple (at least two) binding sites for $NADP^+$ on the enzyme protein, and that the binding of the first molecule of $NADP^+$ modifies the affinity of one or more other sites for the coenzyme. Thus there is an induction of a conformational change as the concentration of $NADP^+$ is increased giving rise to two sites, a state of low affinity and a state of high affinity for $NADP^+$. Luzzatto (107) therefore defined two dissociation constants for the binding of two molecules of $NADP^+$ by the enzyme molecule :

$$E + S \rightleftharpoons ES_1 \quad ., \qquad Ks_1 = (E)(S)/(ES_1)$$

$$ES_1 + S \rightleftharpoons ES_2 \quad ., \qquad Ks_2 = (ES_1)(S)/(ES_2)$$

Ks_1 = dissociation constant at low $NADP^+$ concentration

Ks_2 = dissociation constant at high $NADP^+$ concentration

and $Ks_1 > Ks_2$.

This kinetics was extended to four other variants B, A^-, Ijebu-Ode and Ita -Bale by Afolayan et al (106), and these authors found that the four variants exhibit sigmoid kinetics. But the degree of conformity of the four variants to this kinetic behaviour varies according to the concentration of $NADP^+$ required by each variant for the induction of a transition from a state of low affinity to that of high affinity for $NADP^+$. Here the authors predicted a quadratic model (106, 107). The reaction rate, v, as a function of $NADP^+$ concentration predicted by the model is :

$$v = \frac{(S)^2 V_{max}}{(S)^2 + (S) Ks_2 + Ks_1 Ks_2} \quad \ldots \quad \ldots \quad 1.5$$

It was possible by the use of this equation to fit the experimental data and to derive the binding constants (Ks_1 and Ks_2) of the substrate for the enzyme in the two postulated states (106).

1.7.3. KINETICS OF G6P BINDING TO G6PD

Up to date, the kinetics of G6P binding to the human erythrocyte G6PD has conformed to the Michealis-Menten type kinetics (149), at saturated NADP concentration. This kinetics of G6P binding have been

widely studied for different variants of erythrocyte G6PD (97, 111, 141, 155-157), and the saturation function for all these variants have been found to be hyperbolic at all pH values and temperatures (97, 141). This may mean that there is no interaction between the G6P binding sites and that they are idential and independent or that there is only one G6P binding site on the molecule.

Lessman et al (47), working on the kinetics of G6P binding to the G6PD from Pseudomonas fluorescens at a concentration range of zero to 35 mM however observed a sigmoid type kinetics. Similarly Senior et al (158) obtained sigmoid kinetics for the binding of G6P to the G6PD from Azotobacter beijerinckii while working at a concentration range of 0 to 5mM. Kuby et al (159) in his studies of G6PD from brewers yeast using equilibrium dialysis method observed two classes of G6P binding sites, a tight binding site with K_D = 29μM, and a weak binding site with K_D = 0.26mM. He noted that in kinetic studies, only the tight binding site was observed.

The concentration range at which the studies on the binding of G6P to human erythrocyte G6PD have been carried out so far, lie between 0 and 1.0mM (97, 137, 156). Thus it could be possible that like the case of the bacterial G6PD, increase in the concentration range of study will swamp out the non-Michealian behaviour. Pettigrew and Frieden (160) had shown that the kinetics of the two substrate system normally obey the Michealian type kinetics up to a particular point beyond which they show substantial differences.

1.7.4. pH EFFECTS ON G6PD

Enzymes, being proteins posses ionizable groups and hence are very sensitive to pH changes. These ionizable groups determine the pattern of electrical charges carried by the protein and regulate the extent of interaction with the coenzyme, substrate or an effector. The variation in the kinetic parameters of enzyme (i.e. V_{max} and K_m) with pH are always interpreted in terms of ionizing groups at the enzymes active site, the coenzyme, substrate, the ES complex or any other substance that may be involved in the enzyme reaction. A lot of what is now known about the nature of the enzyme active centres are derived from the studies of pH changes.

The effects of pH changes on K_ms and V_{max}s have been studied for the human erythrocyte G6PD. The different buffers used have however had significant effects ,on these parameters. The plots of log V_m, log K_m^{G6P} and log K_m^{NADP}, against pH by Soldin et al (156) in kinetic experiments in Tris-maleate and ammediol-HCl buffers yielded pK_a values of 6.6, 6.7 and 9.1., and 6.2 and 9.0. respectively. These were taken to be indicative of an imidazole and sulfhydryl group near the G6PD's active centre. Luzzatto et al (141) also indicated the presence of imidazolium group of histidine in variant B, which was absent in A. The B was also said to have a cysteine residue near its active site (141). Evidence for the presence of sulfhydryl group and imidazolium group of histidine near the active site has also been advanced by Babalola et al (97) in the study of G6P binding in tris-borate and triethylamine

borate buffers to G6PD A, B and A^-. Luzzatto (145) reported a pH profile of log K_m^{G6P} for the three common polymorphic variants A, B and A^-. The pH dependence of this kinetic parameter shows familiar trends in the acid and alkaline regions as already reported by the above workers (97, 155-156). But the pH dependence shows a most peculiar and sharp profile in the narrow pH range between 7.0 and 7.6. Luzzatto (145) explained that the peaks at the narrow pH range could be related to the transition of the enzyme from the tetrameric form at acid pH to the dimeric form at alkaline pH. This theory was fortified by the results of gel filtration experiment as a function of pH by Babalola et al (97). Here it was observed that the pH dependence of the dimer-tetramer equilibrium corresponds closely to the narrow pH range (pH 7.2 for variant A and B, and 7.4 for variant A^-). Kahn et al (137) also using tris-borate buffer for the pH ranges of 5.5 to 10.0 obtained a pK_a value of 6.5 ± 0.5 corresponding to an imidazole group for G6PD A, A^-, B and Madrona.

1.7.5. TEMPERATURE EFFECT ON KINETIC PARAMETERS

For the determination of thermodynamic parameters in enzyme kinetics, the values of kinetic parameters are always determined at different temperatures. With this, the heat change for the reaction, ΔH may then be obtained using Van't Hoff's isochore:

$$\frac{d \log K_m}{d(1/T)} = \frac{-\Delta H}{2.303\ R} \quad \ldots \quad \ldots \quad 1.6$$

The most convenient way of using equation 1.6 is to make a plot of log K_m against 1/T. The slope of the straight line thus obtained is then $-\Delta H/2.303R$, where R is the gas constant. The free energy of the reaction at a particular temperature is calculated using the equation

$$\Delta G = - RT\ln K_m \quad \ldots \quad \ldots \quad \ldots \quad 1.7$$

Here it is assumed that the K_m equals the dissociation constant. The entropy of the process can be obtained using the standard thermodynamic equation of state

$$\Delta G = \Delta H - T\Delta S \quad \ldots \quad \ldots \quad \ldots \quad 1.8.$$

In order to obtain the energies of activation it is necessary to make use of the theory of absolute reaction rates (161). The central point of this theory is that the rate of any reaction at a given temperature depends only on the concentration of an energy rich activated complex which is in equilibrium with the unactivated reactants. According to the theory all activated complexes break down at a rate given by $k_B T/h$, where k_B is the Boltzmann constant (the gas constant per molecule) and h is plancks constant. In other words the rate of breakdown is independent of the nature of the complex. Thus the reaction velocity constant k is given by

$$k = \frac{k_B T}{h} k^* \quad \ldots \quad \ldots \quad \ldots \quad 1.9$$

where k^* is the equilibrium constant for the equilibrium between the activated complex and the unactivated molecules. Starred symbols are used to indicate quantities concerned in the activation process. The ordinary thermodynamic equations can be applied to this equilibrium so that

$$\Delta G^* = \Delta H^* - T\Delta S^* = -RT\ln k^*$$

Substituting this into equation 1.9, we have

$$k = \frac{k_B T}{h} e^{-\Delta G^*/RT} = \frac{k_B T}{h} e^{-\Delta H^*/RT + \Delta S^*/R}$$

Assuming that ΔS^* does not vary with temperature this gives by taking logrithms and differentiating

$$\frac{d\ln k}{dT} = \frac{1}{T} + \frac{\Delta H^*}{RT^2} = \frac{\Delta H^* + RT}{RT^2}$$

This may be compared with the empirical Arhenius equation

$$\frac{d\ln k}{dT} = \frac{Ea}{RT^2} \quad \ldots \quad \ldots \quad 1.10$$

where Ea is called the activation energy. It will be noted that $Ea = \Delta H^* + RT$. As with equation 1.6, the most convenient method to obtain Ea is to plot log k versus 1/T. Equation 1.10 thus becomes

$$\frac{d\log k}{d(1/T)} = -\frac{Ea}{2.303R}$$

The slope of the plot will be -Ea/2.303R. It is important to note that this plot gives Ea and not ΔH^*. In practice, k the reaction velocity constant can be replaced by the maximum velocity

of reaction, V_{max}. This will merely affect the position, but not the slope of the line.

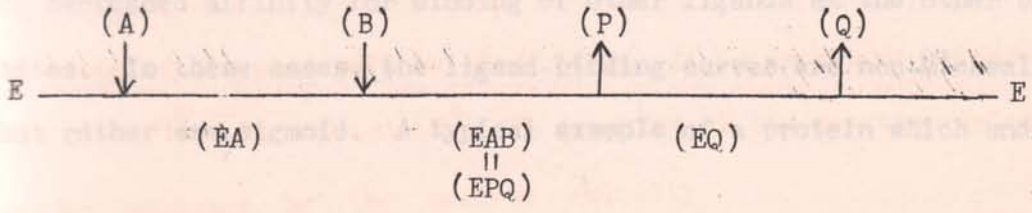
1.7.6. KINETIC MECHANISM OF G6PD ACTION

Results obtained from studies on the kinetic mechanism of glucose-6-phosphate dehydrogenase indicate an ordered, sequential mechanism in which $NADP^+$ is bound first and NADPH released last (102, 106, 156, 162, 163).

Kanji et al (162) in their kinetic studies on pig liver G6PD used initial velocity patterns to rule out a ping-pong mechanism, while product inhibition studies and the use of a competitive inhibitor ruled out a rapid equilibrium random mechanism with dead end complexes. The use of a competitive inhibitor further established the order of substrate binding with $NADP^+$ and glucose-6-phosphate being the first and second substrates respectively.

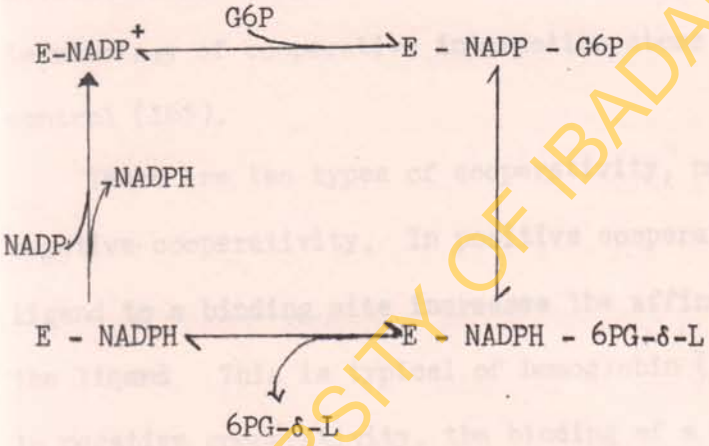
Product inhibition studies using the first product could also be used to rule out a Theorell-Chance mechanism, however, 6-phosphogluconolactone is highly unstable in aqueous solution (half life, 1.5s) (164).

In the notation of Cleland (148) the ordered Bi-Bi mechanism can be depicted as follows :



where E, A, B, P. Q represent enzyme, $NADP^+$, G6P, 6-PG-δ-L and NADPH respectively.

Because of the low dissociation constants for the G6PD-$NADP^+$, and G6PD-NADPH complexes however, and the fact that G6PD is stabilized by $NADP^+$ (83, 84, 96), all the reacting enzyme will be associated with $NADP^+$. Thus the reaction may be without any free enzyme being produced, by way of direct substitution of NADPH by $NADP^+$. The sequence of the reaction will thus be as follows :



1.8.1. COOPERATIVITY

Cooperativity is the process by which the binding of a ligand at a binding site, by an allosteric protein composed of subunits having interacting multiple ligand binding sites, results in an increased or decreased affinity for binding of other ligands at the other binding sites. In these cases, the ligand binding curves are non-Michealian but rather are sigmoid. A typical example of a protein which undergoes

this phenomena is hemoglobin which is composed of four similar polypeptide chains. Here the binding of the first molecule of oxygen to the hemoglobin results in an increased affinity for subsequent oxygen molecules to be bound, that is there is an increased affinity with increase in saturation. A similar cooperativity of substrate binding is found to occur with some enzymes leading to sigmoid plots of velocity against substrate concentration. These enzymes are usually the regulatory or control enzymes on metabolic pathways whose activities are subject to feedback inhibition or activation. The terminology of cooperative interaction stems from the studies on control (165).

There are two types of cooperativity, namely positive and negative cooperativity. In positive cooperativity the binding of the ligand to a binding site increases the affinity of the other sites for the ligand. This is typical of hemoglobin (166) and a host of enzymes. In negative cooperativity, the binding of a ligand to a binding site decreases the affinity of the other binding sites for the same ligand. This is typical of oligomeric enzymes composed of identical subunits e.g. tyrosyl-tRNA synthetase (167) and glyceraldehyde-3-phosphate dehydrogenase (168) from Bacillus Stearothermophilus.

Some theoretical models have been proposed to explain off cooperative phenomena. These are the Monod-Wyman-Changeux (MWC) concerted mechanism (169), the Koshland-Nemethy-Filmer (KNF) sequential mechanism (170), another which combines the two above (171) and an electrostatic model (172). In the MWC model, the existence of the protein

in either of two conformational states, T (= tense), the predominant form when unligated, and R (= relaxed) are assumed. The two states differ in the energies and numbers of bonds between the subunits so that the T state is constrained compared to the R state. The T state has a low affinity for ligands. Ligand first binds to the T state, resulting in the shift of the T-R equilibrium toward the R state, that with the higher affinity. The MWC model can be used to explain off positive but not negative cooperativity. The KNF sequential model assumes that the progress from T to the ligand -bound R state is a sequential process. The conformation of each subunit changes in turn as it binds ligand, and there is no dramatic switch from one state to another. The two assumptions in the KNF model are(a) in the absence of ligands the protein exists in one conformation, (b) upon binding, the ligand induces a conformational change in the subunit in which it is bound. This change may be transmitted to neighbouring vacant subunits via the subunit interfaces. This model embodies Koshlands earlier idea of "induced fit", which postulates that the binding of a substrate to an enzyme may cause conformation changes that align the catalytic groups in their correct orientations. In sacrificing simplicity, the KNF model is more general and is probably a better description of some proteins than is the MWC model. Negative cooperativity cannot be accounted for on the MWC model. The KNF model however accounts for it by the binding of ligand to one site causing a conformational change that is transmitted to a vacant subunit

(assumption (b) of the KNF model). The third model is the general model which combines both the KNF and MWC extremes, along with the dissociation of subunits (171). A fourth is the electrostratic model. Here it is assumed that the binding sites are in proximity and that the two ligands mutually interfere through electrostatic interactions (172).

The concerted two-state allosteric model proposed by Monod et al (169) is extended to expressions representing the initial velocity as a function of substrate concentration for two substrate enzyme systems. It is shown that when the two different conformational states have different affinities for both substrates, kinetic behaviour which is different from that expected from simple analogy to the single substrate case may be obtained. In a two substrate reaction, the importance of one substrate with respect to the allosteric kinetic behaviour of the other substrate has not been generally recognized in spite of the fact that the majority of allosteric enzymes involve two or more substrates. Thus there are at present only three treatments of the kinetics of two-substrate allosteric enzymes: that of Ainsworth (173) which is limited to the dimer case and to some limiting cases of the kinetic behaviour which arises from interactions between the substrates, that of Kirtley and Koshland (174), which is an extension of the sequential model of Koshland et al (170) for allosteric proteins, and that of Sumi and Ui (175) which is limited to enzymes with a ping-pong mechanism (148). There are at least

two reasons why the kinetic behaviour of two substrate allosteric enzymes have not been extensively examined. First, it is usually assumed that as long as the concentration of one substrate is maintained constant the two substrate case can be regarded as being analogous to the single substrate case. It has however been shown that the analogy holds only up to a point, beyond which the two substrate case shows substantial differences from the single substrate case (160). Secondly, while a general treatment is desirable, the mathematical expressions for such treatment become extremely complex, as is characteristic of all enzymatic reactions involving more than a single substrate. Thus a general system to describe allosteric kinetic behaviour would include consideration of a large number of intermediates reflecting cooperativity between sites or between substrates at the same or different sites. The resulting equations would almost be too cumbersome to handle and would give very little information about the kinetic behaviour to be expected for these systems.

The equation derived by Monod et al (169) to describe the binding of a single ligand, A, by an allosteric protein may be converted to the following expression for the velocity of a reaction catalysed by an allosteric enzyme (176) :

$$\frac{V_o}{nE_o} = \frac{K\alpha(1+\alpha)^{n-1} + K' LC\alpha(1+C\alpha)^{n-1}}{(1+\alpha)^n + L(1+C\alpha)^n} \quad \ldots\ldots \quad 1.11$$

Here V_o is the initial velocity, n is the number of substrate binding sites per molecule of enzyme, E_o is the total enzyme concentration, L is $(E)/(E')$, the constant which describes the equilibrium between two conformational states of the enzyme in the absence of substrate, K and K' are rate constants for product formation by the two conformational

states, C is equal to to K_A/K_A' and is the affinity ratio which expresses the difference in the affinities of the conformational states for substrate, and α is equal to $(A)/K_a$, the reduced substrate concentration. K_A and K_A' are thermodynamic dissociation constants describing the binding of A to each of the enzyme conformational states, E and E′, respectively. The derivation of this equation assumes the existence of a rapid, reversible equilibrium between two conformational states of an enzyme which have n independent sites for substrate. The particular derivation of the kinetic equation further assumes that the conformational states may differ in intrinsic catalytic activity and that the reaction velocity is proportional to the concentration of the enzyme substrate complex with all steps prior to formation of the enzyme - substrate complex being in rapid equilibrium (169, 176). Because the model of Monod et al (169) assumes the existence of an equilibrium between two conformational states which have different affinities for a ligand, the kinetic behaviour of two substrate allosteric enzymes may be divided into several classes depending on which of the conformational states has the greater affinity for which substrate. Here it is assumed for simplicity that the substrates Michealis constants are independent of the concentration of the other substrates.

Paulus et al (177) and Goldbeter (178) have analysed the single substrate case of the model of Monod et al (169) with respect to the occurrence of kinetic negative cooperativity. The kinetic negative cooperativity only occurs under the conditions where the conformational

state with the lower catalytic activity has the greater affinity for the substrate. Furthermore, the observed velocities may be much less than the maximum velocity of the reaction. Kinetic negative cooperativity has been observed in the extension of the Monod et al model (169) to the two substrate case (160). The velocities obtained are much less than the V_{max} of the reaction. Optimum conditions for the observation of kinetic negative cooperativity occurred when both enzyme conformational states have different affinities for both substrates. The occurrence of kinetic negative cooperativity is dependent on the concentration of the non-varied substrate in a manner analogous to the action of effectors in the single substrate case (178). The conclusions reached concerning the occurrence of kinetic negative cooperativity in the single substrate case of Monod et al (169) are thus apparently equally applicable to the extension of the model to the two substrate case (160).

In the case of the enzyme G6PD, the kinetic behaviour at variable $NADP^+$ concentration is always positively cooperative (88, 179, 180). Luzzatto (107) explained this kinetic behaviour as suggestive of low affinity for $NADP^+$ at low concentrations and the affinity increasing sharply as the concentration of this substrate is increased. This behaviour was explained in terms of an enzyme molecule bearing multiple binding sites for $NADP^+$, the binding of the first molecule of $NADP^+$ modifying the affinity of one or more other sites for this substrate. Luzzatto (107) in his evaluation of the kinetic

parameters used a simple quadratic model for the binding of two molecules of substrate by the enzyme molecule, with different dissociation constants thus :

$$E + S \rightleftharpoons ES_1 \qquad Ks_1 = (E)(S)/(ES_1)$$

$$ES_1 + S \rightleftharpoons ES_2 \qquad Ks_2 = (ES_1)(S)/(ES_2)$$

With $Ks_2 < Ks_1$. Then the velocity, V as a function of the substrate concentration, (S), would be given as in equation 1.5 :

$$v = \frac{(S)^2 \, V_{max}}{(S)^2 + (S)Ks_2 + Ks_1Ks_2}$$

In the reciprocal form we have :

$$\frac{V_{max}}{v} = 1 + \frac{Ks_2}{(S)} + \frac{Ks_1 Ks_2}{(S)^2} \quad .... \quad .... \quad 1.12$$

The dissociation constants could be deduced from this equation by inserting two pairs of values of V_{max}/v and (S), rewritten twice and solving the equation simultaneously.

At variable G6P concentration, a hyperbolic kinetic behaviour has been observed so far. Babalola et al (97) has however postulated cooperativity between the ionizable groups responsible for the binding of this substrate on the enzyme because of the type of variation of K_m with pH observed for variants A, A^- and B. Again it should be pointed out that the concentrations of G6P that have been used in these studies have been much less than or equal to 1mM (97, 137, 156). Thus it could also be possible that at higher concentrations,

cooperativity might be observed as is the case at variable $NADP^+$ concentrations.

For many proteins, it is convenient to express the ligand binding properties by the Hill's equation (181) :

$$\log (Y/(1-Y)) = n\log(S) - \log K \quad \ldots \quad \ldots \quad 1.13.$$

in which Y, the "fraction saturation" is the fraction of the total number of binding sites occupied by ligand., (S), is the free ligand concentration, and K and n are the dissociation constant of the protein-ligand complex, and the Hill coefficient respectively. The plot of $\log(Y/(1-Y))$ against $\log(S)$ is usually known as the Hills plot. From this, the degree of cooperation among substrate molecules can be conveniently ascertained from the slope, n, the Hills coefficient of the plot. This coefficient is used extensively as a measure of homotropic interactions between substrates in multisubunit enzymes. Whereas Michealian rate curves are characterized by a Hill coefficient of one, the sigmoid kinetics of regulatory enzymes generally yield numbers larger or lesser than unity. The Hill equation may be extended to kinetic measurements by replacing Y by v/V_{max} so as to give

$$\log(v/(V_{max}-v)) = n\log(S) - \log(K)$$

1.8.2. ALLOSTERIC EFFECTORS

Allostery implies a special type of activation or inhibition, and allosteric effectors therefore are the substances which are not substrate analogues and which are not apparently attached to the enzyme at the active sites to which the substrates bind. Allostery

does not provide an explanation for sigmoid kinetics as is often misconstrued in literature but it can affect the degree of substrate binding and therefore cooperativity. The effector therefore acts by inducing conformational changes which alter the activity at the catalytic site. The study of the interaction of G6PD with ions have been reported (83, 107, 111, 179). The aim might be to know the specific effects of these effectors on the association-dissociation equilibrium of the enzyme molecule and how these effects affect the regulatory functions of the enzyme. Kuby et al (180) has studied the effect of EDTA and $NADP^+$ on the macromolecular association phenomena in brewers yeast G6PD; while Cohen et al (92) has studied the influence of monovalent and divalent cations on human erythrocyte G6PD. Luzzatto (107) has also studied the influence of buffer ions and $MgSO_4$ on the Hills constant of $NADP^+$ binding to human erythrocyte G6PD enzyme.

1.9.1. AIM OF PRESENT WORK

The elucidation of the functional properties of enzymes for several decades has relied primarily on kinetic studies. More recently however, the knowledge of the structure of some proteins, such as lysozyme, has yielded insight into the mechanism of substrate binding and therefore of enzyme structure (182). For other proteins such as the quasi-enzyme, hemoglobin, a combination of structural, chemical and functional data have led to an understanding of important properties of the molecule such as cooperativity and the Bohr effect (183). Further

comparative analysis of various homologous molecular species which differ in very limited portions of the protein has shown that it is possible to attribute specific functional and reactivity differences to known structural differences. While this approach has been very fruitful with hemoglobins from different sources, it has not been fully explored in the case of human G6PD enzyme variants.

In West Africa, most especially, while comparative studies have been extensively reported for the common polymorphic variants, A, B and A^- (97, 137, 184), relatively little has been reported on the sporadic variants. Some of the few works on sporadic variants are those of Usanga et al (140), where characterization of new variants was carried out using column chromatography, Luzzatto et al (141) and Kahn et al (137).

In view of previous studies, which indicate that genetically different types of G6PD from human erythrocytes confer different metabolic properties on the red cell (138, 184), and that the different variants are due to amino acid substitution at the structural locus (69, 70), this work has been designed to investigate whether a comparative analysis of the kinetic and thermodynamic parameters of G6P binding to the Mould and B variants will help in the characterization of the Mould variant on one hand and in elucidating the differences in the molecular structure and catalytic functions between the Mould and the normal variant on the other hand. The Mould variant is a new sporadic G6PD variant which was detected in a 69 year old Nigerian

woman who was diagnonized for polycythaemia rubra vera (PRV) (142). This variant was found to be associated with a slow electrophoretic mobility and a slightly higher G6PD red blood cell activity than the normal B.

Although Babalola et al (97) had previously worked on the G6P binding for the three common polymorphic variants A,B and A⁻ his concentration range was between 0.2 to 1.0mM G6P, the normal concentration range utilized by most workers who have worked on the kinetics of G6P binding to the human erythrocyte G6PD. Lessman et al (47) working on the kinetics of G6P binding to the G6PD from Pseudomonas fluorescens, at a concentration range of 0 to 34mM observed a sigmoid type kinetics. Similarly, Senior et al (158) obtained sigmoid kinetics for the binding of G6P to the G6PD from Azotobacter beijerinckii. Kuby et al (159) in his studies on G6PD from brewers yeast using equilibrium dialysis method observed two classes of G6P binding sites: a tight binding site with K_D = 29uM and a weak binding site with K_D = 0.26mM. He also noted that in kinetic studies only the tigher binding site was observed. Thus in the work in this thesis, apart from the characterization of the new sporadic variant and the comparative studies between the new sporadic variant and B variant, the kinetics of G6P binding to human G6PD variants was studied at much higher concentrations than previous concentrations used in order to ascertain if the kinetics of G6P binding is Michealian at all concentrations, or only to a particular point for the human erythrocyte G6PD. Pettigrew et al (160) have shown that the assumption that the two substrate case can be regarded as being analogous to the single substrate case so long as the

concentration of one substrate is maintained constant, holds up to a particular point, beyond which the two substrate case shows substantial difference. Sigmoid type kinetics had been observed by some workers in the binding of $NADP^+$ to G6PD (106, 107, 180) in human erythrocytes.

It was initially thought that this occurrence was due to the interaction of the enzyme with the buffer used (102). However, it was later shown that the buffer used had no effect on the enzyme (97). Babalola et al (97) in his study of G6P binding also observed cooperative ionization of groups at the narrow pH range where there is the dissociation of tetramers to dimers.

The comparative kinetic and thermodynamic studies of G6P binding to the B and Mould variants were carried out on enzyme preparations purified using affinity chromatography. Other workers, for example, Babalola et al (97) who had previously determined the kinetics of G6P binding to the A, B and A^- erythrocyte G6PD types had purified their enzymes according to the techniques of Cohen et al (112), Chung et al (89), and Rattazzi (65). These techniques took as much as ten to sixteen days to yield a "purified" enzyme despite the general lability of enzymes from human sources (58). Babalola et al (97) in the study, obtained an enzyme preparation with a specific activity of about 120 units per milligram. In our purification of the B and Mould enzyme using the simpler, shorter and biospecific affinity method a specific activity of about 170 to 210u/mg was obtained. Thus in view of the previously observed discrepancies in

the kinetic and thermodynamic results for G6PD variants obtained, due to workers using different methods of purification on one hand and enzymes of varying purity on the other hand, it was decided in this study that the kinetic and thermodynamic parameters of the new sporadic Mould variant with the normal B enzyme be compared using enzyme obtained by the same purification procedure. The method of purification in this study was the combination of the modified form of the DeFlora et al (115) and the Craney et al (117) methods, which yielded a highly purified enzyme with a homogenous band by the criteria of polyacrylamide gel electrophoresis using the more sensitive photometric silver staining method.

The kinetic studies in this work were carried out in tris-borate and tris glycine buffers. Yoshida (102) had initially shown that borate ions inhibit the enzyme, and thus stated that kinetic studies in the borate system was unsuitable. However, consequent studies by Luzzatto (141) and Babalola et al (97) showed that the borate ions had no effect on the kinetics of G6PD. Levy et al (185) have shown that borate ions interact with NAD^+ but not $NADP^+$ when used as a buffer in the reactions of G6PD from Leuconostoc mesenteroides. The tris-glycine has not been found to have any effect on the kinetics of G6PD. Using the tris borate and tris glycine buffers, the dependence of K_m and V_{max} on pH and temperature were determined. From these, enthalpy change and activation energies were calculated at each pH. These determinations were then used to show the differences in structure of the G6PD variants in a better perspective just like electrophoretic studies (128, 129).

The solvent is an important functional component of macromolecules. However, despite the extensive accumulation of experimental data and theory (186-188), the role has remained poorly understood. From structural, energetic and dynamic points of view, description of the interactions between the protein and surrounding solvent molecules are still primarily qualitative. This is not to say that the general area of protein hydration and/or solvation has been ignored. Rather it is the fundamental aspects of protein solvent interaction that remain essentially uncharacterized. The effect of changes in water activity on the equilibrium between a protein and the water is of functional relevance. However, this effect could be obscured by the effect of ionic species in solution as is the case of hemoglobin in which its oxygen binding is normally obscured by allosteric effects of ionic species in solution (189). Thus a potentially more useful approach toward probing the energetic dynamic and structural aspects of the interaction between a protein and its aqueous enviroment is to partially replace water with a non-aqueous solvent in which the protein remains functional. Investigations of proteins in mixed aqueous solvents are potentially useful in the study of many aspects of enzyme chemistry. From a kinetic point of view, such systems allow continuous variation in the reaction medium for the study of reaction mechanism (190) and forces involved in enzymatic reactions (191, 192). Our effort in the investigation of behaviour of G6PD in mixed aqueous solvent (water-glycerol system) has been directed at the correlation

of physical properties of mixed aqueous solvent systems with changes in kinetic constants. Put in another way it can be said that this study, in water-glycerol binary system is intended to test the hypothesis that individual water molecules play an integral part in the function of the proteins and that functional parameters can be influenced by change in bulk water activity. This work was carried out in tris-glycine buffer pH 9.00.

At temperatures of about 50°C and above, the properties of water are those characteristic of a normal liquid (193). However, at lower temperatures, its physical properties and thermodynamic response functions become anomalous. All these irregularities are accompanied by anomalies in the temperature dependence of most transport properties and relaxation times (194). Since protein-solvent interactions could be influenced by alterations in the properties of the solvent, the anomalous properties of water could thus have some effects on protein properties. Hence for water and deuterium oxide (heavy water) at around 4°C and 11°C respectively the temperatures of maximum density for these solvents, where fluctuations in internal energy with volume become negative, it will be expected that the variations in protein, and thus enzyme properties will become affected. In this work therefore, it has been decided that the kinetic and thermodynamic properties of G6PD in water and deuterium oxide at temperatures around their maximum densities, that is, about 8°C - 0°C for water, and 14°C - 3°C for deuterium oxide be determined.

These studies were carried out at pH/pD 6.55/6.95 and 9.00/9.40 in tris-borate and tris glycine buffers respectively. From the results obtained, it will then be deduced if the variations in solvent properties have direct effect on solvent-protein interactions.

CHAPTER TWO

EXPERIMENTAL

2.1.1. REAGENTS:

Nicotinamide adenine dinucleotide phosphate, disodum salt ($NADP^+$), D-glucose-6-phosphate, monosodium salt, trizma base · glycine, phenazine methosulphate, nitroblue tetrazolium, lysozyme (Grade 1), 2N phenol reagent, ε-amino caproic acid were obtained from Sigma Chemical (St. Louis, Montana), CM-Sephadex-C50, 2′, 5′ ADP Sepharose 4β were obtained from Pharmacia Fine Chemicals Inc. (Uppsala, Sweden), acrylamide, bisacrylamide, N, N, N, N-tetramethylethylenediamine (TEMED) and ammonium persulphate were obtained from Eastman (U.S.A.), Bio-Gel P-150 was obtained from Bio-Rad Laboratories (Richmond, California), Conaught hydrolysed starch was obtained from Conaught Laboratories (Canada), Titan III Cellulose acetate plates, Chamber wicks, Supraheme buffers were obtained from Helena Laboratories (Beaumont, Texas), methanol, ethanol, Formalin were obtained from Fisher Scientific Co. (U.S.A.), HCl, HNO_3, $AgNO_3$, K_2Cr_2O, Na_2CO_3, NaH_2PO_4, K_2HPO_4, Na_2HPO_4, KH_2PO_4, NaCl, KCl, β-mercaptoethanol, NaOH, KOH, NaAc and all other reagents used were all of analytical grades from the British Drug Houses Ltd (Poole, England).

2.1.2. INSTRUMENTS.

Optical density measurements were made with CARY 219 Spectrophotometer with an in-built recorder, and a constant temperature cell compartment. PYE UNICAM Model 290 MK 2 pH meter was used in the measurment of pH, a refrigerated MSE High speed 21 centrifuge was used for centrifugation: Ultra filtration membranes (PM 30) and cell (50 ml) from Amicon (Lexington, Mass) were used for concentration, Agla micrometer syringes and gilson pipetmen were used for the measurement of volumes while lambda pipettes were used in the measurement of enzyme volumes.

Other instruments included a fraction collector, peristaltic pump and K16/20 column from Pharmacia, high precision digital thermometer, quartz micro and macro cuvettes, dialysis tubings and others.

2.1.3. B U F F E R S :

1. SODIUM PHOSPHATE BUFFER, pH 6.0

   200cm^3 of 0.1M NaOH

   800cm^3 of 0.1M NaH_2PO_4

2. POTASSIUM ACETATE BUFFER, pH 5.8

   350cm^3 of 0.1M Acetic acid

   380$_{cm}{}^3$ of 0.1M KOH

3. SEPHAROSE BUFFERS

   (a) BUFFER A, pH 6.1

   0.1M Potassium Acetate

   0.1M Potassium Phosphate

(b) BUFFER B, pH 7.85

0.1M Potassium Acetate

0.1M Potassium Phosphate

(c) BUFFER C, pH 7.85

0.1M Potassium Chloride

0.1M Potassium Phosphate.

All the above buffers contain 0.2% mercaptoethanol and 1mM EDTA. pH is adjusted with either 1M KOH or 1M CH_3COOH.

4. SEPHADEX BUFFER

SODIUM ACETATE BUFFER, pH 6.25

0.05M Sodium Acetate (6.804g in 1 dm^3).

1.0M Acetic acid (used to make up to desired pH)

20 uM $NADP^+$

0.1mM EDTA

0.2% (v/v) β-mercaptoethanol

All were made up to a total volume of 1 dm^3.

5. BIO-GEL P-150 BUFFER

SODIUM PHOSPHATE BUFFER pH 6.25

0.02M Sodium dihydrogen phosphate

10 μM $NADP^+$

0.1mM EDTA

0.1% (v/v) β-mercaptoethanol

Sodium phosphate (used to make up to desired pH)

All were made up to a total volume of 1 dm^3.

2.1.4. IDENTIFICATION OF G-6-PD

This was performed by either starch gel electrophoresis according to the methods of Smithies and Boyer et al (128, 129), or by cellulose acetate electrophoresis according to the methods of Sparkes et al and Adamson et al (195; 196).

For the starch gel electrophoresis the concentration of $NADP^+$ in the starch gel was 12.5 µM and a voltage of 200V and current of 10mA was applied for fifteen hours. The buffer used was tris-borate-EDTA buffer pH 8.6.

The cellulose acetate electrophoresis was performed using the Helena electrophoresis kit. The cellulose acetate strip used was the Titan III type (Helena lab, 3023). Buffer used was the "Supra heme buffer" containing EDTA, boric acid and tris at pH 8.4. A current of 10mA at 350V was applied for fiteen minutes.

Staining for both methods was by the same method. The starch gel/cellulose acetate strip were stained under incubation in a staining solution of 1M tris-HCl buffer pH 8.6 containing $NADP^+$, G6P, nitroblue tetrazolium, phenazine methosulphate and magnesium sulphate. After staining the gel/strip, the G6PD variants were recognized by visual inspection.

2.1.5. A S S A Y:

G6PD was routinely assayed for spectrophotometrically using CARY 219. The assay mixture contained :

0.18 cm^3	WHOmix solution
0.02 cm^3	5mM $NADP^+$
0.09 cm^3	Water
0.01 cm^3	Enzyme

giving a final concentration of 0.333mM $NADP^+$, 4mM G6P, 0.01M $MgCl_2$ and 0.1M tris-HCl. The WHOmix solution consists of 5 cm^3 of 1M tris-HCl buffer pH 8.0, 0.5 cm^3 1.0M $MgCl_2$, 2.5cm^3 of 0.08M G6P and 22cm^3 of water. The reaction was followed at 340 nm at 25 ± 0.1°C. The formation of NADPH produced a linear increase in absorbance readings for periods of three minutes or more All activities were recorded as change in absorbance per minute and later converted to concentrations per minute using an extinction coeffificent of 6.22 $mM^{-1}cm^{-1}$.

$$\text{Activity (I.U units)} = \frac{\Delta OD_{340}/\text{min}}{6.22 \times (\text{enz.vol})/(cm^3 \text{of reaction mixt})}$$

A unit of activity of G6PD is the amount of enzyme which catalyses the reduction of one micromole of $NADP^+$ per minute at 25°C (147).

2.1.6. PROTEIN DETERMINATION

This was determined using the Folin Cocaltuea assay with conditions and reagents as described by Clark and Switzer (197; 198), with lysozyme as a standard.

The quantitative Folin Gocaltuea (Lowry) test (198) is applied

to proteins in solution, and even dried materials The method is also sensitive: samples containing as little as 5 µg of protein can be analyzed readily. The colour formed by the Folin-Ciocalteu reagent is caused by the reaction of protein with the alkaline copper in the reagent (as in the biuret test) and the reduction of the phosphomolybdate-phosphotungstate salts in the reagent by the tyrosine and tryptophan of proteins.

Many substances have now been however found to interfere with the determination of protein (personal observations, 199), when using the method of Lowry et al (198) and its modifications (197). β-mercaptoethanol and others are some of interfering compounds. Since the purification of G6PD involves the use of β-mercaptoethanol (used for the prevention of the oxidation of sulfhydryl groups in the enzyme), it therefore means that there will be interferences in protein determination using this method. This problem was however overcome by dialysing out the β-mercaptoethanol before protein determination.

2.1.7. PURITY CRITERIA·

This was performed using SDS polyacrylamide gel electrophoresis. The overall approach was that of Fairbanks (200) with several modifications.

Gel is prepared by polymerizing acrylamide using N,N -methylene - bisacrylamide as crosslinkers, ammonium persulfate as free radical source (initiates polymerisation), TEMED as catalyst.

Stock solutions and buffers were prepared and mixed as below

BUFFER A, pH 8.8

48 cm^3 of 1M HCl

36.6g tris base

0.23 cm^3 TEMED

Water to 100 cm^3

BUFFER B, pH 6.8

9.35 cm^3 of 1M HCl

1.2g tris base

0.1 cm^3 TEMED

Water to 20 cm^3

BUFFER C, pH 8.5 (x 10)

6g tris base

28.8g glycine

Water up to 1 dm^3

SOLUTION D

20% SDS

SOLUTION E

40g acrylamide

1.5g bis acrylamide

Water up to 100 cm^3.

SOLUTION F

1.5% ammonium persulfate

The composition of the gels is as below :

RUNNING GEL (7.5%)

Buffer A	4 cm^3
Solution E	6 cm^3
Water	6 cm^3
Solution F	16 cm^3
Solution D	0.16 cm^3

STACKING GEL

Buffer B	2.00 cm^3
Solution E	1.20 cm^3
Water	4.80 cm^3
Solution F	8.00 cm^3
Solution D	0.08 cm^3

Solution F in both cases was always added last since it initiated polymerisation.

Suspensions of protein were prepared for electrolysis by adding the following to the stated final concentration: 1% SDS, 10% glycerin, 10mM tris-HCl buffer pH 8.0, 1mM EDTA, 5% β-mercaptoethanol and 10 µg/ml bromophenol blue (tracking dye). Protein was denatured by boiling the protein suspension, and then applied on polymerized gel. Electrophoresis was then carried out by applying a current of 5 - 10mA with a voltage gradient of 7-8V/cm for four hours.

Gels were stained using the photochemically derived silver stain instead of Coomasie blue stain, by the method of Merril et al (201).

This method could be used to detect as little as 0.01 nanogram of protein per square millimeter; and a hundredfold increase in sensitivity was achieved over coomasie blue stain (201). Proteins were fixed in a solution of 50 percent methanol and 12 percent acetic acid for a minimum of twenty minutes, and excess SDS was removed from the gels by three 200 cm^3, 10 minutes rinses of a solution containing 10 percent ethanol and 5 per cent acetic acid. Gels were then soaked for five minutes in a 200 cm^3 solution of 0.0034M potassium dichromate and 0.0034M nitric acid. They were washed four times, for 30 seconds in 200 cm^3 of deionized water and placed in 200 cm^3 of 0.012M silver nitrate for 30 minutes. This was followed by rapid rinsing with two 300 cm^3 portions of the image developer solution containing 0.28M sodium carbonate and 0.5 cm^3 of commercial formalin per litre. The gels were gently agitated in a third portion of this solution until the image had reached the desired intensity. Development was stopped by discarding the developer and adding 100 cm^3 of one percent acetic acid. The gels were washed twice in 200 cm^3 of water before storage. Maximum sensitivity was achieved when the gel was exposed to relatively intense uniform light during the first five minutes in silver nitrate. A fluorescent light source of uniform intensity gave the best results.

2.2.1. PURIFICATION PROCEDURE

The method used in this work is that of Adediran (180), basically a modification of that of DeFlora et al (115, 116). Here the hemolysate is loaded direct on the 2',5'-ADP Sepharose 4B affinity column, eluted after washing the column, concentrated, dialysed, and then passed on the CM-Sephadex C-50 column, where it is eluted using a salt gradient. A test of homogeniety using the silver stain (201), instead of the coomasie blue stain normally used however showed more than a single band. Hence the inclusion of a third chromatographic step, the gel filtration step using Bio-gel P-150, as used by Craney et al (117). With this, there was homogeneity using the silver stain.

Blood for the purification was obtained from University College Hospital, Ibadan in the case of the Mould enzyme, and from the University of Minnesota, Minneapolis in the case of the B variant.

L Y S I N G

Blood was washed three times with normal saline (0.85% NaCl) so as to remove the plasma. The red blood cells were hemolysed with four volumes of water, and the stromas were eliminated by centrifugation at 15000g for twenty minutes. Hemolysate was assayed for activity after which the following were added

1. 1 in 40 sodium phosphate buffer, pH 6.0
2. 1 in 40 potassium acetate buffer, pH 5.8
3. 0.2% β-mercaptoethanol (v/v)

4. 1mM EDTA

The pH of the mixture was adjusted to pH 6.1 using 1M acetic acid.

2',5' ADP SEPHAROSE 4B COLUMN

4gm of freeze dried 2',5' ADP Sepharose 4B resin was reconstituted by swelling in phosphate buffer (0.1M, pH 7.0), and washing in the same buffer (100 cm^3 per gram of dry powder) so as to remove the materials added prior to freeze drying. It was then packed into a K16/20 Pharmacia column with thermostated jacket, and connected to a peristaltic pump for the control of flow rate. Column was then equilibrated by passing 100cm^3 of Sepharose Buffer A at a flow rate of 70cm^3 per hour.

Hemolysate preparation was loaded onto the column so as to bind. Effluent was then collected and tested for activity. If effluent has activity it signifies that the enzyme did not bind well. This is however dependent on the degree of activity got. If it has no activity it is discarded.

The column was washed with 200cm^3 of buffer A, 200cm^3 of buffer B, and with buffer C (about 800cm^3) until the absorbance reading at 280nm was zero. Elution was achieved by applying to the column 100cm^3 of buffer C containing 50μM $NADP^+$ at a flow rate of 50cm^3 per hour. Elution was achieved by the change in the concentration of the $NADP^+$ originally present in the column. This change in concentration disturbs the original $NADP^+$-enzyme complex, consequently resulting in the elution of the enzyme.

Twenty five tubes containing 4.5 cm^3 each of eluate were collected with the aid of a fraction collector. Those containing about 90 percent of the enzyme were mixed, concentrated with amicon ultrafilter under nitrogen and then dialysed in 500 cm^3 of sodium acetate buffer pH 6.25 containing $NADP^+$, EDTA and β-mercaptoethanol.

CM SEPHADEX C-50 COLUMN

The Sephadex resin was swollen in 0.05M sodium acetate buffer pH 6.25 for two days and equilibrated in the same buffer containing 30uM $NADP^+$, 1mM EDTA and 0.2% β-mercaptoethanol (Sephadex buffer). The resin was then packed into a column of 2.5 x 14.0cm.

Enzyme preparation previously dialyzed was loaded into the column. It was then washed with the sephadex buffer, and then eluted pulsewise with the same buffer containing 0.1, 0.2 and 0.3 M NaCl respectively. About 25 tubes of 3.6 cm^3 each of eluate was collected. Tubes containing the highest activities were pooled together, concentrated and dialyzed in 0.02M sodium phosphate buffer pH 6.25 containing 1mM EDTA, 0.1% β-mercaptoethanol and 10^{-5}M $NADP^+$ (Bio-gel P-150 buffer).

BIO-GEL P-150 COLUMN

Bio-Gel P-150 resin was swollen in 0.02M sodium phosphate buffer pH 6.25 for a day, packed into a 250cm^3 column (2.5 x 56.0cm), and equilibrated in the column with the same buffer.

Dialysate from Sephadex column was then packed on column, and then eluted with the Bio-Gel P-150 buffer. 30 tubes of about 2 8cm^3 each

were collected with the aid of a fraction collector. Fractions showing significant G6PD activity were combined, concentrated to about 10cm^3 by ultrafiltration and stored at 4°C until ready for use.

Enzyme activity and protein concentration were determined as earlier described in all the steps, while protein purity was also determined.

All experiments were carried out at 4°C in the cold room, except assay at 25°C.

2.3.1. THERMOSTABILITY STUDIES:

This was performed as previously described (96) for the Mould variant The enzyme preparation was dialysed before use for three hours against two changes of 500 volumes of 20mM potassium phosphate buffer pH 7.0 containing 0.2M KCl, 0.1mM EDTA and 10μM $NADP^+$, and then for two hours against two changes of 500 volumes of a buffer of the same composition, except that the $NADP^+$ concentration was only 1nM. After dialysis, aliquots of the enzyme solution were adjusted to the desired $NADP^+$ concentration (50μM $NADP^+$ for this case), then incubated at the desired temperature for seven minutes, chilled on ice, and assayed in a CARY 219 spectrophotometer. The composition of the assay mixture is as previously described except that Mg^{2+} was omitted.

2.4.1. KINETIC MEASUREMENTS:

Data from previous work by Luzzatto et al (141) and Babalola et al (97) have shown that most of the buffers used in the study of G6PD have

effects on either the K_m or V_{max}. It was established that borate, and tris had no effect on either of these two parameters and these reagents were therefore used to prepare all the buffers from pH 5.85 to 8.50. That for pH 9.00 was prepared from tris and glycine. Regarding ionic strength, there is evidence from other investigators (111) that this can affect substrate binding. Furthermore, it is known that the molecular aggregation state of G6PD is affected by ionic strength (84, 111). It was therefore necessary to ensure that determinations at all pH values were carried out at the same ionic strength, which is 0.01.

For this work, tris-borate buffers were prepared making use of the method of Babalola et al (97). 0.01M tris glycine buffer however has the composition as in table 2.1.

TABLE 2.1

COMPOSITION OF BUFFERS USED FOR KINETIC DETERMINATIONS (I = 0.01)

MOLARITY OF BORIC ACID	MOLARITY OF TRIS	MOLARITY OF GLYCINE	APPROXIMATE pH
0.790	0.033		5.85
0.720	0.041		6.00
0.650	0.048		6.30
0.590	0.055		6.50
0.530	0.062		6.75
0.490	0.073		7.00
0.360	0.107		7.50
0.240	0.139		8.00
0.094	0.164		8.50
	0.0086	0.010	9.00

All reaction velocity measurements were carried out with a CARY 219 recording spectrophotometer with thermostated cell compartments. PYE UNICAM Model 290 MK 2 pH meter was used in measuring the pH of the reaction mixture, before and after a particular run. All volumes were measured with Gilson pipetmen and Agla micrometer syringes.

The enzyme used for these measurements were those prepared as described earlier. The final preparation was however dialysed in the appropriate buffer to be used for a particular run, before it was used. For each run, 0.01 cm^3 of enzyme, already diluted to give a change in absorbance of approximately 0.075 ΔOD_{340}/min was used.

A typical composition of the reaction mixture for a set of runs can be seen in table 2.2.

TABLE 2.2

REACTION MIXTURE COMPOSITION AT VARIABLE G6P CONCENTRATION

X(cm^3)	G6P (mM)									
	3.00	2.50	2.00	1.50	1.00	0.50	0.20	0.15	0.12	0.10
Buffer pH X	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
G-6-P	0.199	0.167	0.133	0.100	0.067	0.333*	0.133*	0.100*	0.080*	0.067*
$NADP^+$	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Water	0.711	0.743	0.777	0.810	0.843	0.577	0.777	0.810	0.830	0.843
Enzyme	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Concentration of G6P = 0.06M

$G6P^*$ = 0.006M

$NADP^+$ = 5.00mM.

For Mould G6PD, X = 5.85, 6.00, 6.50, 6.75, 7.00, 7.50, 8.00, 8.50, 9.00

For the B G6PD, X = 6.00, 6.30, 6.50, 6.75, 7.00, 7.50, 8.00, 8.50, 9.00

For each set of pHs, experiments were performed at 20°, 27° and 34°C.

CHAPTER THREE

EXPERIMENTAL RESULTS

3.1.1. PURIFICATION OF B AND MOULD G6PD VARIANTS USING AFFINITY CHROMATOGRAPHY METHOD

The electrophoretic mobility of the Mould G6PD, a new sporadic variant was determined before the purification. Plate 3.1 shows the result of the electrophoresis on starch gel after staining as described in experimental. From this it can be seen that the Mould G6PD migrated less than the normal B variant.

The patterns of elution and separation from the proteins of the B and Mould G6PD variants from the various chromatographic columns are shown in figures 3.1 and 3.2.

Table 3.1 (a & b) summarize the purification steps of the two variants by the affinity method. These tables show that the final specific activities of both preparations are 174 units per mg protein for the B enzyme, and 206.7 units/mg protein for the Mould enzyme. The yield is 6.95 percent for the B enzyme while it is 7.13 percent for the Mould enzyme.

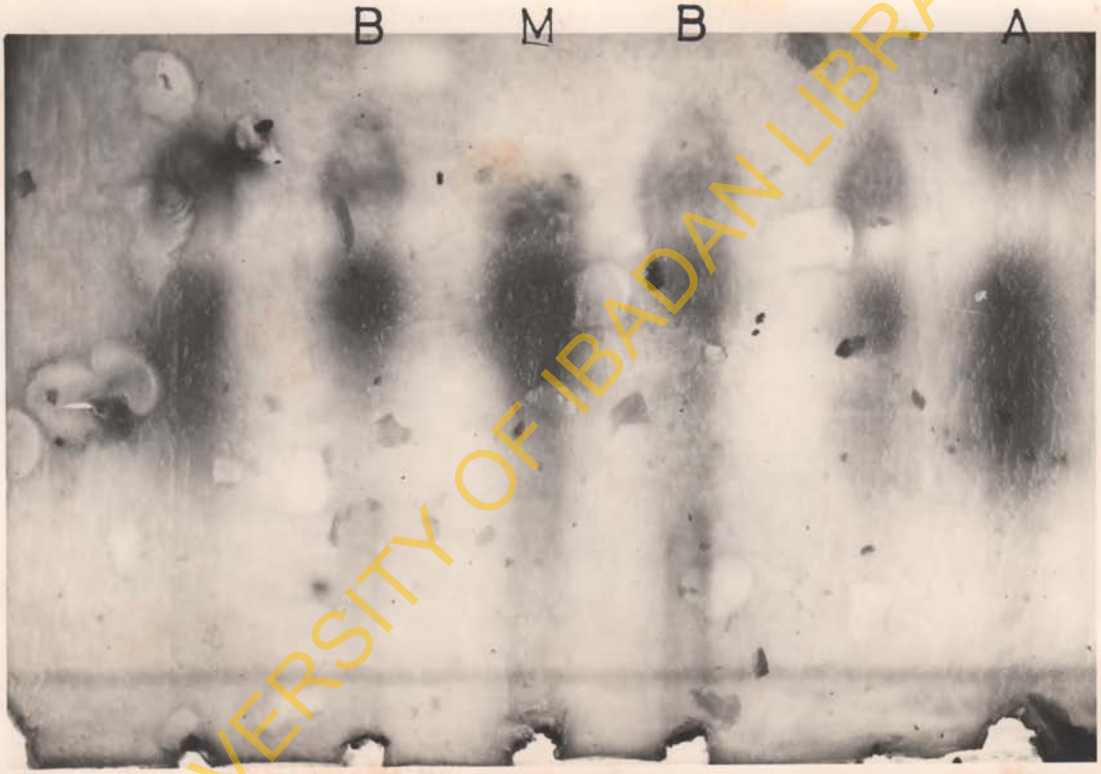


Plate 3.1 : Identification of G6PD enzyme types using starch gel electrophoresis. A represents the G6PD A; B, the normal G6PD B; and M, the new 'Mould" G6PD.

