KINETICS AND THERMODYNAMIC STUDIES OF $NADP^+$ BINDING REACTIONS OF GENETIC VARIANTS OF HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE.

BY

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A B S T R A C T

Inherited Glucose-6-Phosphate dehydrogenase (G6PD) deficiency in humans results in hemolytic anaemia. The enzyme G6PD provides a crucial link in a series of biochemical reactions which occur in the red blood cell that leads to the steady state accumulation of NADPH, reduced glutathione by glutathione reductase, and the removal of potentially dangerous organic peroxides which, if not scanvenged, may result in the formation of radical species of oxygen which damage the energy generation system, which in turn may result in swelling, lysis and hemolytic anaemia. The objective of this thesis was to investigate the human variants of the enzyme G6PD in order to provide a better understanding of the molecular basis of the enzyme activity and factors affecting the onset of the human disease. Human Erythrocyte Glucose-6-Phosphate dehydrogenase has been known to occur in many genetic variants and the catalytic active enzyme of each variant are tetramers and dimers in acidic and alkaline solution respectively. The question then is whether there would be differences in the reactivities of these variants and whether there are differences in the reactivities of the two

active forms of the enzyme from the same variant.

A comparative analysis of the kinetic and thermodynamic studies of $NADP^+$ binding reactions of these variants under controlled and well-defined experimental conditions of pH and ionic strength was therefore undertaken. The binding reaction of $NADP^+$ to G6PD B^+ was also studied as a function of ionic strength of the buffers in order to evaluate the effect of these variations in the buffer system on the co-operative interactions of the $NADP^+$ binding sites on the enzyme.

The findings show that there are two binding sites on each of the enzyme variants and these were identified as imidazolium groups of histidine and sulfhydryl groups. The logKm versus pH curves show a broad plateau between pH 6.7 and 8.2 interrupted by a sharp minimum at pH 7.1 for all the enzyme variants. An explanation of this behaviour in terms of co-operative ionization of groups on the enzyme and enzyme-substrate complex which may be linked to the association - dissociation behaviour of the enzyme is proposed. In agreement with G6P binding data, the plot of the enthalpy of the dissociation of enzyme - $NADP^+$ complex against pH shows the shape of a two U-shaped curves consistent with the existence of a tetrameric form of enzyme at acidic pH and dimeric form at alkaline pH. A similar plot of the activation energy of the reaction for each

variant shows a consistent decrease of the activation energy with increase in pH, the activation energy at the pH 5.8 being almost halved at the alkaline pH of 9.0. This behaviour is explained to arise from the dimer enzyme being more reactive than the tetramer.

There are two schools of thought about the existence and nature of cooperativity among the $NADP^+$ binding sites on G6PD subunits. The study reported in this thesis has unequivocally solved the controversy between the two schools. It is now established that the tetrameric form of the enzyme shows no cooperativity while the dimeric form is cooperative. The diagreement between the two schools of thought has been explained in the variable experimental conditions used by the workers in the two schools. We have shown that an experimental condition that favours tetramer formation therefore favours non-cooperativity while a condition that favours dimer formation favours cooperativity.

The inhibition study by primaquine phosphate shows a complex interaction of this effector with G6PD. There is activation of the G6PD activity at low effector concentration and inhibition at high concentration. This interaction may be due to oxidation of NADPH at low primaquine concentration resulting in generation of more $NADP^+$ which increases the activity of the enzyme. Such a situation might account for the increased hemolysis in variant subjects with low intracellular NADPH concentration which will result in low level of reduced glutathione. Reduced glutathione is necessary for the maintenance of the integrity of the red cells.

A C K N O W L E D G E M E N T

It has been my good fortune to have worked under the supervision of Dr. G.B. Ogunmola. He always made himself available to discuss problems with me - both academic and personal, offering advice as a senior partner in academics not as a boss. He has influenced my life so profitably that I now realise that both academic and social lives have great difficulties which with courage and perseverance are easily surmountable. I wish to express my sincere thanks and profound gratitude to him.

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DEDICATION

This thesis is dedicated to the memory of late Niyi Babalola, a close friend and companion, who shared in my difficulties during the period of my undergraduate course. He lost his life in a boat accident in 1973. I know he should have liked me to join him in the formidable task of G6PD structure analysis. May his soul rest in perfect peace.

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

I certify that this work was carried out by Mr. S.A. Adediran in the Department of Chemistry, University of Ibadan between October 1974 and April 1978.

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G6PD	:	Glucose 6-Phosphate Dehydrogenase
$NADP^+$	:	Nicotinamide Adenine Dinucleotide Phosphate.
G6P	:	Glucose 6-phosphate.
EDTA	:	Ethylene Diamine Tetra Acetic acid.
TRIS	:	TRIS (hydroxymethyl) amino methane.
DPG	:	2,3-diphosphoglycerate.
εACA	:	ε amino caproic acid.
NADPH	:	Reduced Nicotinamide Adenine Dinucleotide Phosphate.
CM	:	Carboxy methyl.
DEAE	:	Diethyl aminoethyl.
Hb	:	Hemoglobin.
ADP	:	Adenosine diphosphate.
6GP	:	6-phosphogluconate.
GSH	:	Reduced glutathione.
PCMB	:	para chloro mercuribenzoate.
p-OHMB	:	para hydroxy mercuribenzoate.
WHO	:	World Health Organisation.

G3PD	:	Glyceraldehyde 3-phosphate Dehydrogenase.
NAD	:	Nicotinamide Adenine Dinucleotide.
GSSG	:	Oxidised glutathione.
6PgD	:	6-phosphogluconate Dehydrogenase.
6PG-δ-L	:	6-phosphogluconо-δ-lactone.
MWC	:	Monod, Wyman and Changeux (Model).

CHAPTER ONE

INTRODUCTION

The principal objective in the study of biological macromolecules is the elucidation of the relationship between the physical and chemical properties and biological function. Application of this goal to the investigations of oligomeric enzymes and other proteins has led to the recognition of some common phenomena which seem to regulate the functions of these proteins and enzymes. Mechanisms proposed to explain the regulation of activity of many oligomeric enzymes place great importance upon interaction among the subunits with attendant structural changes. Although our present knowledge of the structure and subunit interaction in G6PD is very limited, we may assume as in other proteins that changes in the structure of the enzymes are involved in the regulation of the reactivity of the molecule. The occurrence of genetic variants of the enzyme affords us excellent opportunity to correlate the altered reactivity of the variant enzymes to specific structural changes in terms of the mutation in the different genetic variants of the human erythrocyte enzymes.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) catalyses the initial step in the pentose phosphate pathway of carbohydrate metabolism causing reduction of $NADP^+$ to NADPH. The discovery that drug or food induced hemolytic anemia was associated with an inherited deficiency of glucose-6-phosphate dehydrogenase in erythrocytes (1) led to many investigations of the genetic variants of this enzyme in man. Burch et al (2) showed that an adequate amount of NADPH is necessary for the reduction of methemoglobin, as well as to ensure an adequate amount of stable reduced glutathione (GSH). This reduced glutathione is necessary for the maintenance of sulfhydryl groups within the erythrocyte and perhaps in the erythrocyte surface. Thus severe genetic deficiency of this enzyme is frequently associated with a low level of glutathione and with hemolytic anemia. Interest in G6PD derives from these genetic deficiencies found in erythrocytes of persons suffering from various congenital anemias and from attempts to identify structurally altered enzyme molecules in such persons.

The work of Chung et al (4) showed that an intimate relationship exists between the erythrocyte enzyme and its cofactor $NADP^+$. The coenzyme stabilizes the crude enzyme (5,6), activates it and