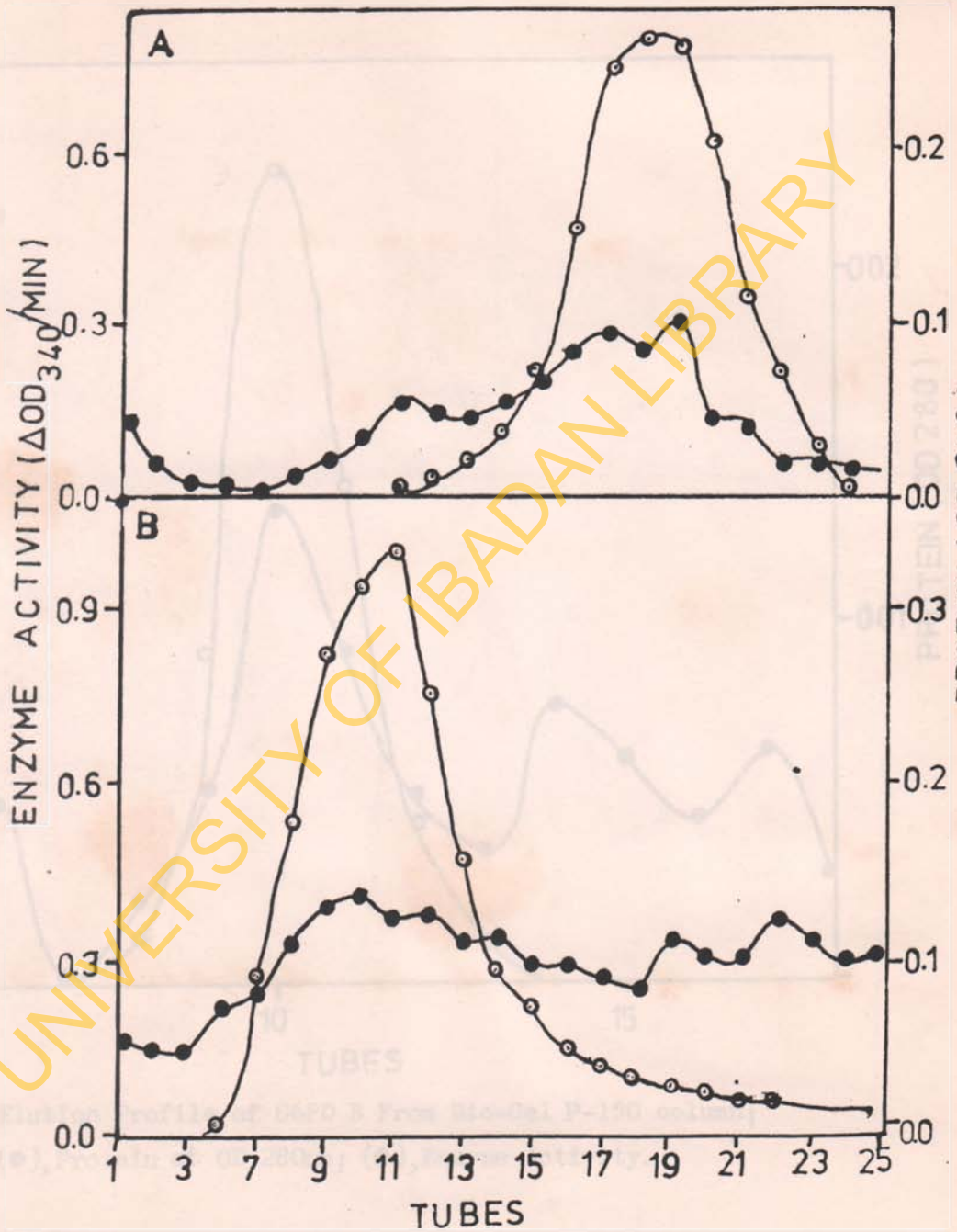


Fig. 3.1(a): Elution Profile of G6PD B from two Chromatographic Columns. (●) Protein at OD280; (⊙) Enzyme Activity.
(a) CM-Sephadex C-50 column.
(b) 2′, 5′ A D P Sepharose 4β Column.

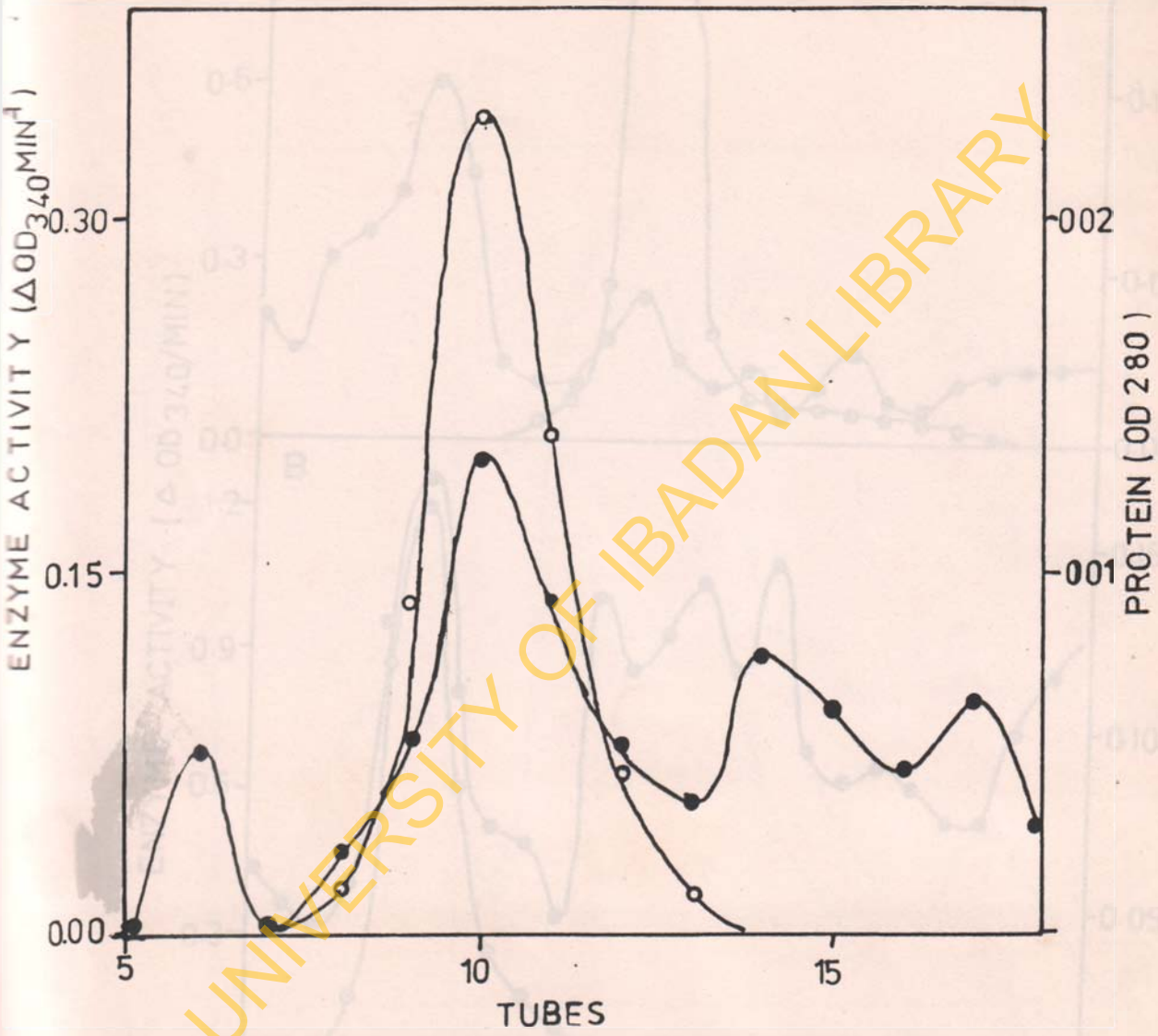


Fig. 3.1(b): Elution Profile of G6PD B From Bio-Gel P-150 column; (●), Protein at OD 280nm; (○), Enzyme Activity.

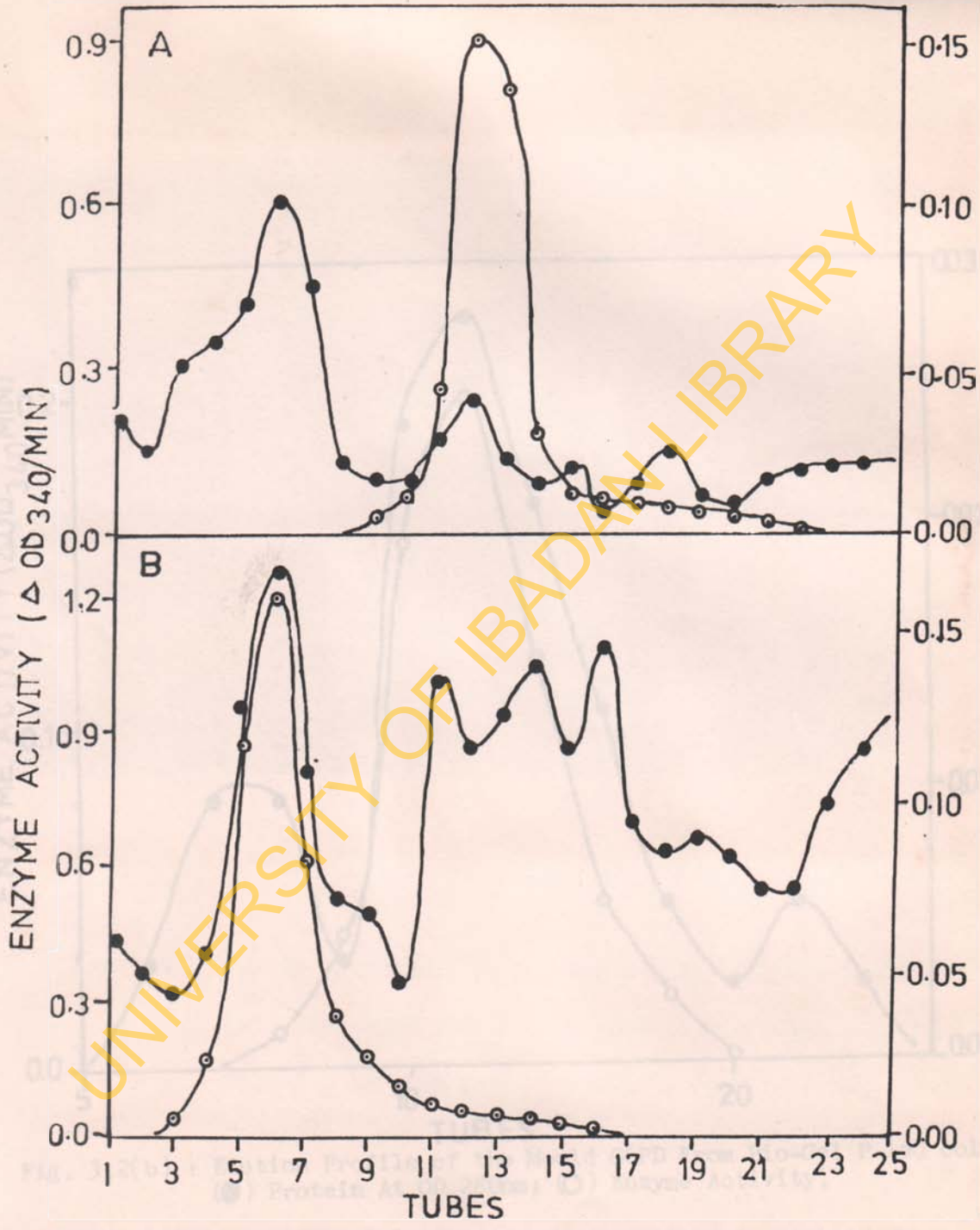


Fig. 3.2(a): Elution Profile of the Mould G6PD From Two Chromatographic Columns. (●), Protein At OD 280; (⊙), Enzyme Activity.
(a) CM-Sephadex C-50 Column.
(b) 2′, 5′ - A D P Sepharose 4B Column.

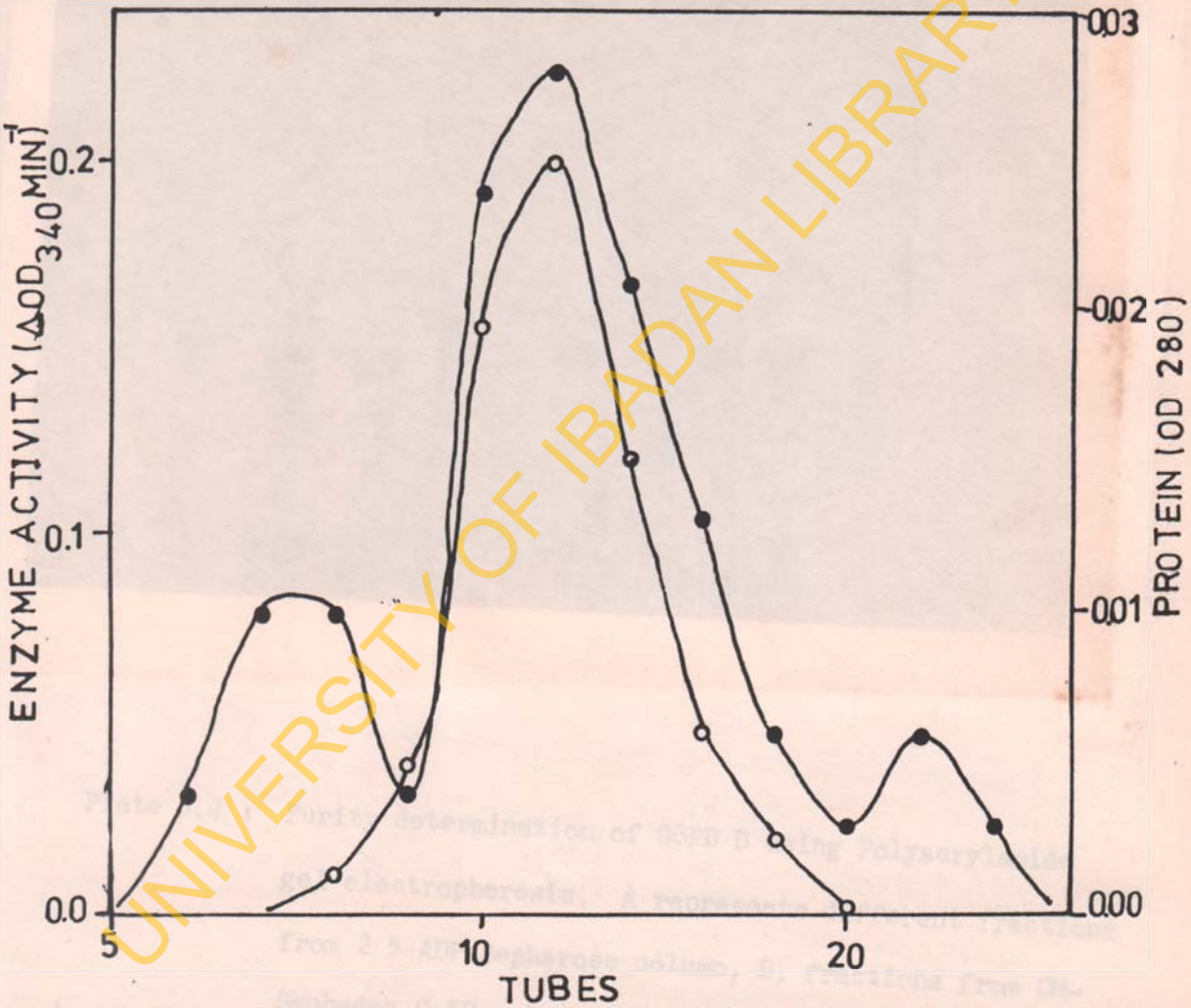


Fig. 3.2(b) : Elution Profile of the Mould G6PD From Bio-Gel P-150 column; (●) Protein At OD 280nm; (○) Enzyme Activity.

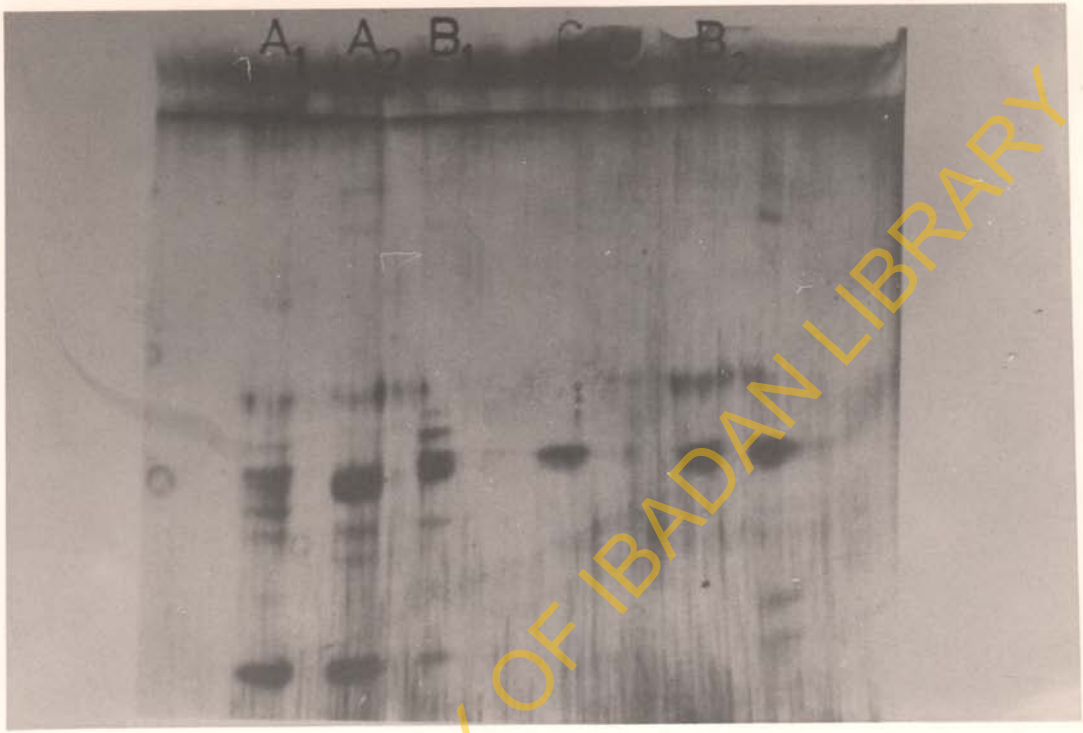


Plate 3.2 : Purity determination of G6PD B using Polyacrylamide gel electrophoresis. A represents different fractions from 2 5 ADP Sepharose column, B, fractions from CM-Sephadex C-50 column, and C, fraction from Bio-Gel P150 column.

TABLE 3.1 (a)

G6PD B PURIFICATION TABLE

STEP	ACTIVITY (UNITS/ CM^3)	TOTAL UNITS	PROTEIN (MG/CM^3)	SPECIFIC ACTIVITY (UNITS/ MG)	YIELD (PERCENT)	PURI-FICATION FACTOR
HEMOLY-SATE	0.47	260.00	41.360	0.011	100.00	1
IX-SEPHA-ROSE	4.43	80.12	0.074	59.860	30.80	5219
EX-SEPHADEX	3.91	48.20	0.036	108.610	18.54	9527
EX-BIO-GEL P-150	1.74	18.04	0.010	174.000	6.95	15263

TABLE 3.1 (b)

MOULD G6PD PURIFICATION TABLE

STEP	ACTIVITY (UNITS/ CM^3)	TOTAL UNITS	PROTEIN (MG/CM^3)	SPECIFIC ACTIVITY (UNITS/ MG)	YIELD (PERCENT)	PURI-FICATION FACTOR
HEMOLY-SATE	0.53	291.50	34.060	0.016	100.00	1
EX-SEPHA-ROSE	4.77	105.34	0.083	57.470	36.67	3684
EX-SEPHA-DEX	3.62	63.69	0.048	75.360	21.86	4831
EX-BIO-GEL P-150	1.45	20.83	0.007	206.700	7.13	13250

The G6PD preparation using this procedure gave a single band in SDS polyacrylamide gel electrophoresis as can be seen in plate 3.2. The temperature inactivation profile for the Mould G6PD at 50μM $NADP^+$ is shown on figure 3.3. The transition temperature obtained is 47.5°C.

The purified G6PDs were usually obtained within two to three days of the start of the isolation procedure.

3.2.1. DEPENDENCE OF V_{max} ON pH AND TEMPERATURE

It has been previously pointed out that when the Lineweaver-Burk plot type (150) is made use of in kinetic data analysis, data points are squeezed together as concentration tends to infinity, while the Dixon (151) type plot squeezes the data points as concentration tends to zero. Wilkinson (154) also pointed out that the Eadie plots which are somewhat more reliable when it comes to fitting parameters also squeeze the data about as badly athough in a different form.

Thus in this study, estimation of the kinetic parameters, V_{max} and K_m, was done using both the Lineweaver-Burk and Dixon plots for the same sets of points, and then determining the average of pairs of values obtained. Estimations of kinetic parameters and their standard errors were obtained using the least square curve fit computer program, LSFITW (See appendix 1).

The Lineweaver-Burk and Dixon type plots at the various pH values at a constant ionic strength of 0.01M and at different temperatures for the enzyme variants investigated gave non-Michealian type plots. Two

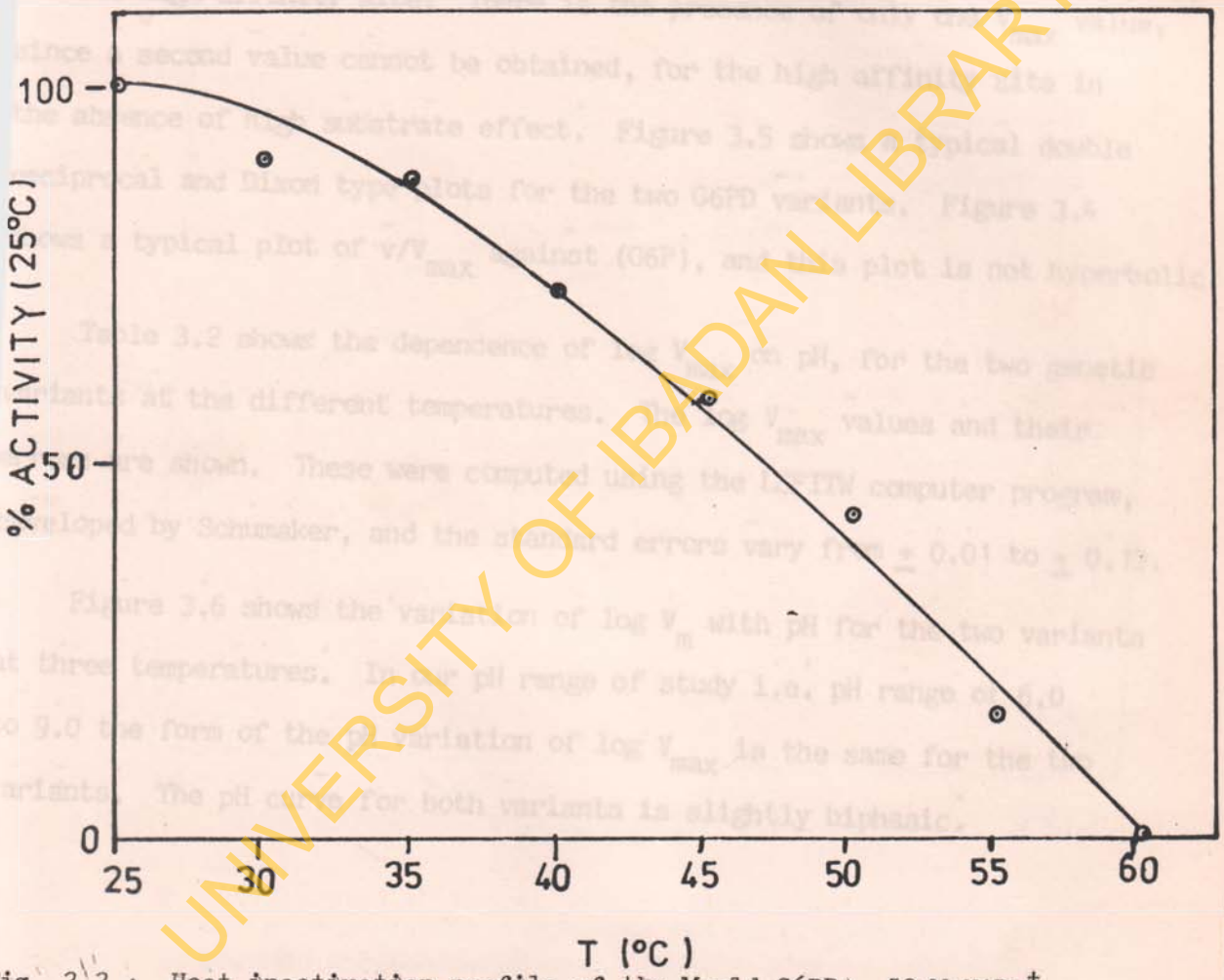


Fig. 3.3.: Heat inactivation profile of the Mould G6PD; 50μM $NADP^+$, No $MgCl_2$.

straight lines were obtained showing the presence of cooperativity in the binding of G6P to the enzyme variants at the low ionic strength of 0.01M and at the concentration range used. Thus for each pH value, we obtained two K_m values, log $K_{m_1}^{G6P}$, for the low affinity site and log $K_{m_2}^{G6P}$, for the high affinity site. There is the presence of only one V_{max} value, since a second value cannot be obtained, for the high affinity site in the absence of high substrate effect. Figure 3.5 shows a typical double reciprocal and Dixon type plots for the two G6PD variants. Figure 3.4 shows a typical plot of v/V_{max} against (G6P), and this plot is not hyperbolic.

Table 3.2 shows the dependence of log V_{max} on pH, for the two genetic variants at the different temperatures. The log V_{max} values and their errors are shown. These were computed using the LSFITW computer program, developed by Schumaker, and the standard errors vary from $\pm$ 0.01 to $\pm$ 0.13.

Figure 3.6 shows the variation of log V_m with pH for the two variants at three temperatures. In our pH range of study i.e. pH range of 6.0 to 9.0 the form of the pH variation of log V_{max} is the same for the two variants. The pH curve for both variants is slightly biphasic.

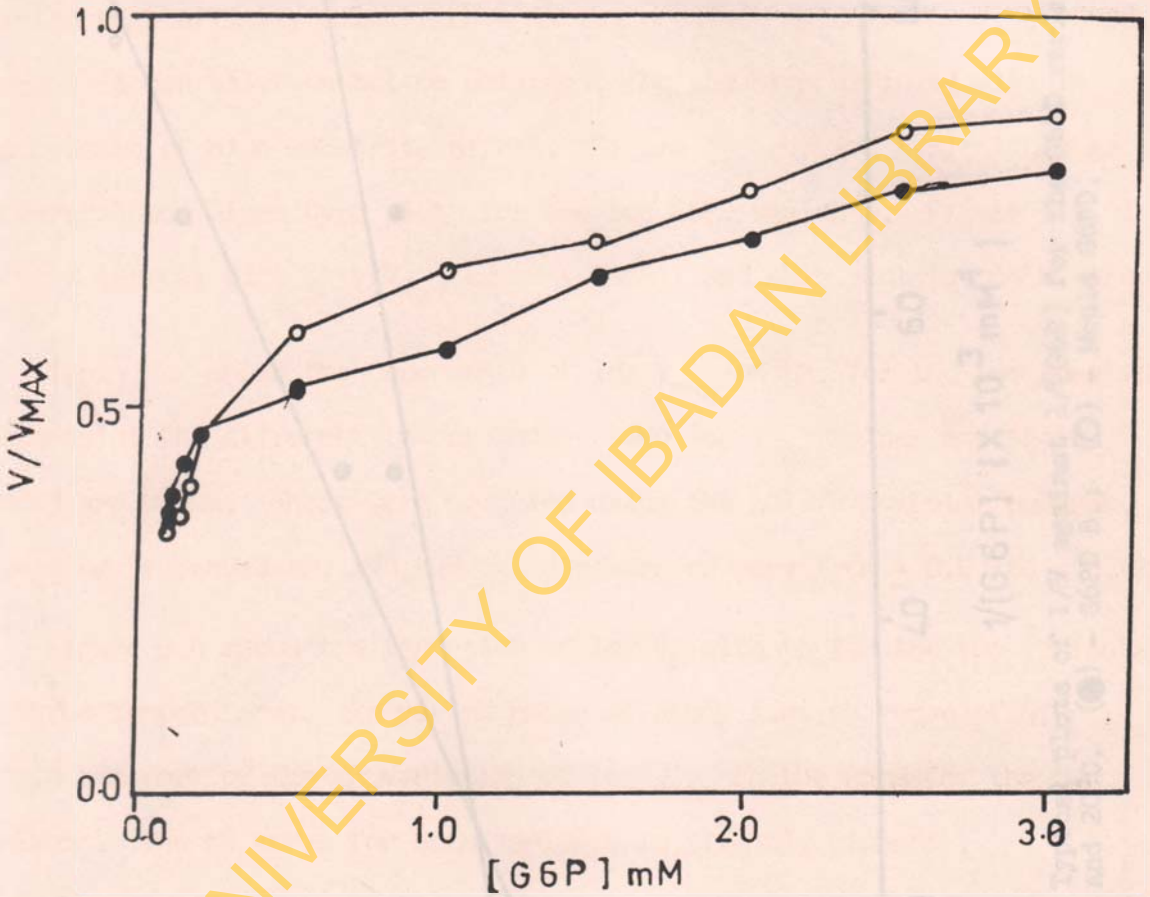


Fig. 3.4 : Typical plot of normalized reaction velocity against G6P concentration at 20°C for the Mould G6PD. (○) - Acidic pH, (●) - Alkaline pH.

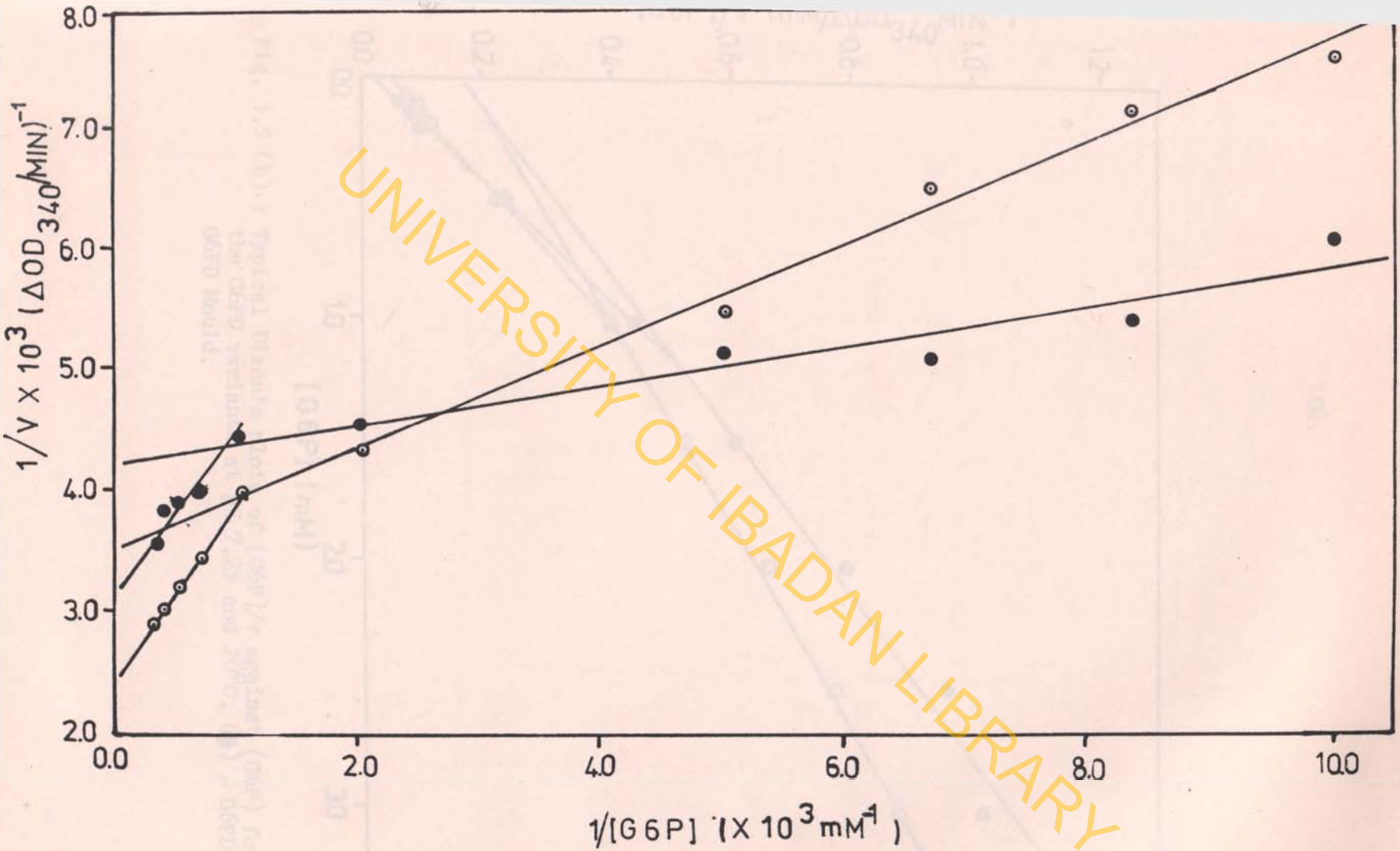


Fig. 3.5 (a) : Typical plots of 1/V against 1/(G6P) for the G6PD variants at pH 7.25 and 20°C. (●) - G6PD B.; (○) - Mould G6PD.

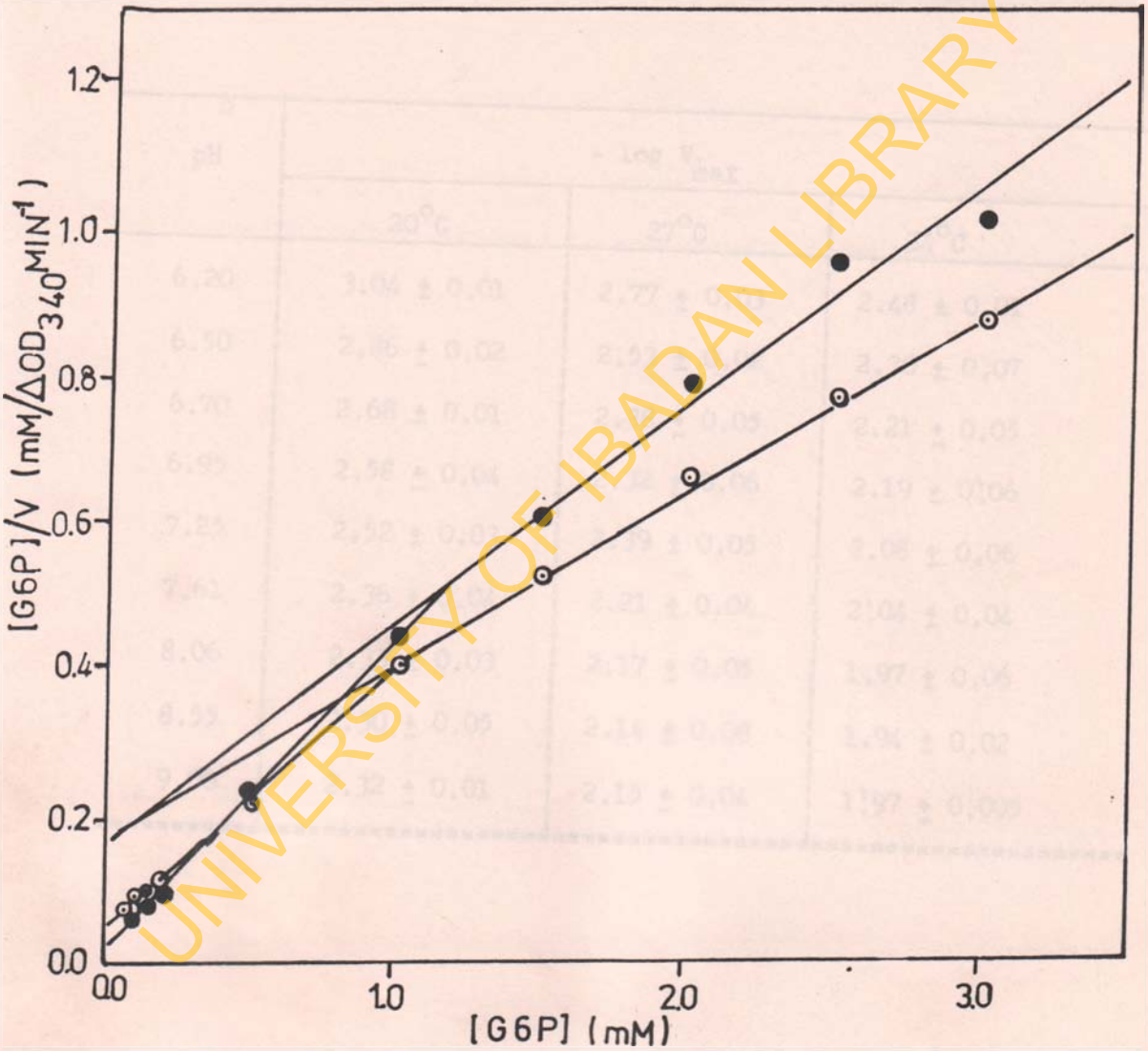


Fig. 3.5 (b) : Typical Dixon's plots of (G6P)/v against (G6P) for the G6PD variants at pH 7.25 and 20°C. (●) - G6PD B., (O) - G6PD Mould.

TABLE 3.2 (a)

DEPENDENCE OF LOG V_{max} ON pH AND TEMPERATURE FOR G6PD \B

pH	- log V_{max}		
	20°C	27°C	34°C
6.20	3.04 ± 0.01	2.77 ± 0.03	2.48 ± 0.01
6.50	2.86 ± 0.02	2.53 ± 0.02	2.30 ± 0.07
6.70	2.68 ± 0.01	2.38 ± 0.05	2.21 ± 0.05
6.95	2.58 ± 0.04	2.32 ± 0.06	2.19 ± 0.06
7.25	2.52 ± 0.03	2.39 ± 0.05	2.08 ± 0.06
7.61	2.36 ± 0.04	2.21 ± 0.04	2.04 ± 0.04
8.06	2.33 ± 0.03	2.17 ± 0.05	1.97 ± 0.06
8.55	2.30 ± 0.05	2.14 ± 0.08	1.94 ± 0.02
9.00	2.32 ± 0.01	2.15 ± 0.04	1.97 ± 0.005

TABLE 3.2 (b)

DEPENDENCE OF LOG V_{max} ON pH AND TEMPERATURE FOR THE MOULD G6PD

pH	- log V_{max}		
	20°C	27°C	34°C
6.05	2.97 ± 0.06	2.71 ± 0.06	2.51 ± 0.04
6.20	2.70 ± 0.11	2.51 ± 0.10	2.29 ± 0.04
6.70	2.47 ± 0.07	2.43 ± 0.13	2.17 ± 0.04
6.95	2.44 ± 0.06	2.28 ± 0.07	2.15 ± 0.07
7.25	2.37 ± 0.01	2.22 ± 0.04	2.10 ± 0.05
7.61	2.30 ± 0.10	2.17 ± 0.04	2.05 ± 0.06
8.06	2.28 ± 0.02	2.12 ± 0.03	1.97 ± 0.04
8.55	2.22 ± 0.03	2.06 ± 0.04	1.94 ± 0.06
9.00	2.28 ± 0.07	2.14 ± 0.06	1.98 ± 0.07

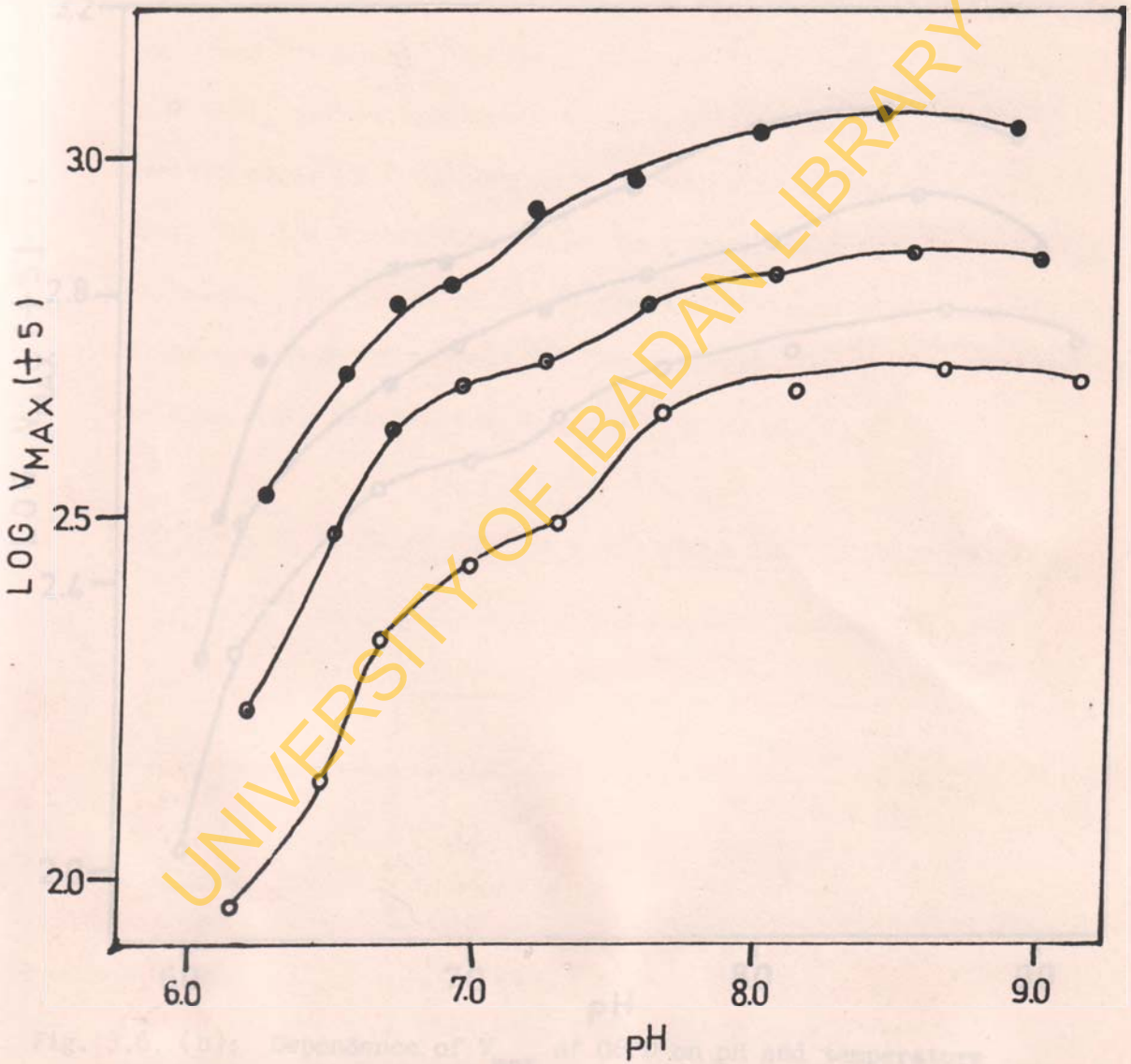


Fig. 3.6 (a): Dependence of V_{max} of G6PD on pH and Temperature for G6PD B. (O) , 20°0; (⊙) , 27°C; (●) , 34°C.

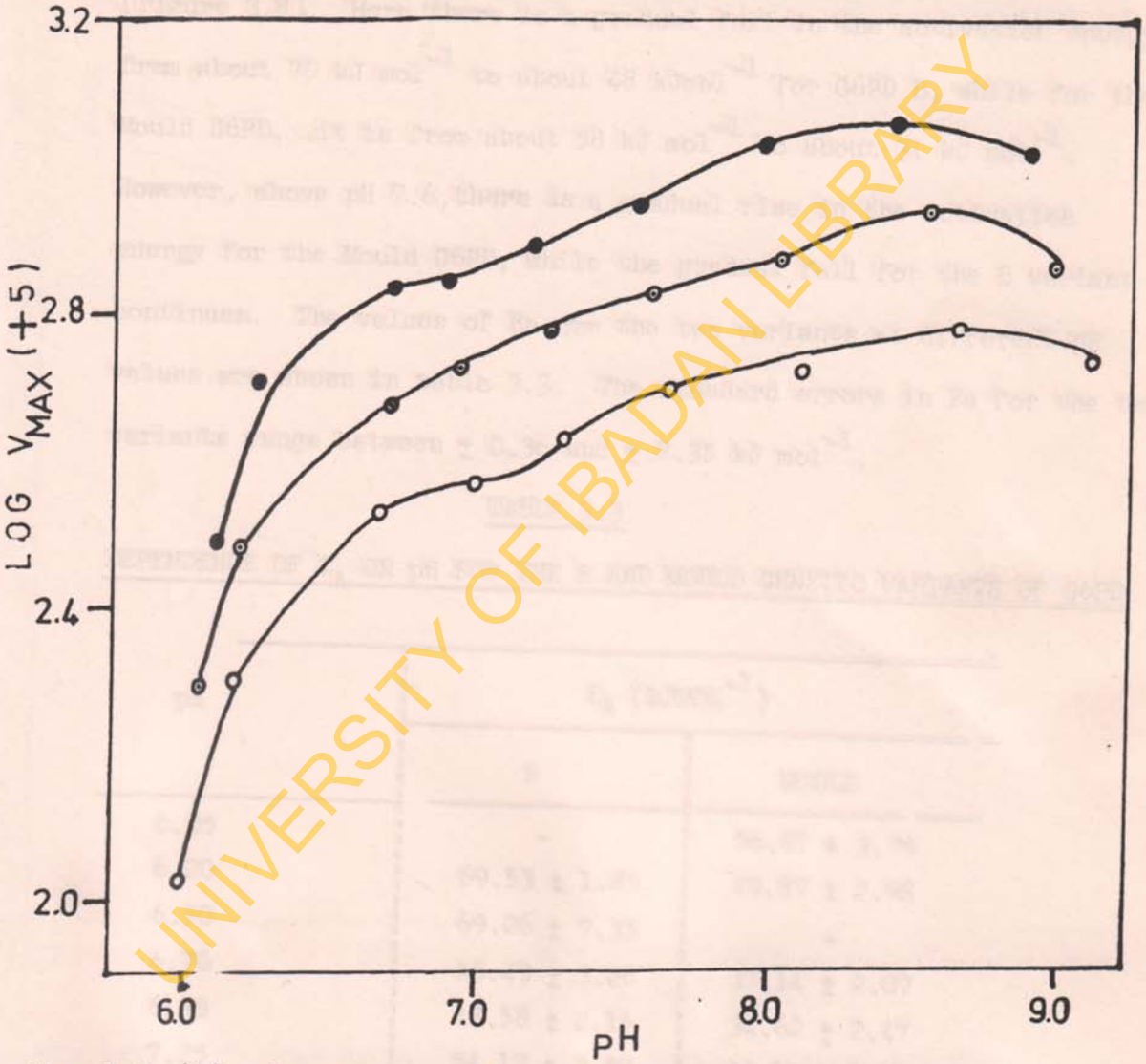


Fig. 3.6. (b): Dependence of V_{max} of G6PD on pH and temperature for the Mould variant. (O), 20°C; (⊙), 27°C; (●), 34°C.

Activation energies, Ea, were calculated from Arrhenius plots of values of log V_{max} at constant pH (figure 3.7). The two enzyme types show very similar dependence of Ea on pH from the pH range of 6.0 to 7.6 (figure 3.8). Here there is a gradual fall in the activation energy from about 70 kJ mol^{-1} to about 48 kJmol^{-1} for G6PD B, while for the Mould G6PD, it is from about 58 kJ mol^{-1} to about 31 kJ mol^{-1}. However, above pH 7.6, there is a gradual rise in the activation energy for the Mould G6PD, while the gradual fall for the B variant continues. The values of Ea for the two variants at different pH values are shown in table 3.3. The standard errors in Ea for the two variants range between ± 0.36 and ± 7.35 kJ mol^{-1}.

TABLE 3.3

DEPENDENCE OF E_A ON pH FOR THE B AND MOULD GENETIC VARIANTS OF G6PD

pH	E_A (kJMOL^{-1})	
	B	MOULD
6.05	-	56.87 ± 3.79
6.20	69.53 ± 1.85	49.87 ± 2.98
6.50	69.26 ± 7.35	-
6.70	58.49 ± 3.26	37.14 ± 2.07
6.95	48.58 ± 2.16	34.62 ± 2.47
7.25	54.17 ± 3.80	33.50 ± 2.11
7.61	39.51 ± 1.72	31.38 ± 0.36
8.06	44.19 ± 2.91	38.54 ± 0.43
8.55	44.57 ± 2.57	34.37 ± 2.18
9.00	43.46 ± 1.76	36.79 ± 2.13

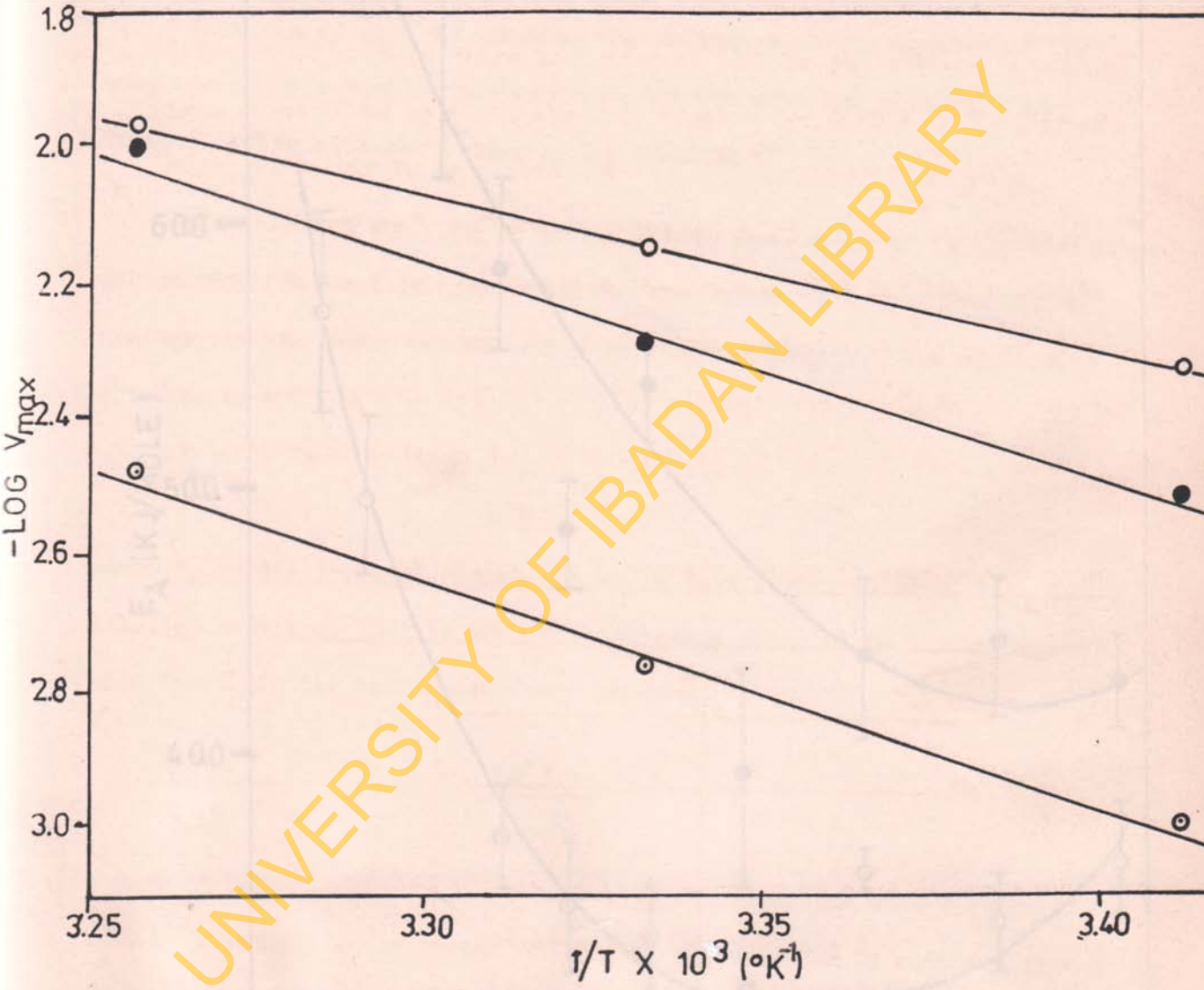


Fig. 3.7 : Typical Arrhenius plots of log V_{max} against 1/T: for variant B. (○), pH 6.20; (●), pH 7.25; (⊙), pH 9.00.

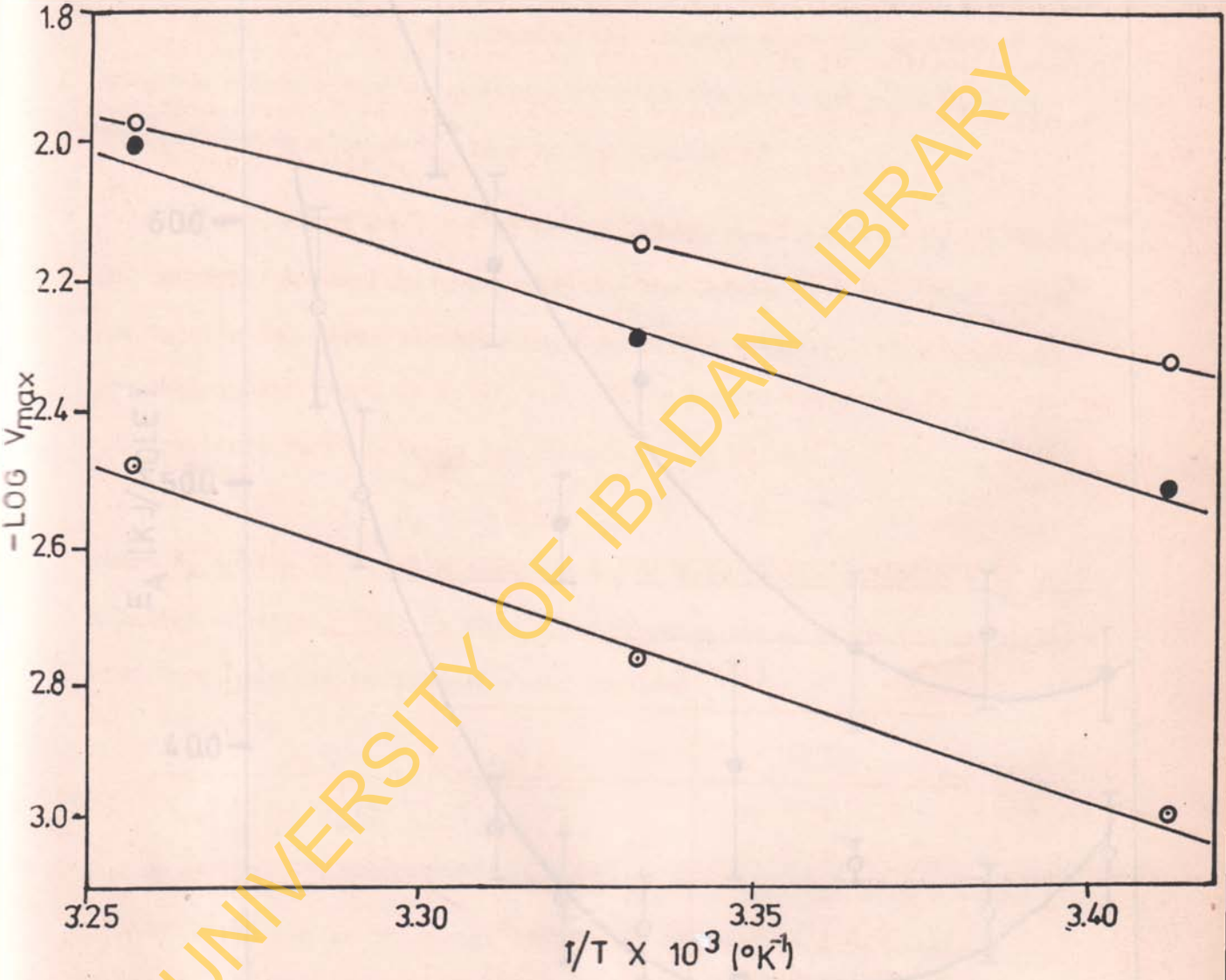


Fig. 3.7 : Typical Arrhenius plots of log V_{max} against 1/T: for variant B. (○), pH 6.20; (●), pH 7.25; (⊙), pH 9.00.

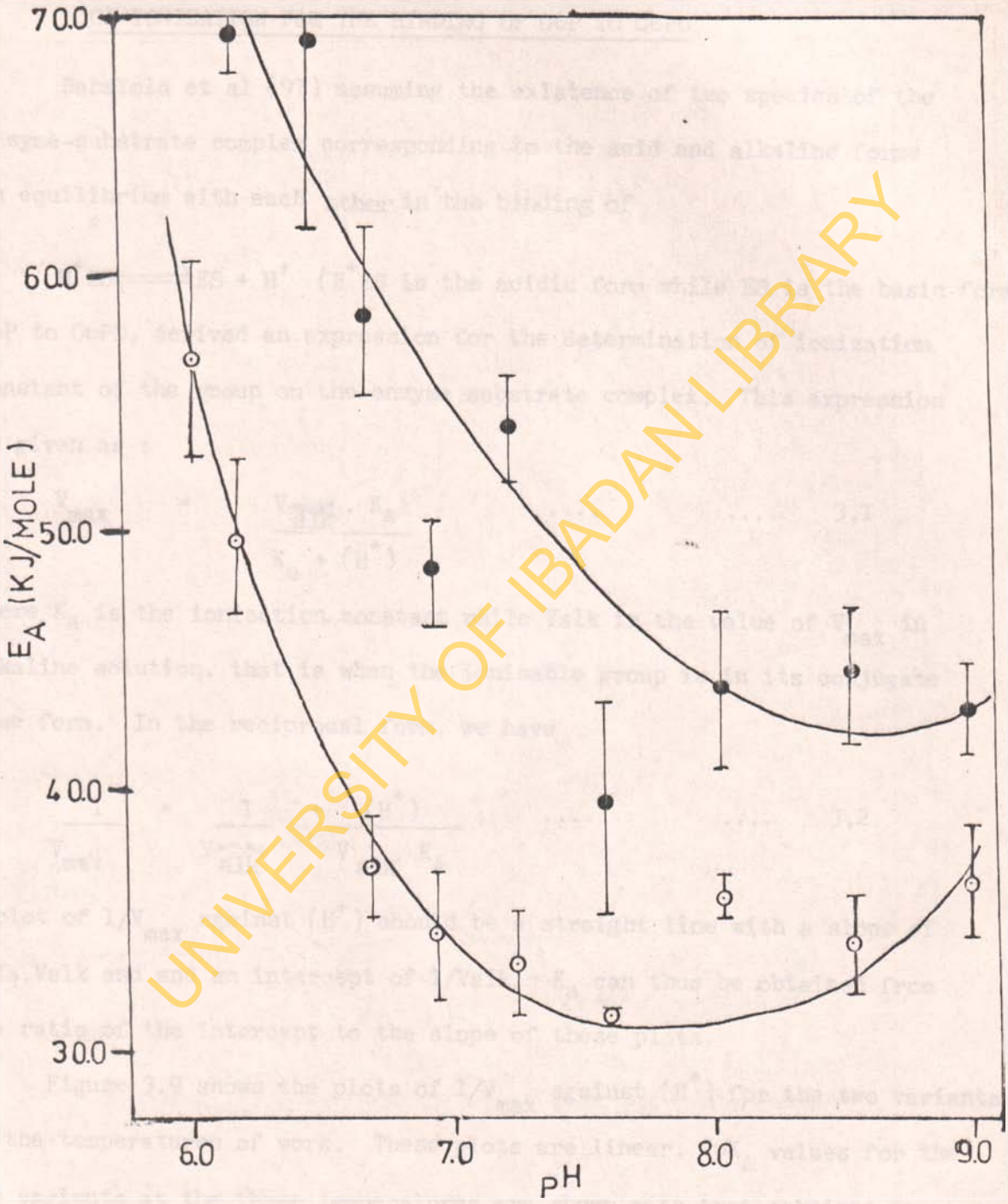


Fig. 3.8: Dependence on pH of the activation energy of G6PD reaction. (●) - G6PD B., (○) - Mould G6PD.

3.2.2. DETERMINATION OF THE IONIZATION CONSTANT AND ENTHALPY OF IONIZATION FOR THE BINDING OF G6P TO G6PD

Babalola et al (97) assuming the existence of two species of the enzyme-substrate complex corresponding to the acid and alkaline forms in equilibrium with each other in the binding of

$H^+ES \rightleftharpoons ES + H^+$ (H^+ES is the acidic form while ES is the basic form)

G6P to G6PD, derived an expression for the determination of ionization constant of the group on the enzyme substrate complex. This expression is given as :

$$V_{max} = \frac{V_{alk} \cdot K_a}{K_a + (H^+)} \quad \ldots \quad \ldots \quad 3.1$$

where K_a is the ionization constant while Valk is the value of V_{max} in alkaline solution, that is when the ionizable group is in its conjugate base form. In the reciprocal form, we have

$$\frac{1}{V_{max}} = \frac{1}{V_{alk}} + \frac{(H^+)}{V_{alk} \cdot K_A} \quad \ldots \quad \ldots \quad 3.2$$

A plot of $1/V_{max}$ against (H^+) should be a straight line with a slope of ./Ka.Valk and and an intercept of 1/Valk. K_a can thus be obtained from he ratio of the intercept to the slope of these plots.

Figure 3.9 shows the plots of $1/V_{max}$ against (H^+) for the two variants t the temperatures of work. These plots are linear. pK_A values for the wo variants at the three temperatures are shown with that obtained by

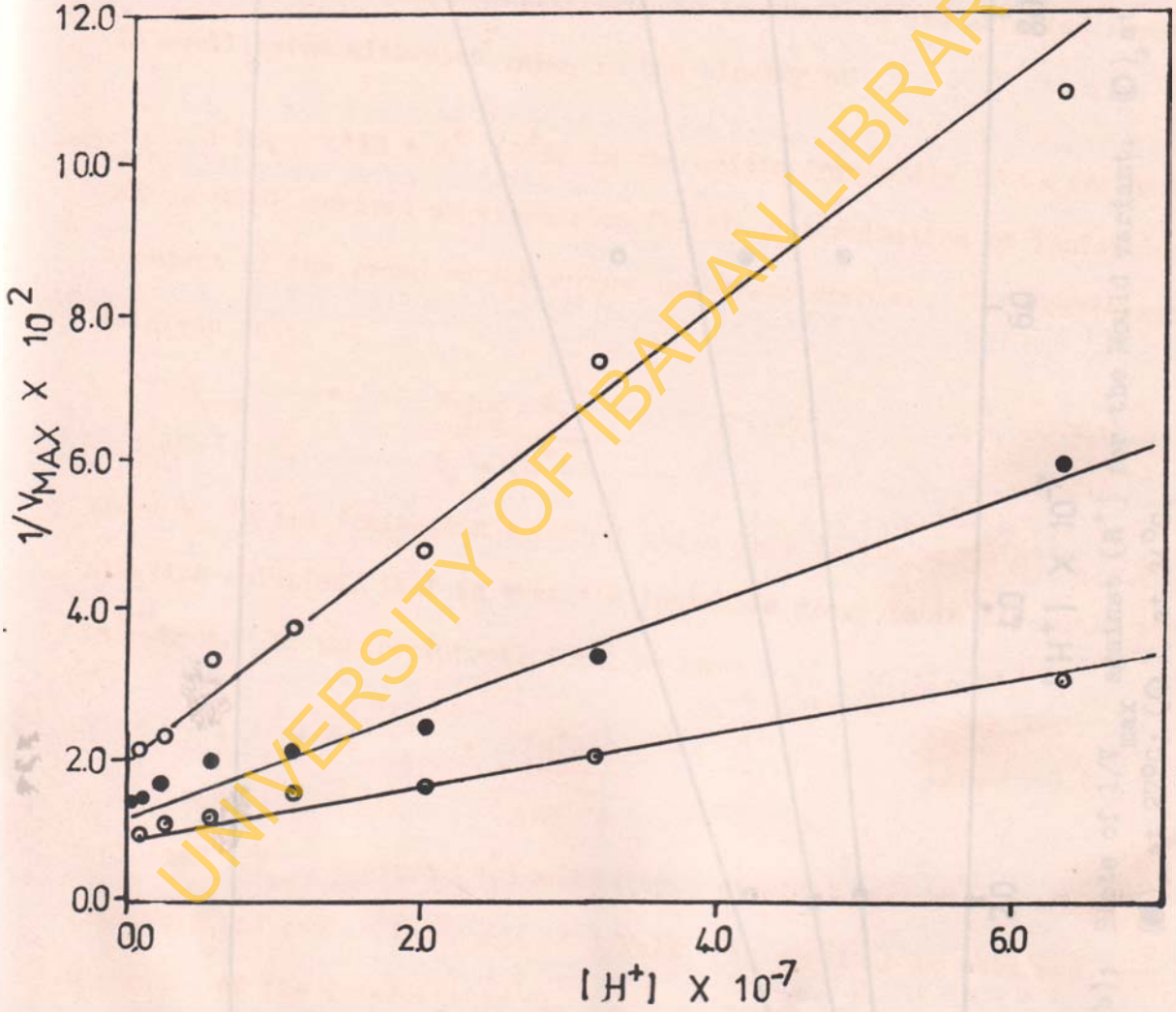


Fig. 3.9 (a): Plots of $1/V_{max}$ against (H^+) for the B variant; (O), at 20°C; (●), at 27°C; (⊙), at 34°C.

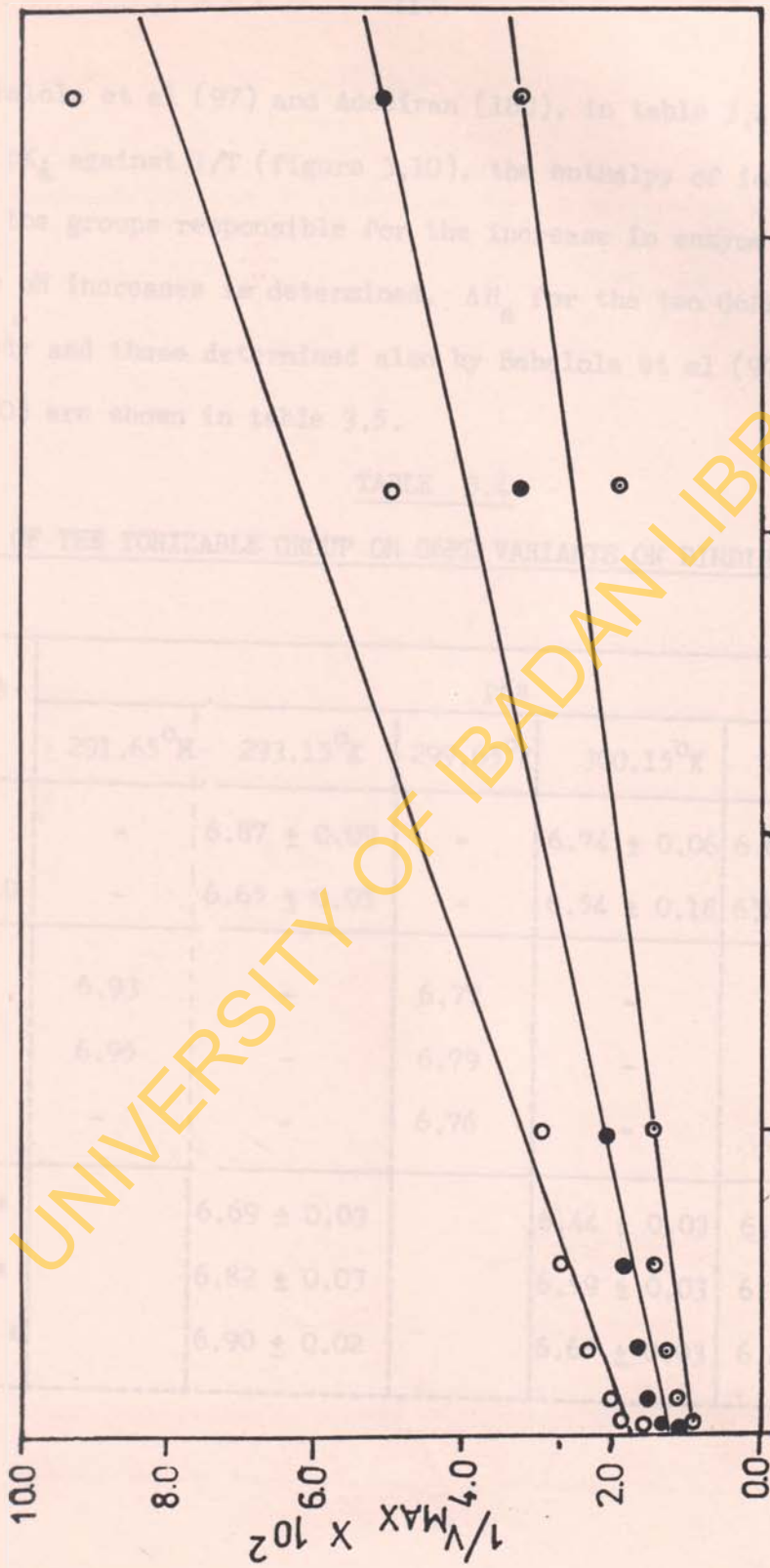


Fig. 3.9 (b): Plots of $1/V_{max}$ against (H^+) for the Mould variant. (○), at 20°C; (●), at 27°C; (⊙), at 34°C.

Babalola et al (97) and Adediran (180), in table 3.4. From the plot of pK_a against 1/T (figure 3.10), the enthalpy of ionization ΔH_a of the groups responsible for the increase in enzyme activity as the pH increases is determined. ΔH_a for the two G6PD variants in this study and those determined also by Babalola et al (97) and Adediran (180) are shown in table 3.5.

TABLE 3.4

pK_a OF THE IONIZABLE GROUP ON G6PD VARIANTS ON BINDING G6P (AND $NADP^+$)*

G6PD	pKa					REFERENCE
	291.65°K	293.15°K	299.65°K	300.15°K	307.15°K	
B	-	6.87 ± 0.09	-	6.74 ± 0.06	6.64 ± 0.11	THIS
MOULD	-	6.65 ± 0.05	-	6.54 ± 0.18	6.47 ± 0.13	STUDY
A	6.93	-	6.77	-	6.66	
B	6.95	-	6.79	-	6.65	97
A^-	-	-	6.76	-	6.64	
A *		6.69 ± 0.03		6.44 ± 0.03	6.39 ± 0.03	
B *		6.82 ± 0.03		6.59 ± 0.03	6.38 ± 0.02	180
A^- *		6.90 ± 0.02		6.68 ± 0.03	6.45 ± 0.03	

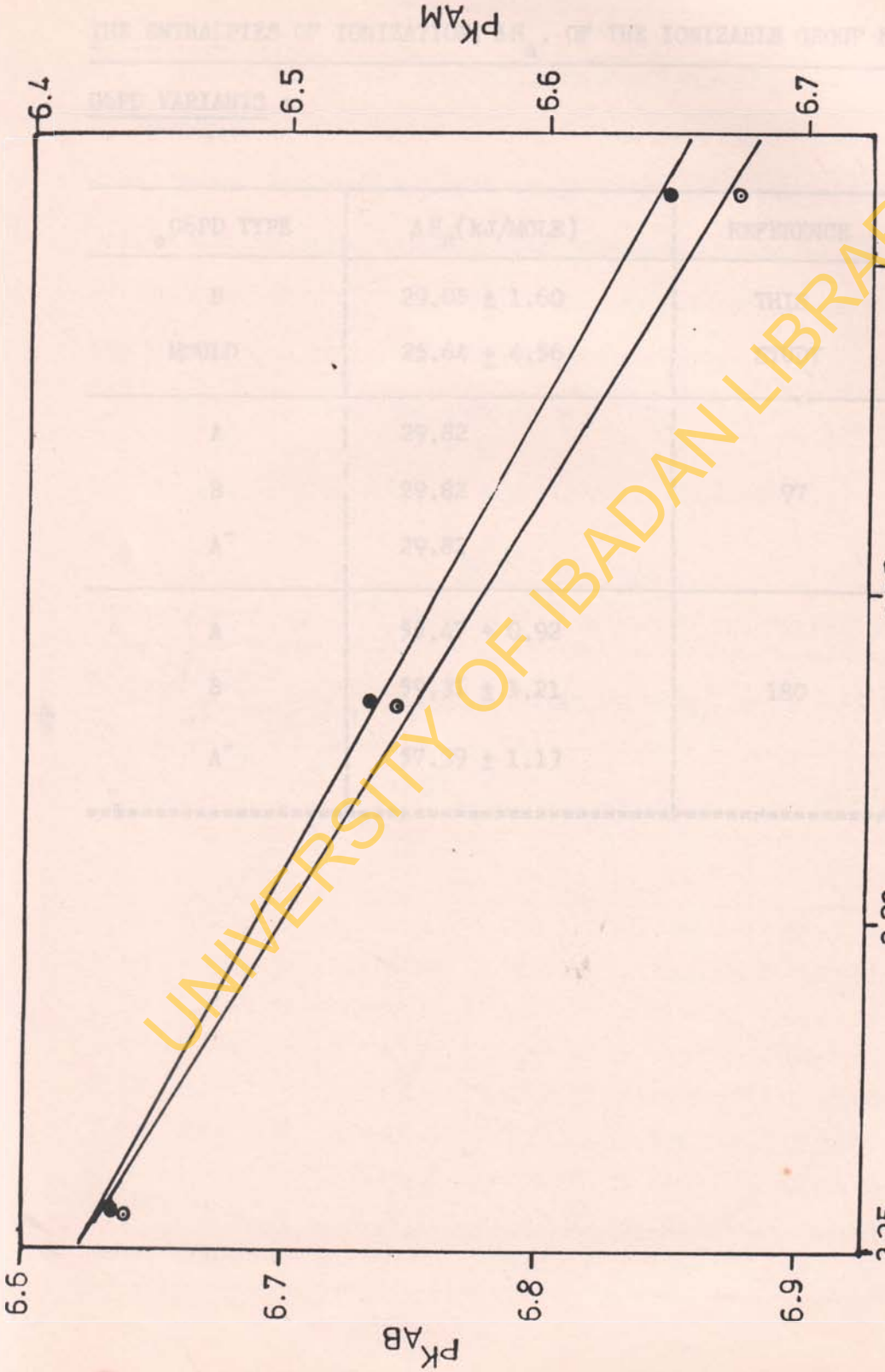


Fig. 3.10 : Plots of pKa against 1/T. (⊙), variant B; (●), the Mould variant.

TABLE 3.5

THE ENTHALPIES OF IONIZATION, ΔH_a, OF THE IONIZABLE GROUP FOR THE G6PD VARIANTS :

G6PD TYPE	ΔH_a(kJ/MOLE)	REFERENCE
B	29.05 ± 1.60	THIS
MOULD	25.64 ± 4.56	STUDY
A	29.82	
B	29.82	97
A^-	29.82	
A	55.47 ± 0.92	
B	59.31 ± 1.21	180
A^-	57.39 ± 1.13	

3.3.1. DEPENDENCE OF K_m FOR G6P ON pH AND TEMPERATURE

Values of K_m, the Michealis constant for G6P, were determined by averaging of the values obtained from both the Lineweaver-Burk and Dixon plots (see figure 3.5 for typical plots). Non Michealian curves were obtained as previously noted, resulting in the presence of two K_m values, $K_{m_1}^{G6P}$ and Km_2^{G6P}. The standard error was ± 0.01 to 0.2 in log K_m for both affinities for the two variants.

Table 3.6 shows the dependence on pH of log $K_{m_1}^{G6P}$ and log $K_{m_2}^{G6P}$ for the two G6PD variants at the different temperatures.

TABLE 3.6 (a)

DEPENDENCE OF LOG Km_1^{G6P} AND LOG Km_2^{G6P} ON pH AND TEMPERATURE FOR G6PD B VARIANT:

pH	- LOG Km_1^{G6P}			- LOG $K_{m_2}^{G6P}$		
	20°C	27°C	34°C	20°C	27°C	34°C
6.20	3.64±0.11	3.39±0.12	3.20± 0.01	4.39±0.14	4.19±0.02	4.02±0.03
6.50	3.58±0.14	3.30±0.08	3.16±0.06	4.25±0.06	4.11±0.06	4.00±0.07
6.70	3.51±0.05	3.29±0.12	3.14±0.13	4.21+0.11	4.09±0.10	3.96±0.06
6.95	3.48±0.07	3.20±0.15	3.09±0.12	4.17±0.06	4.07±0.09	3.94±0.06
7.25	3.58±0.18	3.33±0.16	3.10±0.12	4.26±0.18	4.10±0.08	3.97±0.07
7.61	3.44±0.12	3.24±0.11	3.07±0.08	4.18±0.05	4.05±0.01	3.95±0.05
8.06	3.38±0.05	3.20±0.15	3.05±0.10	4.14±0.08	4.01±0.10	3.93±0.04
8.55	3.35±0.21	3.16±0.12	3.03±0.03	4.14±0.06	3.99±0.04	3.90±0.05
9.00	3.31±0.05	3.14±0.03	2.96±0.09	4.13±0.03	3.98±0.08	3.87±0.05

TABLE 3.6 (b)

DEPENDENCE OF LOG Km_1^{G6P} AND LOG Km_2^{G6P} ON pH AND TEMPERATURE FOR THE MOULD G6PD VARIANT

pH	- LOG Km_1^{G6P}			- LOG Km_2^{G6P}		
	20°C	27°C	34°C	20°C	27°C	34°C
6.05	3.40±0.16	3.21±0.07	3.07±0.09	4.38±0.10	4.22±0.14	4.10±0.03
6.20	3.26±0.16	3.11±0.09	3.03±0.08	4.27±0.03	4.15±0.03	4.05±0.03
6.70	3.24±0.11	3.13±0.14	3.05±0.09	4.27±0.04	4.17±0.04	4.07±0.07
6.95	3.23±0.09	3.09±0.13	3.00±0.13	4.26±0.05	4.13±0.06	4.04±0.09
7.25	3.32±0.04	3.13±0.12	3.02±0.09	4.33±0.08	4.18±0.13	4.06±0.06
7.61	3.18±0.14	3.05±0.09	2.97±0.08	4.24±0.04	4.13±0.13	4.03±0.03
8.06	3.16±0.05	3.06±0.07	2.96±0.07	4.19±0.06	4.09±0.11	4.02±0.05
8.55	3.15±0.08	3.03±0.09	2.92±0.09	4.15±0.03	4.05±0.03	3.94±0.02
9.00	3.12±0.11	2.99±0.09	2.88±0.08	4.11±0.07	4.00±0.09	3.90±0.04

The variations of logKm_1^{G6P} and log Km_2^{G6P} with pH for the B and Mould G6PD variants at the different temperatures can be seen in figure 3.11. In contrast to the behaviour of log V_{max}, the variation of Km with pH is quite complex. For variant B, there is an increase of Km with pH between 6.2 and 6.95, which is then interupted abruptly by a "valley", with a sharp minimum at pH 7.25. As from pH 7.61, there is a gradual

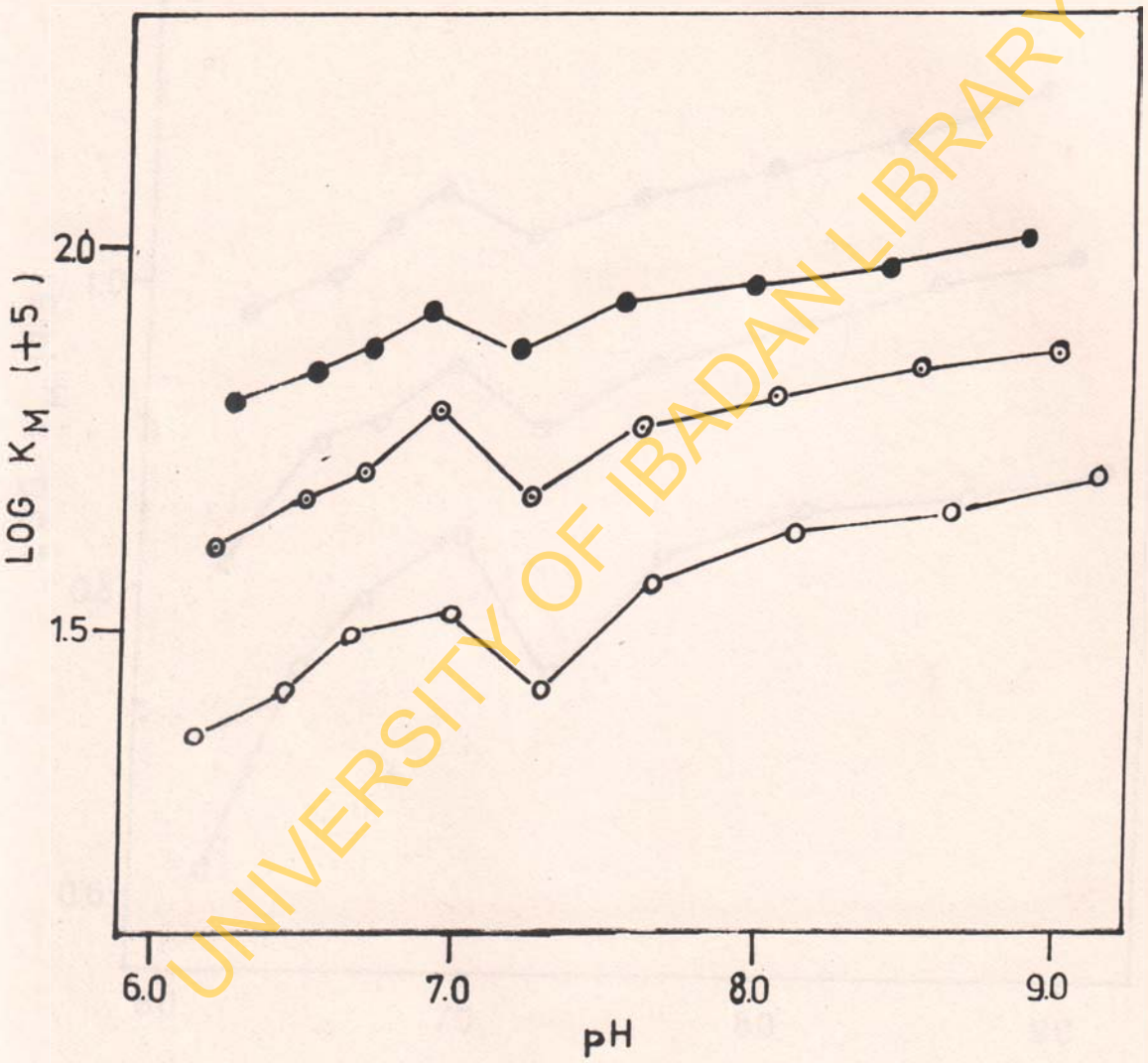


Fig. 3.11 (a) : Dependence on pH and Temperature of Km_1^{G6P} of G6PD B. (O) , 20°C; (⊙) , 27°C; (●) , 34°C.

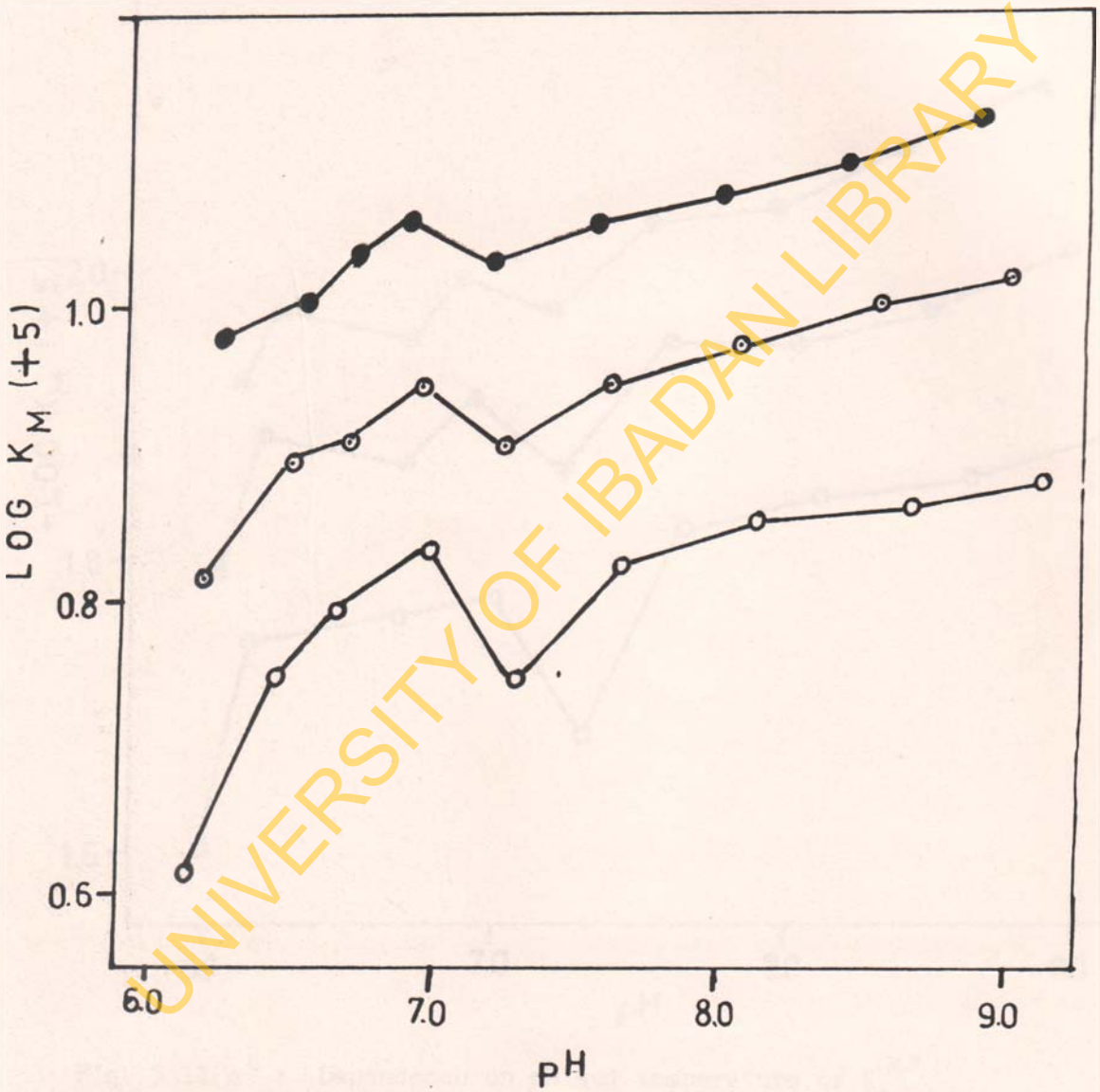


Fig. 3.11 (b) : Dependence on pH and Temperature of $K_{m_2}^{G6P}$ of G6PD B. (O) , 20°C; (⊙) , 27°C; (●) , 34°C.

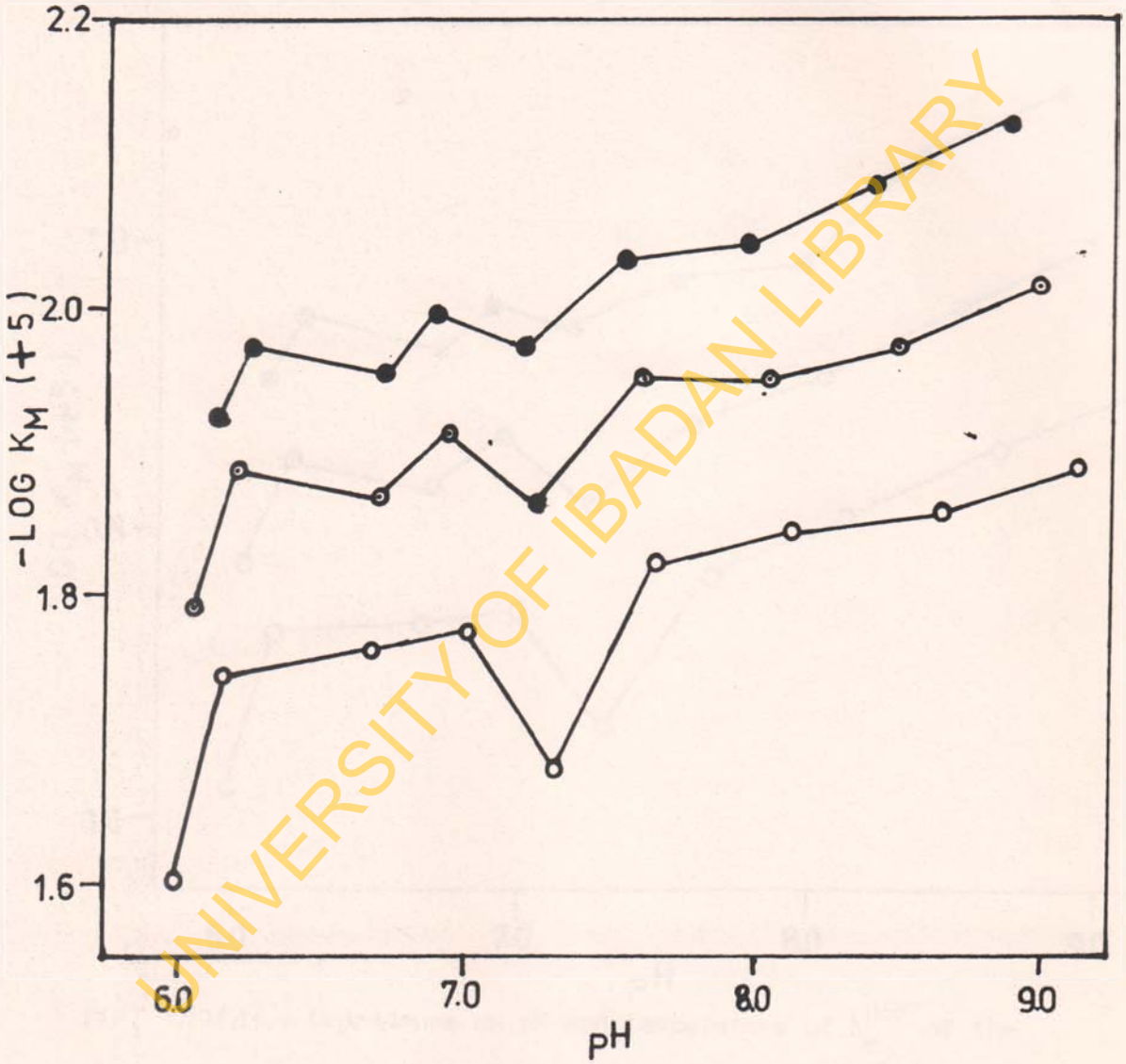


Fig. 3.11(c) : Dependence on pH and temperature of K_m^{G6P} of the Mould G6PD. (O) , 20°C; (⊖) , 27°C; (●) , 34°C.

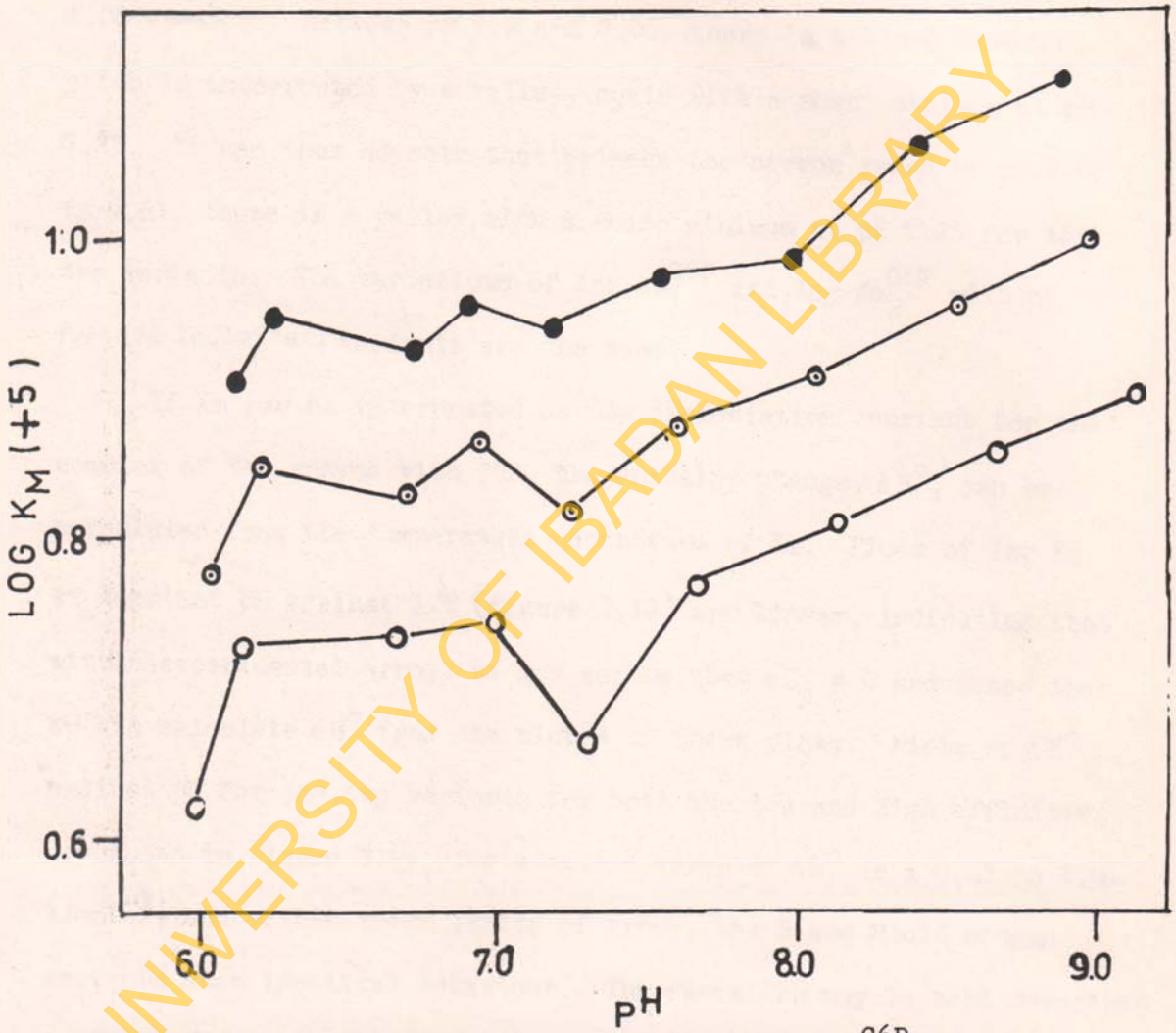


Fig. 3.11(d) : Dependence on pH and temperature of $K_{m_2}^{G6P}$ of the Mould G6PD. (O) , 20°C; (⊙) , 27°C ; (●) , 34°C.

increase in pH till about 9.0. For the Mould variant, there is a sharp increase of Km with pH between 6.05 and 6.20, and again from 8.06 upwards. Between pH 6.2 and 8.06, there is a broad plateau which is interrupted by a valley, again with a sharp minimum at pH 7.25. It can thus be seen that between the narrow range of pH 6.95 to 7.61, there is a valley with a sharp minimum at pH 7.25 for the two variants. The variations of log Km_1^{G6P} and log Km_2^{G6P} with pH for the individual variants are the same.

If Km can be interpreted as the dissociation constant for the complex of the enzyme with G6P, the enthalpy change, ΔH°, can be calculated from the temperature dependence of Km, Plots of log Km at constant pH against 1/T (figure 3.12) are linear, indicating that within experimental error, we may assume that $\Delta C_p = 0$ and hence that we can calculate ΔH° from the slopes of these plots. Plots of ΔH° against pH for the two variants for both the low and high affinities are shown in figure 3.13. The standard error of ΔH° is ± 0.05 to 6.01 $kJmol^{-1}$, and within these limits of error, the B and Mould enzyme variants show identical behaviour. The variation may be best described as two U-shaped curves intersecting at pH 7.25, the same pH at which we have a sharp minimum for both G6PD types.

Table 3.7 shows the dependence on pH of ΔH° for the two G6PD variants.

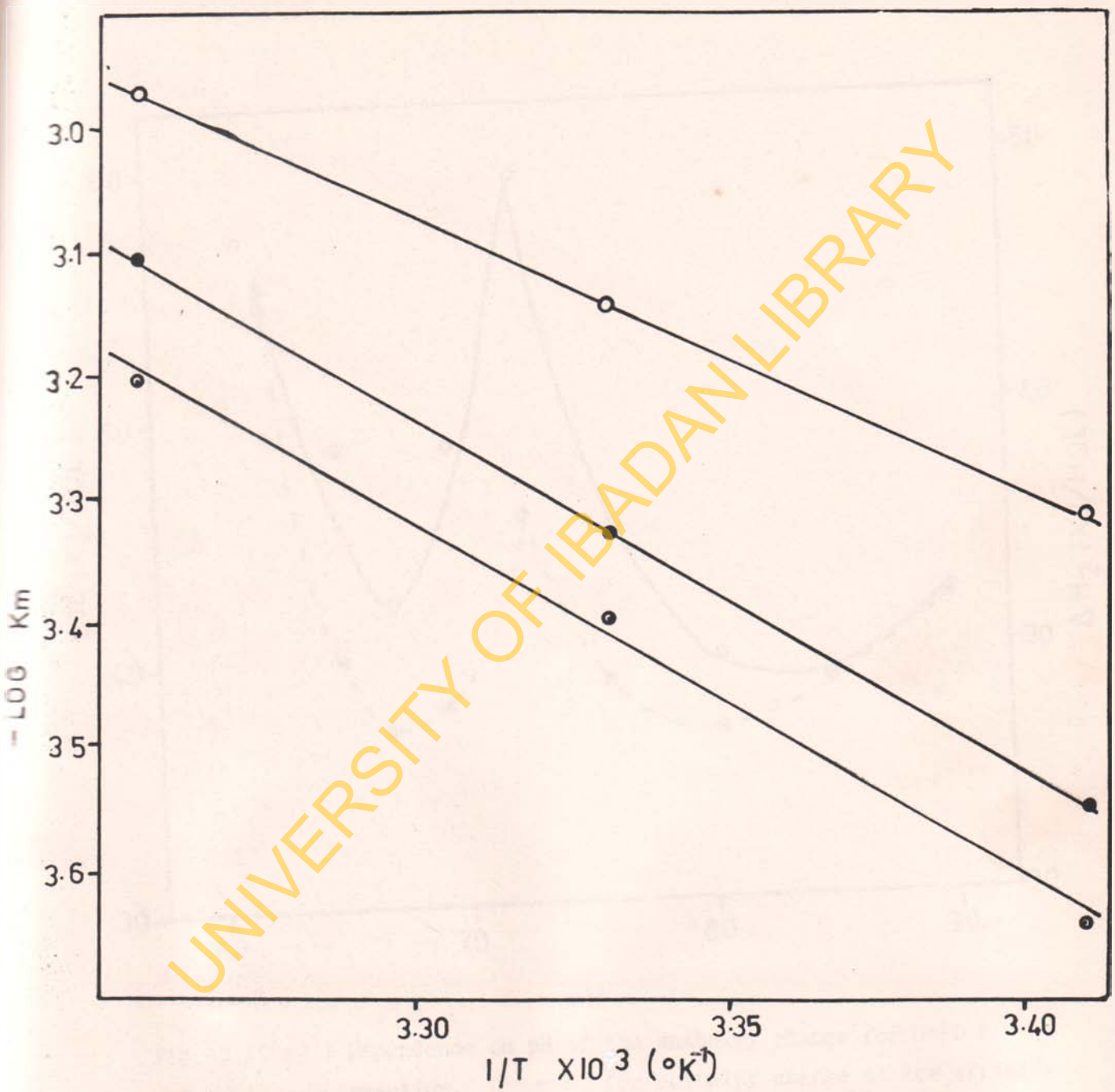


Fig. 3.12 : Typical plots of log K_m against 1/T for G6PD B at 3 pHs. (⊖) , pH 6.20; (●) , pH 7.25; (○) , pH 9.00.

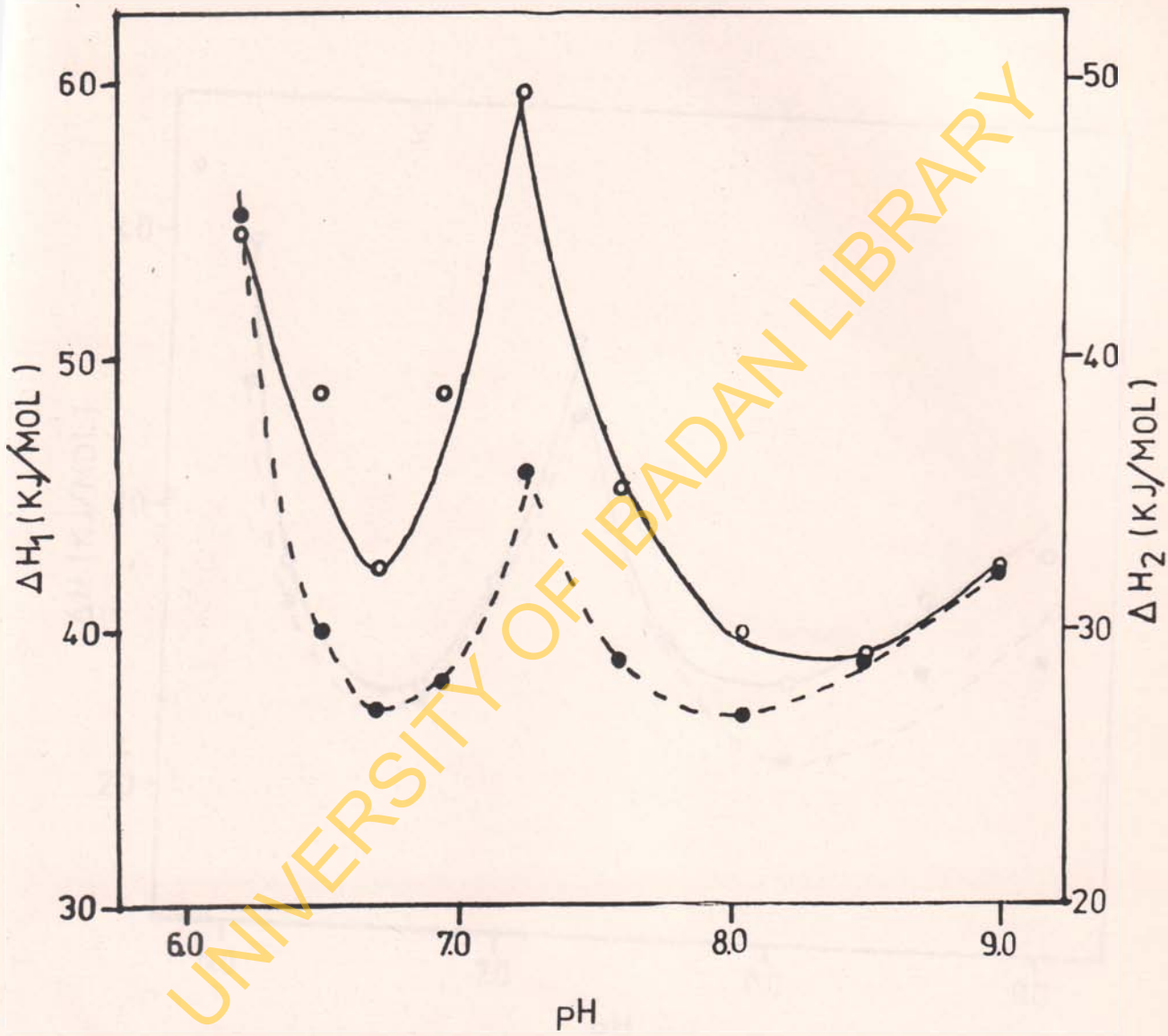


Fig. 3.13(a) : Dependence on pH of the enthalpy change for G6PD B reaction. O——O, for enthalpy change at low affinity (ΔH_1); ●- - -●, for enthalpy change at high affinity (ΔH_2).

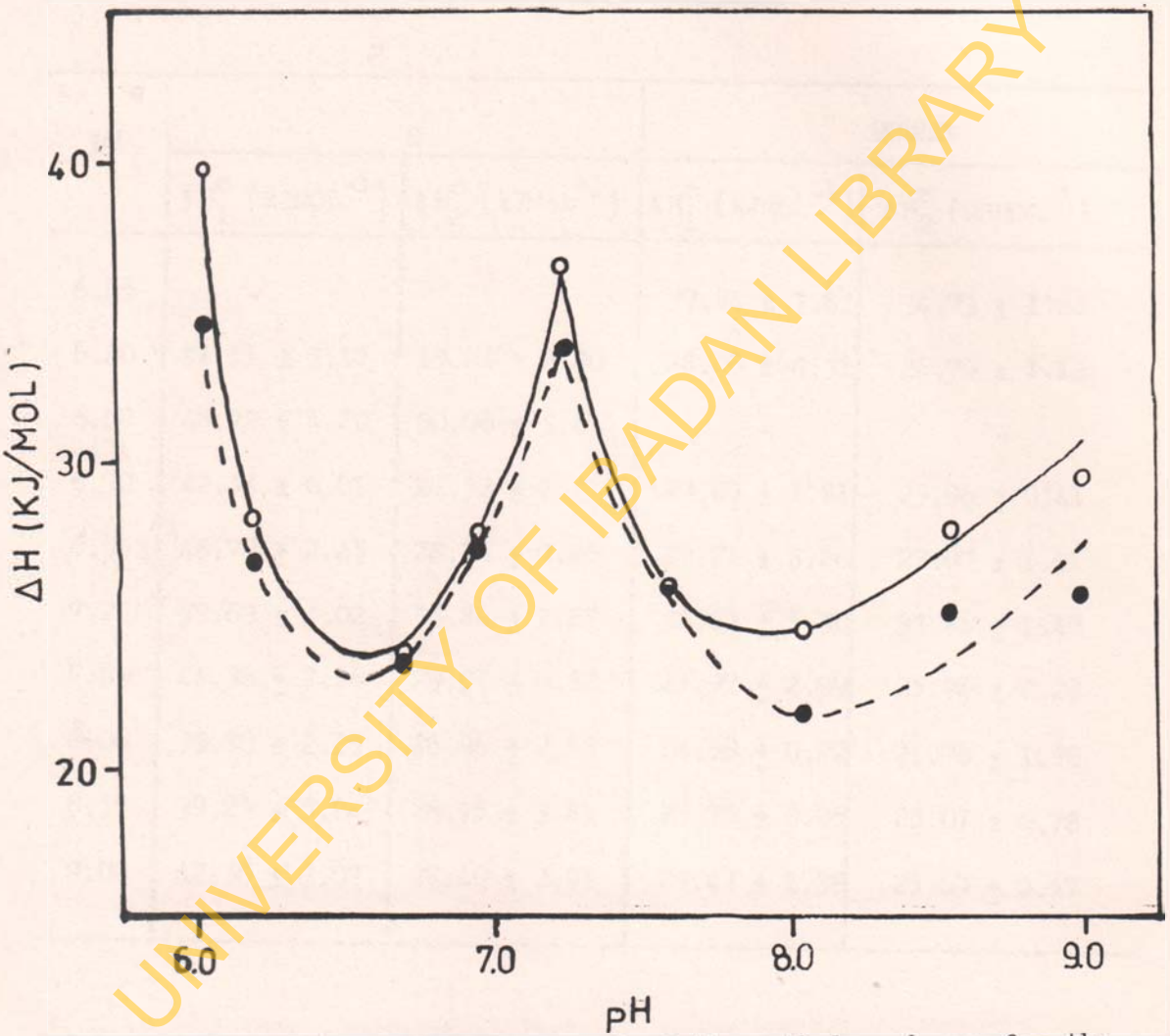


Fig. 3.13 (b) : Dependence on pH of the enthalpy change for the Mould G6PD. (O), for enthalpy change at low affinity (ΔH_1); (●), for enthalpy change at high affinity (ΔH_2).

TABLE 3.7

DEPENDENCE ON PH OF ΔH° FOR THE B AND MOULD G6PD VARIANTS AT BOTH LOW AND HIGH AFFINITIES

pH	B		MOULD	
	ΔH_1° ($kJMOL^{-1}$)	ΔH_2° ($kJMol^{-1}$)	ΔH_1° ($kJMOL^{-1}$)	ΔH_2° ($kJMOL^{-1}$)
6.05	-	-	39.94 ± 3.62	34.73 ± 1.82
6.20	54.51 ± 3.17	45.24 ± 2.20	28.09 ± 4.31	26.70 ± 1.13
6.50	48.79 ± 4.20	30.06 ± 3.45	-	-
6.70	42.44 ± 6.01	27.32 ± 1.21	23.49 ± 1.81	23.96 ± 0.41
6.95	48.79 ± 2.45	28.16 ± 0.46	27.71 ± 3.26	27.07 ± 1.91
7.25	59.63 ± 4.02	35.84 ± 1.88	36.62 ± 5.30	33.86 ± 1.47
7.61	45.36 ± 2.26	29.04 ± 0.88	25.97 ± 2.99	25.94 ± 0.28
8.06	39.80 ± 2.25	26.96 ± 2.55	24.58 ± 0.22	21.76 ± 1.90
8.55	39.25 ± 5.04	28.95 ± 3.81	27.79 ± 0.05	25.07 ± 0.78
9.00	42.30 ± 3.07	32.40 ± 2.91	29.42 ± 1.38	25.69 ± 0.57

3.4.1. DEPENDENCE OF INTERACTION COEFFICIENT (n) ON pH

Working at a concentration range of 0.1 to 3.0mM (G6P) at an ionic strength of 0.01, cooperativity was observed with regards to G6P binding, resulting in two Km values.

The interaction coefficient, n, a measure of cooperativity was obtained from the slope of the plot of log $v/(V_{max}-v)$ against log(G6P), according to the Hills equation :

$$\log v/(V_{max}-v) = n \log(G6P) - \log K.$$

Figure 3.14 shows a typical Hills plot for the determination of the interaction coefficient for G6PD B.

Figure 3.15 shows the variation of the interaction coefficient, n, with pH. The values of n obtained are less than one for both variants, showing that the enzyme exhibits negative cooperativity with respect to G6P binding to the enzyme. The two enzyme types show very similar dependence of n on pH, there being a gradual fall of the interaction coefficient from about 0.7 to 0.4 in the pH region of 6.0 to 9.0.

Table 3.8 shows the dependence on pH of the interaction coefficient for the two variants at 27°C.

TABLE 3.8

DEPENDENCE OF INTERACTION COEFFICIENT ON pH FOR THE B AND MOULD G6PD

pH	INTERACTION COEFFICIENT	
	B	MOULD
6.05	-	0.70 ± 0.04
6.20	0.66 ± 0.09	0.60 ± 0.05
6.50	0.53 ± 0.06	-
6.70	0.50 ± 0.04	0.65 ± 0.06
6.95	0.48 ± 0.06	0.55 ± 0.06
7.25	0.41 ± 0.06	0.53 ± 0.06
7.61	0.41 ± 0.03	0.50 ± 0.03
8.06	0.40 ± 0.03	0.42 ± 0.04
8.55	0.45 ± 0.05	0.40 ± 0.04
9.00	0.37 ± 0.04	0.43 ± 0.04

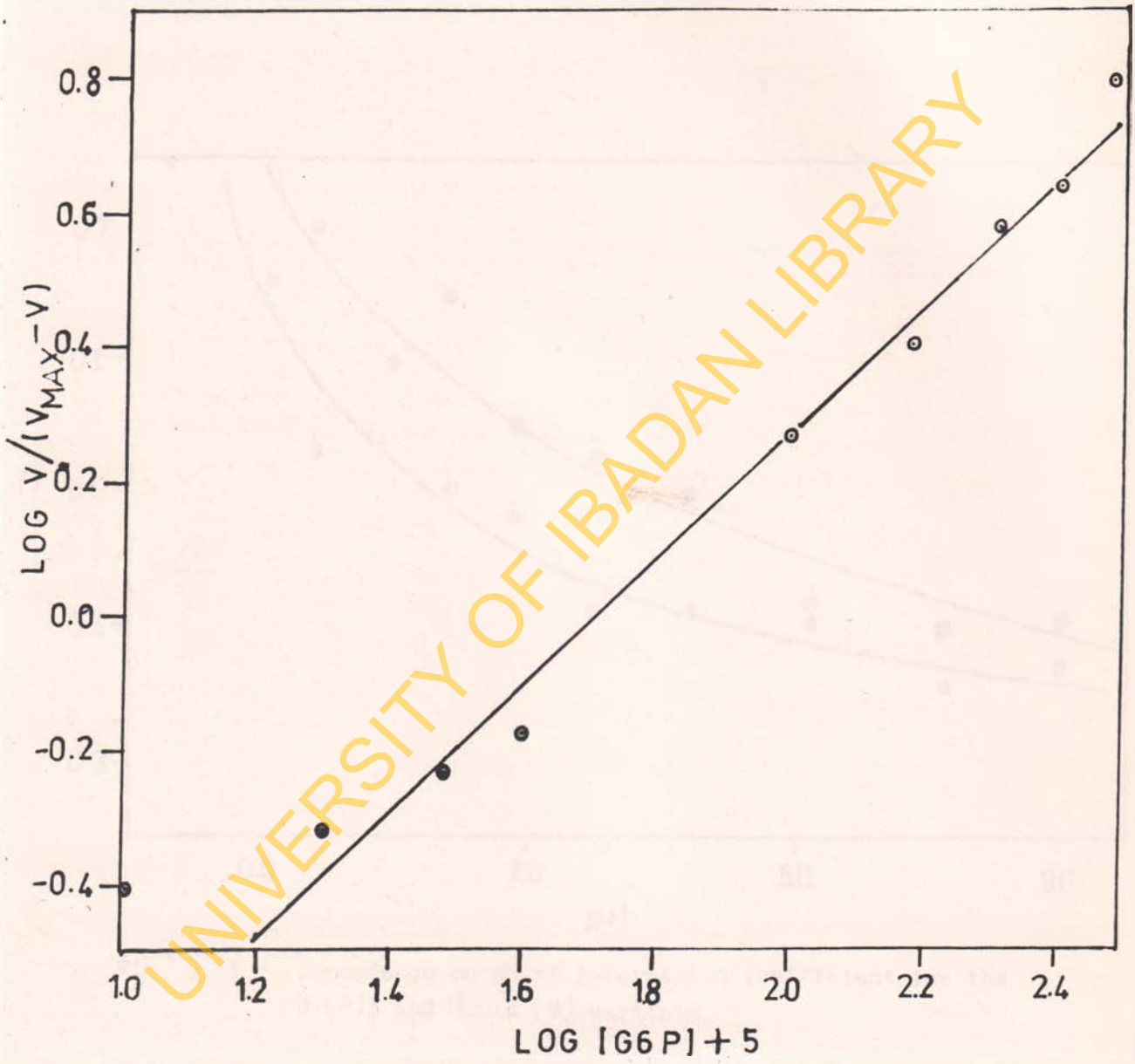


Fig. 3.14 : Typical Hills plot for the determination of the interaction coefficient for G6PD B.

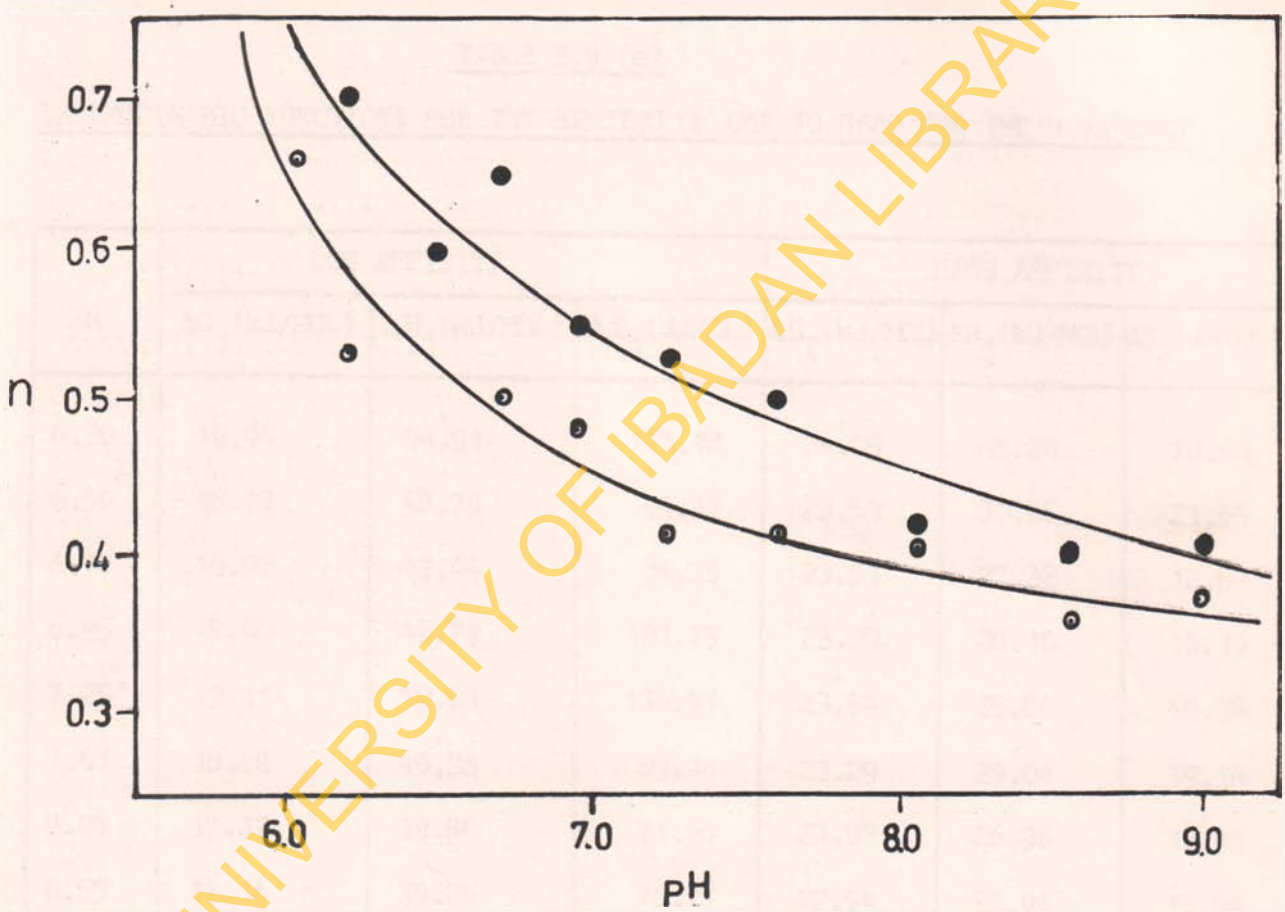


Fig. 3.15 : Dependence on pH of interaction coefficient for the B (0); and Mould (●) variants.

3.5.1. SUMMARY OF THERMODYNAMIC FUNCTIONS FOR THE BINDING OF G6P TO G6PD

The summary of the thermodynamic functions (ΔH, ΔS and ΔG) for the binding of G6P to G6PD is given in table 3.9 (a & b) for the Mould and B G6PD variants, at the different pH values and temperatures, for the two affinities.

TABLE 3.9 (a)

THERMODYNAMIC FUNCTIONS FOR THE BINDING OF G6P TO G6PD FOR THE B VARIANT

pH	LOW AFFINITY			HIGH AFFINITY		
	ΔG_1 (kJ/MOL)	ΔH_1 (kJ/MOL)	ΔS_1 (J/MOL)	ΔG_2 (kJ/MOL)	ΔH_2 (kJ/MOL)	ΔS_2 (J/MOL)
6.20	19.94	54.51	115.18	24.05	45.24	70.60
6.50	18.98	48.79	99.32	23.59	30.06	21.55
6.70	18.92	42.44	78.36	23.51	27.32	12.69
6.95	18.40	48.79	101.25	23.30	28.16	16.19
7.25	19.11	59.63	134.99	23.54	35.84	40.98
7.61	18.60	45.36	89.16	23.29	29.04	19.16
8.06	18.37	39.80	71.40	23.07	26.96	12.96
8.55	18.16	39.24	70.23	22.94	28.94	19.99
9.00	18.04	42.30	80.83	22.87	32.40	31.75

TABLE 3.9 (b)

THERMODYNAMIC FUNCTIONS FOR THE BINDING OF G6P TO G6PD FOR THE MOULD VARIANT

pH	LOW AFFINITY			HIGH AFFINITY		
	ΔG_1(kJ/MOL)	ΔH_1(kJ/MOL)	ΔS_1(J/MOL)	ΔG_2(kJ/MOL)	ΔH_2(kJ/MOL)	ΔS_2(kJ/MOI
6.05	18.43	39.94	71.66	24.27	34.73	34.84
6.20	17.88	28.09	34.01	23.85	26.70	9.49
6.70	17.99	23.49	18.32	23.97	23.96	-0.03
6.95	17.76	27.71	33.15	23.76	27.07	11.03
7.25	18.00	36.62	62.04	24.04	33.86	32.72
7.61	17.53	25.97	28.12	23.72	25.94	7.40
8.06	17.57	24.58	23.35	23.50	21.76	- 5.80
8.55	17.42	27.79	34.55	23.26	25.07	6.03
9.00	17.16	29.42	40.85	23.01	25.69	8.93

CHAPTER FOUR

DISCUSSION

4.1.1. PURIFICATION OF G6PD VARIANTS

The method described in this study, the combination of the modified form of the Deflora et al method (115) and the Craney et al method (117) is one of the simplest and most rapid way so far available of purifying G6PD from human erythrocytes. It is possible to obtain homogenous enzyme within two to three days of the start of the isolation procedure. For usual preparations not exceeding 500 ml of blood, a convenient work schedule involves for the first day, preparation and processing of the hemolysate till adsorption onto 2', 5' ADP-Sepharose 4B column; then a first and second washing of the resin overnight as described under experimental, and during the second day, third washing and elution, concentration and dialysis of enzyme, adsorption, washing and elution of enzyme from CM-Sephadex C-50 column, and dialysis overnight, and during the third day, passage on Bio-Gel P-150 column, elution, concentration and final storage.

Another advantage of this procedure, besides its rapidity and simplicity, lies in the fact that it avoids the specific elution of G6PD from CM-Sephadex by its substrate, G6P. This step, first introduced by Rattazzi(65), although undoubtedly representing an

important simplification of previous methods of purification appears to modify significantly the content of the tightly bound structural $NADP^+$

The final yield of enzyme in the preparations using this procedure were usually low about seven percent, despite the simplicity and overall rapidity of the method. DeFlora et al (115) had a yield of 51 percent without the inclusion of the CM-Sephadex C-50 step, while Craney et al (117), had a final yield of 66 percent. The first step in the DeFlora et al (115) and Craney et al (117) procedure after hemolysis was basically batchwise chromatography on DEAE-Sephadex and NADP Sepharose 4B resins respectively. This resulted in the quick elimination of the red cells and other dehydrogenases present in the hemolysate. The use of the NADP affinity gel by Craney et al (117) also resulted in greater specificity for the enzyme by the affinity gel. The yield obtained by these workers after this first step was 97 percent for DeFlora et al (115) and 88 percent for Craney et al (117). Thus they both still had large quantities of enzyme to work with after the elimination of the red cells.

In this work, the hemolysate was packed directly onto the 2', 5' ADP Sepharose 4B column. The 2', 5' ADP affinity ligand is not specific for only G6PD. It is also specific for other proteins and dehydrogenases like alcohol, glutamate, 6-phosphogluconate dehydrogenases and glutathione reductase (202). The binding capacity of the affinity gel measured in 0.1M Tris-HCl buffer pH 7.6 containing EDTA and β-mercaptoethanol

by batch method using crystalline G6PD is approximately 0.4 mg enzyme per ml swollen gel (2, 5 ADP Sepharose 4B handbook). The presence of all the red blood cells and the other dehydrogenases will not however allow for the effectiveness of this binding capacity. The passage of the hemolysate through the column always took about seven to nine hours, long time enough for some of the enzymes to be denatured. All these shortcomings are therefore probably responsible for the low yield of the enzyme, about 30 to 40 percent from the sepharose column, and consequently the final overall low yield.

The specific activity of the final preparation of the Mould G6PD variant was 207 units per mg. That for the B G6PD was 174 units per mg. These compared well with the values obtained by other workers (65, 115, 117). The specific activity coupled with the homogeneity on SDS PAGE show that the final preparations are of very high purity.

The final enzyme preparation was very stable over a long period of time, having as much as 95 percent of the original activity after about a month and half period of storage.

4.1.2. COMPARISONS BETWEEN THE VARIANTS

From table 3.1, we can see that the yields of the Mould and B G6PD, types are 7.13 and 6.95 percent respectively. The transition temperature for the Mould variant (fig. 3.3) and the B G6PD (96) at 50μM $NADP^+$ are 47.5 and 47.8°C respectively. These show that the two

G6PDs have about the same stability.

The purification factor for the Mould and B G6PD types are 13250 and 15263 respectively. This thus mean that the B enzyme is purified much more than the Mould variant although the Mould variant has the higher specific activity.

Electrophoretic mobility on starch gel (Plate 3.1) and cellulose acetate plate show that the Mould variant is a slower variant than the normal B enzyme (142). It has a mobility of 93.15 percent of the normal B enzyme. The red blood cell activity of the Mould G6PD is also about 113 percent of the B G6PD, a higher activity of the Mould enzyme over the normal type. In mobility the Mould G6PD is similar to other variants like the Baltimore-Austin and Madrona G6PD (132, 137), although all these have lower activity than the B G6PD unlike the Mould variant (142).

4.2.1. pH DEPENDENCE OF V_{max}

The variation of V_{max} with pH shows the effect of ionization of groups on those enzyme forms present at infinite substrate concentrations. Figure 3.6 shows an apparent drop in log V_{max} in the low pH region (6.0 - 7.5), but no clearcut drop at high pH values. According to Babalola et al (97), the hypothesis which can best explain the progressively steep decrease of log V_{max} as the pH decreases is that there is an ionizable group, which in its conjugate acid form, renders the enzyme substrate complex inactive. This leads to equation 3.4 :

$$\frac{1}{V_{max}} = \frac{1}{V_{alk}} + \frac{(H^+)}{V_{alk}\ K_a}$$

The pK_a of the ionizable group determined from the ratio of the intercept to the slope of the plots of $1/V_{max}$ against (H^+) (Figure 3.9) is 6.64 ± 0.11 and 6.47 ± 0.13 at 34°C for the B and Mould variants respectively. Values of the pK_a at various temperatures plotted against 1/T for all the variants gave straight lines (Figure 3.10), and within experimental error the pK_a has the same temperature dependence for all variants, and the enthalpy of ionization calculated from the slope of the best line through the points is 29.05 ± 1.60 kJ $mole^{-1}$ for the B enzyme and 25.64 ± 4.56 kJ $mole^{-1}$ for the Mould enzyme. Thus, the ionization constant and the enthalpy of ionization of the groups which distinguish these variants is not affected by the amino acid substitution. Tables 3.4 and 3.5 show the ionization constant and the enthalpy of ionization of the ionizable group for the two enzyme types in this work, and that obtained also for the binding of G6P to G6PD by Babalola et al (97), and for the binding of $NADP^+$ to G6PD by Adediran (180).

If the ionization of the group responsible for the activation of the enzyme is not accompanied by a configurational change in the protein, values obtained in this work for the pK_a and for the enthalpy of ionization suggest that the group is not the secondary phosphate group of either substrate, since ΔH_a^o values for the secondary ionization of sugar phosphates is close to zero. The pK_a of the

secondary phosphate ionization of G6P is 6.565 at 20°C, while the standard enthalpy of ionization is -3.70kJ mole^{-1} (97). Both the pK_a and enthalpy of ionization are lower than the typical values of 7.6 to 8.4, and 42.0 to 54.6kJ mole^{-1} respectively for a terminal amino acid. Thus the most likely group implicated would seem to be the imidazolium group of a histidine residue. This is because the pK of the imidazole group ranges between 5.6 and 7.0, with an ionization enthalpy of 28.84 to 31.35 kJ mole^{-1} (203). This same group had also been previously indicated in a similar study by Babalola et al (97), and by Adediran (180) in the binding of $NADP^+$ to G6PD.

The variation of the activation energy with pH (figure 3.8) can be readily accounted for in terms of the enthalpy of ionization of the group responsible for the activation of the enzyme. It has been shown by Babalola et al that since $(H^+)/(\bar{K}_a + (H^+))$ is the fraction of the group that is dissociated at any pH value,

$$E_a = E_{alk} + \frac{(H^+)}{\bar{K}_a + (H^+)} \Delta H^o_a \quad \ldots\ldots \quad \ldots\ldots \quad 4.1.$$

where $\bar{K}_a$ is the average ionization constant over the temperature range used in the measurement of the activation energy, and E_{alk} is the activation energy when all the enzyme is in the conjugate base form. A best estimate of E_{alk} was obtained as follows :

$$V'_{max} = \frac{K_a + (H^+) \cdot V_{max}}{K_a}$$

V'_{max} is the maximum velocity that would be obtained if all the enzyme

remained in the conjugate base form throughout the pH region. Because we have shown that the variation of log V_{max} with pH can be ascribed to a single ionizable group, log V_{max} should be pH invariant at all temperatures. Apart from small variations noted for the two G6PD types, this is found to be the case. For each variant, we take the average of these values as the best value of V_{alk}. E_{alk} is then calculated from these values of V_{max}. Figure 4.1 shows the plots of E_a calculated from equation 4.1 against pH for the Mould and B G6PD types. Superimposed are the experimental points, Table 4.1 gives the values of E_{alk}, $\bar{K}_a$ and ΔH_a^o for G6PD B and Mould. The variations of the fits with pH for the two G6PD types are similar, although the calculated values for the B enzyme fit the experimental points better especially in the alkaline region. The experimental values for the Mould enzyme deviate appreciably from the calculated values at the alkaline region.

TABLE 4.1

TABLE OF E_{ALK}, K_A AND ΔH_a^o FOR THE TWO VARIANTS, B AND MOULD

G6PD TYPE	E_{ALK} (kJ MOLE^{-1})	$\bar{K}_A$ (x 10^{-7})	ΔH_a^o (kJ MOLE^{-1})
B	44.67	1.82	29.05
MOULD	29.51	3.24	25.64

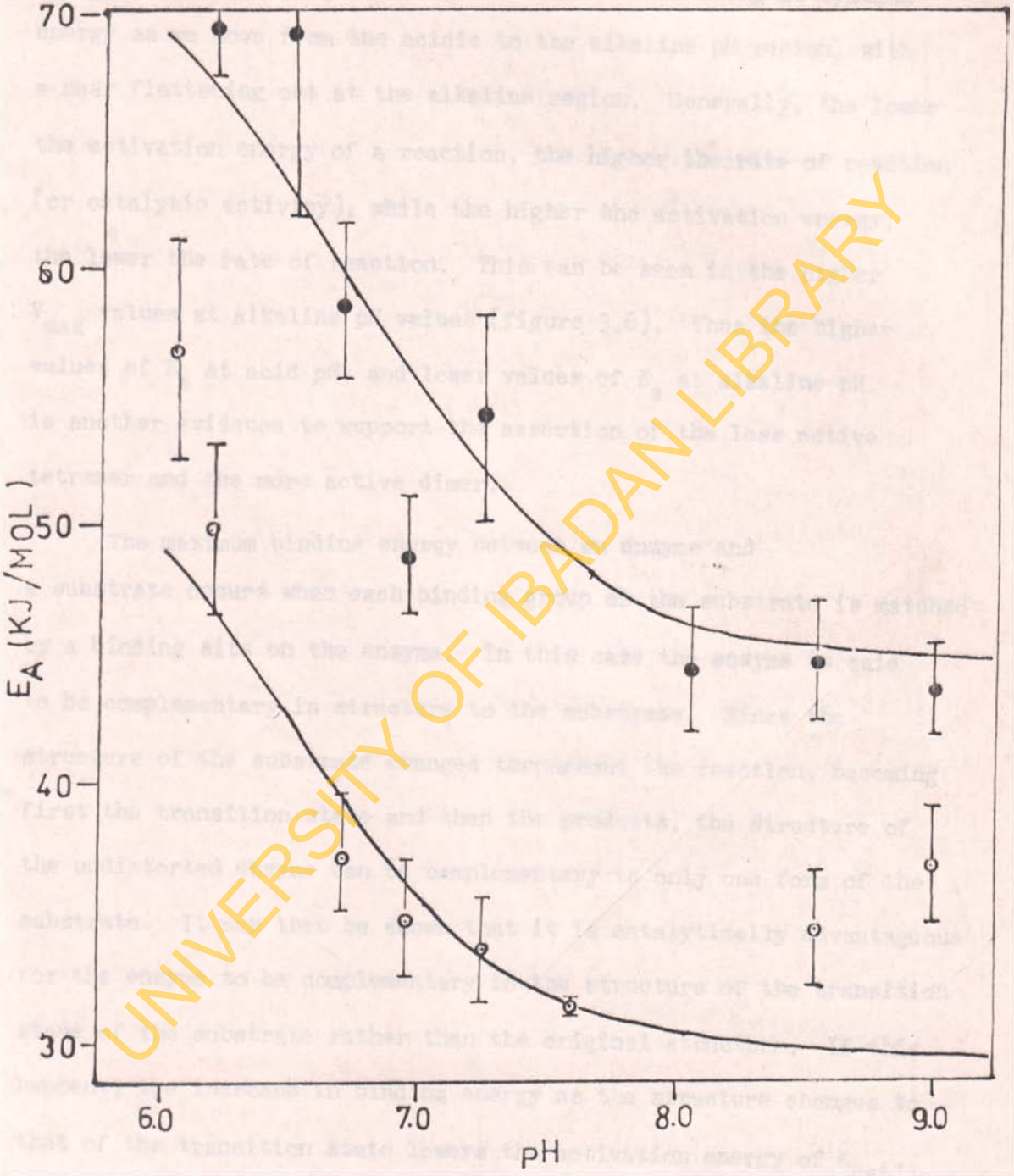


Fig. 4.1: Plots of E_A calculated from equation 4.1 against pH. Experimental points are (●) for G6PD B; and (0) for Mould G6PD.

The general trend of these plots is that of decreasing activation energy as we move from the acidic to the alkaline pH region, with a near flattening out at the alkaline region. Generally, the lower the activation energy of a reaction, the higher the rate of reaction (or catalytic activity), while the higher the activation energy, the lower the rate of reaction. This can be seen in the higher V_{max} values at alkaline pH values (figure 3.6). Thus the higher values of E_a at acid pH, and lower values of E_a at alkaline pH is another evidence to support the assertion of the less active tetramer and the more active dimer.

The maximum binding energy between an enzyme and a substrate occurs when each binding group on the substrate is matched by a binding site on the enzyme. In this case the enzyme is said to be complementary in structure to the substrate. Since the structure of the substrate changes throughout the reaction, becoming first the transition state and then the products, the structure of the undistorted enzyme can be complementary to only one form of the substrate. It may then be shown that it is catalytically advantageous for the enzyme to be complementary to the structure of the transition state of the substrate rather than the original structure. If this happens, the increase in binding energy as the structure changes to that of the transition state lowers the activation energy of K_{cat}. Conversely, if the enzyme is complementary to the structure of the unaltered substrate, the decrease in binding energy on the formation

of the transition state will increase the activation energy of K_{cat}. The concept of enzyme - transition state complementarity was introduced by Haldane (204) and elaborated upon by Pauling (205).

When the enzyme is complementary to the initial substrate, so that the binding of the initial enzyme - substrate will be good, the K_m will be low. However, the formation of the transition state will lead to a reduction in binding energy as the substrate goemetry changes to give a poorer fit, resulting in the lowering of K_{cat}. When the enzyme is complementary to the transition state an adverse energy term in the initial enzyme - substrate complex will increase K_m, but the gain in binding energy as the reaction reaches the transition state will increase K_{cat}. In this study, in the acid region (pH 6.0 - 7.0) where we have predominantly tetramers for the binding of G6P to G6PD at saturated $NADP^+$ concentration, we obtained low values for K_m and V_{max}, and high values for the activation energy, of K_{cat} ($V_{max} \propto K_{cat}$). In the alkaline region, where we have predominantly dimers (pH 8.0 - 9.0), we obtained comparatively, high values for K_m, V_{max} and low values for the activation energy. Thus going by the concept of enzyme - transition state complementarity, it could be possible that in the acid region where we have tetramers, the enzyme is complementary to the initial substrate. However, in the alkaline region where we have predominantly dimers, complementarity shifts from the initial

substrate to the transition state complex. This shift could be due to the differences in size and ternary structure between the dimers and tetramers.

Comparison of the activation energies obtained for the Mould and B G6PD types shows that the values obtained for the Mould are always lower than that for the B G6PD. This thus shows the greater reactivity of the Mould G6PD than the B G6PD.

4.3.1. pH DEPENDENCE OF K_m

The variations of K_m with pH usually indicate the effects of ionizations in the free substrates and the enzyme forms reacting with them.

It was pointed out by Dixon (151) that if a graph of log K_m is plotted against pH, the results could easily be interpreted by a few simple rules. He stated that the graph will consists of straight line sections joined by short curves, and that each bend will indicate the pK of an ionizing group in one of the components, and the straight line portions when produced intersect at a pH corresponding to the pK. Each pK of a group situated in the ES complex produces an upward bend, i.e. an increase of positive slope with increase of pH, or in other words, a bend with the concave side upwards while each pK of a group situated in either the free enzyme or the free substrate produces a downward bend.

Any interpretation of the variation of log K_m with pH will depend on whether K_m is the dissociation constant of the enzyme-substrate complex or whether it is a ratio of velocity constants. There is evidence that K_m for the binding of $NADP^+$ can be identified with a dissociation constant (106, 107, 179). Babalola et al (97) in their study of the binding of G6P to G6PD also did assume the same (for an $NADP^+$ saturated enzyme). Thus like Babalola et al (97) the same assumption will be made in this study. Inspection of the plots of log K_m against pH for the two G6PD types (figure 3.11) show that the variations of log $K_{m_1}^{G6P}$, and log $K_{m_2}^{G6P}$ are similar for both variants. This is an evidence which shows that the sites for the low and high affinity states are close to each other, and are also not independent.

In the acid region (pH 6.05 - 6.95) (figure 3.11), there is a general increase in log K_m with pH, with a maximum at about pH 6.95 for the B G6PD, while for the Mould variant, there is a sharp increase in log K_m with pH between 6.05 and 6.20, and then a plateau between 6.20 and 6.95. According to Dixon's rules, the downward bend at around pH 6.2 and 6.95 for the Mould and B G6PD types respectively represent the pK of one of the substrates or of a group on the enzyme. Soldin et al in two different works using plots of log K_m versus pH had previously obtained pK values of 6.2 (206) and 6.8 (156) for G6P binding to human erythrocyte G6PD, and these they associated with the involvement of a histidine residue in the interaction of the free enzyme with its substrate. Thus the observed pK values in this

work are most probably associated with a histidine residue also.

In the alkaline region, there is a gradual increase in log K_m with pH as from pH 8.06. This is especially noticeable for the Mould variant. According to Dixon's rules, this upward bend at about pH 8.06 represent the pK of groups on the ternary complex of G6PD-$NADP^+$-G6P. The pK of sulfhydryl groups in proteins have been given as being between 8.0 to 9.0 (203). Thus the observed pK is probably associated with a sulfhydryl group of a cysteine residue being involved in the catalytic mechanism.

Babalola et al (97) and Soldin et al (156, 206) had previously independently implicated the presence of a sulfhydryl and imidazole groups in the binding of G6P to G6PD.

The valley with the minimum at pH 7.25 observed for the two variants in the pH range of 6.90 to 7.60 require a more complex explanation. Generally, K_m will show a pH dependence if there are ionizable groups, the pK values of which are different on the enzyme and on the enzyme-substrate complex. The observed minimum implies that more than one ionizable group is involved, and the fact that the values of log K_m in acid and alkaline solution just around the valley are about the same implies that the net uptake of protons across the pH range where the extremum occurs is about zero. The pH range over which log K_m varies, however is so narrow that it cannot be accounted for by any number of independent ionizable groups. Thus cooperativity between ionizable

groups is highly probable. Babalola et al (97) also observed this phenomena in their similar study, and also postulated cooperativity between the ionizable groups.

Babalola et al (97) noted that there are different conditions under which the observed variation of log K_m could arise. However, the one most plausible is that if there is a set of cooperatively ionizing groups on the enzyme, and if upon binding of the substrate the degree of cooperativity changes, log K_m will show an extremum with pH, the sharpness of which will still depend on the number of groups and on the degree of cooperativity. The pH at which the extremum occurs will depend on the intrinsic pK of the ionizing groups. If the cooperativity decreases upon binding of substrate, log K_m will show a minimum with pH; if cooperativity increases upon binding of a substrate, the log K_m will show a maximum with pH.

Babalola et al (97) described the cooperative ionization of the linked ionizable groups by an empirical equation analogous to the Hill equation used to describe the cooperative binding of oxygen to hemoglobin (207). This equation is given as :

$$\log K_m = \log K_m^a + \frac{n}{p} \log (K_E + (H^+)^p) - \frac{n}{q} \log (K_{ES} + (H^+)^q) \quad .. \quad 4.2.$$

where log K_m^a is the value of log K_m at large (H^+) and is given by the plateau value of log K_m on the acid side of the extremum; n is the number of ionizing groups, and p and q are "Hills constants"

for the ionizable groups on the enzyme and on the enzyme-substrate respectively. K_E and K_{ES} are the ionization constants for the enzyme and enzyme-substrate complex respectively and are given by :

$$K_E = (H^+_{min})^p$$

and $$K_{ES} = K_E^{\,q/p}$$

where H^+_{min} is the value of the (H^+) at the position of the minimum value of K_m, K_m^{min}.

Values can be chosen arbitrarily for n and p while q is calculated using the expression.

$$\log K_m^{min} - \log K_m^a = \frac{n(q-p)}{pq} \log 2 \quad \ldots\ldots \quad 4.3.$$

For this work, values of log K_m as a function of pH were calculated using the known values of H^+_{min}, log K_m^{min} and log K_m^a for the various choices of n and p. Figure 4.2 shows the fitted curves for the Mould and B G6PD types at 20°C, superimposed on their respective data. For both variants, n = 8 and p = 6.5. The calculated value of q is 5.34 and 5.11 for the Mould and B G6PDs respectively. In molecular terms, this means that there are eight ionizable groups, which ionize cooperatively with a Hill constant of 6.5 in the enzyme for both variants, and 5.34 and 5.11 in the enzyme-substrate complex for the Mould and B variants respectively. Table 4.2 gives the values of K_E, K_{ES}, n, p and q for the two G6PDs

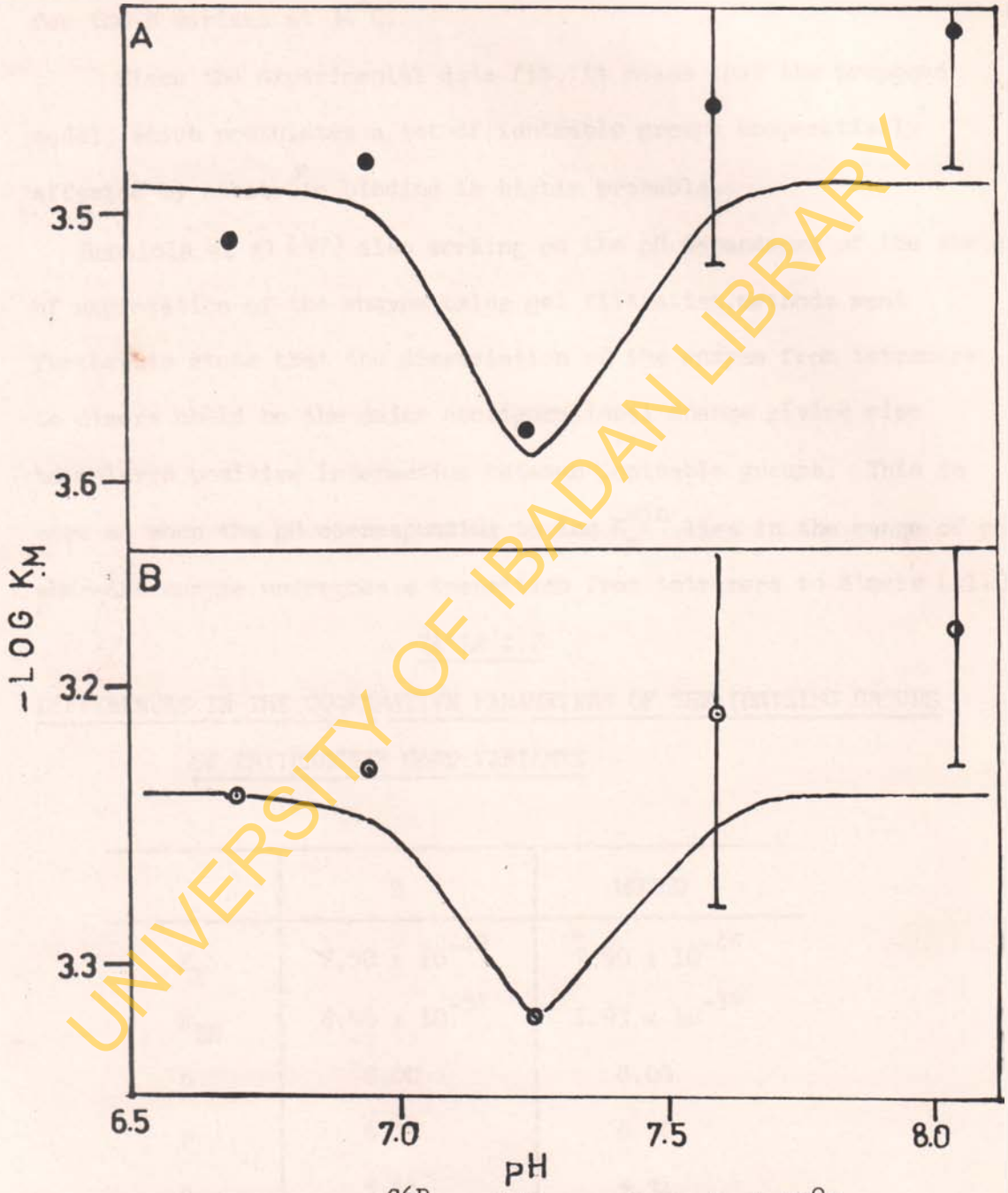


Fig. 4.2: Data fit of log $K_{m_1}^{G6P}$ variation against pH at 20°C with experimental points superimposed on plots. (●) - B variant; (0) - Mould Variant.

at 20°C. The values of n = 8 and p = 6.5, but q = 4.11 had been previously used by Babalola et al (97) for the binding of G6P to G6PD for the B variant at 34°C.

Since the experimental data fit, it means that the proposed model, which postulates a set of ionizable groups cooperatively affected by substrate binding is highly probable.

Babalola et al (97) also working on the pH dependence of the state of aggregation of the enzyme using gel filtration methods went further to state that the dissociation of the enzyme from tetramers to dimers could be the major configurational change giving rise to a large positive interaction between ionizable groups. This is more so when the pH corresponding to log K_m^{min} lies in the range of pH wherethe enzyme undergoes a transition from tetramers to dimers (111).

TABLE 4.2

DIFFERENCES IN THE COOPERATIVE PARAMETERS OF THE IONIZING GROUPS OF ERYTHROCYTE G6PD VARIANTS

	B	MOULD
K_E	7.50×10^{-48}	7.50×10^{-48}
K_{ES}	8.96×10^{-38}	1.93×10^{-39}
n	8.00	8.00
p	6.50	6.50
q	5.11	5.34

The observed variation of enthalpy change with pH for the enzyme - G6P complex (figure 3.13) for the two affinities for each variant on one hand, and for the two variants on the other hand are similar. They show two minima with one maximum at pH 7.25. The position of this extremum correspond to the position of the abrupt changes in the log K_m versus pH profile for the two variants. With the dissociation of the tetramer to the dimer at this near neutral pH (111), the regions of the two minima in the ΔH plot might be associated with the regions of the pH range where there is a preponderance of tetramers at acidic pH and dimers at alkaline pH. Although the variations of enthalpy with pH for the G6PD B and Mould variants are the same, the magnitude of the enthalpies for the B variant are consistently higher than that of the Mould variant at all pH values.

In summary, the observed pH variation of log K_m for the two variants can be explained as follows . In the acid region at about pH 6.0 to 6.95, there is a pK value associated with the involvement of a histidine residue in the interaction of the free enzyme with its substrate. In the alkaline region the variants have a pK value of about 8.1 which is probably for sulfhydryl group of a cysteine residue, associated with the binding of G6P to the enzyme. At the ionic strength of 0.01, the tetramers of the two variants saturated with $NADP^+$, but not with G6P dissociate into dimers in the pH region of 6.9

to 7.6. This dissociation is accompanied by the cooperative ionization of at least eight groups.

Comparing therefore the kinetics and thermodynamics of the two variants studied in this work, it can be seen that there is no significant difference in the variation of log K_m, log V_m, ΔH and E_a with pH, both for the two enzymes. However, in terms of the magnitude of energies involved, there is an appreciable difference between those of the Mould and B variants. Genetic differences have been ascribed to single amino acid replacements between the A, B and Hektoen G6PD variants (69, 70). Single amino acid replacements have also been implicated in the detectable differences in the thermodynamics of G6P binding to G6PD variants A, B and A^-. Thus the appreciable differences in the magnitude of energies involved in the binding of G6P to the Mould and B variants could be attributed to single-amino acid replacements in the Mould enzyme. There is also no reason to believe that the replaced amino-acid is physically a part of the binding site; if it were we would expect differences in the variation of K_m and ΔH with pH.

4.4.1. COOPERATIVITY IN G6P BINDING TO G6PD

The kinetics of G6P binding to G6PD at saturated $NADP^+$ concentrations studied by most workers so far (97, 111, 141, 155) have always conformed to the Michealis Menten kinetics. The saturated function in all these studies have been hyperbolic at all pH values and temperatures. With these findings, these workers postulated

independent and identical or only one binding site for G6P binding to the enzyme molecule.

However, the results obtained in this study using higher G6P concentrations (0.1mM to 3.0mM) show otherwise. Figure 3.4 shows a typical plot of v/V_{max} against (G6P). These plots are not hyperbolic but sigmoid. Figure 3.5 also shows typical plots of $1/v$ against $1/S$, and S/v against S. These plots all give two straight lines with two K_m values thus showing the presence of cooperative type of kinetics, instead of the normal Michealian kinetics. Determination of the interaction coefficient n, using the Hills plot of log $v/(V_{max} - v)$ against log (G6P) gave n values of magnitude less than one. These values thus show the existence of negative cooperativity in G6P binding to G6PD. All these show that in the binding of G6P to G6PD more than one site is invloved. Again these sites are not independent, since if they were, they will not interact with each other.

It is easily assumed that as long as the concentration of one substrate is maintained constant, the two substrate case can be regarded as being analogous to the single substrate case. Pettigrew and Frieden (160) have however shown that this analogy only holds up to a point, beyond which the two substrate case shows substantial differences from the single substrate case. Paulus et al (177) and Goldbeter (178) in the analysis of the single substrate case of the model of Monod et al (169) showed that kinetic negative cooperativity is related to conditions where the enzyme state with the lower

catalytic activity has the greater affinity for the substrate. Pettigrew et al (160) in their observation of kinetic negative cooperativity in the extension of the Monod et al model (169) to the two substrate case noted that optimum conditions for the observation of kinetic negative cooperativity occurred when both enzyme states have different affinities for both substrates, and that the occurrence of kinetic negative cooperativity is dependent on the concentration of the non-varied substrate. Thus they concluded that the occurrence of kinetic negative cooperativity in the single substrate case of Monod et al (169) are thus apparently equally applicable to the extension of the model to the two substrate case. Results obtained for the B and Mould $G6P_D$ types in this work have shown that when the enzyme is in the tetrameric form the affinity for G6P is high while in the more active dimeric form, the affinity for G6P becomes lower. Again, the affinities of G6P and $NADP^+$ for the enzyme differ, for the two enzyme states. With these observations therefore, the occurrence of negative cooperativity in the binding of G6P to the enzyme is not abnormal.

The kinetics of $NADP^+$ binding to G6PD has been found to be positively cooperative (106, 107, 180). The binding of G6P to G6PD has also been found to be negatively cooperative in this study. This difference in the types of cooperativity exhibited by G6P and $NADP^+$ is not strange however, since Pettigrew et al (160) in their studies on two substrate cases have found instances where one

substrate exhibited positive cooperativity, while the other negative cooperativity. It has already been observed that the binding of G6P and $NADP^+$ to G6PD at the pH range of 6.9 to 7.6 involve the cooperative ionization of groups on both the enzyme and enzyme-substrate complex (97, 180). This cooperative ionization of groups in this pH range has also been observed in this work.

It is possible that the non observation of cooperativity in the binding of G6P to G6PD by the other workers can be associated with the concentration range in which they carried out their kinetic measurements. Most of these workers had worked in concentration ranges less than or equal to 1mM (97, 137, 156). An inspection of the plot of v/V_{max} against (G6P) in this study (figure 3.4) shows that at about 1mM or less, the binding of G6P to the enzyme will be at the high affinity site. Thus the data presented by the other workers is for the high affinity state.

Chung et al (89) have shown that an intimate relationship exists between the erythrocyte enzyme and its cofactor $NADP^+$. The coenzyme stabilizes the enzyme (4, 5), activates it and protects it from inactivation by various agents (5). There have been some evidence that the coenzyme is tightly bound to the enzyme (96) and that this bound nucleotide determines the stability and structural integrity of the catalytically active enzyme molecule (89). G6P especially at high concentrations, on the other hand, induces the dissociation of the active dimer to the inactive monomer (83, 89).

Wrigley et al (83) in their electron microscope studies on the structure, and subunit association and dissociations, effected the dissociation of the dimer with about 1.0 to 3 mM G6P. The decrease in affinity of the enzyme for G6P in the concentration range of about 1.0mM and above in our kinetic studies therefore, could probably be a control mechanism by which the enzyme prevents its dissociation from the active form to the inactive form. This change from high affinity to the lower affinity state as the concentration of G6P is increased will also give the negative type of cooperativity. Omachi et al (208) and Yoshida (102) have shown that in human erythrocytes the concentration of $NADP^+$ is about 2μM, a concentration which favours the dimeric form of the enzyme. High concentrations of G6P at this low $NADP^+$ concentration will obviously result in the dissociation of the enzyme to the inactive form. Thus the decrease in affinity of the enzyme for G6P, as observed in this study, is probably the manner such dissociation will be prevented in the erythrocyte.

Table 3.8 shows the values of the interaction coefficient at different pH values for both the Mould and B G6PD variants. Figure 3.15 shows the variation of interaction coefficient with pH at a temperature of 27°C and ionic strength of 0.01. The interaction coefficient decreases with pH (reflecting increase in cooperativity), and reaches about a minimum in the predominantly alkaline region. pH had been shown to be a very critical parameter in the determination of molecular forms of G6PD (83, 84). Hence at acid pH, the tetrameric form is present predominantly and at alkaline pH, the dimer is the

dominant enzyme form. Cancedda et al (96) and Bonsignore et al (179) working independently on the binding of $NADP^+$ to G6PD, observed that the cooperative enzyme with two dissociation constants for the G6PD-$NADP^+$ complex is the dimer while the non-cooperative enzyme with a single dissociation constant is the tetramer. Results obtained could therefore be interpreted to mean that in the dimeric form, the binding sites of G6P on the enzyme are very accessible to each other for interaction, while in the tetrameric form, the sites become less accessible to each other for interaction. This is probably due to change in the conformation of the enzyme in moving from the dimeric to the tetrameric form.

At constant temperature and ionic strength, n decreases with pH (meaning increase in cooperativity). Increase in pH therefore promote the dimeric form of the enzyme, suggesting that groups that tend to ionize as pH increases are linked to dimer formation in the enzyme.

It can thus be concluded from this work that at high enough G6P concentrations negative cooperativity is observed in the binding of G6P to G6PD at saturated $NADP^+$ concentration. Also while the tetrameric form is moderately cooperative, n for them almost approaching one as temperature and pH is decreased, the dimeric forms are highly cooperative.

4.5.1. COMPARISON OF THE MOULD G6PD VARIANT WITH OTHER G6PD VARIANTS FROM WEST-AFRICA

So many glucose-6-phosphate dehydrogenase variants have been

described that it has become very difficult to determine whether or not a newly discovered variant is distinct from any other. This difficulty can be partially overcome by determining a number of physico-chemical properties and comparing the values with those already reported for the human variants.

Table 4.3 shows most of the G6PD variants from West-Africa which have been characterized up to date, according to the W.H.O. (130) recommendations, and those characterized in this study (i.e. B and Mould types). Although the ubiquitous B enzyme has been characterized previously by a number of authors, it was characterized again in this study, for comparative studies with the new sporadic variant, the Mould G6PD, using the same purification procedure and thus the same enzyme purity. It was further rationalized that the comparison of the properties of B obtained in this study with B from previous studies will indicate if the Mould variant can be compared with the other previously characterized variants from West Africa, considering the higher purity of enzymes prepared by us to those of previous workers.

Carrying out a side by side comparison of the B variant from this study and previous studies (Table 4.3), it can be seen that the electrophoretic mobility, red blood cell G6PD activity, K_m^{G6P}, pH optimum and activation energy are all similar. K_m^{NADP} for our study is 4.9± 0.7 M while that for previous studies is about 2.9 to 4.4 M. These however are not significantly different. While 58.5°C was

obtained as the transition temperature by Usanga et al (140) from their thermostability studies on G6PD B, the value obtained by Cancedda et al (96), whose conditions were mimicked in determining the transition temperature of the Mould enzyme was 47.8°C. While the conditions of determination of the transition temperature by Cancedda et al (96) (in terms of $NADP^+$ concentration etc) were not the same as those of Usanga et al (140), Cancedda et al (96) also pointed out that the differences in the degree of purity (and therefore presence of contaminating proteins) did also contribute to the difference in transition temperature.

From the similarities obtained between the properties of the B enzyme characterized previously and in this study, the Mould enzyme can therefore be compared with the other already characterized West African G6PD variants, so as to confirm if the Mould G6PD is really a new variant or the same type as one of the previously described variants.

A side by side comparison of the Mould enzyme with other West African G6PD variants (Table 4.3) shows that it has a higher red blood cell G6PD activity than any of the previously described variants. In terms of electrophoretic mobility, it also differs from all the other variants apart from the Abeokuta and Lanlate variants which have similar mobility as it. The affinity of the enzyme for G6P is similar to those of the A, A^-, Ijebu-Ode and Ibadan - Austin variants, while

TABLE 4.3

PROPERTIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE VARIANTS FROM WEST AFRICA

Enzyme Type	R.B.C. G6PD ACT (%)	Electro-Phoretic Mobility (%)	K_m^{G6P} (M)	K_m (2-deoxy G6P) (%)	K_m^{NADP} (M)	K_i^{NADPH} (M)	Transition Temp. (°C)	pH Optimum	Activation Energy (kJ Mole^{-1})	References
B	100	100	72	< 4	2.9-4.4	30	58.5	Normal	45.02*	23,128, 140,180*
A	90	110	63.10	< 4	2.9-4.4	16	Normal	Normal	32.82*	
A$^-$	8 - 20	110	64.6	< 4	3.0-4.5	-	Normal	Normal	42.59*	
Adame	100	96	46	8.7	19.0	10	58.5	-	-	140
Abeokuta	100	91	81	1.9	7.7	35	57.0	-	-	140
Lanlate	100	91	40	4	20.6	-	-	-	-	140
Ekiti	100	72	53	-	-	-	-	-	-	140
Ilesha	25	75	83	-	-	-	-	-	-	140
Ijebu-Ode	100	84	60	-	24.0	38	58.0	-	-	106, 141
Ita-Bale	100	65	91	2.5	11.0	-	57.0	-	-	106, 141
Mali	1-5.2	100	8-20	18-43	3.2-5.5	-	-	-	77.80	137
Dakar	4 - 5	110	27-30	< 6	3.5-5.5	-	-	-	49.14	137
B2	100	100	57± 5	4 - 8	12.0	-	Normal	-	-	209
A2	90	110	47	5	12.0	-	Normal	-	-	209
Gambia	78	85	58	3.9	5.3	-	Normal	Normal	-	138
Ibadan-Austin	72	80	62-72	< 4	3.3	-	Normal	Normal	-	132
Mould	113	92	64.3±3.7	-	18.5±1.7	-	47.5	Normal	38.54	This Work
B	110	100	71.7±6.0	-	4.9±0.7	-	47.8*	Normal	44.19	This Work, 96*

the affinity for $NADP^+$ is different from all but the Adame and Lanlate G6PD variants. The transition temperature is normal that is similar to that of the B variant just like the A, A^-, Adame, Ijebu-Ode, A2, B2 Gambia and Ibadan - Austin variants. The pH optimum is also normal when compared with the B variant. The activation energy of the Mould enzyme is quite different from that of all other West African variants determined so far. Apart from the A variant with an activation energy of 32.82 kJ $mole^{-1}$, it has the lowest activation energy with a value of 38.54 kJ $mole^{-1}$ at the same pH. From these observations therefore, it can be seen that though the Mould variant is similar to the other variants in some of its properties, it is not similar to any of the variants in all of its properties. Thus it can be positively concluded that the Mould G6PD variant is a new G6PD variant, with its own unique properties.

4.6.1. CONCLUSION

The isolation of the B and Mould G6PDs using the affinity chromatography method gave a yield of about 7 percent and specific activity of about 170 to 210 units per milligram for the two G6PDs. From the data obtained, it was observed that the two G6PDs have about the same stability although the Mould variant is a slow variant associated with a slightly higher red cell G6PD activity.

The variation of V_{max} with pH for the B and Mould G6PD enzymes was similar. There was a sharp rise in V_{max} in the acid region followed by a gradual flattening out in the alkaline region. On

the average, the maximum velocity values for the Mould variant were always higher. As had been previously shown by other workers (97, 156, 206) the variation of V_{max} with pH and temperature in this study were compatible with the presence of a single ionizable group. The pK obtained for this group was 6.64 and 6.47 at 34°C for the B and Mould G6PD respectively, while the enthalpy of ionization was 29.05 ± 1.60 and 25.64 ± 4.56 kJ $mole^{-1}$ for the B and Mould G6PD respectively. The probable group associated with these pK values was the imidazole group of a histidine residue.

The dependence on pH of Km for the two G6PDs was also similar, except in the acid region where there were some differences. For the Mould enzyme, there was a sharp increase in Km from about pH 6.0 to 6.2, after which there was a plateau till about pH 6.95 followed by an extremum with a minimum at pH 7.25. For the B enzyme, there was a gradual increase in Km till about pH 6.95 after which there was also an extremum at pH 7.25. For the two G6PDs, there was a gradual increase in Km in the alkaline region, although the increase was sharper for the Mould enzyme. The Km variations for the two G6PDs also showed that the enzyme has a higher affinity for G6P in the acid region (when in the tetramer form), than in the alkaline region (when in the dimer form). The variations of Km_1^{G6P}, and Km_2^{G6P} for each enzyme type were also similar. The variation of Km with pH for the two G6PD types like the report of Babalola et al (97) and Soldin et al

(156, 206) for B type enzyme indicated the presence of an imidazole group in the binding of the free enzyme to the substrate in the acid region, and a sulfhydryl group on the enzyme-substrate ternary complex in the alkaline regiln.

The pH dependence of Ea for the two G6P types were similar. The variation of Ea with pH involved a gradual decrease in Ea as we moved from the acid to the alkaline region. The Ea values obtained for the Mould enzyme were however always lower than those obtained for the B enzyme, showing the greater reactivity of the Mould enzyme, when compared to the B enzyme. The pH dependence of ΔH for the two affinity states of each variant and for the two variants were also similar. The variation of ΔH with pH gave rise to two U shaped curves intersecting at pH 7.25, the pH of dissociation of the tetramer to the dimer.

Two types of cooperativity were observed for G6P binding to the Mould and B G6PDs in this study. In the pH region of 6.9 to 7.6, the region of the dissociation of the enzyme from tetramer s to dimers, cooperative ionization of groups on the enzyme and enzyme-substrate complex was observed. This type of cooperativity had been previously reported by Babalola et al (97) and Adediran (180). However, in the pH region of 6.0 to 9.0, negative cooperativity was observed in in the binding of the substrate, G6P, to the enzyme. This implied that there are at least two G6P binding sites which interact with each other on the enzyme molecule. This cooperative phenomenom

found among the G6P binding sites in this study have not been observed before. The kinetics of G6P binding to G6PD in human erythrocytes have always been assumed to be Michealian in nature. This assumption however is due to the concentration ranges utilized in kinetic determinations of G6P binding to the enzyme. The negative cooperativity observed in this study, has been associated with the probable mechanism by which the dissociation of the enzyme to the inactive monomer form is prevented at high G6P concentrations. Wrigley et al (83) and Chung et al (89) have previously observed that high concentrations of G6P cause the dissociation of the active form of the enzyme to the inactive form. Variation of the interaction coefficient with pH, gave results indicative of a more cooperative dimer and a less cooperative tetramer.

The properties of the Mould G6PD variant were compared with those of other G6PD variants that have been characterized in West Africa, other than the polymorphic G6PD B enzyme in this study. Differences in its red blood cell G6PD activity, electrophoretic mobility, K_m^{G6P}, K_m^{NADP}, Ea, and its other properties when compared to their's, showed that it is quite distinct, establishing the fact that it is a new sporadic variant. Similarities in the variations of both its kinetics and thermodynamic functions with pH when compared to the B normal enzyme however, suggested that the structural locus i.e. the position of amino acid changes that differentiate between the G6PD

variants, is not part of the binding site for G6P but away from it.

In general therefore, it can be concluded that a new sporadic G6PD variant, the Mould G6PD, associated with polycythaemia rubra vera (PRV) has been identified. The kinetics of the G6P binding to G6PD B and Mould is complex and show negative cooperativity.

CHAPTER FIVE

SOLVENT EFFECTS ON THE THERMODYNAMICS AND KINETIC REACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

5.1.1. INTRODUCTION

Over the years, a lot of work has been done involving proteins. Structural information about proteins including the conformational extremes and the interactions between the proteins and their ligands have been refined to high resolution by means of X-ray crystallography (210–212). Studies of molecular dynamics of proteins using both low temperature ligation measurements (213), hydrogen exchange kinetics (214) and energy minimization calculations have provided insight into protein function. Kinetic and thermodynamic studies of enzymes, and proteins in general using spectroscopic techniques (162) have also shed light on basic mechanisms of protein function.

The solvent is an important determinant of the overall structure of macromolecules. The experimental works on protein dynamics (186, 188) and existing theoretical models (187, 188, 215-217) indicate a more intimate interaction between the protein and solvent than heretofore appreciated. This interaction is of great importance, when we also regard the possible heterogeneity and

variability of intracellular microenviroments.

Despite the extensive accumulation of experimental data and theory however, the role of the solvent as a functional component remains poorly understood. From structural, energetic and dynamic points of view, descriptions of interactions between the protein and surrounding solvent molecules are still primarily qualitative. This is not to say that the general area of protein "hydration" and/or "solvation" has been ignored. Rather it is the functional aspects of the protein solvent interactions that remain essentially uncharacterized. Thus in the case of hemoglobin or an enzyme like G6PD, one may ask if it is possible to define the role that protein-water interactions play in the transfer of free energy between the binding sites of the allosteric effectors and oxygen binding sites, at the heme groups in the case of hemoglobin, and between say the binding sites of the different ligands in the case of G6PD.

Although the equilibrium between a protein and its solvent is likely to be of functional relevance, the effect of changes in water activity on the protein could be obscured by the effects of ionic species in solution, for example the case of hemoglobin in which its oxygen binding is normally obscured by allosteric effects of ionic species in solution (189). Thus a potentially more useful approach toward probing energetic, dynamic and structural aspects of the interaction between a protein and its aqueous enviroment is to partially replace water with a non-aqueous solvent in which the protein remains

functional. Investigations of proteins in mixed aqueous binaries are potentially useful in the study of many aspects of enzyme chemistry. From a kinetic point of view such systems allow continuous variation in the reaction medium for the study of reaction mechanisms, an approach used in organic chemistry (212). Aqueous organic solvents can also be used in the study of forces involved in enzymatic reactions (191, 192).

Normally, protein-solvent interactions are influenced by alterations in local viscosity, internal pressure and changes in mass composition of solvents. Again it is assumed that the protein is in equilibrium with the solvent at constant temperature, pressure and composition. Thus the variation of, say, temperature which normally results in the alterations of the intrinsic properties of solvents should influence the interactions between proteins and solvents. This variation of temperature therefore could be another useful way of also probing the energetic, dynamic and structural aspects of the interaction between a protein and its aqueous enviroment. This is moreso when it is known that the different types of water that is H_2O and D_2O, exhibit anomalous properties at low temperatures.

In this aspect of this work therefore, our efforts have been directed towards probing the effect of aqueous solvents on the properties of the G6PD enzyme, by the correlation of the physical properties of the different solvent systems employed with the changes in the kinetic and thermodynamic functions of the enzyme. Put in

another way, it could be said that this report is intended to test the hypothesis that individual water molecules play an integral part in the function of proteins, and that functional parameters can be influenced by change in bulk water activity. The solvent systems employed in this study are :

1. Water - glycerol binary system : Here, by the variation of the composition of the water - glycerol mixed aqueous system, bulk solvent properties like dielectric constant, viscosity and surface tension could easily be altered.
2. H_2O or D_2O system : At low temperatures, the physical and thermodynamic response functions of these solvents become anomalous. Therefore, at about $4^{\circ}C$ and $11^{\circ}C$, the temperatures of maximum density for water and deuterium oxide respectively, where the fluctuations in their internal energy with volume become negative, it would be expected that the variations in the enzyme properties will become affected. Thus the variation of their physical properties, most especially internal pressure which measures the change in internal energy with volume, by the variation of temperature at these points (around their temperatures of maximum density) should reflect the influences of these solvents on the G6PD molecule.

The G6PD enzyme type used for this study was the B.

5.1.2. G L Y C E R O L

Glycerol, the organic solvent utilized in our binary mixture study, was discovered in 1779 by the Swedish Chemist Scheele. It however, owes its name, "glycerine" (an adaption of the Greek word "glukos", meaning sweet), to Chevreuil, a French scientist.

Glycerol is the simplest trihydric alcohol. It is a colourless, viscous liquid odorless when pure and has a warm, sweet taste. It is neutral to indicators

Much work has been done on physical properties of glycerol over the years. The specific gravity which has long been one of the principal means of estimating the purity of the distilled glycerol, has been determined for the anhydrous and solutions of glycerol at different temperatures. At 15°C the specific gravity of anhydrous glycerol is 1.26557g/cm^3, while at the same temperature, the specific gravity at 1% (w/w) is 1.00240g/cm^3.

The boiling point of pure glycerol has been determined at different pressures. At 760mm Hg, it is 290°C. The freezing point has also been determined by a number of workers, with results ranging from 17 - 18°C. The most precise value however, is 18.17°C.

When glycerol and water are mixed there is a slight decrease in volume and a slight rise in temperature (218). The amount of temperature rise will vary with the concentration of the glycerol, but will reach a maximum of 5°C at 58 percent (w/w). Campbell (219)

who studied the vapour pressure and specific volume of binary mixtures observed that glycerol forms at least one hydrate. He observed that when glycerol and methanol are mixed there is a drop in the temperature and volume.

The dilution of glycerol to a specified concentration can be calculated thus (218) :

$$x = 100\,(a-b)/b$$

$$y = 100\,((a-b)/b)D$$

$$k = 100 - (V(D-1) + 100d)$$

where

x = parts by wt. of water to be added to 100 parts of glycerol

y = parts by vol. of water to be added to 100 vol. of glycerol.

k = % contraction based on the final volume

a = % (wt or vol) of glycerol in the original material.

b = % (wt or vol) of glycerol in the desired concentration

D = density of the original material

d = density of the desired concentration

V = volume of the original material.

The high viscosity of glycerol is one of its distinctive characteristics and is the basis of some of its uses. Consequently, many measurements of its viscosity have been taken. The viscosity of glycerol solutions over almost the entire region of 0 to 100 percent glycerol and 0 to 100°C has been determined and can be found in various tables. The absolute viscosity of zero percent glycerol is

1.005 centipoise at 20°C. Viscosity decreases as temperature increases. It also increases rapidly with increasing pressure. Electrolytes dissolved in anhydrous glycerol and glycerol solution also increase the viscosity of glycerol with the exception of ammonia bromide and iodide which have the opposite effect.

The surface tension of glycerol is less than that of water, though higher than that of the majority of organic liquids. It diminishes with increasing temperature. The surface tension of glycerol is raised slightly by the addition of water.

The dielectric constants of glycerol-water solutions at 25°C have been obtained using a current having a frequency of 0.57×10^6 cycles/second. The dielectric constant of the anhydrous glycerol is decreased by increasing temperature.

Because of its hydroxyl groups, glycerol has solubility characteristics similar to those of water and the simple aliphatic alcohols. It is completely miscible with most alcohols and even phenol. The heats of solution of mixing are quite small and exothermic and gets smaller as dilution with water is increased.

Glycerol of any concentration when exposed to air, will absorb or lose moisture until a time when its moisture content is in equilibrium with the moisture in the air.

Colvin (220) has shown that the addition of as much as 40 percent glycerol (v/v) to water does not change the dissociation constant of water, nor does the ionic activity product show

appreciable change. The addition of glycerol even in large amounts to aqueous acetate or phosphate buffered solutions or to dilute hydrochloric and sulphuric acid solutions has only slight effect on the H^+ concentration (221).

5.1.3. STRUCTURAL INTEGRITY OF PROTEINS (G6PD) IN GLYCEROL

Shifrin et al (222) have shown that glycerol at concentrations of up to 30 percent (v/v) do not destroy the catalytic activity nor the tertiary or quaternary structure of native L-asparaginase. They also showed that glycerol and other polyhydric alcohols reduce the dissociation of this enzyme to its inactive form.

Levy (56) in his study on rat mammary gland G6PD, gave evidence to show that glycerol at concentrations of up to 50 percent (v/v) did not cause the denaturation of the enzyme. This fact was suggested by the identical fluorescence titrations in aqueous and 50% glycerol solutions. He also observed that glycerol protects the enzyme agaiinst inactivation at neutral and alkaline pH, thus suggesting that it stabilizes the enzyme by preventing the dissociation of the dimer.

Bolen et al (223) in their kinetic studies on adenosine deaminase in mixed aqueous solvent showed that at up to 70 percent (v/v), ethylene glycol (another polyhydric alcohol), the enzyme is not irreversibly denatured, and also does not undergo any structural change.

In general, it has been reported that glycerol, sucrose and other polyhydric alcohols stabilize a variety of oligomeric proteins

(224, 225) and also enhance the extent to which denatured subunits reassemble into functional proteins (226).

From all these observations therefore, it could be deduced that the G6PD enzyme will function in the glycerol-water binary mixture as it will in an aqueous solution, especially up to the maximum glycerol concentration (50% v/v) utilized in this study. Its kinetic parameters could however be subjected to the bulk solvent properties of the mixture.

5.1.4. PROPERTIES OF WATER AND DEUTERIUM OXIDE

Water is one of the very common solvents found in the world. Biologically it is the most common medium in which macromolecules function. Thus as has been previously noted, its properties will affect the functioning of proteins to no small extent.

At about 50°C and above, the properties of water are those characteristic of a normal liquid (193). For most liquids, these normal trends continue to the lowest temperatures at which measurement can be made - that is until they freeze or vitrify. Water on the other hand, differs in that most of its physical properties and thermodynamic response functions become anomalous at lower temperatures. The thermal expansibility, $\alpha = V^{-1}(\partial V/\partial T)_p$, becomes negative at 4°C., the isothermal compressibility, $K = -V^{-1}(\partial V/\partial P)_T$, and isobaric heat capacity, $C_p = T(\partial S/\partial T)_p$ reach a minimum, and start increasing as the temperature is reduced, the density, p, reaches a maximum at

$4^{\circ}C$ and start decreasing as the temperature is reduced (193, 194, 227, 228, 229-231). All these anomalies are accompanied by anomalies in the temperature dependence of most transport properties and relaxation times (194). Figure 5.1 shows some of the temperature dependence of the thermodynamic response function of liquid water at atmospheric pressure (194, 227, 229-231).

Deuterium oxide, D_2O, is the heavier form of normal water, H_2O. Its physical properties are quite different from that of water. It has a maximum density at a temperature of about $11.0^{\circ}C$, a melting point of $2.82^{\circ}C$, a boiling point of $101.42^{\circ}C$; results which indicate that at the same temperature, D_2O solutions are more structured than water solutions. Another difference between H_2O and D_2O is their relationship to life processes. While H_2O is necessary for life, D_2O is poisonous to all but the lowest forms.

Despite the above difference in their properties, there are still similarities in their p-V-T properties. Fine and Millero (232) examined the effect of temperature on the specific volume compressibility, expansibility and specific heats at constant volume and pressure for D_2O and H_2O. The plot of specific volume against temperature at two pressure values ($p = 0$ and 1000 bars) for the two solvents were similar in shape. The compressibility curves for both D_2O and H_2O were also similar, with a minimum at the middle temperature range (about $50^{\circ}C$) at both pressures. The expansibility curves for the two solvents were also similar, with the curves at the

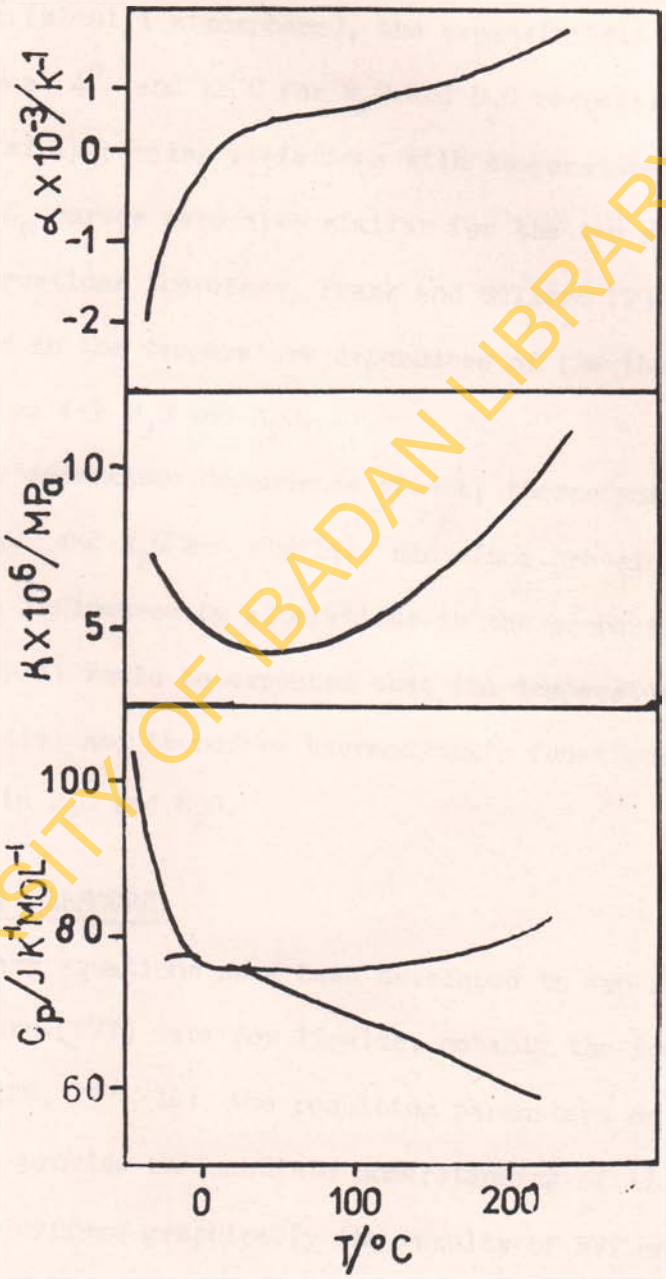


Fig. 5.1 : Temperature dependence of the thermodynamic response functions of liquid water at atmospheric pressure (references : 94, 227, 229-231).

two pressures (0 and 1000 bar) for each solvent converging at about 50°C. At 0 bar (about 1 atmosphere), the expansibility values became negative at 4° and 11°C for H_2O and D_2O respectively. Cv curves showed fairly similar variations with temperature for the two solvents. C_p curves were also similar for the two solvents. With these observations therefore, Frank and Millero (232) established the similarities in the temperature dependence of the thermodynamic response functions for D_2O and H_2O.

Since the temperature dependence of most thermodynamic response functions for D_2O and H_2O are similar, and since protein - solvent interactions are influenced by alterations in the properties of the solvent, then it would be expected that the temperature dependence of the G6PD kinetic, and therefore thermodynamic functions will be similar both in D_2O and H_2O.

5.1.5. INTERNAL PRESSURE

Although many equations have been developed to express pressure-volume -temperature (PVT) data for liquids, notably the isothermal equations (227, 228, 232-234), the resulting parameters or constants do not in general provide an immediate understanding of liquid properties. It is possible to express graphically the results of PVT measurements in such a way that immediate understanding of liquid properties and comparison between different liquids are apparent. This approach was described by Hildebrand and Scott (233, 234), but has since been

rather neglected. This interpretation of PVT data for liquids is made in terms of the equation below :

$$dU = T.dS - p.dV \quad .... \quad .... \quad 5.1.$$

which is called a "thermodynamic equation of state" (235) because it provides a relationship between pressure, p, molar volume, V, temperature, T, and the molar internal energy, U, which is valid for all substances and which is closely related to oridinary PVT data.

The appearance of equation 5.1 indicates that U changes in a simple way when S and V are changed ($dU \alpha dS$ and $dU \alpha dV$). This suggests that the most sensible way of regarding U is as a function of S and V. Thus U (S,V). The mathematical consequence of U being a function of S and V is that dU can be related to changes in S and V by the relation

$$dU = (\partial U/\partial S)_V . dS + (\partial U/\partial V)_S . dV \quad .... \quad 5.2.$$

Comparing equations 5.1 and 5.2 we notice that

$$(\partial U/\partial S)_V = T, \text{ and } -(\partial U/\partial V)_S = P$$

Dividing equation 5.2 by ∂V, and imposing a constant temperature we get

$$(\partial U/\partial V)_T = (\partial U/\partial S)_V (\partial S/\partial V)_T + (\partial U/\partial V)_S$$

$$= T.(\partial S/\partial V)_T - p$$

But by Maxwell's relation

$$(\partial S/\partial V)_T = (\partial P/\partial T)_V$$

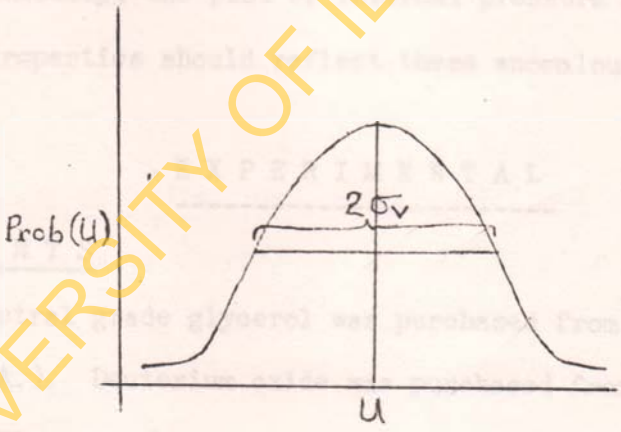
Therefore

$$(\partial U/\partial V)_T = T. (\partial P/\partial T)_V - p \quad .... \quad .... \quad 5.3$$

$(\partial U/\partial V)_T$, the isothermal internal energy volume coefficient is often called the internal pressure. For ideal gases this is zero. However, for imperfect gases and liquids, it becomes appreciable, and is frequently much greater than the external pressure, p.

Internal pressure is a function of temperature at constant volume for most liquids (236). This can be seen in the case of water where it shows a very considerable temperature dependence in ambient conditions (236) (attributable to the open H-bonded structure in water under ordinary conditions).

In the different types of water, there are always fluctuations of internal energy with volume, giving a distribution of the form :



In the variance form, changes in internal energy with volume can be stated thus :

$$\left(\frac{\partial U}{\partial V}\right)_T = \frac{\sigma_{uv}^2}{\sigma_v^2}$$

σ_{uv} is called the double variance.

When σ_{uv} is high relaxation is fast for water, in the order of pico seconds. But when it is low, relaxation is slow.

For water, U and V always have the same sign. However, at $4^{o}C$ (probably $11^{o}C$ for D_2O), effects are reversed. Internal energy, U, then increases as volume decreases. Thus under this condition internal pressure, $(\partial U/\partial V)_T$, becomes negative (changes sign).

The implications of this phenomenom are obvious. Since protein-solvent interactions are influenced by changes in solvent properties, it would then mean that at about $4^{o}C$ in water (about $11^{o}C$ for D_2O) the trends in any property of the protein will be affected. Thus graphically, the plot of internal pressure against any of the protein properties should reflect these anomalous effects.

EXPERIMENTAL

REAGENTS

Spectral grade glycerol was purchased from Fisher Scientific Co. (U.S.A.). Deuterium oxide was purchased from Aldrich Co. (U.S.A.).

INSTRUMENTS

CARY 219 spectrophotometer with an inbuilt recorder and thermostated cell compartment was used for kinetic measurements for the experiments in water-glycerol binary mixture.

For the experiments in water/D_2O solvents at low temperatures, kinetic measurements were carried out with CARY 118 spectrophotometer

with a thermostated cell compartment, interfaced to a PDP 11 computer. CARY 1605 spectrophotometer with a thermostated ' cell compartment, hooked onto a Sargent Welch recorder was used for the continuous assay of enzyme activity in the course of the kinetic measurements. NESLAB endocal refrigerated circulating bath model RTE-5DD was used to control the experimental temperatures, while Fluke digital thermometer calibrated at the temperature of the triple point of water (0.01°C) and connected to a temperature probe was used to determine the exact temperature in the cell compartment of the CARY 118 spectrophotometer in the course of kinetic determinations.

PYECAM pH meter was used in measuring the pH. All volume measurements were effected using agla micrometer syringes and Lambda pipets.

5.2.1. KINETIC DETERMINATIONS IN WATER-GLYCEROL SYSTEM

Experiments were carried out at pH 9.0 using tris-glycine buffer (I = 0.01). The dilution of glycerol to a specified concentration was achieved using the equation :

$$y = 100\ ((a-b)/b)D$$

For the set of runs at 20°C, a was 100, b was variable depending on glycerol concentration while D was 1.2611g/cm^3.

For a set of runs at a particular glycerol concentration and temperature, reaction mixture was made up as in Table 5.1.

TABLE 5.1a

REACTION MIXTURE COMPOSITION AT A PARTICULAR GLYCEROL CONCENTRATION AND TEMPERATURE

(cm^3)	G6P (mM)						
	2.50	2.00	1.50	1.00	0.50	0.20	0.10
BUFFER + GLYCEROL	3.00 cm^3						
G6P	0.167	0.133	0.100	0.067	0.333*	0.133*	0.067*
NADP	0.08 cm^3						
WATER	0.743	0.777	0.810	0.843	0.577	0.777	0.843
ENZYME	0.01 cm^3						

For table 5.1, concentration of G6P = 0.06M, $G6P^*$ = 0.006M and $NADP^+$ = 5.00mM. Table 5.1b shows the amount of glycerol to buffer at each glycerol concentration.

TABLE 5.1b

GLYCEROL - BUFFER RATIO

	% GLYCEROL (v/v)						
	10.0	20.0	25.0	30.0	35.0	40.0	50.0
BUFFER	2.60	2.20	2.00	1.80	1.60	1.40	1.00
GLYCEROL	0.40	0.80	1.00	1.20	1.40	1.60	2.00

For each of the runs, 0.01cm^3 of enzyme diluted to give ΔOD_{340}/ minute of about 0.075 was used. G6PD B purified as described in Chapter two was used for these experiments.

The kinetic measurements were determined at 20.0, 27.0 and 34.0°C.

5.2.2. KINETIC DETERMINATIONS IN WATER/D_2O SYSTEM

For the experiments in water, all buffers and solutions were prepared using millipore water. Tris borate buffer pH 6.55 (I = 0.01) and 0.01M tris glycine buffer pH 9.0, were used for these experiments.

For the experiments in D_2O, all buffers and solutions were prepared in D_2O. Experiments were performed using tris-borate buffer pD 6.95 and tris glycine buffer pD 9.40, (Note that pD = pH + 0.4).

Kinetic measurements were carried out in a 1 cm^3 quartz cell in CARY 118 spectrophotometer. Kinetic data acquired during each measurement was immediately analysed by the PDP 11 computer interfaced to the spectrophotometer using the program G6PDH, an enzyme kinetic data acquisition and analysis programme developed by Roger Gregory. (See appendix).

The composition of the reaction mixture was as follows: Buffer 0.75cm^3; 5mM NADP, 0.02cm^3; enzyme solution, 0.005cm^3; water (or D_2O) and the substrate G6P were added in different volumes to give a final volume of 1.0cm^3. The final G6P concentrations were 3.0, 2.5, 2.0, 1.5, 1.0, 0.5, 0.4, 0.3, 0.2 and 0.1mM.

Temperatures at which kinetic parameters were determined are as follows : H_2O experiments - 0.5, 1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0 and 8.0°C. D_2O experiments - 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0 and 14.0°C.

For each run, 0.005cm^3 of enzyme diluted to give a velocity of 0.075 ΔOD_{340}/min was used. Enzyme type B purified as described in Chapter two was used for these experiments.

RESULTS AND DISCUSSION

5.3.1. EFFECT OF WATER-GLYCEROL BINARY SYSTEM ON G6PD

Experiments for this binary system were performed in tris-glycine buffer pH 9.0. This was because glycerol did not have any effect on the tris glycine system. A previous attempt to use the tris-borate buffer system resulted in the lowering of the pH by about two units indicative of possible interactions between the borate ions and glycerol.

When glycerol and water are mixed there is a slight decrease in volume and a slight rise in temperature (23). In our experimental range however, (0 to 50 percent glycerol (v/v)), the contraction is less than one percent, thus it is assumed negligible. The problem of rise in temperature was solved by pre-equilibration of the reaction mixture. Glycerol of any concentration when exposed to air will gain or lose moisture until a concentration is reached when it is in equilibrium with the air. Thus the reaction mixture, whether in the equilibration vial or in the cuvette inside the spectrophotometer was always kept closed.

Values of V_{max} and Km for G6P were determined by standard double reciprocal plots of 1/v against 1/(G6P), and Dixon's plots of (G6P)/v against (s) (see figure 5.2 for typical plots) as was done in the case of the variation of V_{max} and Km with pH in the fully aqueous system. Averages were then taken. The linearity of these

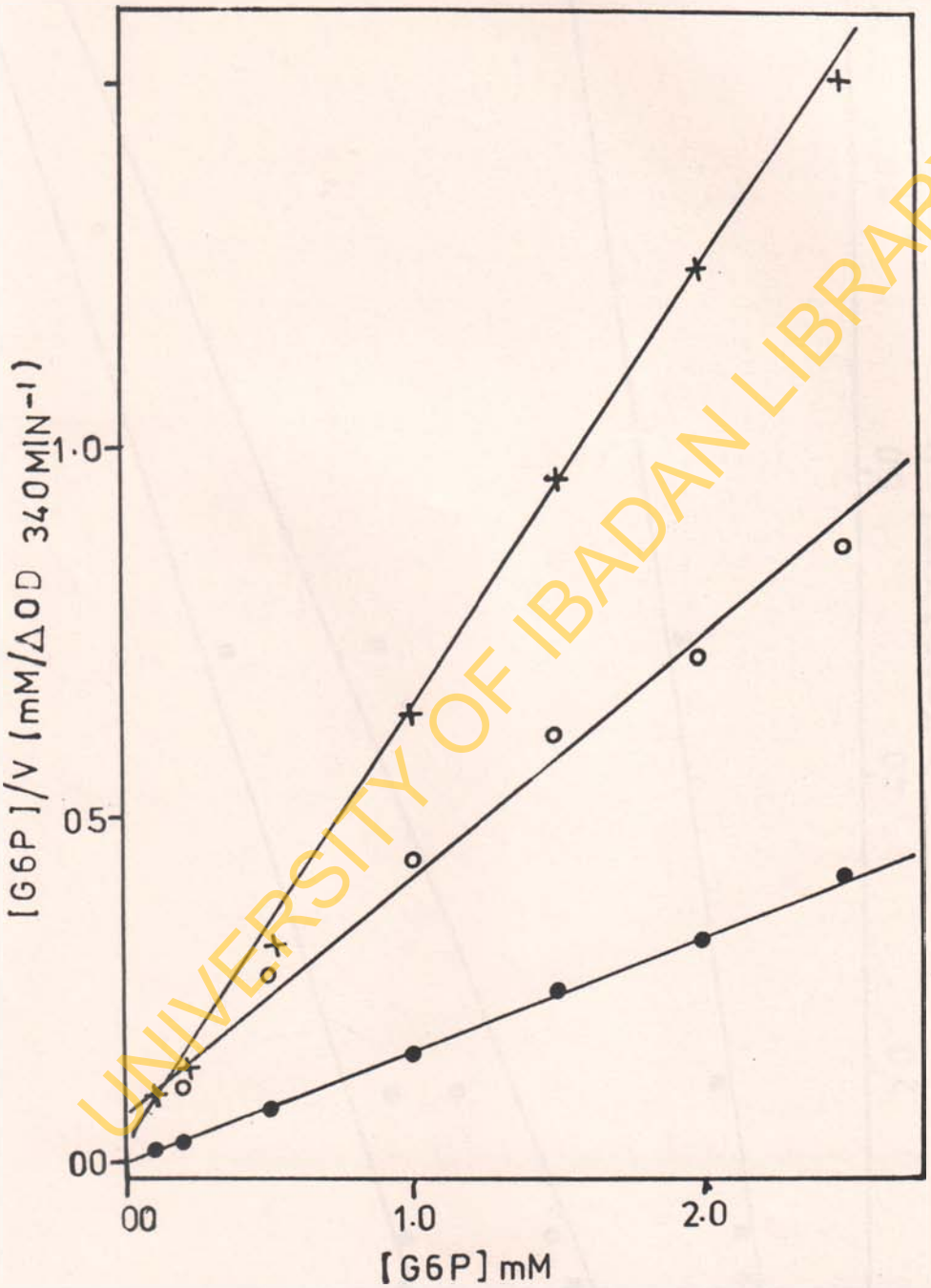


Fig. 5.2(a) : Typical Dixon's plot of (G6P)/V against (G6P) at pH 9.0 at different glycerol concentrations; (●), 10% (v/v); (o) 30% (v/v); and (X), 50% (v/v).

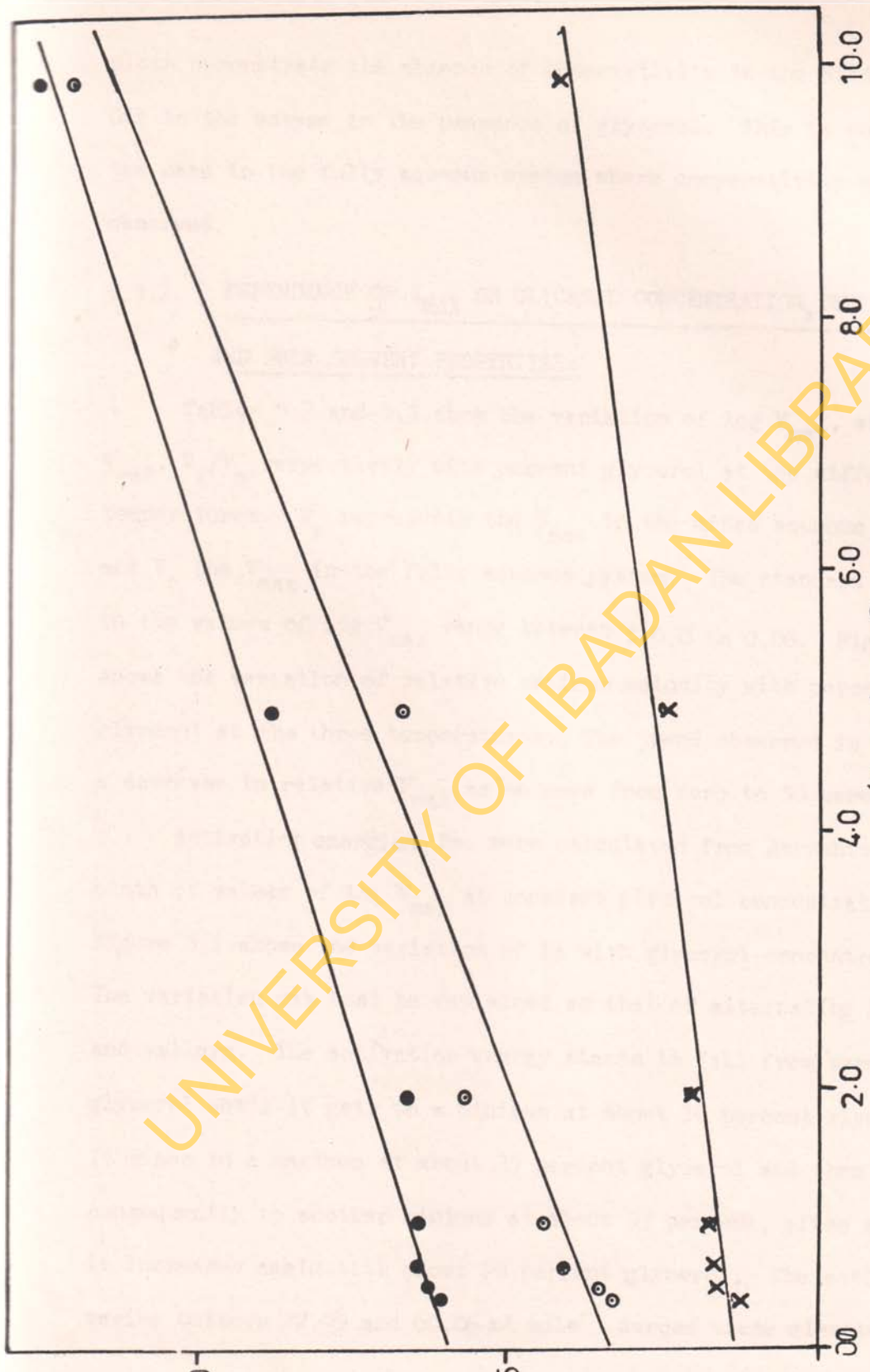


Fig. 5.2(b) : Typical plot of 1/v against 1/(G6P) at pH 9.0 at different glycerol concentrations. (X), 10% (v/v); (⊙), 30% (v/v); and (●), 50% (v/v).

plots demonstrate the absence of cooperativity in the binding of G6P to the enzyme in the presence of glycerol. This is unlike the case in the fully aqueous system where cooperativity was observed.

5.3.2. DEPENDENCE OF V_{MAX} ON GLYCEROL CONCENTRATION, TEMPERATURE AND BULK SOLVENT PROPERTIES:

Tables 5.2 and 5.3 show the variation of log V_{max}, and relative V_{max}, V_x/V_o, respectively with percent glycerol at the different temperatures. V_x represents the V_{max} in the mixed aqueous system and V_o the V_{max} in the fully aqueous system. The standard errors in the values of log V_{max} range between +-0.0 to 0.06. Figure 5.3 shows the variation of relative maximum velocity with percent glycerol at the three temperatures. The trend observed is a decrease in relative V_{max} as we move from zero to 50 percent glycerol.

Activation energies, Ea, were calculated from Arrhenius plots of values of log V_{max} at constant glycerol concentration. Figure 5.4 shows the variation of Ea with glycerol concentration. The variation can best be explained as that of alternating hills and valleys. The activation energy starts to fall from zero percent glycerol until it gets to a minimum at about 10 percent glycerol. It rises to a maximum at about 25 percent glycerol and then consequently to another minimum at about 33 percent, after which it increases again till about 50 percent glycerol. The activation energy varies between 27.09 and 60.06 kJ $mole^{-1}$ across these glycerol concentrations.

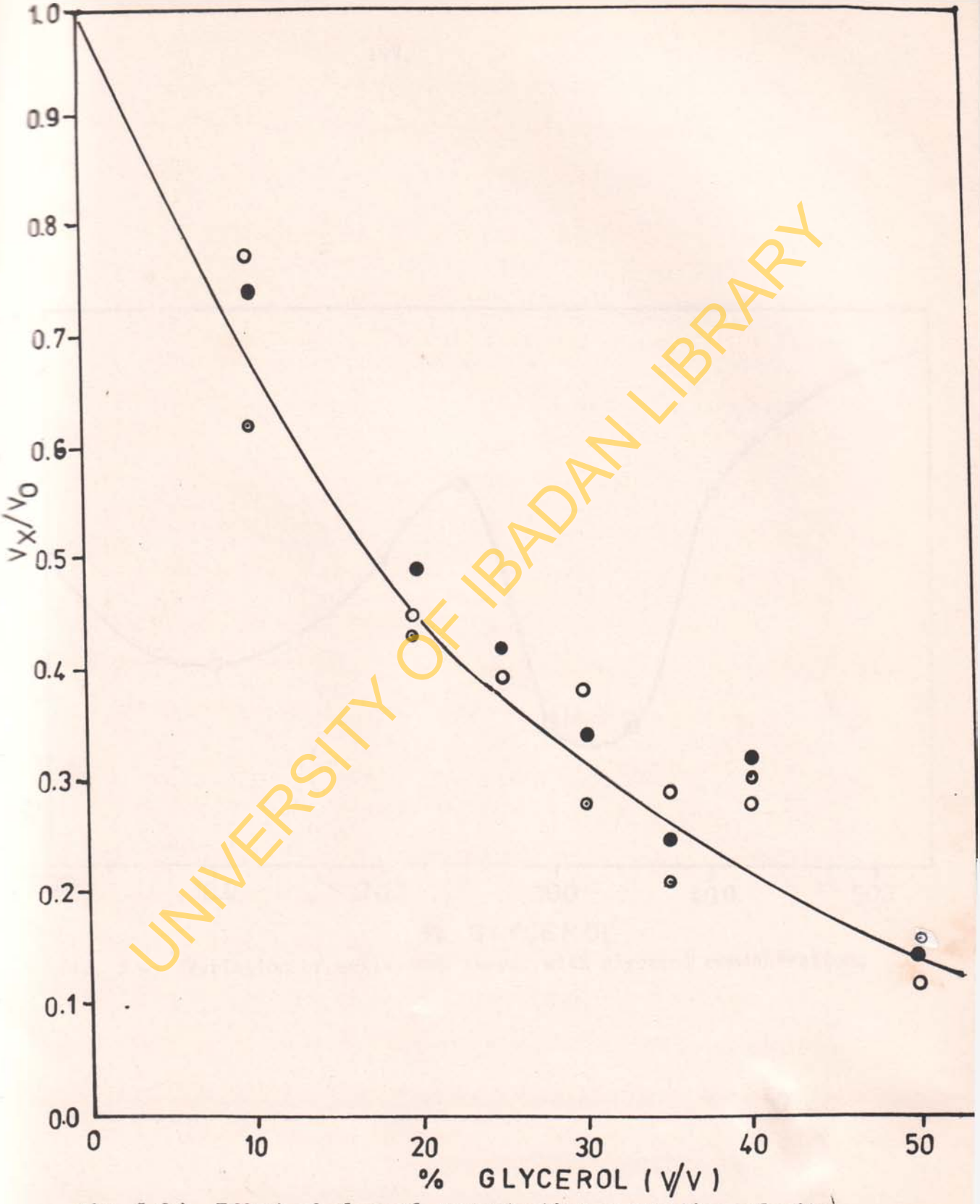


Fig. 5.3 : Effect of glycerol concentration on reaction velocity. (O), at 20°C ; (●), at 27°C; and (⊙), at 34°C.

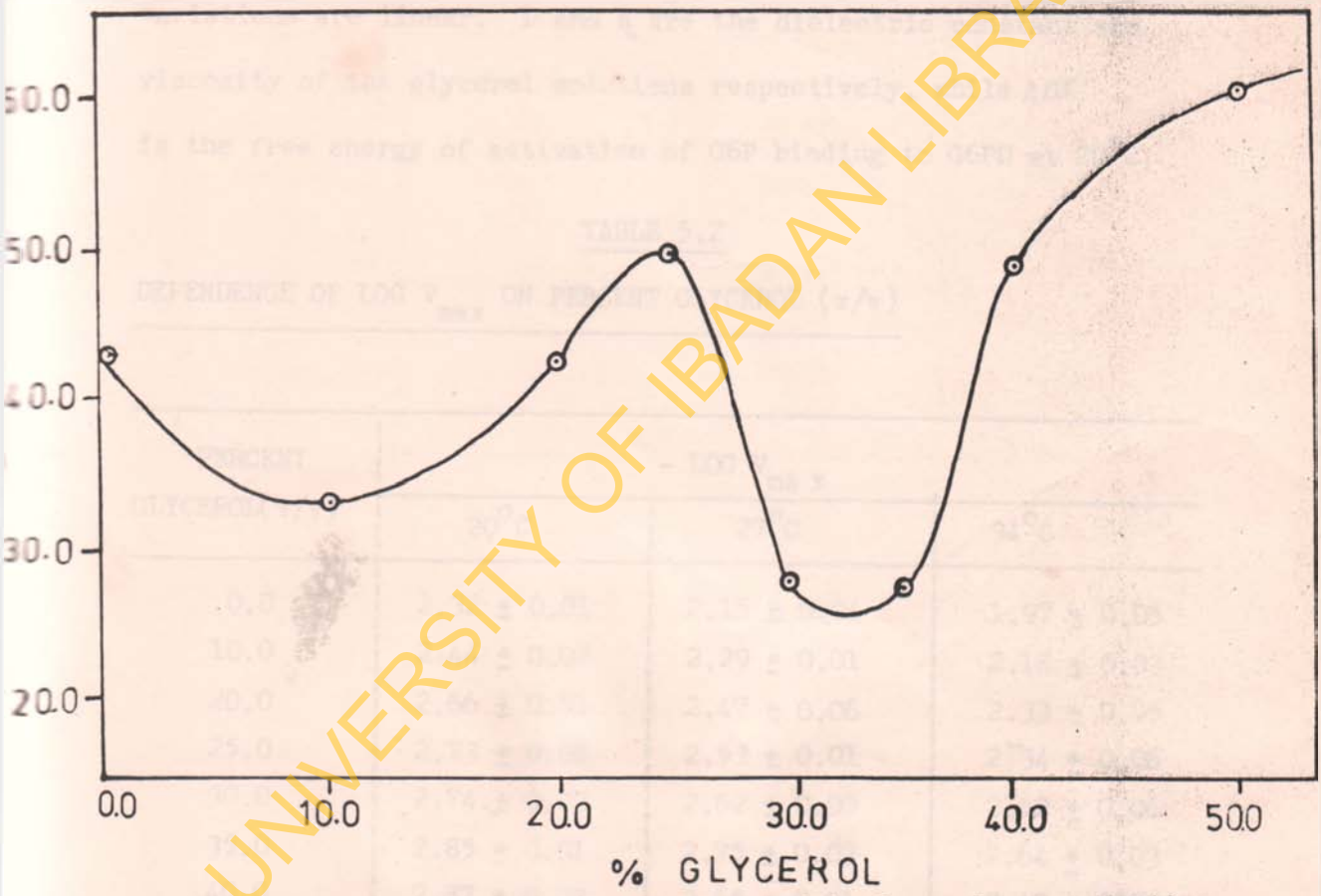


Fig. 5.4: Variation of activation energy with glycerol concentration.

The errors in activation energy are ± 0.94 to 3.18 kJ mole^{-1}.

Table 5.4 shows the variation of Ea with glycerol concentration.

Figures 5,5, 5.6 and 5.7 show the variations of log (V_x/V_o) with 1/D at 20° and 27°C, Vx/Vo with 1/η at 20° and 27°C, and ΔG* with surface tension at 20°C, respectively. All these variations are linear. D and η are the dielectric constant and viscosity of the glycerol solutions respectively, while ΔG* is the free energy of activation of G6P binding to G6PD at 20°C.

TABLE 5.2

DEPENDENCE OF LOG V_{max} ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL(v/v)	- LOG V_{max}		
	20°C	27°C	34°C
0.0	2.32 ± 0.01	2.15 ± 0.04	1.97 ± 0.05
10.0	2.44 ± 0.02	2.29 ± 0.01	2.18 ± 0.03
20.0	2.66 ± 0.01	2.47 ± 0.06	2.33 ± 0.05
25.0	2.73 ± 0.06	2.53 ± 0.01	2.34 ± 0.06
30.0	2.74 ± 0.03	2.62 ± 0.05	2.52 ± 0.06
35.0	2.85 ± 0.01	2.75 ± 0.03	2.64 ± 0.03
40.0	2.87 ± 0.03	2.65 ± 0.01	2.49 ± 0.03
50.0	3.24 ± 0.03	3.02 ± 0.02	2.76 ± 0.02

TABLE 5.3

DEPENDENCE OF RELATIVE MAXIMUM VELOCITY (V_x/V_o) ON PERCENT GLYCEROL (v/v) :

PERCENT GLYCEROL(v/v)	Vx/Vo		
	20°C	27°C	34°C
0.0	1.00	1,00	1,00
10.0	0.77	0,74	0,62
20.0	0.45	0.49	0,43
25.0	0.39	0.42	0,42
30.0	0.38	0.34	0,28
35.0	0.29	0.25	0,21
40.0	0.28	0.32	0,30
50.0	0.12	0.14	0,16

TABLE 5.4

DEPENDENCE OF ACTIVATION ENERGY, Ea ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL(v/v)	Ea ($KJ\ MOLE^{-1}$)
0.0	43.46 ± 1.76
10.0	33.25 ± 2.31
20.0	42.50 ± 2.62
25.0	49.37 ± 3.03
30.0	27.97 ± 1,26
35.0	27.09 ± 0.94
40.0	48.85 ± 3.18
50.0	60.06 ± 2.68

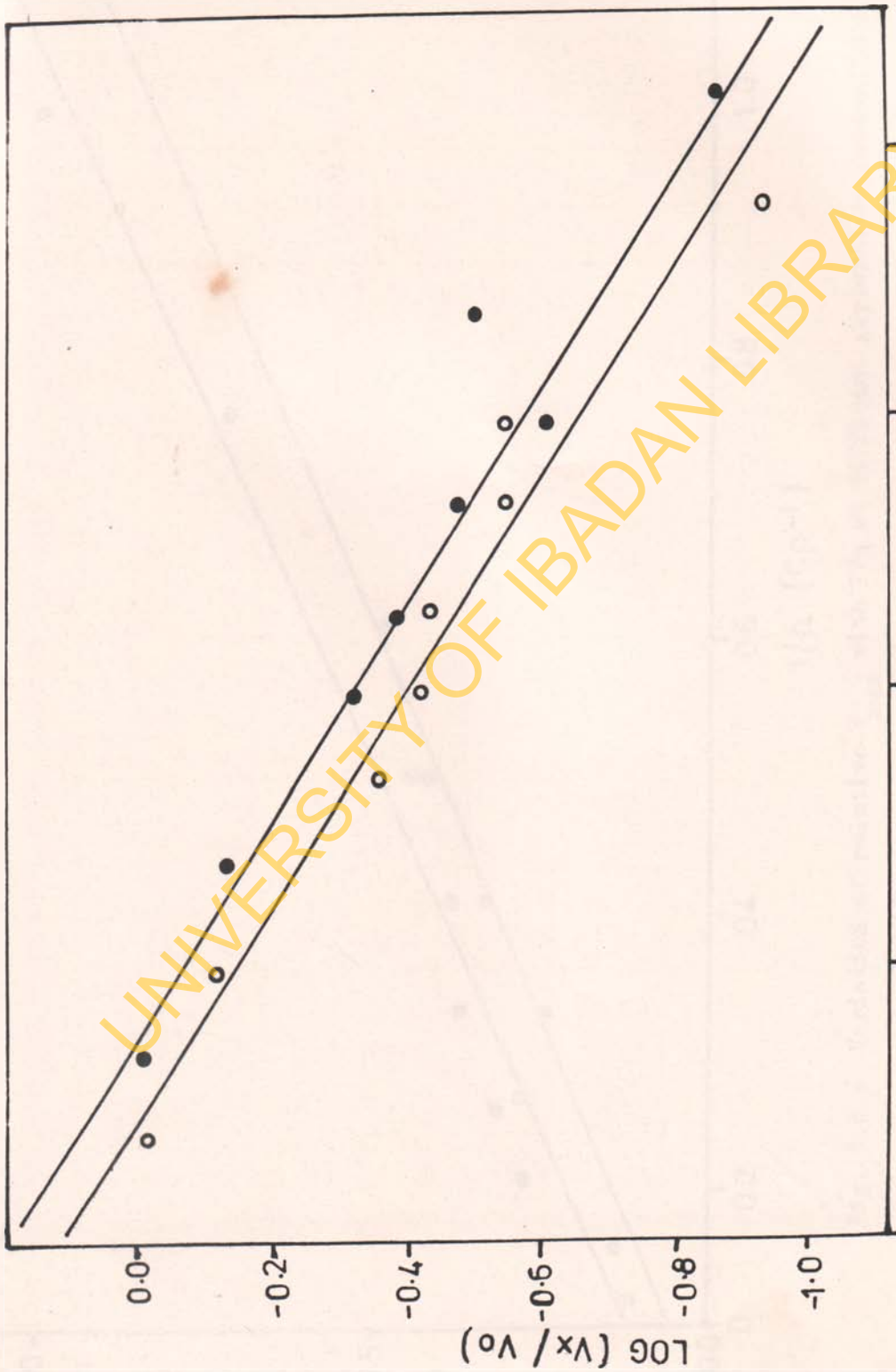


Fig. 5.5 : Variation of log (Vx/Vo) with 1/D at different glycerol concentrations, at 20°C (O); and 27°C (●).

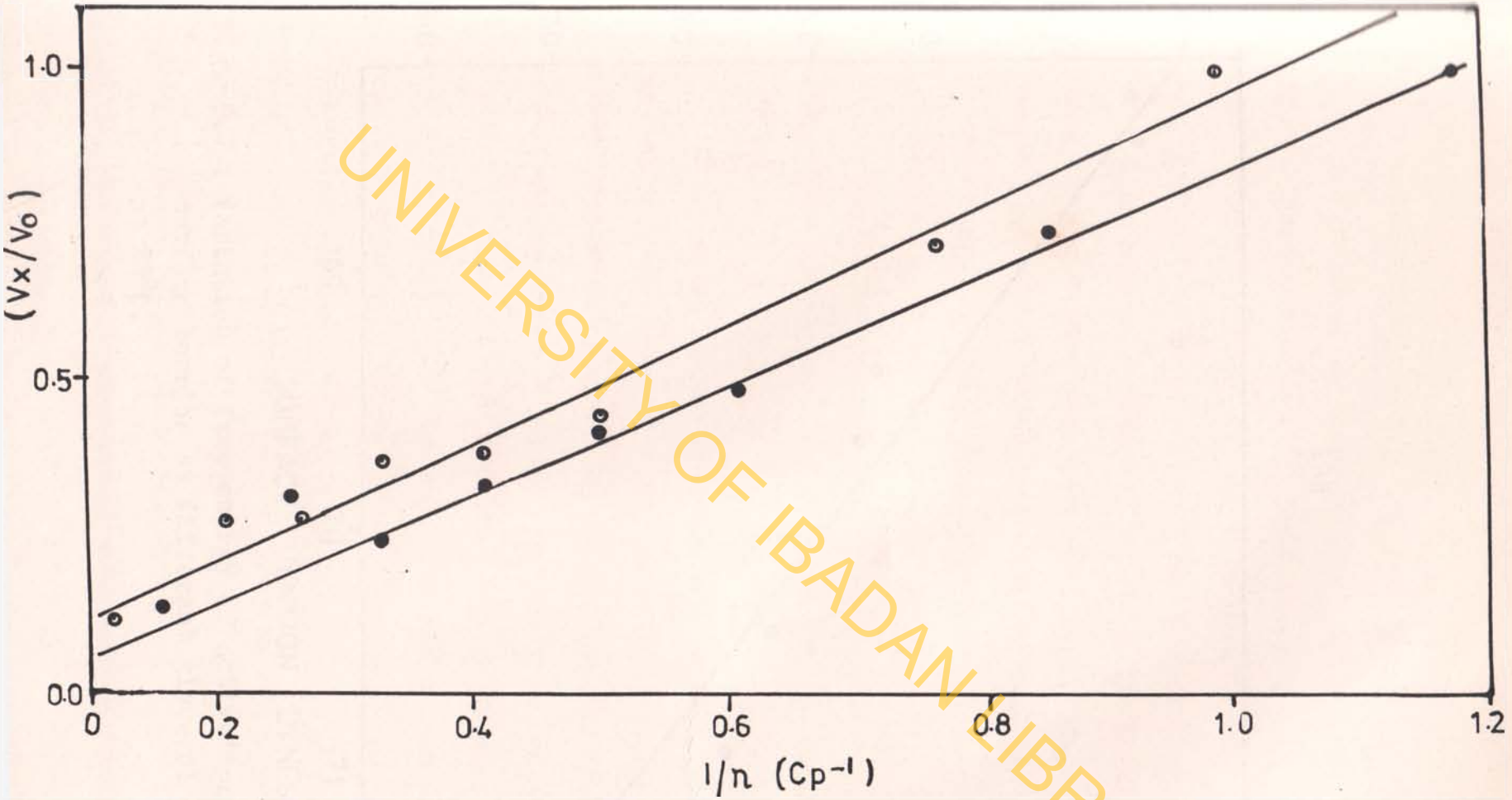


Fig. 5.6 : Variation of relative V_{max} with $1/\eta$ at different glycerol concentrations at 20°C (⊖); and 27°C (●).

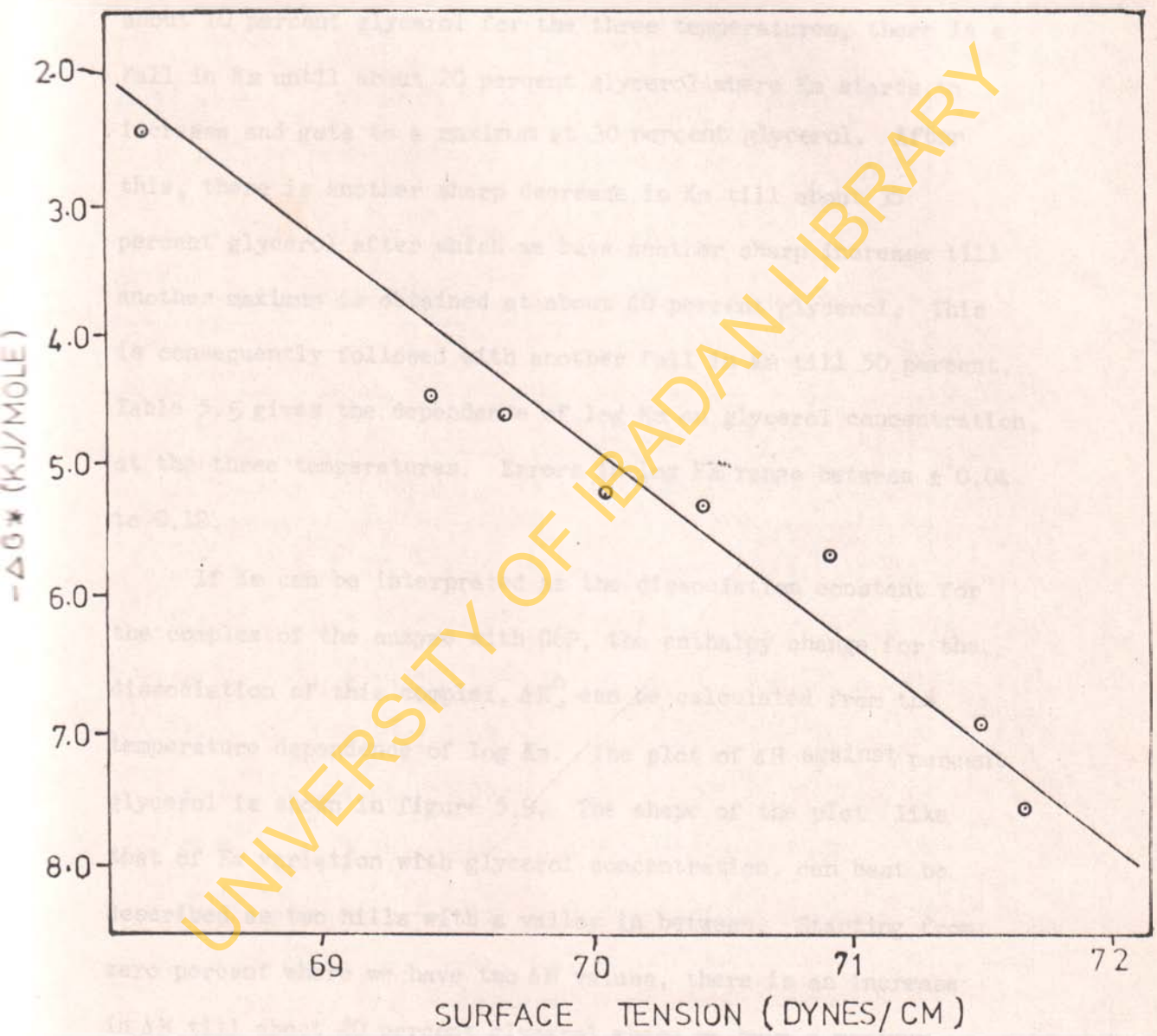


Fig. 5.7 : Variation of free energy of activation with surface tension at different glycerol concentration, at 20°C.

5.3.3. DEPENDENCE OF Km ON GLYCEROL CONCENTRATION AND TEMPERATURE

In contrast to the behaviour of V_{max}, the variation of Km with glycerol concentration is quite complex (figure 5.8). Starting from about 10 percent glycerol for the three temperatures, there is a fall in Km until about 20 percent glycerol where Km starts to increase and gets to a maximum at 30 percent glycerol. After this, there is another sharp decrease in Km till about 35 percent glycerol after which we have another sharp increase till another maximum is obtained at about 40 percent glycerol. This is consequently followed with another fall in Km till 50 percent. Table 5.5 gives the dependence of log Km on glycerol concentration, at the three temperatures. Errors in log Km range between ± 0.04 to 0.12.

If Km can be interpreted as the dissociation constant for the complex of the enzyme with G6P, the enthalpy change for the dissociation of this complex, ΔH°, can be calculated from the temperature dependence of log Km. The plot of ΔH against percent glycerol is shown in figure 5.9. The shape of the plot like that of Ea variation with glycerol concentration. can best be described as two hills with a valley in between. Starting from zero percent where we have two ΔH values, there is an increase in ΔH till about 20 percent glycerol where we have a maximum, followed by a decrease till about 30 percent glycerol where we have a minimum. After this we have another increase till about 38 percent glycerol after which we have a decrease in ΔH until

50 percent glycerol. The values of ΔH across the glycerol concentrations, range between 22.43 to 85.43 kJ mole^{-1}. The error in ΔH is ± 2.31 to 14.03 kJ mole^{-1}. Table 5.6 gives the variation of ΔH with percent glycerol.

TABLE 5.5

THE DEPENDENCE OF LOG Km ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL(v/v)	- LOG Km		
	20°C	27°C	34°C
10.0	4.10 ± 0.12	3.87 ± 0.06	3.82 ± 0.12
20.0	4.41 ± 0.09	3.96 ± 0.12	3.74 ± 0.10
25.0	4.37 ± 0.12	3.98 ± 0.04	3.81 ± 0.09
30.0	3.75 ± 0.07	3.64 ± 0.07	3.58 ± 0.08
35.0	4.51 ± 0.09	4.22 ± 0.08	3.88 ± 0.04
40.0	4.14 ± 0.09	3.73 ± 0.05	3.49 ± 0.04
50.0	4.22 ± 0.11	4.07 ± 0.07	3.98 ± 0.05

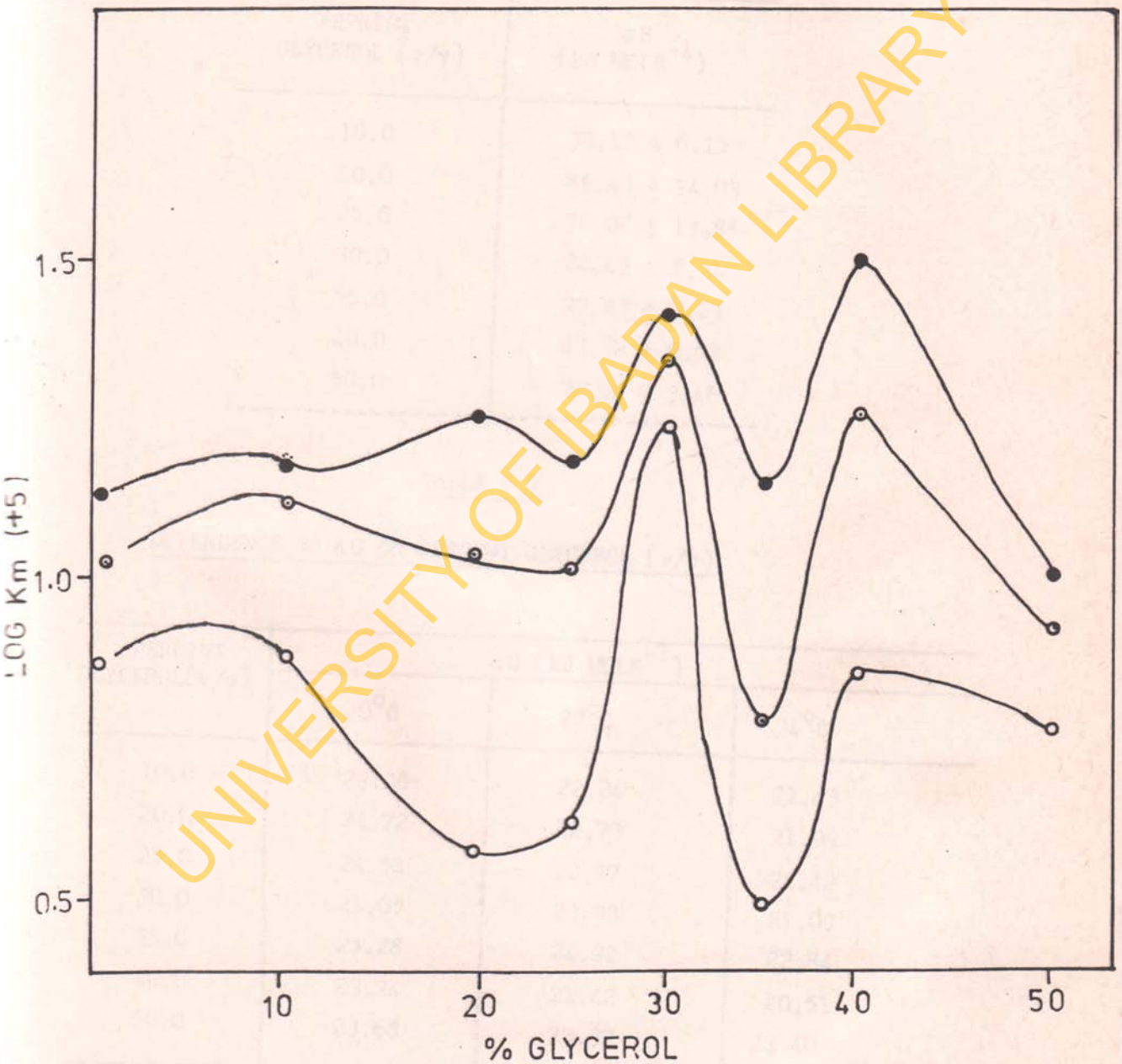


Fig. 5.8 : Dependence on glycerol concentration of log K_m^{G6P} at 20°C, (○); 27°C, (⊖); and 34°C, (●); at pH 9.0.

TABLE 5.6

DEPENDENCE OF ΔH ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL (v/v)	ΔH ($kJ\ MOLE^{-1}$)
10.0	38.12 ± 6.15
20.0	85.43 ± 14.03
25.0	72.02 ± 13.88
30.0	22.43 ± 2.31
35.0	79.83 ± 3.23
40.0	83.72 ± 9.52
50.0	30.64 ± 3.46

TABLE 5.7

DEPENDENCE OF ΔG ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL(v/v)	ΔG ($kJ\ MOLE^{-1}$)		
	20°C	27°C	34°C
10.0	23.06	22.24	22.43
20.0	24.72	22.73	21.99
25.0	24.54	22.87	22.42
30.0	21.05	20.93	21.03
35.0	25.28	24.22	22.84
40.0	23.24	21.42	20.51
50.0	23.68	23.37	23.40

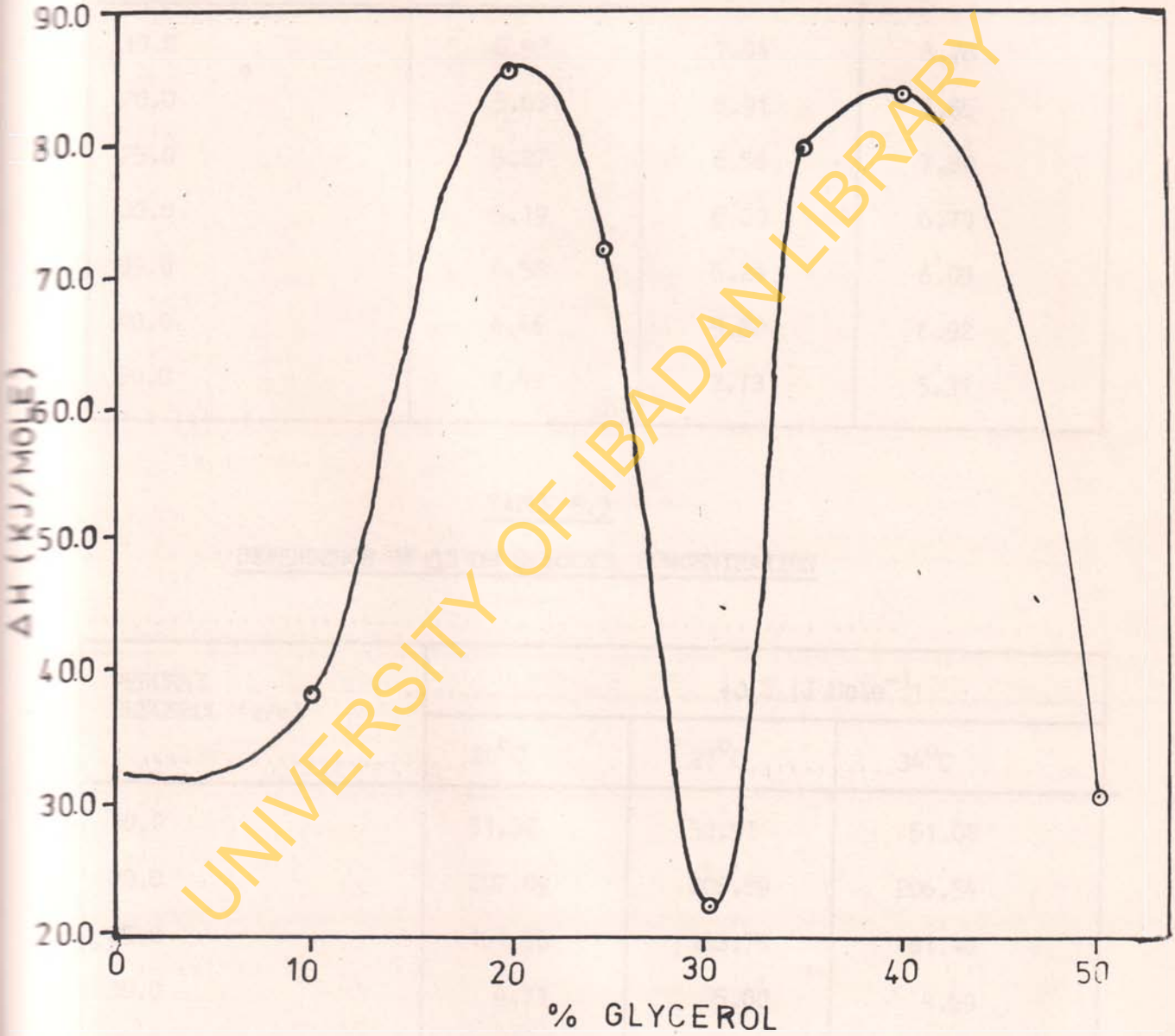


Fig. 5.9 : Variation of ΔH with glycerol concentration.

TABLE 5.8

DEPENDENCE OF ΔG^* ON PERCENT GLYCEROL (v/v)

PERCENT GLYCEROL (v/v)	$-\Delta G^*$ (kJ MOLE^{-1})		
	20°C	27°C	34°C
10.0	6.92	7.94	8.78
20.0	5.63	6.91	7.85
25.0	5.27	6.56	7.80
30.0	5.19	6.00	6.73
35.0	4.58	5.24	6.05
40.0	4.46	5.82	6.92
50.0	2.42	3.73	5.31

TABLE 5.9

DEPENDENCE OF ΔS ON GLYCEROL CONCENTRATION

PERCENT GLYCEROL (v/v)	$+\Delta S$ (J Mole^{-1})		
	20°C	27°C	34°C
10.0	51.37	52.91	51.08
20.0	207.09	208.89	206.54
25.0	161.96	163.75	161.48
30.0	4.71	5.00	4.59
35.0	186.08	185.27	185.54
40.0	206.31	207.56	205.79
50.0	23.19	23.62	23.57

TABLE 5.10

DEPENDENCE OF ΔH* AND ΔS* ON GLYCEROL CONCENTRATION

PERCENT GLYCEROL (v/v)	20°C		27°C		34°C	
	ΔH*(KJ/MOLE)	ΔS*(J/MOLE)	ΔH*(KJ/MOLE)	ΔS*(J/MOLE)	ΔH*(KJ/MOLE)	ΔS*(J/MOLE)
10.0	30.81	128.71	30.75	128.90	30.69	128.50
20.0	40.06	155.86	40.00	156.29	39.94	155.59
25.0	46.93	178.07	46.87	178.01	49.81	178.12
30.0	25.53	104.79	25.47	104.85	25.41	104.64
35.0	24.65	99.71	24.59	99.38	24.53	99.56
40.0	46.36	173.36	46.30	173.65	46.24	173.08
50.0	57.62	204.81	57.56	204.19	57.50	204.49

TABLE 5:11

TABLE OF SURFACE TENSION; VISCOSITY; AND DIELECTRIC CONSTANT OF WATER-GLYCEROL MIXTURES

PERCENT GLYCEROL (v/v)	Surface Tension At 20°C (Dynes/cm)	VISCOSITY (cP)		DIELECTRIC CONSTANT	
		20°C	27.5°C	20°C	25°C
0.0	71.68	1.01	0.85	80.37	78.50
10.0	71.50	1.39	1.16	76.71	74.80
20.0	70.94	2.00	1.65	73.20	71.32
25.0	70.45	2.45	1.99	71.60	69.81
30.0	70.09	3.00	2.46	69.95	68.20
35.0	69.71	3.71	3.02	68.25	66.71
40.0	69.43	4.83	3.81	66.80	65.10
50.0	68.35	8.55	6.34	63.41	61.76

5.3.4. NON-COOPERATIVITY IN THE PRESENCE OF GLYCEROL

In the fully aqueous solvent system at pH 9.0, sigmoid kinetics was observed for the binding of G6P to G6PD B (figure 3.6). However, in the presence of glycerol at the same pH, the Michealian type kinetics was observed (figure 5.2).

Levy et al (237) in their studies on NAD^+ and $NADP^+$ linked interactions of rat mammary glucose-6-phosphate dehydrogenase also observed a similar phenomenon. They found that in fully aqueous medium the inhibition of NAD+ linked reaction by NADPH gave a sigmoid kinetics. However, in the presence of 40 percent glycerol (v/v) normal Michealis kinetics was observed. This occurrence they explained in terms of the presence of two dimeric forms, X and Y of the enzyme in equilibrium with each other. While the X form wqs taken as the form with greater NAD linked activity, the Y form was taken as the form with greater $NADP^+$ linked activity. The Y form was promoted by the presence of $NADP^+$ and NADPH, while the X form was favoured by the presence of glycerol, which stabilizes it, and NAD^+. Glycerol inhibited the $NADP^+$ linked reaction, while activating on one hand, and counteracting the NADP(H) inhibition on the other hand, of the NAD linked activity. It was thus taken that NAD^+ exhibited a homotropic interaction only in the presence of the antagonistic ligand NADPH in fully aqueous medium.

Levy (56) also observed that addition of glycerol helped to

diminish the relative inhibition of the $NADP^+$ linked reaction by dehydroepiandrosterone. This observation he explained off with the idea that a change in the three dimensional structure of the protein, induced by glycerol, precluded the dehydroepiandrosterone from exerting its inhibitory effect by preventing the steroid-binding site from assuming a position sufficiently close to the active centre. Like dehydroepiandrosterone, glycerol inhibits only the $NADP^+$ linked reaction (56). Again, as is in the case of dehydroepiandrosterone, glycerol does not inhibit by competing with $NADP^+$. Thus the counteraction of the inhibition effect of NADPH on NAD^+ by glycerol was explained off by the same idea, that the presence of glycerol leads to the deformation of the binding site of NADPH (56).

In this study, the presence of at least two binding sites exhibiting a homotropic interaction for G6P binding has been identified by the sigmoid kinetics obtained in the fully aqueous reaction medium. The presence of only one site observed therefore in the presence of glycerol could therefore be due to the deformation of one of the G6P binding sites, as a result of the change in conformation of the G6PD molecule induced by the presence of the glycerol just like the case with the mammary gland G6PD.

5.3.5. BINARY SOLVENT EFFECTS ON RATE PARAMETER OF G6PD

Westhead and Malmstrom (238) studied the effects of a variety of solvents on catalytic activity, using enolase. They found that the reaction velocity was highly dependent upon water concentration

and that the velocities in various solvent systems formed a single line when velocities were plotted as functions of water concentrations. Result of similar studies with G6PD B is shown in figure 5.3. The velocity decrease observed here is not linear with water concentration (and therefore glycerol concentration). The decrease observed is far greater than can be expected on the basis of water concentration alone. Thus the attempt to correlate the properties of the bulk solvent with changes in the maximum velocity of the reaction.

A number of investigators have treated simple ion-ion, ion-dipole and dipole-dipole interactions for those reactions in which electrostatic interactions are more important than non electrostatic ones (239, 240). Barnard and Laidler (192) expanded the concepts of dielectric constant effects derived from reactions of smaller molecules to enzyme kinetics. The various approaches differ in sophistication but predict essentially the same behaviour of reaction rate with dielectric, i.e. the log of velocity should be a linear function of the reciprocal of the dielectric constant for the bulk medium, i.e.

$$\log V \alpha 1/D.$$

Results presented in figure 5.5 show the variation of log Vx/Vo against 1/D at 20°C and 27°C, for G6P binding in a mixture of water and glycerol (no dielectric constants are available for 27°C, thus those for 25°C were used since they are close together). Since a given

composition of these solvent systems give quite different dielectric constants, the close correspondence and linearity of the plots suggest that the overall reaction velocity is quite indeed dependent upon electrostatic forces.

Castaneda-Agullo et al (241) also noted that there existed the possibility that velocity behaviour could also be due to some other property of binaries which varied in parallel manner to dielectric constant. Consequently an attempt was made to find other properties of the bulk solvent which could be correlated with the rate parameter,

A number of organic reactions in mixed aqueous media have demonstrated that non electrostatic forces influence non-enzymatic reactions (240, 242). Thus in this work, we shall consider those forces known to influence organic reactions such as surface tension and viscosity in the G6PD catalyzed reaction.

A model for considering the combination of two molecules in solution has recently been advanced by Sinanoglu an Abdulnur (243). This model provides a convenient way of looking at the interaction of combining molecules solely in terms of a bulk solvent property. This is accomplished by considering the solvent sheath around the molecules as the boundary of a cavity in solution, the solvent side being a macroscopic phase and the cavity being microscopic in character. This model is applicable only in sofar as the molecules which make up the solvent are much smaller than those destined to react, so that the concept of macroscopic surface tension will have

some validity. The gain in free energy accompanying the loss of surface area for the process of two molecules combining to form a complex is approximated by the expression :

$$\Delta G^{*} = \sigma \Delta A$$

where ΔG^{*} is the free energy, σ is the surface tension, and ΔA is the loss of surface area. Figure 5.7 shows the plot of activation free energy, ΔG^{*} against surface tension. It shows a marked linear dependence of the rate parameter upon surface tension. The relation holds upto the highest concentration of glycerol used.

The high viscosity of glycerol is one of its distinctive characteristics and is the basis of some of its uses. At $20^{o}C$, the relative viscosity of water-glycerol mixture is as high as 8 at 50 percent glycerol, while at $27^{o}C$ it is 7.5. Recent studies by Beece et al (244) have suggested that solvent viscosity is an important possibly dominant variable in the rate of reaction between heme proteins and ligands. Beece et al (244) have shown that over a wide range of viscosity the transition rates in heme-CO are inversely proportional to the solvent viscosity and can consequently be described by Kramer's equation (245) :

$$k = \frac{\nu_o \ \nu'_o \ p}{3n} \ e^{- H^{*}/RT}$$

where k is the rate coefficient, ν_o is the undamped frequency in the initial well, ν'_o is the corresponding frequency at the top of the inverted barrier, p is the linear mass density, H^{*} is the barrier

height and η is the viscosity. For our purpose, Kramers equation can be reduced to a simple form thus :

$$V = c\eta^{-1}$$

where V is the reaction rate, c a constant given by $v_o v'_o p e^{-H^*/RT}/3$ Figure 5.6 shows the variation of the relative rate parameter with the reciprocal of viscosity. There is indeed a marked linear dependence of rate on the reciprocal of viscosity at all concentrations of glycerol.

Another likely nonelectrostatic candidate for consideration is the internal pressure of the binary solvent system. But due to the unavailability of compressibility and expansibility data, the internal pressure of the binary solvent system could not be computed. It should however be noted that surface tension and internal pressure are the measures of the cohesive nature of the surface and bulk liquid respectively. Both properties are direct functions of one another in non-polar liquids and are somewhat more loosely related in associated liquids (246). Assuming this relationship we would assume that the relationship between internal pressure and activation free energy (and thus rate parameter) of the enzyme will be similar to that of surface tension.

The variation of activation energy with the concentration of glycerol can be seen in figure 5.4. At low concentration of the organic solvent, that is between 0 to 20 percent glycerol, there is a fall in activation energy, indicative of increased reactivity of

the enzyme. This could mean that the effects due to the differential hydration of the protein in the presence of the glycerol is greater than the changes in the bulk properties of the solvent system like decreased electrostatic interactions, and increased viscosity which might reduce reactivity because of change in water concentration. At this concentration range, it might be that the change in the three dimensional structure induced by the addition of glycerol favours the better interaction between the substrate and the stabilized active site for G6P binding on the enzyme. The variation in activation energy at lower glycerol concentrations, is about 16.5 kJ $mole^{-1}$. However, at higher glycerol concentrations, this variations increase in magnitude to about 35 kJ $mole^{-1}$ accompanied by increasing activation energy. Large relative increases in activation energy in this range indicate decrease in reactivity. It thus could mean that stabilization of the non deformed G6P binding site at those high concentrations of glycerol by differential hydration is no longer enough to offset the effects due to reduced water activity in the reaction medium. Increase in interaction energy between substrate and enzyme due to reduced dielectric constant, and increased viscosity now become appreciable.

These observations at low water concentration (or high glycerol concentration) thus show the important role of water in G6PD action.

5.3.6. BINARY SOLVENT EFFECTS ON AFFINITY PARAMETER (K_m^{G6P}) OF G6PD

The variation of log K_m with glycerol concentration is shown in

figure 5.8. This variation is the same at the different temperatures employed in this study except at the lower glycerol concentration at 34°C. The variation of log K_m with glycerol concentration is basically an oscillation between decreasing and increasing G6P affinity for the enzyme. Starting from about 10 per cent glycerol, there is an increased G6P affinity for the enzyme until about 20 percent glycerol where the affinity starts decreasing again. At about 30 percent glycerol, there is a change in the affinity trend again. The affinity for G6P starts increasing till about 35 percent glycerol where it starts to decrease again, till about 40% where we have another increase in affinity for G6P by the enzyme till 50 percent glycerol.

Haire and Hedlund (247) in their work on the effects of EG on hemoglobin ligation discussed in terms of a model in which EG interacts with hemoglobin in a weak allosteric fashion at the concentration range where there was decreased oxygen affinity, while at other concentration ranges (range of increased oxygen affinity), pertubations of protein hydration lead to stabilization of the high affinity form. Since glycerol does not apparently affect G6P and $NADP^+$ binding to the enzyme directly (56) the observed pattern for G6P binding to the enzyme could thus be explained in terms of a model in which pertubations in protein hydration lead to weak interaction of G6P with the enzyme at some glycerol concentrations, and strong interaction of G6P with the enzyme at other concentration

ranges of glycerol. This pertubation in protein hydration as had been pointed out (247)lead to a change in the three dimensional structure of the enzyme.

A look at the values of K_m in the presence and absence of glycerol show that the stabilized binding site for G6P in the presence of glycerol is most probably the high affinity site.

The Van't Hoff data based on the data presented in figure 5.8 have been illustrated in figure 5.9. It can be seen that points of increased G6P affinity correspond to high enthalpy values while those of decreased G6P affnity correspond to low enthalpy values. The changes in enthalpy vary greatly, from about 25 kJ $mole^{-1}$ to 85 kJ $mole^{-1}$ in the glycerol concentration range used in this study. These variations in enthalpy again reflect changes in the conformation of protein as a result of the differential hydration effect of glycerol on the enzyme.

The K_m parameter was found not to be a linear functions of either reciprocal of dielectric constant, surface tension or reciprocal of viscosity. Since the K_m parameter could be more complex in terms of the rate constants of which it is composed, this lack of correlation is not too surprising.

5.3.7. COMPENSATION PHENOMENA IN G6P BINDING TO G6PD

Data from a variety of experiments suggest the existence of a single very common and phenomenologically simple response of water to changes in solutes, regardless of the way these changes are produced. Phenomenologically, this response appears to be revealed by simple patterns of enthalpy and entropy changes having such similar qualitative characteristics as to suggest that they are all manifestations of the same property of liquid water. These patterns consists of parallel enthalpy and entropy changes which compensate each other to produce minor changes in the free energy of the process under investigation. Such compensation is often remarkably precise. The major experimental finding that suggest that these patterns have a single source is the similarity of the ratio of enthalpy change to entropy change found among the many examples.

Compensation of an enthalpy change produced by change of an independent variable, by entropy change is a common occurrence in small-molecule and protein reactions (248, - 254). Likhtenshtein (254) from his plots of ΔH and ΔS values for apparent Michealis constants, maximum velocity constants, and binding equilibrium constants for a large number of enzymic processes obtained rough linear relationships with Tc values in the range of 260 to 315^{o}K. From these he proposed that enthalpy-entropy compensation play an essential role in enzymic mechanism, and associated it with both the protein conformation and a solvent shell, invoking an energy-chain mechanism whereby energy

released in one complete enzymic process is stored in the water shell and then released to be used for activation in the subsequent catalytic cycle.

In 1953, Vaslow and Doherty (252, 253) also demonstrated enthalpy-entropy compensation in inhibitor binding to α-chymotrypsin, and they attached enough significance to their experimentally observed compensation pattern to propose a catalytic mechanism on the basis of this phenomenom. Specifically they proposed that the thermodynamic changes indicated spontaneous folding of an initially unfolded part of the protein about the inhibitor or substrate to produce enhanced reactivity of substrates through mechanical distortion and a change in the enviroment (dielectric constant, etc.). Other interesting examples of compensation phenomena have been presented in a remarkable set of studies carried out by Beetlestone, Irvine and co-workers (248, 249, 255). In their extensive studies on ferrihemoglobins, enthalpy-entropy compensation was the rule rather than the exception. This phenomena they explained off on the basis of strictly electrostatic model. From their results, the hypothesis that the compensation behaviour is dependent on the charged groups of the proteins no matter where located was supported

Lumry and Rajender (256) in a review of the work done by them and other workers concluded that the existence of the compensation is real, also noting that the compensation temperature lie in a relatively narrow range, from about 250 to 315°K. They also noted that

the compensation pattern is a consequence of the properties of liquid water as a solvent regardless of the solutes and solute properties studied; proposing temperature-independent heat-capacity changes and/or shifts in concentrations of the two phenomenollogically significant species of water as the bases of the effect. The existence of a similar and probably identical relationship between enthalpy and entropy change in a variety of protein reactions, Lumry et al (256) said, suggested that liquid water plays a direct role in many protein processes and may therefore be a common participant in the physiological functions of proteins. They thus proposed that the linear relationship be used as a diagnostic test for the participation of water in protein processes.

So far there have been many ad-hoc explanations for the compensation phenomena. However, the connection of the phenomena with thermodynamics was found by Benzinger (257), and is given below:

Conventionally,

$$\Delta G(T) = \Delta H(T) - T\Delta S(T) \quad \ldots\ldots \quad 5.1.$$

Now integrating $\int_0^T \Delta S(T)\,dT$ by parts, to see $T\Delta S(T)$ in a new light, we obtain

$$\int_0^T \Delta S(T)\,dT = T\Delta S(T) - \int_0^T T' \left\{\frac{\delta \Delta S(T')}{\delta T'}\right\}_p dT$$

$$= T\Delta S(T) - \int_0^T \frac{T'\Delta C_p(T')\,dT'}{T'}$$

$$= T\Delta S(T) - \int_0^T \Delta C_p(T')\,dT'$$

Hence $\underline{T\Delta S(T)} = \int_0^T \underline{\Delta S(T')}dT' + \int_0^T \underline{\Delta C_p}\,(T')\,dT'$ 5.2

Conventionally

$$\underline{\Delta H\,(T)} = \Delta H(0) + \int_0^T \underline{\Delta C_p}\,(T')dT' \qquad 5.3,$$

Thus combining equations 5.1, 5.2 and 5.3, we obtain

$$\underline{\Delta G(T)} = \Delta H(0) + \int_0^T \underline{\Delta C_p}\,(T')\,dT' - \int_0^T \underline{\Delta S(T')}\,dT'$$

$$- \int_0^T \underline{\Delta C_p}(T')dT'$$

$$= \Delta H(0) + \int_0^T \underline{\Delta S(T')}\,dT'$$

The heat integrals have disappeared

Total S or ΔS, H or ΔH and C_p or ΔC_p are underlined. These are the usual experimental quantities.

Lumry (258) in rationalizing Benzingers discovery, stated that fluctuations of 'heat' $\int_0^T \Delta C_p\,(T')dT'$ and of entropy, $1/T \int_0^T \Delta C_p(T)dT$, in a system at equilibrium at T and p constant are limited only by probability considerations. $\int_0^T \Delta C_p\,(T').dT'$ is the average heat of the system. The heat at any instant can vary in value from zero to the sum of system heat and reservoir heat. Large fluctuations are rare but any value of the heat of the system between these limits can occur Fluctuations of equivalent size in G like those in T and p for a large system are relatively so much more rare that they can be ignored Since entropy and enthalpy fluctuations must cancel each other to a degree established by fluctuations in their difference, G, their cancellation is

virtually exact. The integral, $1/T \int_0^T S(T)dT$ measures the expansion of the phase-space cloud due to increase in T from 0 → T; that is the effect of T in the Boltzmann weighting factor $(kT)^{-1}$. It has the high stability against fluctuations found in G(T), T, P and H(0). The second entropy term, $1/T \int_0^T C_p (T')dT'$ measures the average expansion of the phase space cloud due to the average heat, but in systems with significant cooperativity, fluctuations in heat and thus in this entropy term and thence in the volume of the phase-space cloud can be large.

For protein systems there are three general methods for generating the pairs of ΔS - ΔH quantities which provide the points of a compensation plot. The first way is to vary the chemical structure of a parent compound to produce a homologous series of reactants for a particular process. The second way is to vary the solvent composition. In water systems this is by adding increasing amounts of monohydroxyl/poly-hydroxy alcohols. The third way is to vary the hydrogen ion activity of the solution.

In this work, ΔS - ΔH quantities were generated using the second and third methods. For ΔH - ΔS quantities obtained by varying the glycerol concentration, the maximum variation obtained for ΔG, calculated from both K_m and V_{max} parameters at the three temperatures of study was about 4 kJ $mole^{-1}$. Unlike this, variation for ΔH was 22.43 to 85.43 kJ $mole^{-1}$ and 25.41 to 57.62 kJ $mole^{-1}$ for those calculated from K_m and V_{max} respectively, while the variation for ΔS was about 4.56 to 208.89 J $mole^{-1}$ and 99.38 to 204.81 J/mole for those calculated

from K_m and V_{max} parameters respectively. This shows that while G is virtually invariant, ΔH and ΔS vary widely with changes in glycerol concentration. Plots of ΔH (ΔH^*) against ΔS (ΔS^*), for the three temperatures (figure 5.10) were linear, showing the presence of compensation between ΔH and ΔS. Table 5.12 gives the values of the compensation temperature at the three temperatures of experiment, for both the ΔS - ΔH and ΔS^* - ΔH^* plots.

TABLE 5.12

TABLE OF COMPENSATION TEMPERATURES FOR G6P BINDING TO G6PD AT THREE EXPERIMENTAL TEMPERATURES

Plot Type	COMPENSATION TEMPERATURE (Tc)		
	20°C	27°C	34°C
ΔS - ΔH	300.06 ± 3.24	300.06 ± 3.24	300.06 ± 3.24
ΔS^* - ΔH^*	300.42 ± 5.31	300.42 ± 5.31	300.42 ± 5.31

From Table 5.12, it can be seen that the compensation temperatures for the three temperatures of determination are the same, i.e. about 300°K, for both the ΔS - ΔH and ΔS^* - ΔH^* plots.

It has already been shown in this work that the kinetic parameters, especially the rate parameter V_{max} is very dependent upon water concentration and the electrostatic and non-electrostatic properties.

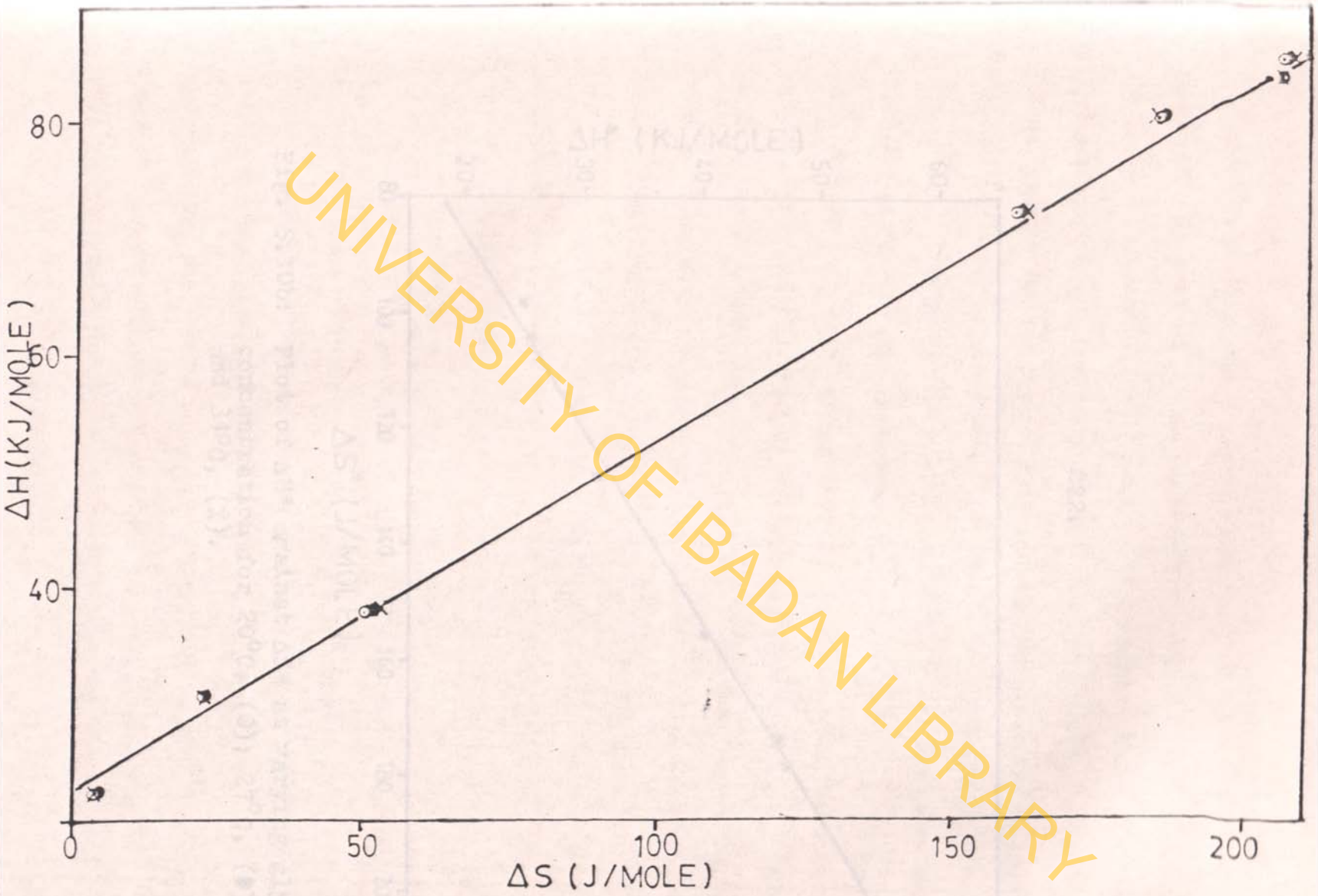


Fig.5.10a: Plot of ΔH against ΔS varying glycerol concentration for 20°C, (⊙); 27°C, (●); and 34°C, (x).

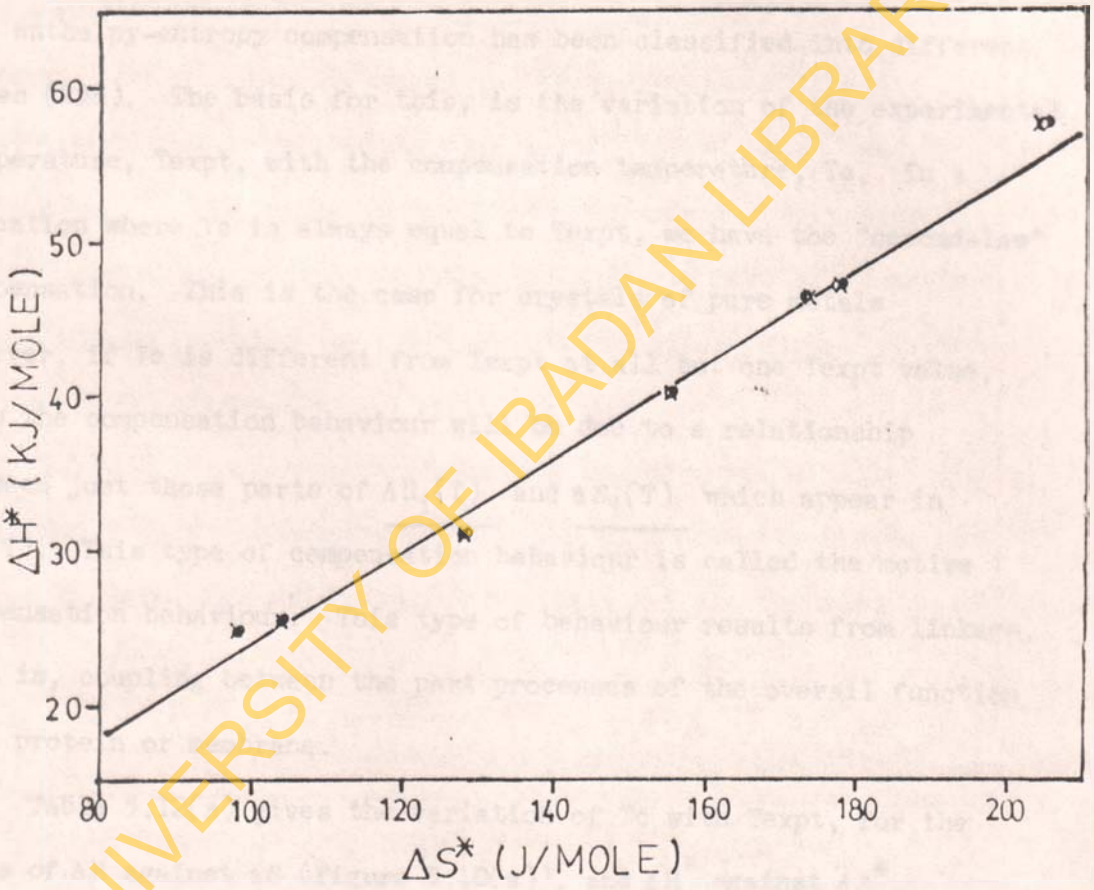


Fig. 5.10b: Plot of ΔH* against ΔS* at varying glycerol concentration for 20°C, (0); 27°C, (●); and 34°C, (X).

of the bulk solvent like dielectric constant, viscosity and surface tension. The presence of the compensation phenomena which has been proposed as diagnostic test for the participation of water in protein processes (256) again solidify the fact that liquid water plays a direct role in the reaction of glucose-6-phosphate dehydrogenase. The enthalpy-entropy compensation has been classified into different types (258). The basis for this, is the variation of the experimental temperature, Texpt, with the compensation temperature, Tc. In a situation where Tc is always equal to Texpt, we have the "second-law" compensation. This is the case for crystals of pure metals However, if Tc is different from Texpt at all but one Texpt value, then the compensation behaviour will be due to a relationship between just those parts of $\Delta H_i(T)$ and $\Delta S_i(T)$ which appear in $\Delta G_i(T)$. This type of compensation behaviour is called the motive compensation behaviour. This type of behaviour results from linkage, that is, coupling between the part processes of the overall function of a protein or membrane.

Table 5.12(a) gives the variation of Tc with Texpt, for the plots of ΔH against ΔS (figure 5.10(a)), and ΔH^* against ΔS^* (figure 5.10(b)). It can be observed that Tc is different from Texpt at all but one temperature, which is 27°C, for the two plots. This means that the type of compensation behaviour found in the G6PD enzyme is the motive compensation. This could therefore be an

evidence for the existence of linkage in G6PD processes.

The variation of the hydrogen ion activity (pH) in the binding of G6P to the Mould and B enzymes, also yielded enthalpy values which were compensated for by the corresponding entropy values. In the pH range of about 6.0 to 9.0, the maximum variation in ΔG for the enzyme-G6P complex was about 1.3kJ $mole^{-1}$ for the two variants (Table 3.9). By contrast, the variations in the corresponding ΔH and ΔS values were about 17.0 kJ $mole^{-1}$ and 60 J $mole^{-1}$ respectively. Thus while ΔG was almost invariant, variations in ΔH were compensated for by variations in ΔS. A plot of ΔH against ΔS was linear, showing the compensation behaviour (figure 5.11) for the two G6PD enzymes.

Table 5.13 gives the compensation temperatures obtained for the Mould and B G6PD enzymes for the low and high affinity states at 27°C. It can be seen that the Tc values obtained for the two G6PD types

TABLE 5.13

G6PD Type	L.A	H.A
B	315.47 ± 7.25	299.64 ± 4.32
MOULD	310.00 ± 5.43	302.20 ± 4.16

are similar, for both the low and high affinity states. Beetlestone and

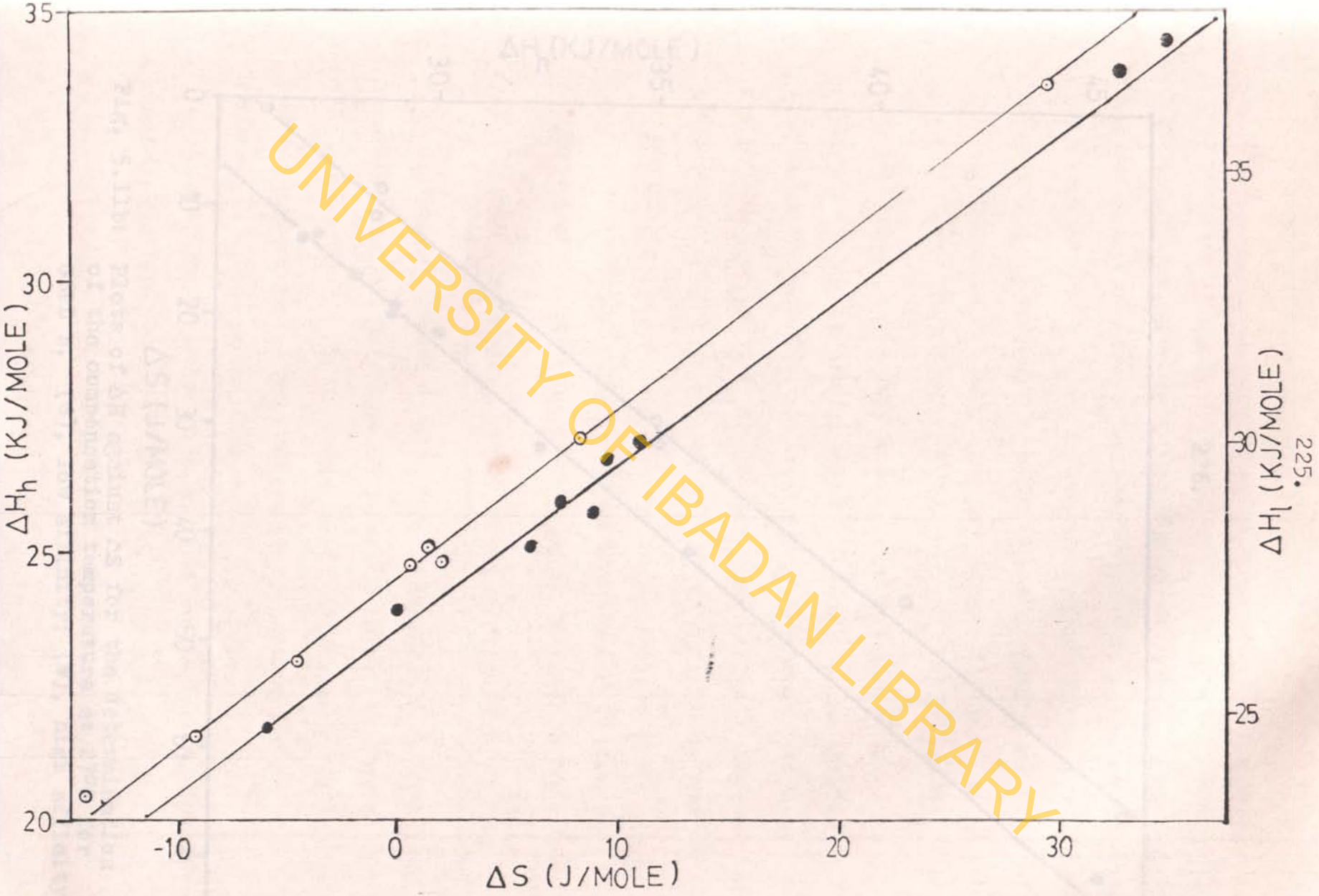


Fig. 5.11(a): Plots of ΔH against ΔS at variable pH at 27°C for the Mould variant. (⊙) - low affinity; (●) - high affinity.

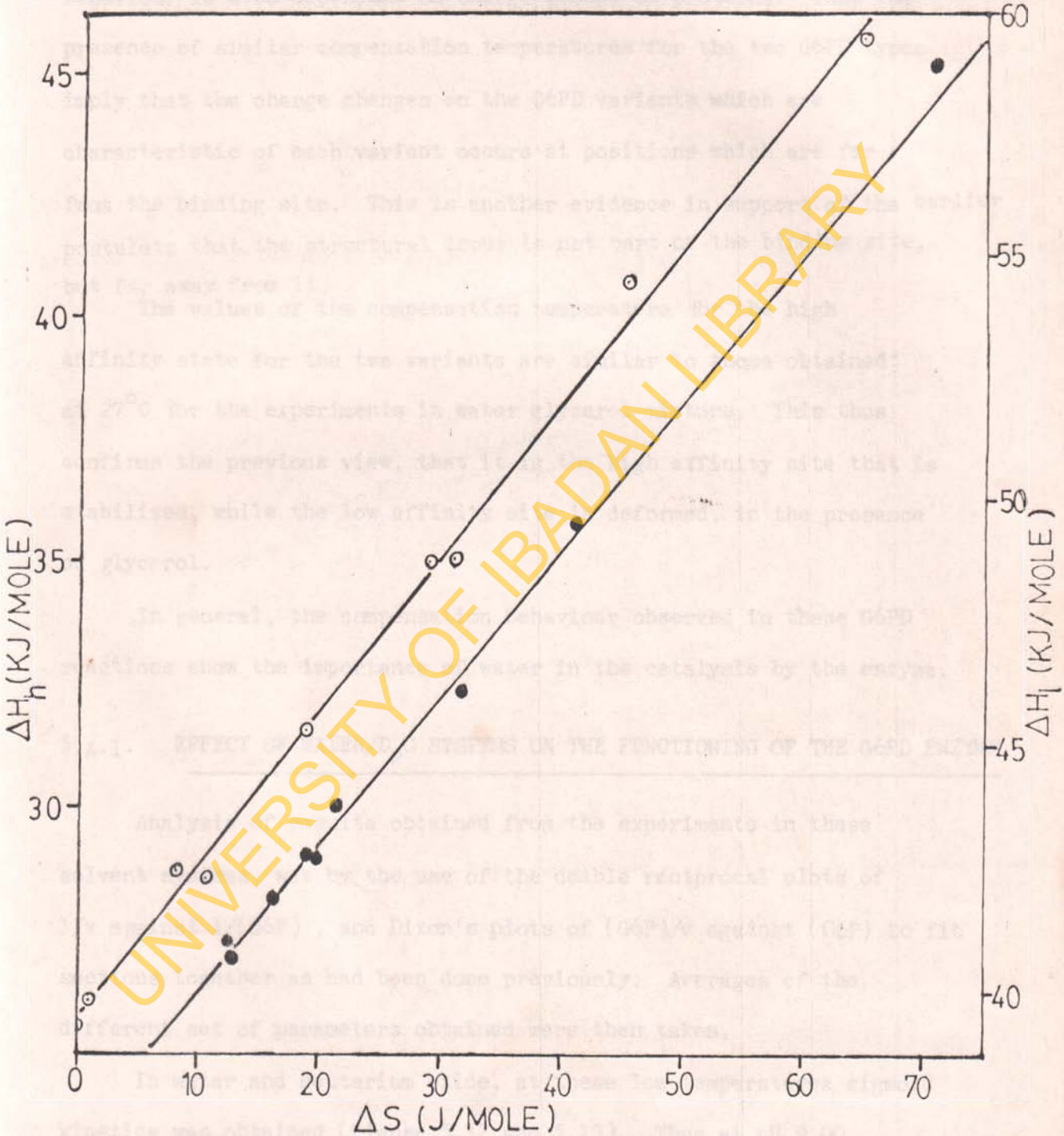


Fig. 5.11b: Plots of ΔH against ΔS for the determination of the compensation temperature at 27°C for G6PD B. (⊖), low affinity; (●), high affinity.

co-workers (248, 255, 256) have previously shown that the compensation behaviour is also dependent on charge groups on proteins. Thus the presence of similar compensation temperatures for the two G6PD types imply that the charge changes on the G6PD variants which are characteristic of each variant occurs at positions which are far from the binding site. This is another evidence in support of the earlier postulate that the structural locus is not part of the binding site, but far away from it.

The values of the compensation temperature for the high affinity state for the two variants are similar to those obtained at 27°C for the experiments in water glycerol mixture. This thus confirms the previous view, that it is the high affinity site that is stabilized, while the low affinity site is deformed, in the presence of glycerol.

In general, the compensation behaviour observed in these G6PD reactions show the importance of water in the catalysis by the enzyme.

5.4.1. EFFECT OF WATER/D_2O SYSTEMS ON THE FUNCTIONING OF THE G6PD ENZYME

Analysis of results obtained from the experiments in these solvent systems, was by the use of the double reciprocal plots of $1/v$ against $1/(G6P)$, and Dixon's plots of $(G6P)/v$ against $(G6P)$ to fit sections together as had been done previously. Averages of the different set of parameters obtained were then taken.

In water and deuterium oxide, at these low temperatures sigmoid kinetics was obtained (figure 5.12 and 5.13). Thus at pH 9.00

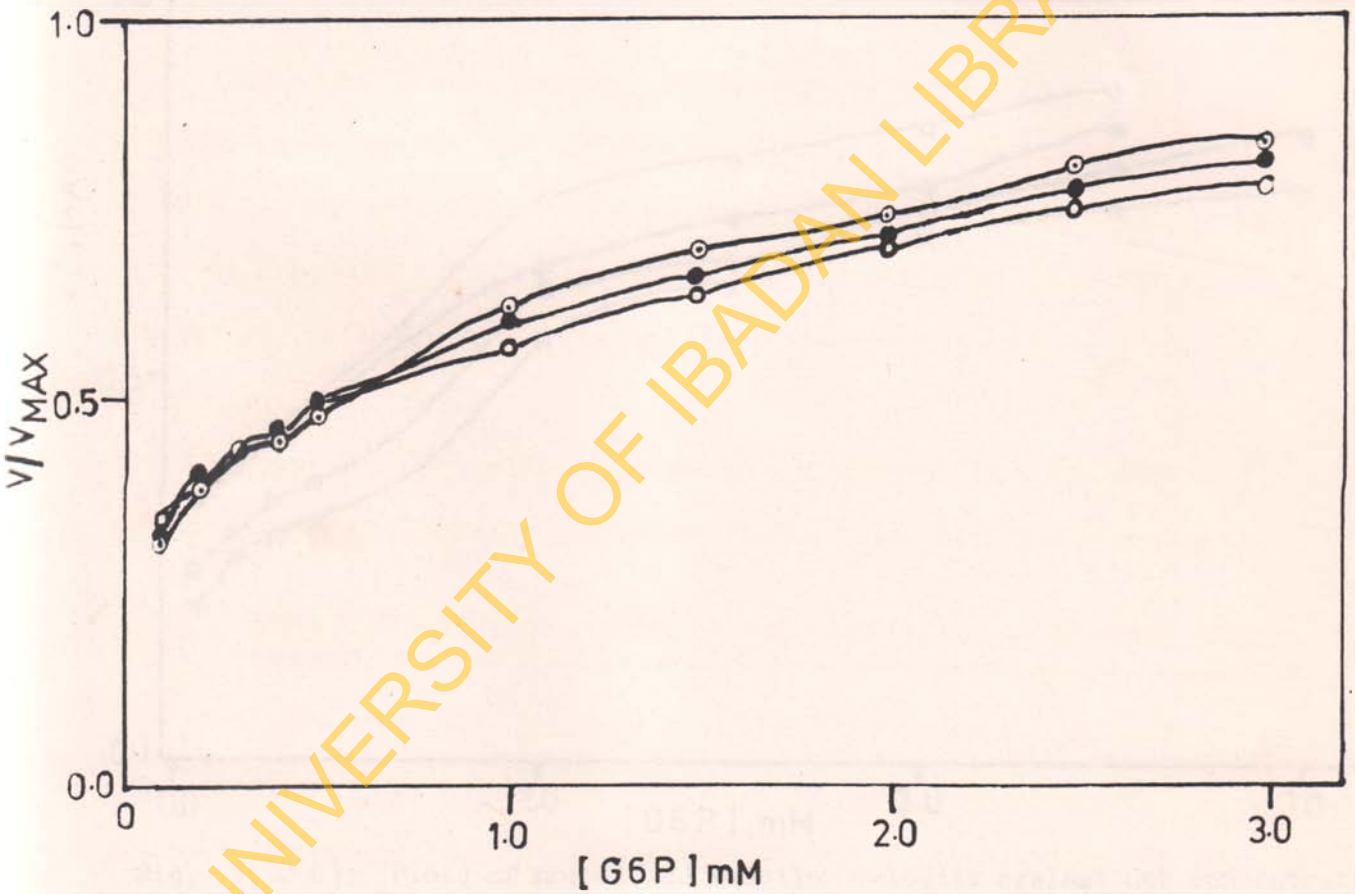


Fig. 5.12(a) : Plots of normalized reaction velocity against G6P concentration at pH 9.0 for water experiment at 8°C (○), 4°C (⊙), 1°C (●).

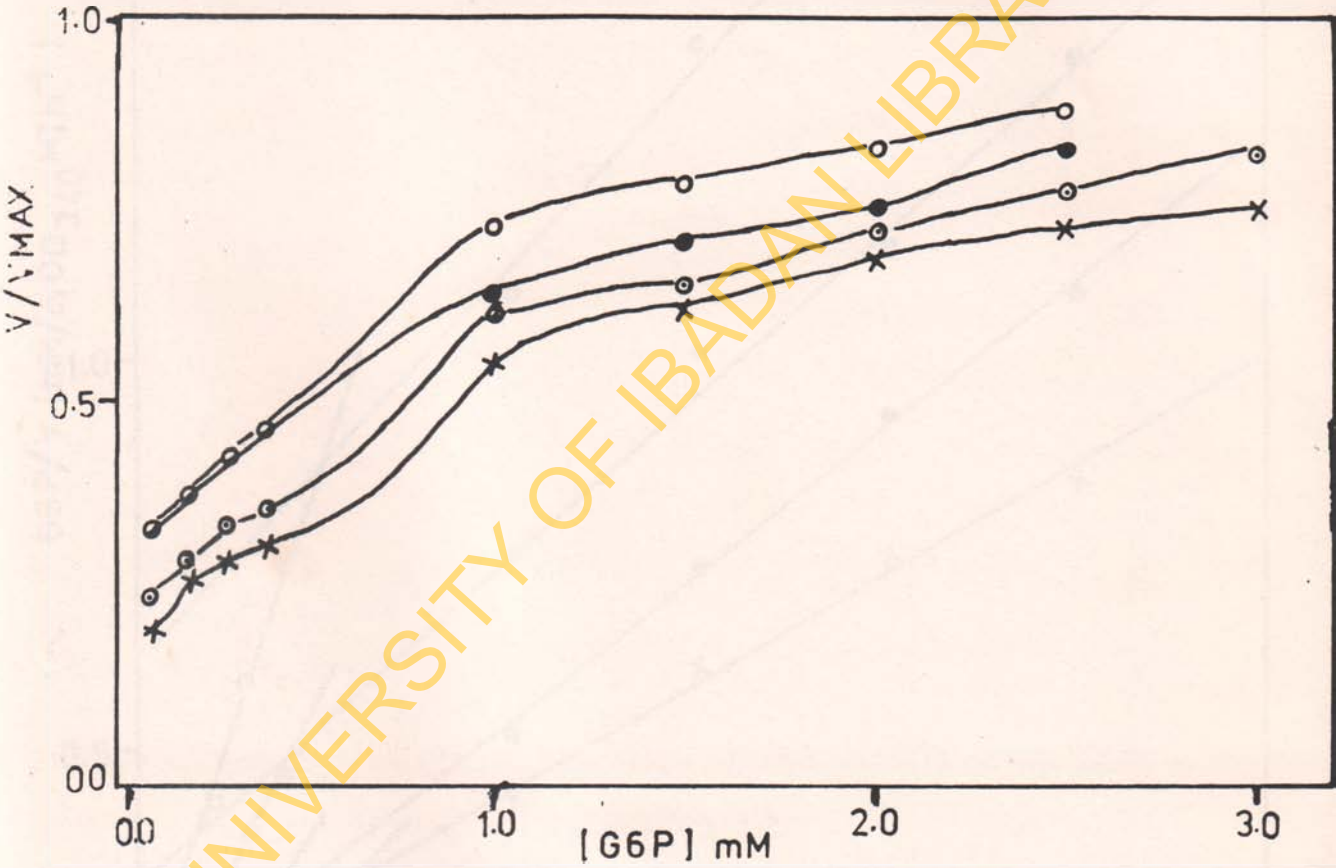


Fig. 5.12(b): Plots of normalized reaction velocity against G6P concentratio at pD 9.4 for D_2O experiments at 14°C (●); 11°C (○), 8°C (⊙) and 5°C (X).

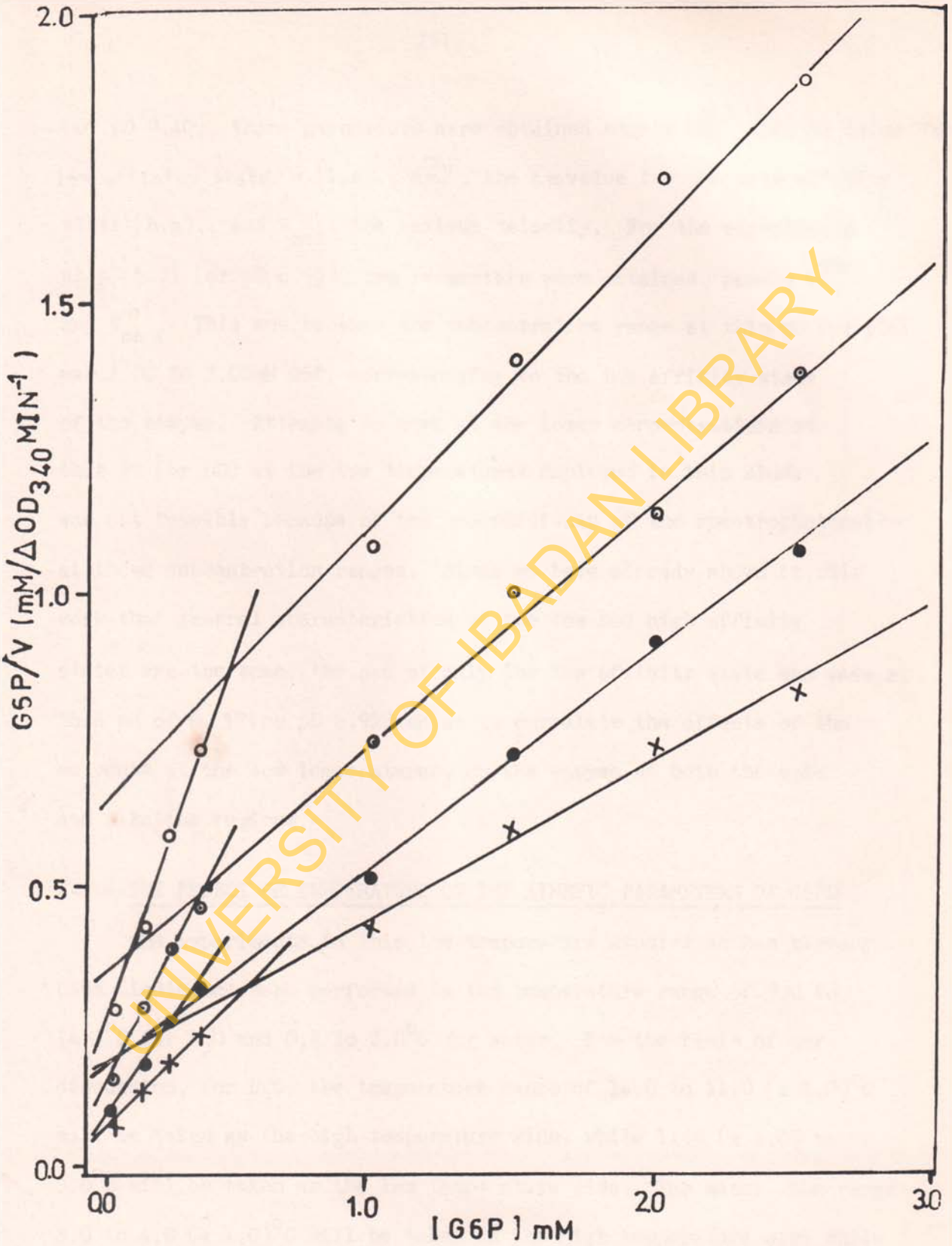


Figure 5.13 : Typical Dixon's plot of (G6P)/v against (G6P) at pD 9.4 for the D_2O experiment at 14°C (X), 11°C (●), 7°C (⊙) and 3°C (○).

(or pD 9.40), three parameters were obtained namely Km_1^{G6P}, the Km value for the low affinity state (l.a)., Km_2^{G6P}, the Km value for the high affinity state (h.a)., and V_{max}, the maximum velocity. For the experiments at pH 6.55 (or pD 6.95), two parameters were obtained, namely Km_1^{G6P} and V_{max}. This was because the concentration range at this pH (or pD) was 1.00 to 3.00mM G6P, corresponding to the low affinity state of the enzyme. Attempts to work at the lower concentrations at this pH (or pD) at the low temperatures employed in this study was not feasible because of the insensitivity of the spectrophotometer at those concentration ranges. Since we have already shown in this work that general characteristics of the low and high affinity states are the same, the use of only the low affinity state was made at this pH of 6.55 (or pD 6.95) so as to correlate the effects of the solvents at the low temperatures, on the enzyme at both the acid and alkaline regions.

5.4.2. THE EFFECT OF TEMPERATURE ON THE KINETIC PARAMETERS OF G6PD

The experiments in this low temperature studies as has already been indicated were performed in the temperature range of 3.0 to 14.0°C for D_2O and 0.5 to 8.0°C for water. For the basis of our discussion, for D_2O, the temperature range of 14.0 to 11.0 (± 1.0)°C will be taken as the high temperature side, while 11.0 (± 1.0) to 3.0°C will be taken as the low temperature side. For water, the range 8.0 to 4.0 (± 1.0)°C will be taken as the high temperature side while

4.0 (± 1.0) to 0.5 °C will be taken as the low temperature side.

11°C is the temperature of maximum density for D_2O while 4°C is that for water.

The temperature dependence of Km and V_{max} for G6P binding to the enzyme at the different pH (D)s are summarized in figures 5.14-5.16. Tables 5.14 to 5.17 give the dependence on temperature of log Km and log V_{max} for the two pH (D)s.

Looking at the results obtained for the water experiments at pH 6.55 and pH 9.00 (figs. 5.14a and 5.15), a general characteristic becomes discernable immediately. Km decreases with temperature until about 4°C (4.24°C for pH 6.55, 3.55°C for pH 9.00 (1.a) and 5.86°C for pH 9.00 (h.a)), where a further decrease in temperature results in increase in Km. The slope of the plot of 1/T against log Km, Δ log Km/Δ(1/T) for the high temperature side is negative, yielding a positive enthalpy, ΔH_1, just like the case at higher temperatures. However, at the low temperature side, the slope, Δ log Km/Δ(1/T) positive yielding a negative enthalpy, ΔH_2. For pH 6.55, ΔH_1 is 84.57 kJ $mole^{-1}$, turn around temperature is 4.24°C, while ΔH_2 is -71.48 kJ $mole^{-1}$. At pH 9.00 (1.a) ΔH_1 is 57.44 kJ $mole^{-1}$, turn around temperature is 3.55°C while ΔH_2 is -81.06 kJ $mole^{-1}$. For pH 9.00 (h.a), ΔH_1 is 73.40 kJ $mole^{-1}$, turn around temperature is 5.86°C while ΔH_2 is -99.57 kJ $mole^{-1}$.

The V_{max} values for pH 6.55 and pH 9.00 also decrease as the temperature decreases. However at about 4.0°C (4.38°C for pH 6.55 and

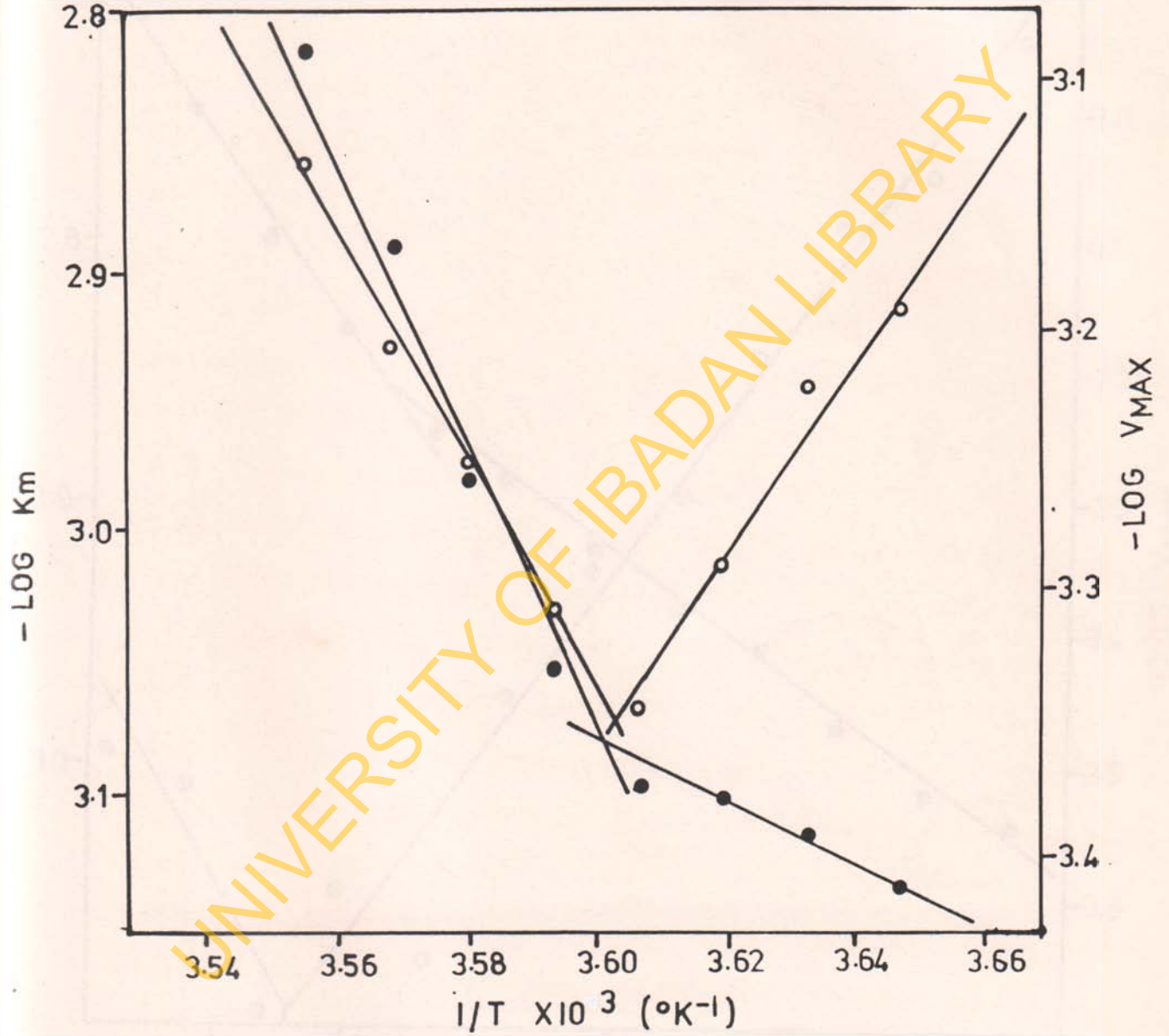


Fig. 5.14(a): Plot of log K_m (V_{max}) against 1/T for the experiment in water at pH 6.55. (O) - log K_m ; (●) - log V_{max}.

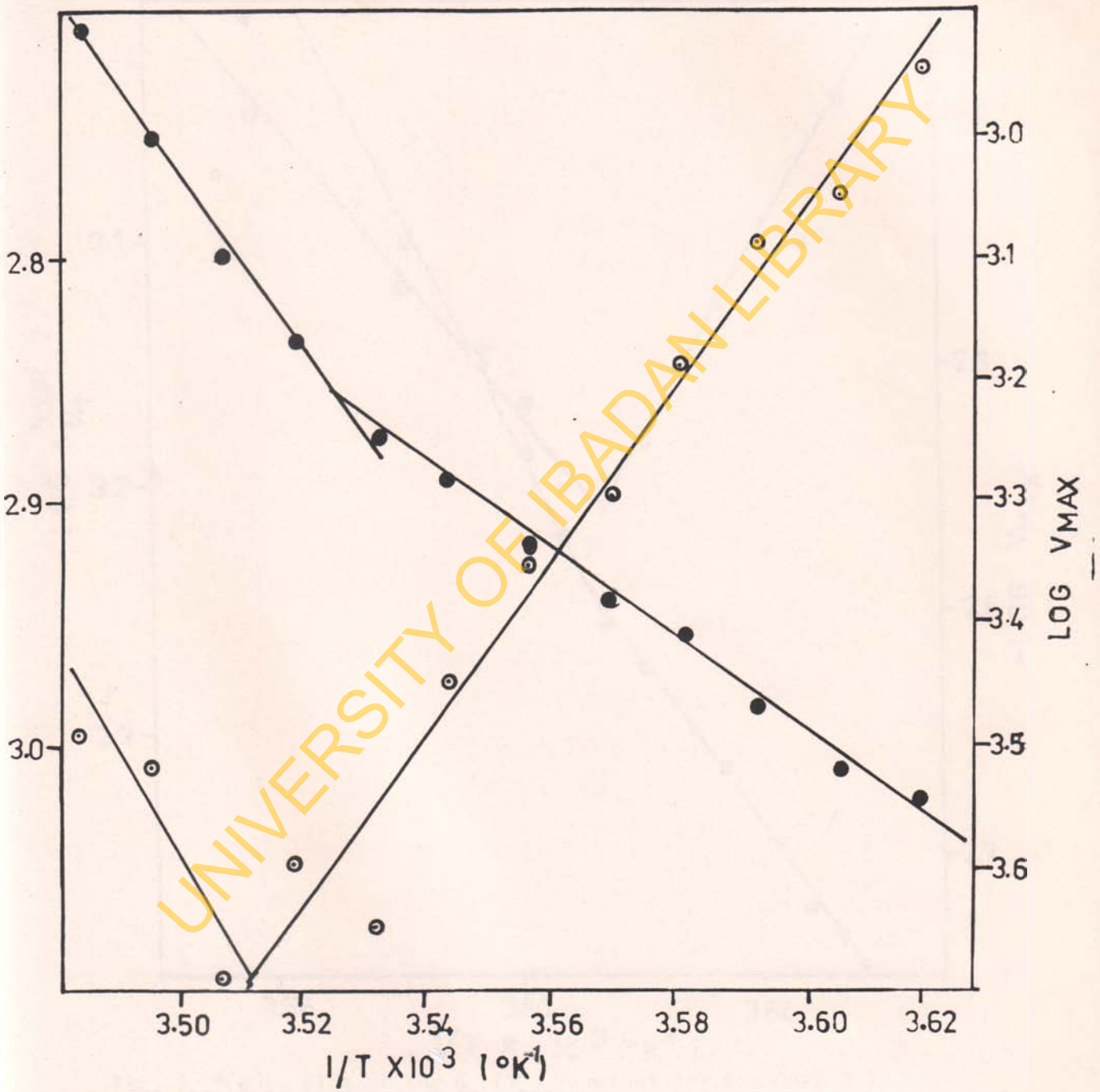


Fig. 5.14(b): Plot of log K_m(V_{max}) against 1/T for the experiment in D_2O at pD 6.95. (⊙) - log K_m; (●) - log V_{max}.

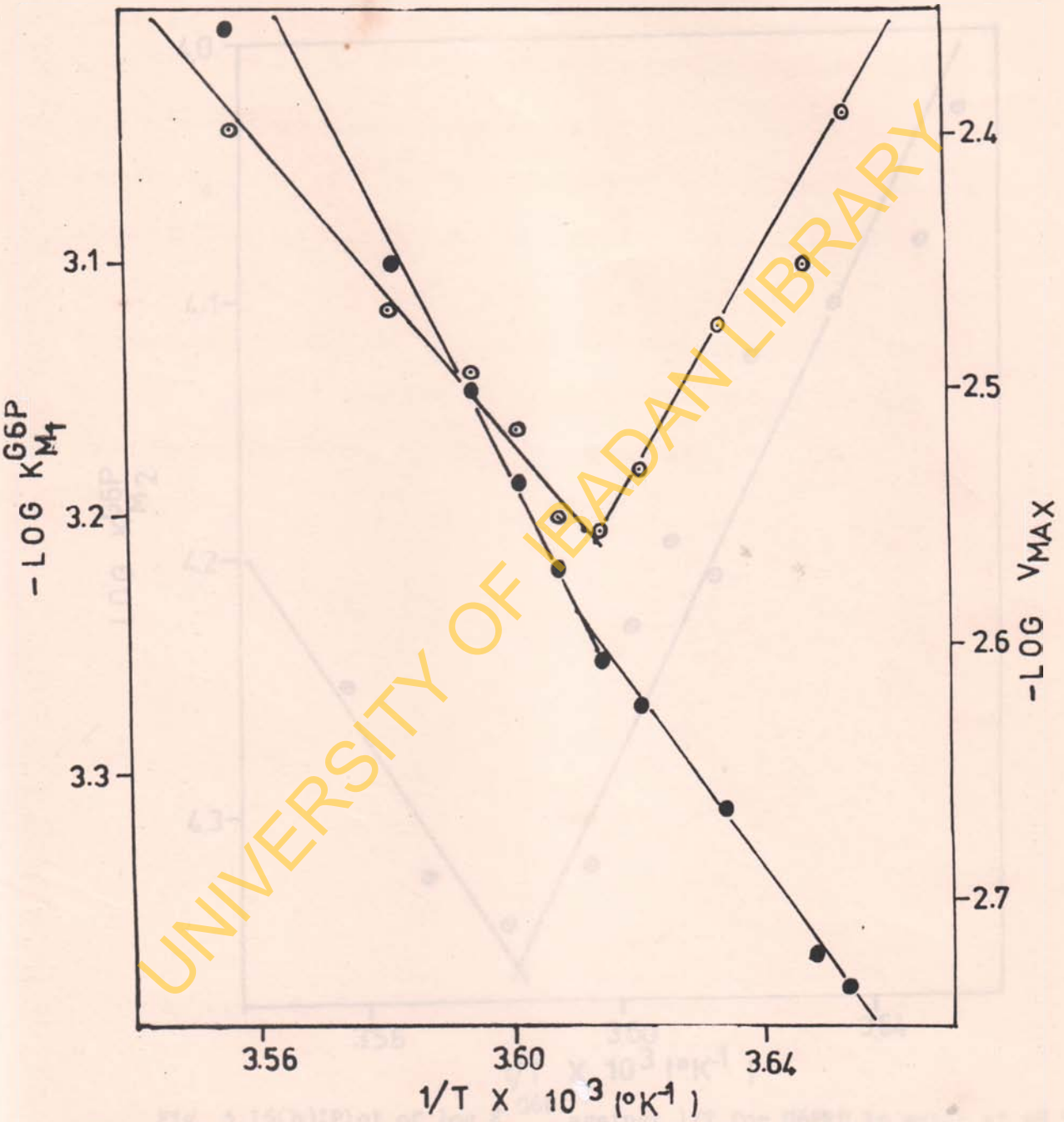


Fig. 5.15(a): Plot of log K_m (V_{max}) against 1/T for G6PD B in H_2O at pH 9.00. (⊙) - log $K_{m_1}^{G6P}$; (●) - log V_{max}

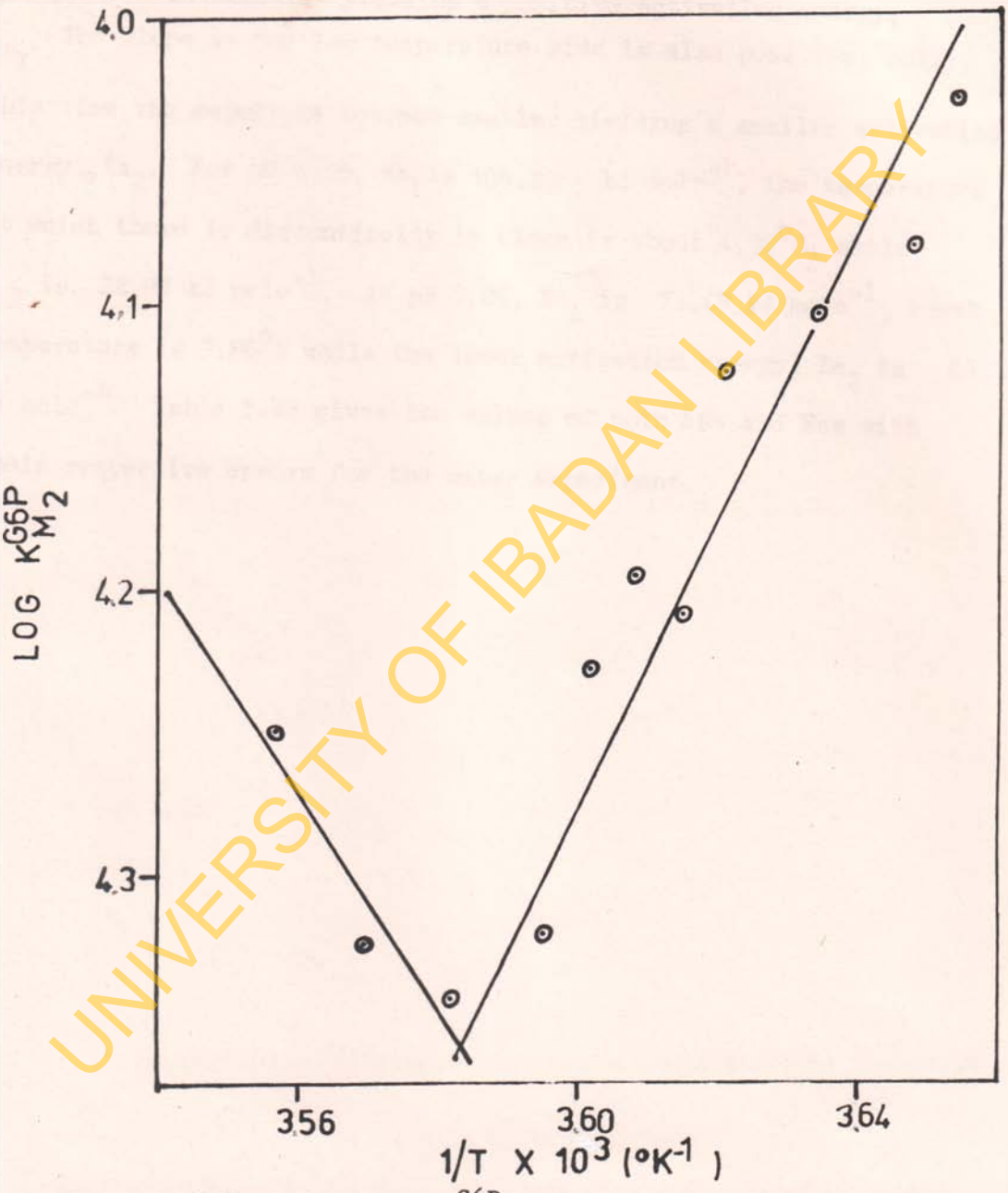


Fig. 5.15(b): Plot of log $K_{m_2}^{G6P}$ against 1/T for G6PDB in water at pH 9.0.

3.86°C for pH 9.0) the rate of decrease changes. The slope of the plot of log V_{max} against 1/T, $\Delta \log V_{max} / \Delta(1/T)$ for the high temperature side is negative yielding a positive activation energy, Ea_1. The slope at the low temperature side is also positive. only this time the magnitude becomes smaller yielding a smaller activation energy, Ea_2. For pH 6.55, Ea_1 is 104.35 kJ $mole^{-1}$, the temperature at which there is discontinuity in slope is about 4.38°C, while Ea_2 is 22.98 kJ $mole^{-1}$. At pH 9.00, Ea_1 is 71.48 kJ $mole^{-1}$, break temperature is 3.86°C while the lower activation energy, Ea_2 is 65.10 kJ $mole^{-1}$. Table 5.18 gives the values of both ΔH_s and Eas with their respective errors for the water experiment.

TABLE 5.14

TABLE OF LOG K_M AND LOG V_{max} FOR WATER EXPERIMENTS AT pH 6.55

(LOW TEMPERATURES)

Temp (°C)	- Log K_{ml}^{G6P}	- Log V_{max}
8.0	2.86 ± 0.04	3.09 ± 0.04
7.0	2.93 ± 0.08	3.19 ± 0.06
6.0	2.97 ± 0.12	3.25 ± 0.07
5.0	3.03 ± 0.06	3.33 ± 0.05
4.0	3.07 ± 0.07	3.37 ± 0.04
3.0	3.01 ± 0.07	3.38 ± 0.04
2.0	2.94 ± 0.03	3.39 ± 0.02
1.0	2.92 ± 0.08	3.41 ± 0.04

TABLE 5.15

TABLE OF LOG K_M AND LOG V_{MAX} FOR D_2O EXPERIMENTS AT PD 6.95

(LOW TEMPERATURES)

Temp (°C)	- Log K_{ml}^{G6P}	- Log V_{max}
14.0	2.99 ± 0.11	2.91 ± 0.06
13.0	3.01 ± 0.15	2.99 ± 0.08
12.0	3.09 ± 0.07	3.10 ± 0.03
11.0	3.05 ± 0.10	3.17 ± 0.05
10.0	3.07 ± 0.12	3.24 ± 0.06
9.0	2.97 ± 0.06	3.28 ± 0.04
8.0	2.93 ± 0.09	3.33 ± 0.06
7.0	2.90 ± 0.09	3.38 ± 0.05
6.0	2.84 ± 0.03	3.41 ± 0.02
5.0	2.79 ± 0.03	3.46 ± 0.02
4.0	2.77 ± 0.06	3.52 ± 0.04
3.0	2.72 ± 0.04	3 54 ± 0.03

TABLE 5.16

TABLE OF LOG K_M AND LOG V_{MAX} FOR WATER EXPERIMENTS AT PH 9.00

(LOW TEMPERATURES)

TEMP (^{o}C)	- LOG K_{m1}^{G6P}	- LOG K_{m2}^{G6P}	- LOG V_{max}
8.0	3.05 ± 0.05	4.25 ± 0.07	2.36 ± 0.02
7.0	3.12 ± 0.14	4.32 ± 0.09	2.45 ± 0.04
6.0	3.12 ± 0.14	4.34 ± 0.03	2.45 ± 0.04
5.0	3.14 ± 0.12	4.32 ± 0.07	2.50 ± 0.03
4.5	3.17 ± 0.14	4.23 ± 0.01	2.54 ± 0.04
4.0	3.20 ± 0.07	4.20 ± 0.02	2.57 ± 0.03
3.5	3.21 ± 0.12	4.21 ± 0.03	2.61 ± 0.03
3.0	3.18 ± 0.08	4.13 ± 0.04	2.62 ± 0.03
2.0	3.12 ± 0.11	4.10 ± 0.04	2.62 ± 0.03
1.0	3.10 ± 0.14	4.08 ± 0.01	2.72 ± 0.05
0.5	3.04 ± 0.06	4.03 ± 0.01	2.73 ± 0.04

TABLE 5.17

TABLE OF LOG K_M AND LOG V_{MAX} FOR D_2O EXPERIMENTS AT PD 9.40

(LOW TEMPERATURES)

TEMP (°C)	- LOG Km_1^{G6P}	- LOG Km_2^{G6P}	- LOG V_{max}
14.0	3.23 ± 0.10	4.16 ± 0.06	2.44 ± 0.07
13.0	3.29 ± 0.06	4.19 ± 0.13	2.48 ± 0.02
12.0	3.33 ± 0.14	4.21 ± 0.10	2.53 ± 0.04
11.0	3.41 ± 0.14	4.19 ± 0.12	2.58 ± 0.03
10.0	3.32 ± 0.09	4.18 ± 0.13	2.59 ± 0.03
9.0	3.23 ± 0.08	4.17 ± 0.12	2.59 ± 0.03
8.0	3.16 ± 0.09	4.14 ± 0.13	2.60 ± 0.06
7.0	3.11 ± 0.06	4.12 ± 0.15	2.62 ± 0.05
6.0	3.10 ± 0.11	4.06 ± 0.01	2.65 ± 0.05
5.0	3.03 ± 0.05	4.09 ± 0.08	2.67 ± 0.02
4.0	3.01 ± 0.04	4.06 ± 0.05	2.70 ± 0.03
3.0	2.96 ± 0.10	4.04 ± 0.01	2.72 ± 0.06

TABLE 5.18

TABLE OF ΔH AND EA FOR THE WATER EXPERIMENT AT PH 6.55 AND 9.00

Energy Type (kJ/mole)	PH		
	pH 6.55	pH 9.00 (L.a)	pH 9.00 (h.a)
ΔH_1	84.57 ± 8.99	57.44 ± 3.85	73.40 ± 14.02
ΔH_2	-71.48 ± 11.95	-81.06 ± 1.24	-99.57 ± 10.22
Ea_1	104.35 ± 9.01	71.48 ± 6.37	
Ea_2	22.98 ± 4.78	65.10 ± 2.77	

TABLE 5.19

TABLE OF ΔH AND E_A FOR THE D_2O EXPERIMENT AT PD 6.95 AND 9.40

Energy Type (kJ/Mole)	PH		
	pD 6.95	pD 9.40 (L.a)	pD 9.40 (h.a)
ΔH_1	80.42 ± 13.88	98.29 ± 10.62	40.21 ± 3.75
ΔH_2	-65.42 ± 2.35	-87.76 ± 7.29	-28.45 ± 1.68
Ea_1	135.31± 5.31	73.39 ± 2.66	
Ea_2	65.10 ± 2.15	26.33 ± 3.54	

Looking also at the results for the D_2O experiments at pD 6.95, pD 9.40 (l.a) and pD 9.40 (h.a) (figures 5.14b and 5.16), we also observe a trend in the variation of Km and V_{max} with temperature, which is similar to those for water. Km decreases with temperature until about 11·0°C (11.67°C for pD6.95, 11.18°C for pD 9.4 (l.a) and 12.03°C for pD 9.4 (h.a)) where a further decrease in temperature results in increase in Km. The slope of the plot of log Km against 1/T for the high temperature side like the case of water is negative yielding a positive enthalpy, ΔH_1. At low temperature side however, a negative enthalpy, ΔH_2 is obtained corresponding to a positive slope. At pD 6.95, ΔH_1 is 80.42 kJ $mole^{-1}$, turn around temperature is 11.67°C while ΔH_2 is -65.42kJ $mole^{-1}$. At pD 9.40 (l.a), ΔH_1 is 98.29 kJ $mole^{-1}$, turn around temperature is 11.18°C while ΔH_2 is -87.76 kJ $mole^{-1}$. At pD 9.40 (h.a), ΔH_1 is 40.21 kJ $mole^{-1}$, turn around temperature is 12.0°C while ΔH_2 is -28.45 kJ $mole^{-1}$.

The V_{max} values for pD 6.95 and 9.40 also decrease with temperature, only that at about 11.0°C (10.75°C for pD 6.95 and 11.35°C for pD 9.40) a change in the rate of decrease becomes discernable. The slopes of the plot of log V_{max} against 1/T for the low and high temperature sides are the same in sign, that is negative, the only difference being a drop in the value of the slope as we move to the low temperature side. At pD 6.95, Ea_1 is 135.31 kJ $mole^{-1}$ break temperature is 10.75°C while Ea_2 is 65.10kJ $mole^{-1}$. At pD 9.40,

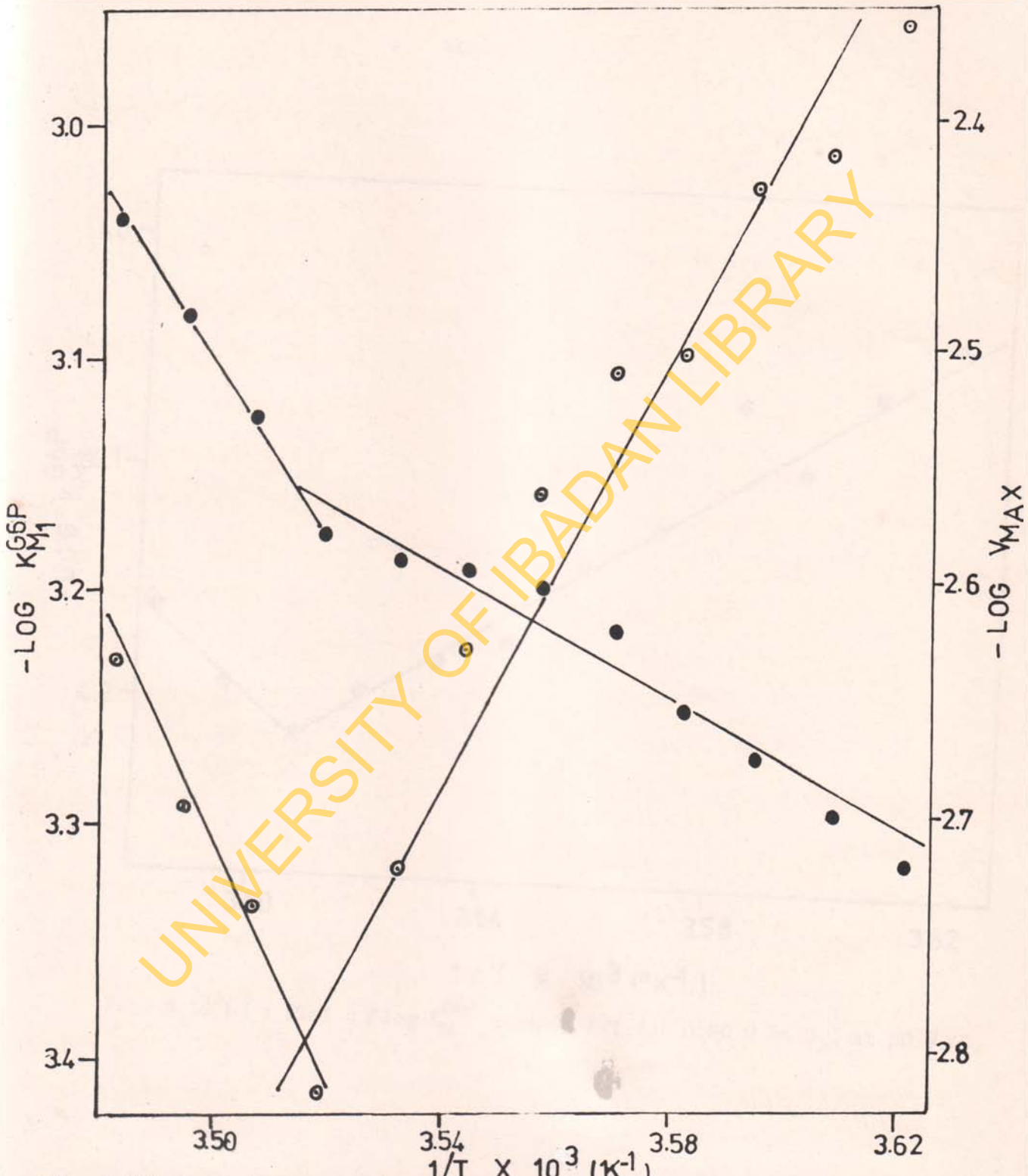


Fig. 5.16(a): Plot of log K_M (V_{MAX}) against 1/T for G6PD B in D_2O at pD 9.4
(⊙) - log $K_{M_1}^{G6P}$; (●) - log V_{MAX}.

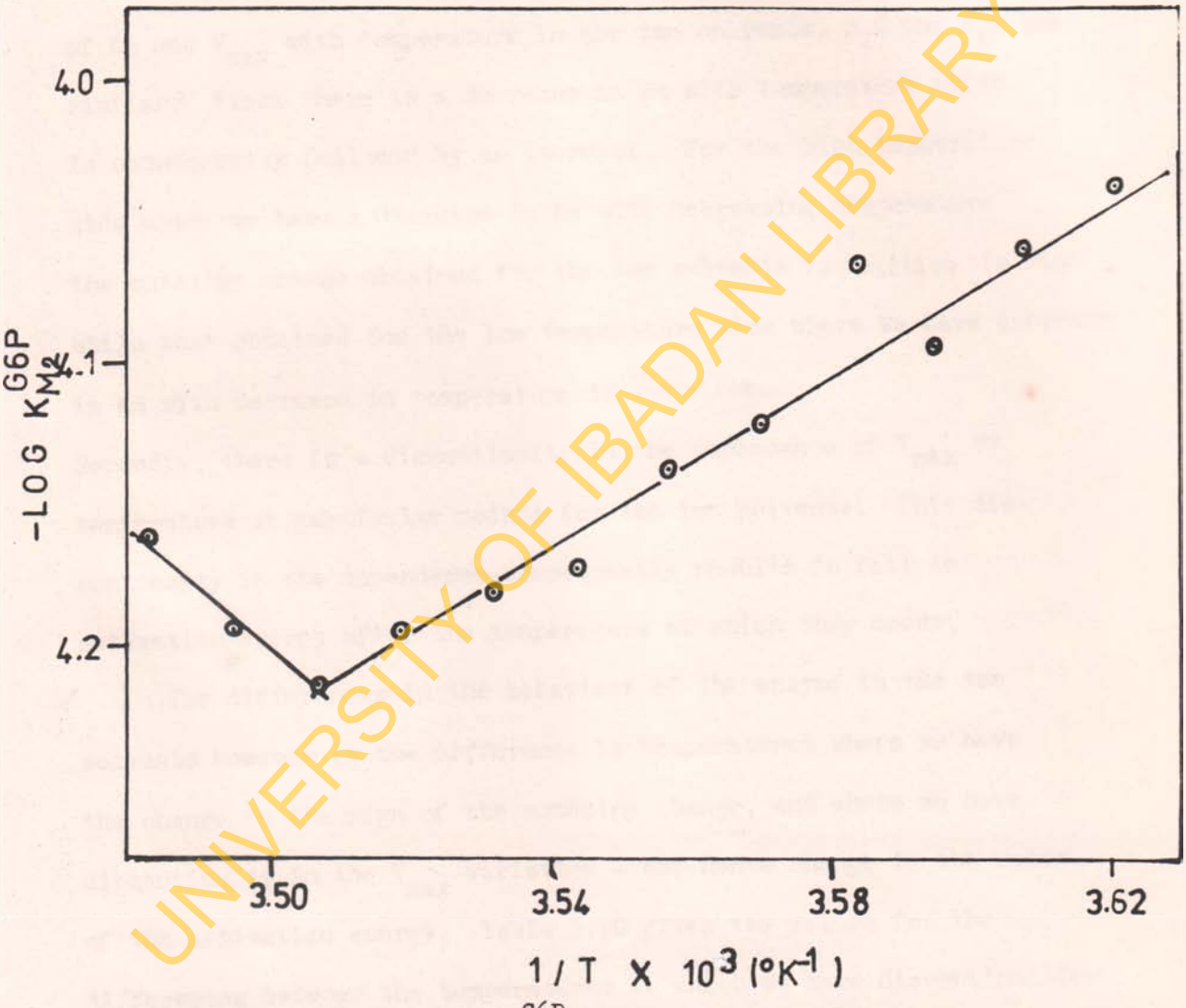


Fig. 5.16(b) : Plot of log $K_{M_2}^{G6P}$ against 1/T for G6PD B in D_2O at pD 9.40.

Ea_1 is 73.39 kJ mole^{-1}, break temperature is 11.35^{o}C while Ea_2 is 26.33 kJ mole^{-1}. Table 5.19 gives the values of ΔH and Ea with their respective errors for the D_2O experiments.

From the above descriptions, we can see that the variations of Km and V_{max} with temperature in the two solvents, D_2O and H_2O are similar. First there is a decrease in Km with temperature which is consequently followed by an increase. For the high temperature side where we have a decrease in Km with decreasing temperature the enthalpy change obtained for the two solvents is positive in sign while that obtained for the low temperature side where we have increase in Km with decrease in temperature is negative.

Secondly, there is a discontinuity in the dependence of V_{max} on temperature at particular points for the two solvents. This discontinuity in the dependence consequently results in fall in activation energy after the temperature at which they occur.

The differences in the behaviour of the enzyme in the two solvents however is the difference in temperatures where we have the change in the sign of the enthalpy change, and where we have discontinuity in the V_{max} variation - and hence change in the value of the activation energy. Table 5.20 gives the values for the differences between the temperatures at which we have discontinuities for both solvents at the same pHs and affinities, for the enthalpy changes and energy of activation. The differences observed is about 7^{o}C, the difference in the temperatures of their maximum density. This shows that the dependence of the G6PD properties on solvents

is unique for each particular solvent.

TABLE 5.20

DIFFERENCES IN TEMPERATURE OF BREAK POINTS FOR D_2O AND H_2O AT THE SAME pHs AND AFFINITIES

pH/pD	$\Delta T_{(\Delta H)}$ (°C)	$\Delta T_{(Ea)}$ (°C)
pH 6.55/pD 6.95	7.43	6.37
pH 9.00/pD 9.40 (l.a)	7.63	7.49
pH 9.00/pD 9.40 (h.a)	6.17	-

Since density is the measure of the mass composition of solvents, and since the abrupt changes in the normal trend of the kinetic and therefore thermodynamic parameters happened at the point of maximum density for the two solvents, it could therefore be possible that the alterations in mass composition is one of the factors responsible for the observed anomalous behaviour of the enzyme.

Naturally, protein-solvent interactions would be influenced by alterations in the solvent properties. At 4° and 11°C for water and deuterium oxide respectively, it is known that fluctuations of internal energy with volume change sign. Since internal pressure is the measure of the change in internal energy with volume, it could therefore be one of the most probable property of the two solvents

apart from mass composition, which can also account for the type of behaviour observed for the kinetic and thermodynamic parameters of G6P binding to G6PD in this study.

The possible role of the solvent viscosity in determining the dynamic state of the protein through structural fluctuations has been suggested in a number of theoretical models for enzyme action (259 - 262). There have also been observed effects of viscosity on enzyme rate parameters (244). Thus correlation of the solvent viscosity with the enzymic rate can be used to determine if the observed trends in these low temperature experiments is as a result of the alterations in viscosity as was the case in the glycerol experiment.

A look at the variations of Km and V_{max} with temperature for the solvents show that variations of Km is affected more than that of V_{max}. at the temperatures of maximum density for water and D_2O. Km reaches a minimum at the temperature of maximum density for the two solvents, and then start increasing with continued decrease in temperature, unlike V_{max} which continues to decrease (no minimum) although at a different rate. Since the Km parameter could be more complex in terms of the rate constants of which it is composed however, this observation is not therefore surprising.

5.4.3. THE EFFECT OF VISCOSITY ON THE RATE CONSTANT

Viscosity is the resistance that one part of a fluid offers to the flow of another part of the fluid. It is produced by the shearing effect of moving one layer of the fluid past another and *is quite distinct from intermolecular attractions. It influences* protein reactions, even in aqueous solutions, and it has been suggested that it may play a significant role in the determination of the dynamic state of the protein through structural fluctuations (259 - 262). The expression for the variation of viscosity with rate was given by Kramers (245), and has been simplified to $V = c\eta^{-1}$, for the purpose of this study as has been shown earlier.

Figure 5.17 gives the dependence on viscosity of V_{max} at pH 6.55 and 9.00 for the water experiment. It was observed that V_{max} is dependent on viscosity at the two pHs until about 4°C (4.4°C at pH 6.55 and 3.75°C at pH 9.00) where there resulted discontinuity in the dependence. This discontinuity in viscosity at this temperature implies that viscosity is not the most likely solvent property responsible for the observed anomalous behaviour of V_{max}, and by implication Km. This therefore means that another property of the solvent which has probably been influencing the kinetic parameters of the G6PD enzyme in parallel with viscosity is probably respensible for the observed anomalous behaviour of the enzyme at 4°C and 11°C in water and D_2O respectively. Table 5.21 gives the values of viscosity of water at the low temperatures utilised.

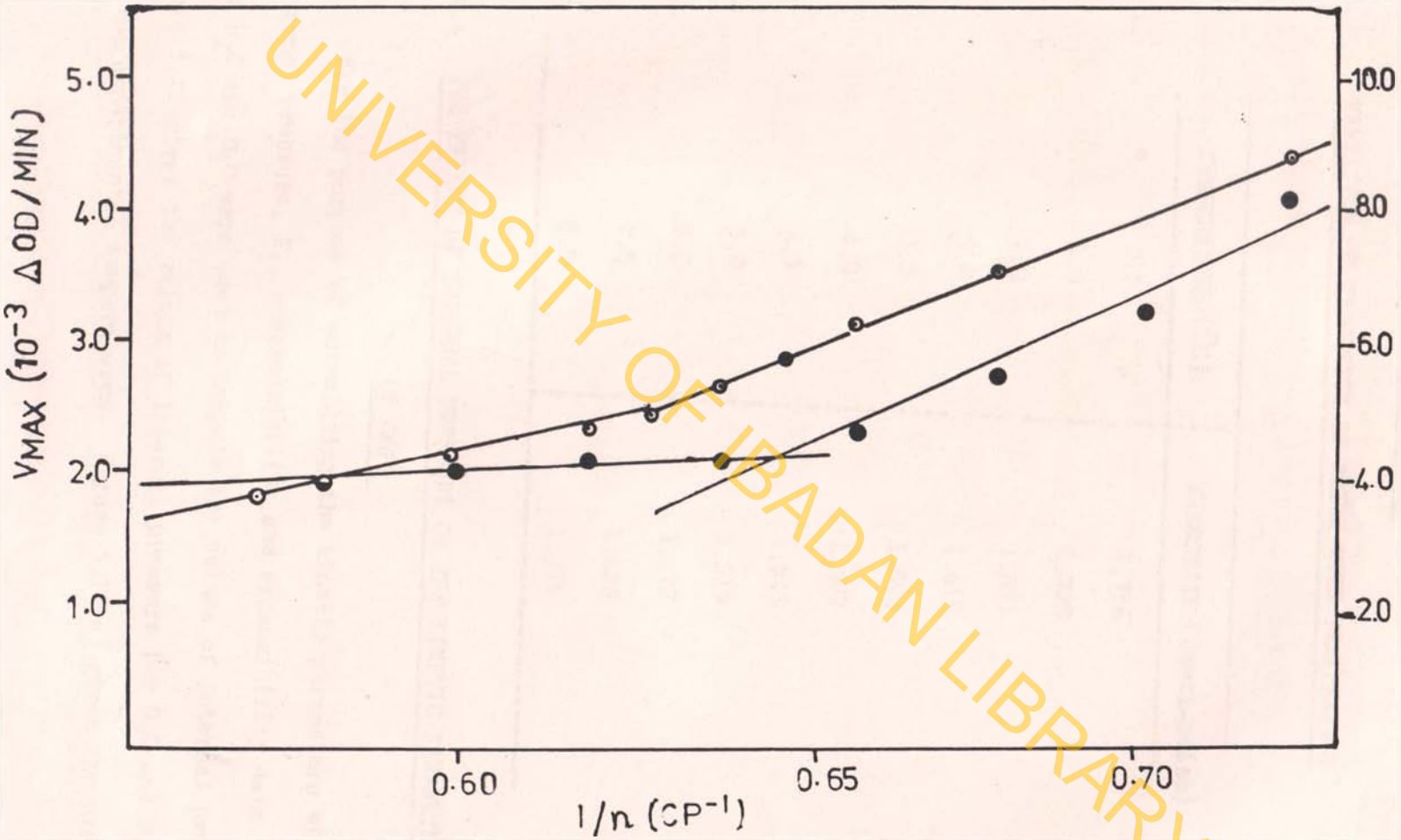


Fig. 5.17 : Dependence on 1/Viscosity of V_{MAX} for water experiment at pH 6.55 (●) and pH 9.00 (⊙).

TABLE 5.21

VARIATION OF VISCOSITY OF WATER WITH TEMPERATURE

TEMPERATURE (^{o}C)	VISCOSITY (Centi-poise)
0.5	1.756
1.0	1.728
2.0	1.671
3.0	1.618
3.5	1.593
4.0	1.567
4.5	1.543
5.0	1.519
6.0	1.472
7.0	1.428
8.0	1.386

5.4.4. THE EFFECT OF INTERNAL PRESSURE ON THE KINETIC PARAMETERS OF G6PD

For the purpose of correlating the kinetic parameters with internal pressure, P_i, compressibility and expansibility data for H_2O and D_2O were used to compute the values of internal pressure. Table 5.22 gives the values of internal pressure for H_2O and D_2O at the experimental temperatures. Figure 5.18(a) gives the plot of

TABLE 5.22

INTERNAL PRESSURE VALUES AT DIFFERENT TEMPERATURES

Temperature (°C)	Internal Pressure (Bar)	
	H_2O	D_2O
0.5	318.93	-
1.0	271.88	-
2.0	179.63	-
3.0	88.54	751.94
3.5	46.75	-
4.0	- 1.51	675.73
4.5	-45.82	-
5.0	-89.94	582.82
6.0	- 178.44	489.14
7.0	- 256.47	407.37
8.0	- 351.37	308.81
9.0	-	231.69
10.0	-	131.84
11.0	-	45.39
12.0	-	- 54.96
13.0	-	- 123.28
14.0	-	- 233.06

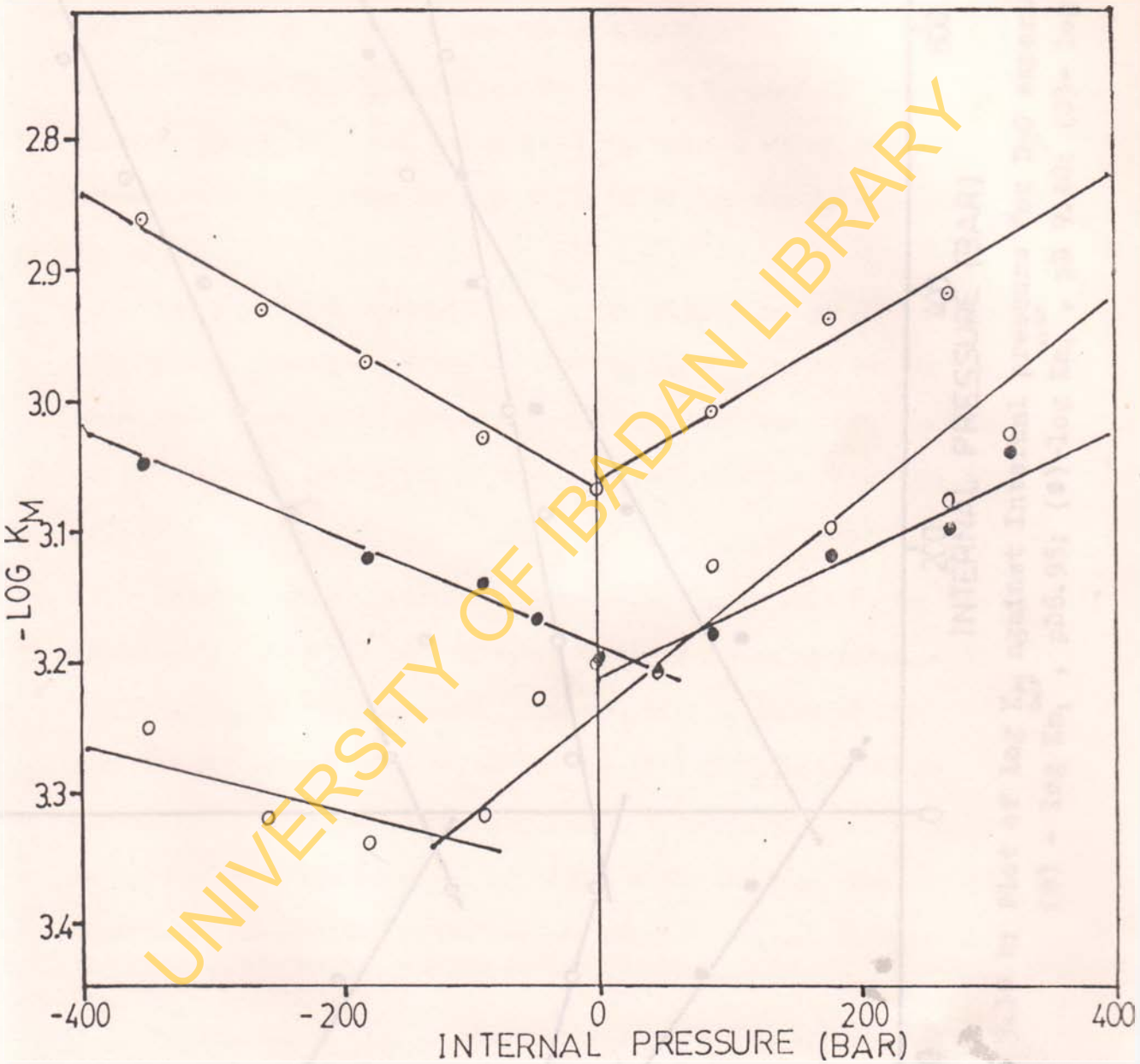


Fig. 5.18 a: Plot of log Km against Internal pressure for water experiment. (0) - log Km^{cap}, pH 6.55; (●) - log Km_1^{cap}, pH 9.00; (0)- log Km_2^{cap} - 1, pH.9.00.

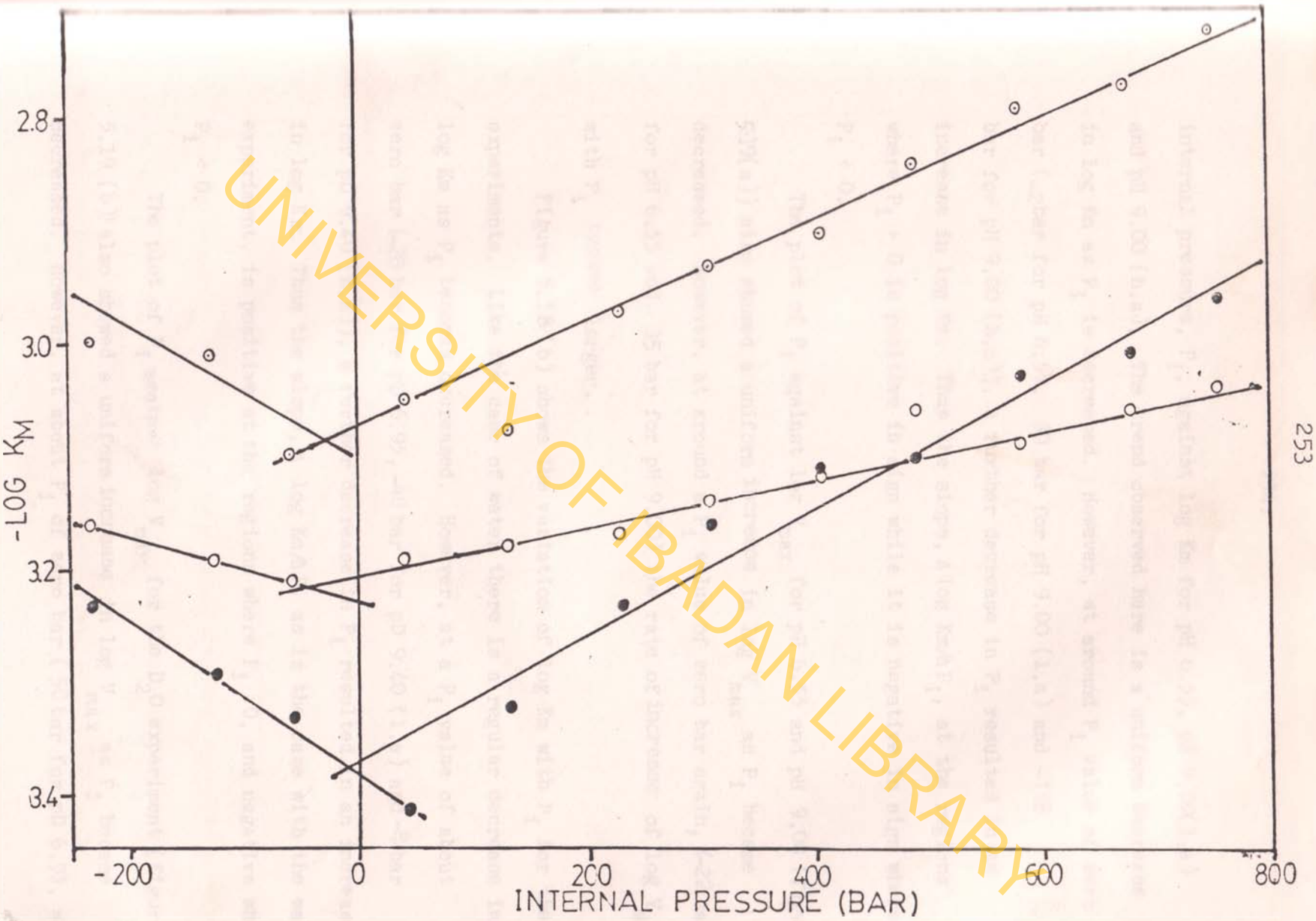


Fig. 5.18 b: Plot of log K_m against Internal pressure for D_2O experiment.
(0) - log Km_1^{G6P}, pD6.95; (●)-log Km_1^{G6P}, pD 9.40; (0)- log Km_2^{G6P}- 1, pD 9.40.

internal pressure, P_i, against log Km for pH 6.55, pH 9.00(l,a) and pH 9.00 (h,a). The trend observed here is a uniform decrease in log Km as P_i is decreased. However, at around P_i value of zero bar (-2bar for pH 6.55, 30 bar for pH 9.00 (l,a) and -118 bar for pH 9.00 (h,a)), a further decrease in P_i resulted in an increase in log Km. Thus the slope, $\Delta \log Km/\Delta P_i$, at the regions where $P_i > 0$ is positive in sign while it is negative in sign where $P_i < 0$.

The plot of P_i against log V_{max} for pH 6.55 and pH 9.00 (figure 5.19(a)) also showed a uniform increase in log V_{max} as P_i became decreased. However, at around a P_i value of zero bar again, (-22 bar for pH 6.55 and 35 bar for pH 9.00) the rate of increase of log V_{max} with P_i became larger.

Figure 5.18 (b) shows the variation of log Km with P_i for the D_2O experiments. Like the case of water there is a regular decrease in log Km as P_i becomes decreased. However, at a P_i value of about zero bar (-28 bar for pD 6.95, -40 bar for pD 9.40 (l,a) and -8 bar for pD 9.40 (h,a)), a further decrease in P_i resulted in an increase in log Km. Thus the slope, Δ log $Km/\Delta P_i$ as is the case with the water experiment, is positive at the regions where $P_i > 0$, and negative where $P_i < 0$.

The plot of P_i against log V_{max} for the D_2O experiment (figure 5.19 (b)) also showed a uniform increase in log V_{max} as P_i became decreased. However, at about P_i of zero bar (50 bar for pD 6.95, and

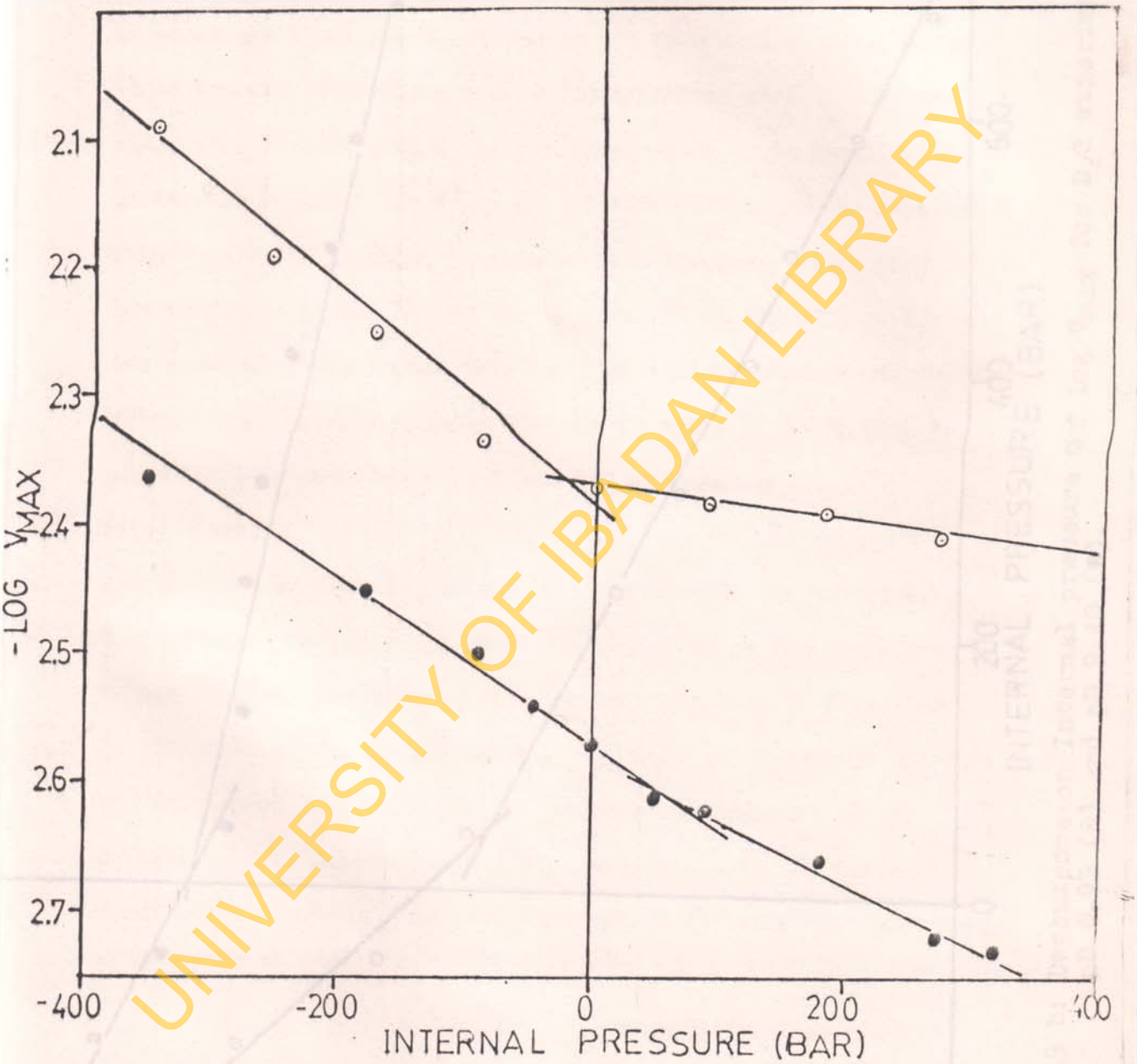


Fig. 5.19 a: Dependence on Internal pressure of log V_{max} for water experiment at pH 6.55 (0) and pH 9.00 (●).

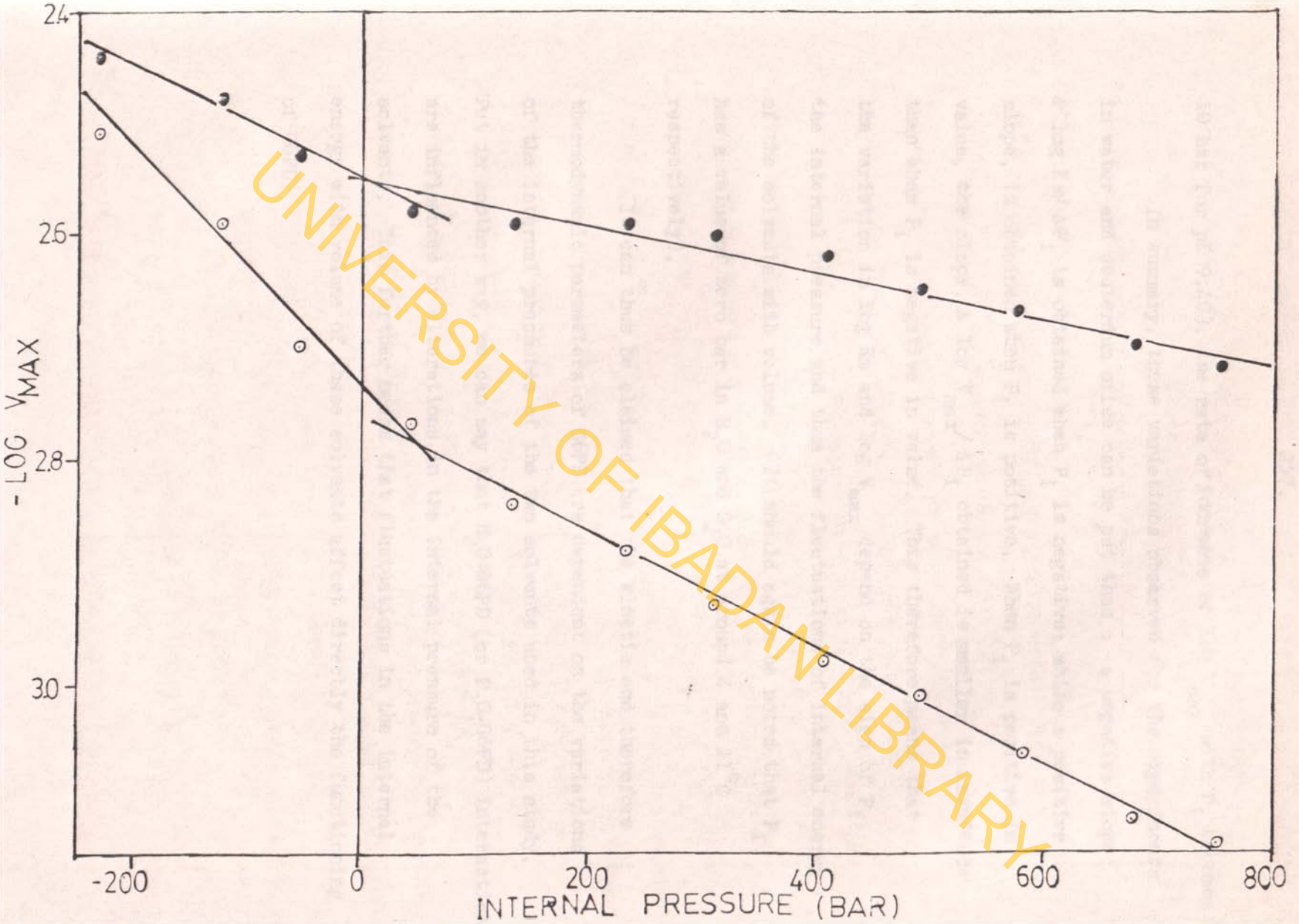


Fig. 5.19 b: Dependence on Internal pressure of log V_{max} for D_2O experiment at pD 6.95 (⊙) and pD 9.40 (●).

10 bar for pD 9.40), the rate of increase of log V_{max} with P_i becomes larger.

In summary, these variations observed for the experiments in water and deuterium oxide can be put thus : a negative slope, $\Delta \log Km / \Delta P_i$ is obtained when P_i is negative; while a positive slope, is obtained when P_i is positive. When P_i is positive in value, the slope, $\Delta \log V_{max} / \Delta P_i$ obtained is smaller in magnitude than when P_i is negative in value. This therefore means that the variation in log Km and log V_{max} depend on the sign of P_i, the internal pressure and thus the fluctuations of internal energy of the solvents with volume. (It should again be noted that P_i has a value of zero bar in H_2O and D_2O at around 4 and 11°C respectively).

It can thus be claimed that the kinetic and therefore thermodynamic parameters of G6PD are dependent on the variations of the internal pressures of the two solvents used in this study. Put in another way, we can say that H_2O-G6PD (or D_2O-G6PD) interactions are influenced by alterations in the internal pressure of the solvents. This further means that fluctuations in the internal energy with volume of these solvents affect directly the functioning of G6PD.

5.4.5. THE EFFECT OF TEMPERATURE ON INTERACTION COEFFICIENT

The variation of interaction coefficient, n, with temperature for the experiments in H_2O and D_2O at pH 9.00/pD 9.40 is shown in figure 5.20.

For the experiment in H_2O medium, n increases from about 0.54 to 0.65 as the temperature is decreased from 8.0 to 4.0°C. However, after about 4°C, there becomes a decrease in n as temperature is decreased. For the experiment in D_2O, the same pattern as that of water is observed, n increases from about 0.72 to 0.88 as the temperature is decreased from 14.0 to 11°C. However, after about 11°C, there becomes a decrease in n as the temperature is decreased further.

Since the type of cooperativity observed in this case of G6P binding to G6PD is the negative type, increase in n implies decrease in cooperativity thus the trend observed for the reaction of G6PD in the two solvents is a decrease in cooperativity as temperature is decreased, until at about 4°C and 11°C for the water and D_2O experiments respectively, where any further decrease in temperature results in increase in cooperativity. These observations therefore imply that interaction coefficient of G6PD is affected by alteration in the mass composition and internal pressure of the two solvents. This again confirms the fact that G6PD action is affected by some of the properties of the solvent in which it functions.

Table 5.23 shows the variation of interaction coefficient with temperature.

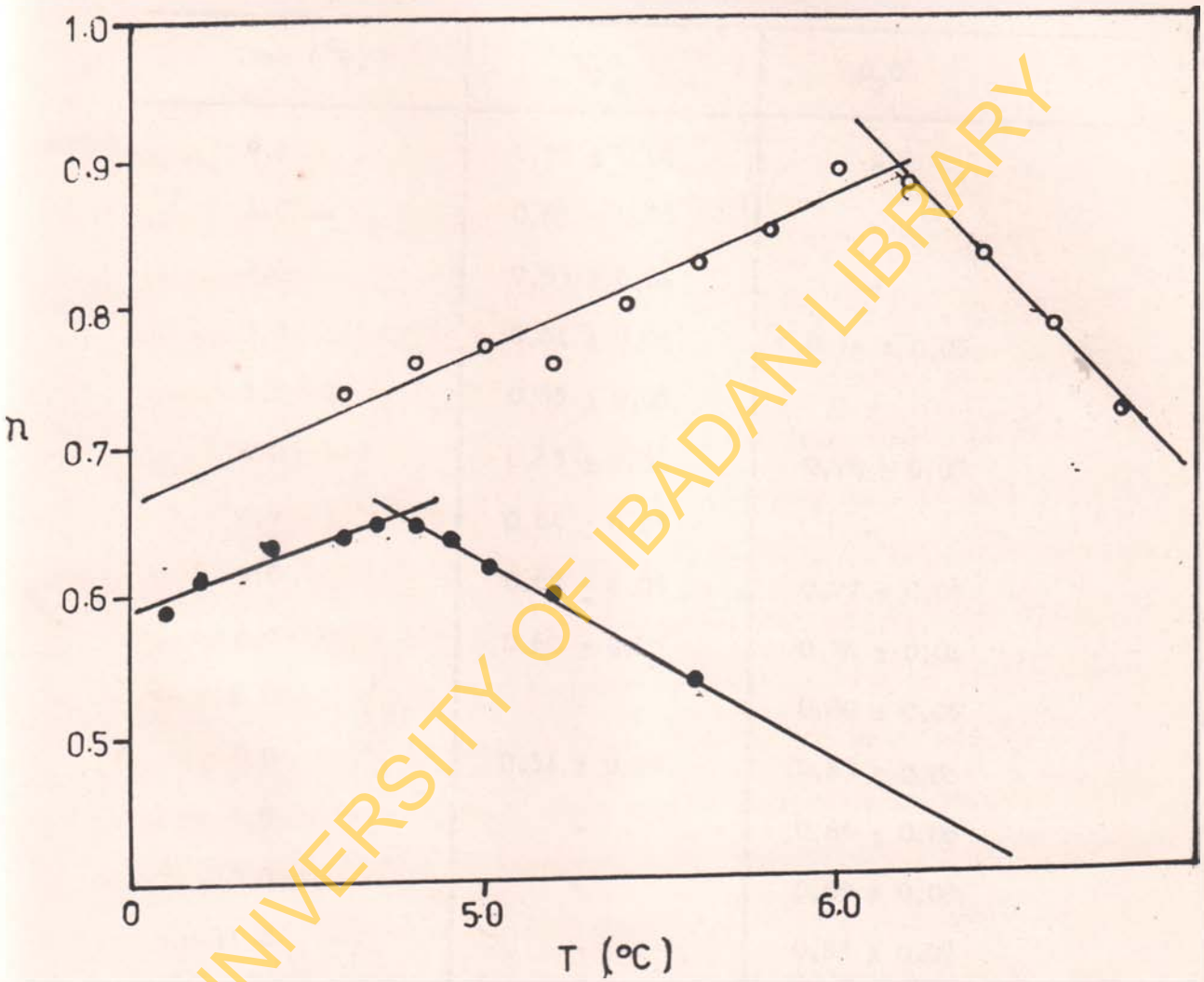


Fig. 5.20 : Variation of Interaction coef ficient with temperature at pH 9.00 (●) and pD 9.40 (○).

TABLE 5.23

DEPENDENCE OF INTERACTION COEFFICIENT ON TEMPERATURE

Temp (^{o}C)	H_2O	D_2O
0.5	0.59 ± 0.04	-
1.0	0.61 ± 0.05	-
2.0	0.63 ± 0.04	-
3.0	0.64 ± 0.04	0.74 ± 0.05
3.5	0.65 ± 0.05	-
4.0	0.65 ± 0.04	0.76 ± 0.05
4.5	0.64 ± 0.05	-
5.0	0.62 ± 0.05	0.77 ± 0.05
6.0	0.60 ± 0.05	0.76 ± 0.04
7.0	-	0.80 ± 0.05
8.0	0.54 ± 0.05	0.83 ± 0.06
9.0	-	0.85 ± 0.06
10.0	-	0.89 ± 0.08
11.0	-	0.88 ± 0.07
12.0	-	0.83 ± 0.07
13.0	-	0.78 ± 0.06
14.0	-	0.72 ± 0.07

5.4.6. FLUCTUATIONS IN THE ENZYME-PROTEIN

Enzymes normally act to reduce the activation energy for their respective chemical reactions. This role is vital, in view of the restricted temperature range suitable for living cells, as well as limitation on intracellular reaction concentrations posed by consideration of cytosolic solvent capacity (263, 264).

Results from previous studies (e.g. X-ray crystallography, thermal denaturation studies, atomic packing-density calculations) (188, 211, 212, 265) have suggested a static, rigid form for the enzyme structure, which serves the singular role of maintaining statically a requisite three-dimensional arrangement of active site residues. This notion is however rapidly becoming outmoded (266).

Accummulating evidence has revealed a rich variety of internal motions (structural fluctuations) in proteins (215, 267), pointing to a rather fluid dynamic structure, involving rapid conformational fluctuations which allow relatively easy, if somewhat transient, accessibility of interior groups to solvent and molecular probes. These classes of fluctuations span a virtual gamut of time domains, and encompasses essentially every part of the protein structure. Of particular interest are fluctuations in the nano-second and subnanosecond range.

The distribution functions for thermodynamic parameters in macroscopic systems are usually extremely sharp and, except in special cases near critical points, deviations of these parameters from the

means are extremely small (268). Individual protein molecules however, are very small systems consisting of relatively few discrete particles (atoms) in comparison to familiar macroscopic objects and in such cases, statistical fluctuations in thermodynamic properties assume much greater importance (186). Of particular interest here, since they are readily calculated from known properties of proteins in solution, are fluctuations about the mean of the internal energy, U, and total volume, V. General expressions for the mean square fluctuations (second moments of the distribution function) of U and V are (268) :

$$\overline{\delta U^2} = k_B T^2 m C_v$$

$$\overline{\delta V^2} = k_B T V \beta_T$$

where m and V are the mass and volume of the system, respectively, C_v is the heat capacity at constant volume, β_T the isothermal bulk compressibility, T the absolute temperature, and k_B is the Boltzmann's constant.

Thermodynamics can tell us little about the precise molecular form of the fluctuations or of their kinetics. Presumably, the majority of fluctuations involve small rapid changes in bond lengths and angles of individual groups in the polypeptide chain but these may readily combine to give gross changes in configuration.

It is apparent from studies involving relaxation processes (269, 270) that sizeable conformational fluctuations are possible, and cover a time range of nanoseconds, or less, up to minutes or hours.

In mitochondria, the role of conformational fluctuations in electron transfer and energy coupling has been discussed in qualitative terms. Conformational fluctuations are probably implicated in maintaining the correct potentials in the redox pools, the redox switches and in the storage and transduction of energy (271).

In general, structural fluctuations play important roles in protein function.

In the 100-year history of enzymology (272), most of what we know concerning the great kinetic power and specificity of enzymes comes from studies focusing on the active site - a three dimensional cleft or crevice on the enzyme molecule to which the substrate is bound. The trenchant efforts of the physical chemist and the organic chemist have produced a wealth of information on the nature of the binding and catalytic events at the enzyme active site. Yet one is left with a bit of a puzzle. The active site, the "scene of the action", occupies a relatively small portion of the total volume of the enzyme molecule, so that most of the amino acid residues in the protein structure actually have no contact with the bound substrate. Thus one is faced with the obvious question : Why are enzymes so big?

It is known that enzymes act to reduce the activation energy for their respective chemical processes. However, even when all the mechanistic effects of enzymes in lowering activation energy have been taken into account, there still remains a finite activation

energy. Thus some energy (viz, free enegy) must be provided "from somewhere" in order for the reactant(s) to enter the transition state. As noted by Ferdinand (273), "this can only come from the translational energy of solute molecules bombarding the enzyme substrate complex because the substrates themselves have become tied down in the active site and have no translational energy with which to enter the translational state". If one assumes that features of the various, known enzymatic reaction mechanisms are linked ultimately to the macromolecular conformation, then thermal fluctuations in conformational variables (as governed by solvent interactions) surely must be involved in catalytic processes (216). In the last couple of years, there have emerged several theoretical models of enzyme action, which suggest that particular classes of fluctuations in the protein structure provide means for collimating thermal energy to produce high free-energy events at the active site.

Transduction is the process by which enzyme-proteins transmit energy down to the active centre. The idea that enzyme-proteins behave as energy transducers is not new (274-276). Thus it could be possible that the other bulk of the enzyme-protein apart from the active centre, have part of their function as that of energy-transducers.

The solvent is an important determinant of the overall structure of macromolecules. Experimental works on protein dynamics (186-188) and theoretical models (187, 188, 215-217, 244, 259-262, 270, 275-278) indicate a more intimate relationship between the solvent and protein than previously recognized. This relationship is important when the heterogeneity and the variability

of intracellular environments are considered. Obviously the ambient solvent-solute system is ultimately responsible for energizing the enzyme-substrate complex. Naturally, protein-solvent interactions (and. therefore, energy transduction) would be influenced by alterations in the local viscosity, mass composition of solvent, etc.

Although the protein is assumed to be in equilibrium with a solvent at constant temperature, pressure and composition, nevertheless certain types of bulk-solvent fluctuations may temper internal protein motions (279). As noted by Ikegami (279), in a true protein solution the temperature, pressure and composition fluctuate, "since the volume of solvent with which a single protein molecule is in thermal equilibrium is probably small". Accordingly, the average fluctuation of the "structural state" of the protein, P, about its mean value, Pm, will be expected to be a function of fluctuations in the three solvent quantities.

Previously in this discussion, we mentioned the emergence of theoretical models of enzyme action. These models all view the enzyme molecule as an energy transducer. Of these models however, only three - the Careri model (187, 188, 215, 216), the Gavish Frauenfelder model (244, 259, 260) and the Somogyi - Damjanovich-Welch (SDW) model (261, 262, 278) - actually approach the energy transduction idea in a quantitative manner. The Careri model maintains that the enzyme structure is "programmed", such that the

temporal course of catalytic events is governed through the space-time fluctuating nature of solvent-surface interactions (e.g. bound water). The Gavish-Frauenfelder and the SDW models analyze the "energization" of the enzyme-substrate complex directly through random collision with solvent particles. The Gavish-Frauenfelder model views the protein matrix as a "Fokker-Planck fluid", using ideas from the Brownian theory of motion in a field force and concerning itself with the local viscosity within the protein structure. The SDW model focuses more on the surface events involved in the collisional "energization" and thus, deals explicitly with the solvent viscosity.

A major achievement thus far, particularly for the Gavish-Frauenfelder and SDW models, is the explanation of the effect of solvent viscosity on enzyme kinetics. There have appeared many isolated reports of observed effects of viscosity on enzyme rate parameters (244).

In addition to the viscosity per se, dependence of enzyme kinetics on the mass composition of the medium is elaborated in the SDW model. The actual exchange of energy between the protein and solvent is by nature a molecular collision process. The mass and velocity of the solvent/solute species are critical parameters in the efficiency of energy transfer (280). A faster-moving small molecule (with a steeper repulsion potential) has a greater probability for a translation-vibration transition upon colliding with

the protein. And, a certain threshold speed is required, the fraction of molecules having speed equal to or greater than this value being dictated by the Maxwell-Boltzmann distribution. At a given temperature where the average energy of the molecules is $3/2k_BT$, the average speed distribution will also depend on the mass distribution, as $3/2k_BT = 1/2\ (\overline{mV^2})$ (v = velocity). According to the SDW model the solvent/solute composition is an important factor.

At the heart of all the theoretical models so far is a basic idea elaborated by Lumry and workers (217, 275, 276), that the protein is a free-energy "linkage device" for coupling chemical processes to the properties of the liquid medium. Lumry has also proposed that the aforementioned enthalpy-entropy compensation phenomena is at the root of free-energy "linkage" in many enzymic processes. It is thermodynamically feasible for an enzyme-protein to change its enthalpy and entropy in a transduction process by large amounts, provided it is done in the above compensatory manner. Accordingly, many features of energy transduction by the protein matrix appear only in those enthalpy and entropy contributions which are not manifest in the measured, overall free-energy change of the combined protein-chemical subsystems of enzyme reaction. Thus such free-energy changes, usually determined at a single temperature value and single solvent composition, generally yield little direct information about the transducing ("linkage") properties of enzymes-particularly if the given enzyme process depends on fluctuations (281).

In this study, results obtained on the effects of H_2O/D_2O and glycerol-water solvent systems on the G6PD enzyme, supported the previous experimental evidence and theoretical models for the interactions of proteins with solvents. In the studies in the water (or D_2O) system at about the temperatures of their maximum densities (where we have the anomalous behaviour of their thermodynamic functions), correlations were found between the rate and affinity parameters of the enzyme with some of the solvent properties - viz : mass composition (density), viscosity and internal pressure. Variation of V_{max} and K_m with temperature (and thus mass composition) showed the dependence on mass composition of the solvents, of the V_{max} and Km of the enzyme. For the Km parameter, there was a decrease in its value until the temperature of maximum density, where there became an increase instead. For the V_{max} there was a change in the magnitude of its dependence at about the temperature of maximum density. These observations support the SDW model which predicted the explicit dependence of enzyme kinetics on the mass composition of the medium.

The rate parameter, V_{max}, was also dependent on the viscosity of the two solvents utilised, as had been shown by Beece et al (244) and predicted by the Gavish-Frauenfelder and SDW models. However, after the temperature of maximum density for the solvents, a discontinuity in the dependence was observed. This discontinuity at that point, implied that viscosity was not the solvent property

affecting the solvent-protein interactions at that point. Instead, another solvent property which the enzyme-protein had been dependent on in parallel with the viscosity was probably responsible for the observed enzyme rate variation at that point. The mass composition has already been implicated in the trends in the enzyme kinetic parameters at these low temperatures.

Strong dependence was observed between the V_{max} and Km of the G6PD enzyme, and the internal pressures of both D_2O and H_2O, again implying that alterations in the solvent internal pressure influence the solvent-G6PD interactions.

It is already known that there are fluctuations of internal energy with volume, for both solvents and enzyme-proteins (186). Again the variation of ΔH with ΔS in this study, had shown a motive type of compensation, implying "linkage" in G6PD processes. Thus the dependence of the kinetic parameters of the G6PD enzyme on the solvent properties, most especially the internal pressure which measures the variation in internal energy with volume strongly suggests the possible coupling of the fluctuations in the properties of the solvent medium to the fluctuations in the protein. Lumry(277) had previously elaborated that the protein is a free-energy "linkage device" for coupling chemical processes to the properties of the liquid medium.

Correlation of the rate parameter of G6PD with the viscosity, dielectric constant and surface tension of the water-glycerol

system also showed the dependence of the rate parameter on the bulk solvent properties of the water-glycerol medium.

All these observations therefore, suggest that G6PD-solvent interactions (and therefore energy transduction) is influenced by the alterations in the solvent properties, due either to change of the medium's temperature or composition. This could also be taken to mean that the properties of the solvent medium influence the different types of fluctuations in the protein structure, which collimate thermal energy to produce high free energy-events at the active site.

5.5.1. C O N C L U S I O N

Unlike the sigmoid type kinetics observed in the fully aqueous medium at pH 9.0, Michealis type kinetics was observed in the presence of glycerol. This observation we attributed to the probable deformation of one of the G6P binding sites, most probably the low affinity site, due to pertubations of the protein hydration in the presence of glycerol.

Study of the rate parameter (V_{max}) at a variety of temperatures in glycerol led to a variety of correlations. Log V_{max} was found to be a linear function of the dielectric constant and surface tension, while V_{max} was found to be a linear function of viscosity. All these correlations show a strong dependence of the reaction rate on the properties of the bulk solvent, and thus the concentration of water. There was no correlation between the

affinity parameter, Km, and the properties of the bulk solvent. Since the Km parameter is complex in terms of the rate constants of which it is composed, this lack of correlation was not too surprising. There were large variations in Km however, oscillating between increasing and decreasing affinity for G6P binding to the enzyme. These variations we attributed to changes in conformation of the enzyme due to the differential hydration caused by different concentrations of glycerol.

Compensation of changes in ΔH by ΔS was observed. The type of compensation observed was the motive type, suggesting the existence of "linkage process" in G6PD functions. The compensation phenomenom generally implicated the water solvent as serving an important role in the functioning of the G6PD enzyme.

Kinetic and thermodynamic functions obtained for the binding of G6P to G6PD both in water and deuterium oxide at low temperatures (0.5 to 8.0°C in water, and 3.0 to 14.0°C in deuterium oxide) exhibited some anomalous behaviour, especially at about 4°C and 11°C, the temperatures of maximum density for water and deuterium oxide respectively.

K_m and V_{max} decreased with decrease in temperature until at about the temperatures of maximum density for the solvents, where any further decrease in temperature resulted in increase in Km, and change in the rate of decrease for V_{max}. ΔH was positive until the temperature of maximum density for the two solvents after which

it became negative in sign. Ea also showed discontinuity in value at these same temperatures in water and deuterium oxide.

Because of the observed anomalous behaviour of the kinetic parameters of the enzyme at about the temperatures of maximum density for water and deuterium oxide, we decided to correlate the kinetic parameters, and thus the thermodynamic parameters with some intrinsic properties of the solvents.

Variation of Km and V_{max} with temperature (and by implication mass composition of solvent) showed that these parameters were dependent on the mass composition of the solvents, implying the possible influence of the mass composition of the solvents on the catalysis of G6PD. A bid to correlate the rate parameter with viscosity changes at the different temperatures for the water experiment at pH 6.55 and 9.00 showed that although V_{max} was dependent on viscosity until about 4°C, a discontinuity in the dependence was observed just about this temperature. This thus implied that another solvent property which probably influenced the enzyme properties in parallel with the solvent viscosity was probably predominantly responsible for the observed variations at the temperatures of maximum density for the solvents. Variation of Km and V_{max} with the internal pressure of the two solvents, showed that these parameters and therefore thermodynamic functions of the enzyme were dependent on the internal

pressure of the two solvents. As has been shown previously, internal pressure is a measure of the variation in internal energy with volume. These variations have been shown to be reversed at about $4^{\circ}C$ for water and $11^{\circ}C$ for deuterium oxide. Thus the exact dependence of both the kinetic and thermodynamic functions at these temperature regions for the two solvents imply that internal pressure is one of the solvent properties which strongly affect the properties of the G6PD enzyme. Fluctuations of internal energy with volume for both solvents and enzyme proteins exist (186). Linkage process in G6PD reactions have been implicated in this study by the observation of motive compensation in the G6PD enzyme. It could therefore be possible that the solvent influences the properties of the G6PD enzyme by coupling the fluctuations in its internal energy with volume to that of the enzyme.

Variation of the interaction coefficient of the enzyme with temperature also showed similar dependence as the Km parameter.

All these observations (both for experiments in water-glycerol, and water (or D_2O) systems) do suggest that the catalytic properties of the G6PD enzyme are very dependent on the intrinsic properties of the solvents in which it functions. This means that the different types of fluctuations in the enzyme structure which collimate thermal energy to produce high free-energy events at the active site are strongly linked, and could be influenced by fluctuations in the solvent properties in which it is functioning.

In general, therefore, it could be said that the solvent plays an important role in the catalytic function of the enzyme.

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APPENDIX 1

LSFITW PROGRAM

```
LIST

 1 PRINT"DO YOU NEED INSTRUCTIONS-(YES,NO)";
 2 INPUT Z$
 3 IF Z$="NO" THEN 95
 4 PRINT"LSFITW IS A LEAST SQUARE CURVE FIT PROGRAM.  IT FITS DATA"
 5 PRINT"TO THE EQUATION Y=A(0)+A(1)*X+A(2)*X^2+...+A(M)*X^M."
 6 PRINT"THE DATA SHOULD BE ENTERED IN DATA STATEMENTS BEGINNING IN LINE 9
 7 PRINT"THIS DATA CONSISTS OF N, THE NUMBER OF X,Y DATA PAIRS,"
 8 PRINT"W, INDICATING THE PRESENCE OF WEIGHTS (1=YES, 0=NO), AND THEN"
 9 PRINT"THE DATA, ENTERED IN SIMPLE X,Y PAIRS IF UN-WEIGHTED, AND EN-"
10 PRINT"TERED IN X,Y PAIRS FOLLOWED BY THE WEIGHT FOR THAT PAIR IF"
11 PRINT"WEIGHTS WERE SELECTED.  ALL OTHER VALUES ARE ENTERED AT 'RUN'"
12 PRINT"TIME.  TO STOP THE PROGRAM , TYPE 'STOP'."
13 PRINT"NOTE: THE DEGREE OF CURVE MUST BE <= 10 AND NO MORE THAN 75"
14 PRINT"DATA PAIRS MAY BE ENTERED OR THE PROGRAM MUST BE MODIFIED."
15  REM  IF WEIGHTS ARE USED THE DATA IS ENTERED AS FOLLOWS:
16  REM
17  REM      910 DATA X(1),Y(1),W(1),X(2),Y(2),W(2),...,X(N),Y(N),W(N)
18  REM
19  REM  FOR DATA WITHOUT WEIGHTS ENTER AS SHOWN BELOW:
20  REM
21  REM      910 DATA X(1),Y(1),X(2),Y(2),.....,X(N),Y(N)
22  REM
23  REM  WHERE:  X(I) = THE VALUE OF X IN THE ITH DATA PAIR
24  REM          Y(I) = THE VALUE OF Y IN THE ITH DATA PAIR
25  REM          W(I) = THE VALUE OF THE WEIGHT TO BE APPLIED
26  REM                 TO THE VALUE OF Y IN THE ITH DATA PAIR
27  REM
28  REM  THE DEGREE OF CURVE IS ENTERED DURING THE EXECUTION OF THE
29  REM  PROGRAM IN RESPONSE TO A QUESTION FROM THE COMPUTER.  DEGREE
30  REM  MAY BE REDUCED OR INCREASED FOLLOWING EACH SOLUTION TO OBTAIN
31  REM  THE COEFFICIENTS FOR A DIFFERENT DEGREE CURVE.
32  REM
33  REM  THE MAXIMUM DEGREE OF CURVE IS 10 AND THE MAXIMUM NUMBER OF
34  REM  DATA PAIRS WITH WEIGHTS IS 75.  SHOULD A HIGHER DEGREE OF
35  REM  CURVE BE DESIRED OR IF MORE DATA IS TO BE ENTERED, RETYPE
36  REM  THE DIM STATEMENT AT LINE 100 SETTING THE PARAMETERS AT THE
37  REM  THE DESIRED LEVEL.
38  REM
39  REM  THE PROGRAM PRINTS THE COEFFICIENTS AND STANDARD DEVIATIONS
40  REM  FOR EACH COEFFICIENT, THE STANDARD DEVIATION OF THE FITTED
41  REM  CURVE, THE Y VALUE, FITTED Y VALUE AND THE DEVIATION BETWEEN
42  REM  THE Y AND FITTED Y VALUES.
43  REM
44  REM  THE PROGRAM COMPUTES, BUT DOES NOT PRINT THE ORTHONORMAL
45  REM  VECTORS P(K,I) FOR K=1 TO M AND I=1 TO N.  IT ALSO COMPUTES
46  REM  THE COEFFICIENTS IN THE EXPANSION OF THE P(K,I) IN TERMS OF
47  REM  THE V(K,I):
48  REM
49  REM      P(K,I)=A(K,1)*V(1,I)+A(K,2)*V(2,I)+...+A(K,K)*V(K,I)
50  REM
51  REM  HOWEVER, P(K,I) IS STORED AS V(K,I) WRITING OVER THE
52  REM  ORIGINAL V(K,I) AND A(K,J) IS STORED AS V(K,N+J) IN THE
53  REM  PROGRAM.
54  REM
55  REM  TO BRING THE PROGRAM TO AN END TYPE 'STOP'
56  REM  FOLLOWING THE QUESTION ASKING FOR DEGREE OF CURVE.
57  REM
58  REM  DEVELOPED BY:  DR. J. SCHUMAKER, NRL.
90 STOP
95 PRINT
```

```
100 DIM X(75), Y(75), V(12,75), W(75)
105 PRINT "TYPE THE DEGREE OF CURVE DESIRED";
110 INPUT M1
115 READ N,W1
120 FOR I=1 TO N
125 READ V(2,I),Y(I)
130 IF W1=0 THEN 145
135 READ W(I)
140 GOTO 150
145 LET W(I)=1
150 LET V(1,I)=1
155 LET X(I)=V(2,I)
160 FOR J = 2 TO M1
165 LET V(J+1,I)=V(J,I)*V(2,I)
170 NEXT J
175 NEXT I
180 LET M = M1+1
185 GOSUB 200
190 RESTORE
195 GOTO 105
200 LET N1 = 0
205 FOR I = 1 TO N
210 LET V(M+1,I) = Y(I)
215 IF W(I) = 0 THEN 225
220 LET N1 = N1 + 1
225 NEXT I
230 PRINT
235 PRINT
240 IF N1 - M < 0 THEN 615
245 FOR I = N+1 TO N+M
250 FOR K = 1 TO M+1
255 LET V(K,I) = 0
260 NEXT K
265 LET V(I-N,I) = 1
270 NEXT I
275 FOR K = 1 TO M+1
280 IF K = M+1 THEN 295
285 LET T = 1
290 GOTO 310
295 LET T = -1
300 GOTO 315
305 LET T = -T
310 IF K = 1 THEN 345
315 FOR L = 1 TO K-1
320 LET S(L) = 0
325 FOR J = 1 TO N
330 LET S(L) = S(L) + V(K,J)*V(L,J)*W(J)
335 NEXT J
340 NEXT L
345 FOR I = 1 TO N+M
350 IF K=1 THEN 370
355 FOR L = 1 TO K-1
360 LET V(K,I) = V(K,I) - S(L)*V(L,I)
365 NEXT L
370 NEXT I
375 IF T < 0 THEN 425
380 LET D(K) = 0
385 FOR I = 1 TO N
390 LET D(K) = D(K) + V(K,I)*V(K,I)*W(I)
395 NEXT I
400 LET D = SQR(D(K))
405 FOR I = 1 TO N+M
410 LET V(K,I) = V(K,I)/D
415 NEXT I
420 IF T = 0 THEN 305
425 NEXT K
430 IF N1+M = 0 THEN 575
```

```
435 LET S = 0
440 FOR I = 1 TO N
445 LET S = S + V(M+1,I)*V(M+1,I)*W(I)
450 NEXT I
455 LET S = SQR(S/(N1-M))
460 PRINT "TERM", "COEFFICIENT", "STD. DEV."
465 PRINT
470 FOR L = 1 TO M
475 LET C(L) = 0
480 FOR K = L TO M
485 LET C(L) = C(L) + V(K,M+L)*V(K,M+L)
490 NEXT K
495 LET C(L) = S*SQR(C(L))
500 PRINT (L-1),-V(M+1,M+L),C(L)
505 NEXT L
510 PRINT
515 PRINT
520 PRINT "STANDARD DEVIATION =";S
525 PRINT
530 PRINT
535 PRINT "X VALUE","Y VALUE","Y ESTIMATE","  RESIDUAL"
540 PRINT
545 FOR I = 1 TO N
550 PRINT X(I),Y(I),Y(I)-V(M+1,I),V(M+1,I)
555 NEXT I
560 PRINT
565 PRINT
570 RETURN
575 PRINT "TERM","COEFFICIENT"
580 PRINT
585 FOR K = 1 TO M
590 PRINT (K-1),-V(M+1,M+K)
595 NEXT K
600 PRINT
605 PRINT
606 PRINT "TRY A LOWER DEGREE CURVE."
607 PRINT
608 PRINT
610 RETURN
615 PRINT "MORE COEFFICIENTS THAN DATA:  NO. OF COEFFICIENTS =";M
620 PRINT "                              NO. OF NON-ZERO WTS.=";N1
625 PRINT
630 PRINT
635 RETURN
900 DATA 5,0
910 DATA 1,1,2,2,3,3,4,4,5,5
99999 END
```

APPENDIX TWO

G-6-PDH PROGRAM

```
RT-11 FORTRAN IVV01B-08     THU 22-DEC-83 16:02:05  PAGE 001

      C*************************************************************
      C
      C     ENZYME KINETIC DATA ACQUISITION AND ANALYSIS
      C
      C12345678901234567890123456789012345678901234567890123456789012345678901234567890
0001        DIMENSION TIME(500),OD(500),RESID(500)
0002        LOGICAL*1 TITLE(65),BLANK(65),LABEL(65)
0003        REAL*8 SUM,SUMX,SUMY,SUMX2,SUMY2,DELTA,RCOEFF,A,B,SIGMAA,SIGMAB,
           1       VARNCE
0004        DATA BLANK/65*'#'/,LABEL/26*' ','R','E','S','I','D','U','A','L',
           1            ' ','P','L','O','T',26*' '/
0005        DATA NWL/3400/,NSLIT/200/,NREC/4/
0006     10 WRITE(6,1000) (BLANK(I),I=1,65)
0007   1000 FORMAT(1H ,'CARY 118 KINETIC DATA ACQUISITION'/1H ,65A1/)
0008     15 WRITE(6,1010)
0009   1010 FORMAT(1H ,'1....TO INITIALIZE CARY'/1H ,
           1           '2....TO ACQUIRE DATA'/1H ,
           2           '3....TO ANALYZE DATA'/)
0010        WRITE(6,1015) (BLANK(I),I=1,65)
0011   1015 FORMAT(1H ,'4....TO STORE DATA'/1H ,
           1           '5....TO RECOVER DATA'/1H ,
           2           '6....TO END'/1H ,65A1)
0012        READ(6,1020,ERR=15) MENU
0013   1020 FORMAT(I1)
0014        IF(MENU.LT.1.OR.MENU.GT.6) GOTO 15
0016        GOTO(100,300,130,200,400,500),MENU
0017    100 WRITE(6,1100)
0018   1100 FORMAT(1H$,'CURRENT WAVELENGTH ? ')
0019        READ(6,1110,ERR=100) NWCAL
0020   1110 FORMAT(I4)
0021        WRITE(6,1120) NWCAL,NSLIT
0022   1120 FORMAT(1H ,'***IH*WD*SO*RD*CD*F#/01/*AG*WC/',I4,'/*SP/',I3,'/**')
0023        CALL ISLEEP(0,0,5,0)
0024        WRITE(6,1130) NWL
0025   1130 FORMAT(1H ,'***W1/',I4,'/*WE*RS*M1***')
0026    300 WRITE(6,1140)
0027   1140 FORMAT(1H ,'ENTER TITLE')
0028        READ(6,1150) (TITLE(I),I=1,65)
0029   1150 FORMAT(65A1)
0030        WRITE(6,1160)
0031   1160 FORMAT(1H$,'TOTAL NO. OF DATA POINTS REQUIRED ')
0032        READ(6,1165) NTOTAL
0033   1165 FORMAT(I3)
0034        WRITE(6,1170)
0035   1170 FORMAT(1H$,'TOTAL TIME OF MEASUREMENT /MINS (F4.2) ')
0036        READ(6,1175) FTIME
0037   1175 FORMAT(F4.2)
0038        FINT=(FTIME*60.0)/FLOAT(NTOTAL)
0039        NINT=INT(FINT*10.0)
0040        IF(NINT.LT.3) NINT=3
0042        IF(NINT.GT.999) NINT=999
0044        WRITE(6,1185) NINT
0045   1185 FORMAT(1H ,'***WD*RT/',I3,'/*DT***')
0046        WRITE(6,1190)
0047   1190 FORMAT(1H ,'KEY (CR) TO START')
0048        READ(6,1200) CUE
0049   1200 FORMAT(A1)
```

```
RT-11 FORTRAN IVV01B-08    THU 22-DEC-83 16:02:05  PAGE 002

0050          WRITE(6,1210)
0051    1210  FORMAT(1H ,'***T0*RE***')
0052          READ(6,1220) DUMMY
0053    1220  FORMAT(A50)
0054          DO 120 I=1,NTOTAL
0055     120  READ(6,1230) TIME(I),OD(I)
0056    1230  FORMAT(2X,F6.1,4X,F7.4)
0057          WRITE(6,1240)
0058    1240  FORMAT(1H ,'***RD***')
0059          CALL PLOT(1,NTOTAL,OD,TIME,TITLE)
0060     130  WRITE(6,1250)
0061    1250  FORMAT(1H ,'INDICATE RANGE OF DATA TO BE ANALYSED')
0062          WRITE(6,1260)
0063    1260  FORMAT(1H$,'STARTING POINT ? ')
0064          READ(6,1270) NSTART
0065    1270  FORMAT(I3)
0066          WRITE(6,1280)
0067    1280  FORMAT(1H$,'ENDING POINT ? ')
0068          READ(6,1270) NEND
0069          NX=NEND-NSTART+1
0070          IF(NX.LT.3) GOTO 130
              C*****************************************************************
              C
              C SEE LINFIT PROGRAM 6-1,BEVINGTON (1969),P105
              C
              C*****************************************************************
0072          SUM=0.0
0073          SUMX=0.0
0074          SUMY=0.0
0075          SUMX2=0.0
0076          SUMXY=0.0
0077          SUMY2=0.0
0078          DO 150 I=NSTART,NEND
0079          SUM=SUM+1.0
0080          SUMX=SUMX+TIME(I)
0081          SUMY=SUMY+OD(I)
0082          SUMX2=SUMX2+TIME(I)*TIME(I)
0083          SUMXY=SUMXY+TIME(I)*OD(I)
0084          SUMY2=SUMY2+OD(I)*OD(I)
0085     150  CONTINUE
0086          DELTA=SUM*SUMX2-SUMX*SUMX
0087          A=(SUMX2*SUMY-SUMX*SUMXY)/DELTA
0088          B=(SUMXY*SUM-SUMX*SUMY)/DELTA
0089          VARNCE=(SUMY2+A*A*SUM+B*B*SUMX2
             1          -2.0*(A*SUMY+B*SUMXY-A*B*SUMX))/FLOAT(NX-2)
0090          SIGMAA=DSQRT(VARNCE*SUMX2/DELTA)
0091          SIGMAB=DSQRT(VARNCE*SUM/DELTA)
0092          RCOEFF=(SUM*SUMXY-SUMX*SUMY)/DSQRT(DELTA*(SUM*SUMY2-SUMY*SUMY))
0093          DO 160 I=NSTART,NEND
0094     160  RESID(I)=OD(I)-A-B*TIME(I)
0095          WRITE(24,1300) (BLANK(I),I=1,65),(BLANK(I),I=1,65)
0096    1300  FORMAT(1H ,65A1,65A1/)
0097          WRITE(24,1310) A,SIGMAA,B,SIGMAB,RCOEFF
0098    1310  FORMAT(1H ,'OD(0)= ',F8.5,' +/- ',F8.5,5X,'RATE OD UNITS/SEC= ',
             1 1PD12.5,' +/- ',1PD12.5,5X,'CORR.COEFF= ',F8.5/)
0099          WRITE(24,1300) (BLANK(I),I=1,65),(BLANK(I),I=1,65)
0100          CALL PLOT(NSTART,NEND,RESID,TIME,LABEL)
```

```
0101          GOTO 15
0102     200  WRITE(6,2000)
0103    2000  FORMAT(1H$,'FILENAME ? ')
0104          CALL ASSIGN(2,'DUMI',-1)
0105          DEFINE FILE 2 (0,NREC,U,ND)
0106          WRITE(2'1) NTOTAL
0107          DO 210 I=1,65
0108     210  WRITE(2'ND) TITLE(I)
0109          DO 220 I=1,NTOTAL
0110     220  WRITE(2'ND) TIME(I),OD(I)
0111          CALL CLOSE(2)
0112          GOTO 15
0113     400  WRITE(6,2000)
0114          CALL ASSIGN(2,'DUMI',-1)
0115          DEFINE FILE 2 (0,NREC,U,ND)
0116          READ(2'1) NTOTAL
0117          DO 410 I=1,65
0118     410  READ(2'ND) TITLE(I)
0119          DO 420 I=1,NTOTAL
0120     420  READ(2'ND) TIME(I),OD(I)
0121          CALL CLOSE(2)
0122          CALL PLOT(1,NTOTAL,OD,TIME,TITLE)
0123          GOTO 15
0124     500  STOP
0125          END
```

```
      C*********************************************************************
      C
      C     PLOTTING ROUTINE
      C
      C*********************************************************************
0001          SUBROUTINE PLOT(NSTART,NEND,Y,X,LABEL)
0002          DIMENSION Y(500),X(500)
0003          LOGICAL*1 LABEL(65),PLOT(101),DOT,STAR,SPACE
0004          DATA DOT,STAR,SPACE/1H.,1H*,1H /
0005          YMAX=Y(NSTART)
0006          YMIN=Y(NSTART)
0007          DO 300 I=NSTART+1,NEND
0008          YMAX=AMAX1(YMAX,Y(I))
0009      300 YMIN=AMIN1(YMIN,Y(I))
0010          WRITE(24,3000) (LABEL(I),I=1,65)
0011     3000 FORMAT(1H ,65A1)
0012          DO 310 I=1,101
0013      310 PLOT(I)=DOT
0014          WRITE(24,3010) YMIN,YMAX
0015     3010 FORMAT(1H ,'MIN. VALUE= ',F8.5,4X,'MAX. VALUE= ',F8.5/)
0016          WRITE(24,3020) (PLOT(I),I=1,101)
0017     3020 FORMAT(1H ,30X,101A1)
0018          DO 320 I=1,101
0019      320 PLOT(I)=SPACE
0020          ZERO=-(YMAX+YMIN)/(YMAX-YMIN)
0021          NZERO=INT(50.0*(ZERO+1.0))+1
0022          IF(NZERO.LE.0) NZERO=1
0023          DO 350 I=NSTART,NEND
0024          PLOT(NZERO)=DOT
0025          POS=(2.0*Y(I)-YMAX-YMIN)/(YMAX-YMIN)
0026          NPOS=INT(50.0*(POS+1.0))+1
0027          PLOT(NPOS)=STAR
0028          WRITE(24,3030) I,X(I),Y(I),(PLOT(J),J=1,101)
0029     3030 FORMAT(1H ,I3,4X,F6.1,5X,F7.4,5X,101A1)
0030      350 PLOT(NPOS)=SPACE
0031          DO 360 J=1,101
0032      360 PLOT(J)=DOT
0033          WRITE(24,3020) (PLOT(I),I=1,101)
0034          REWIND 24
0035          RETURN
0036          END
```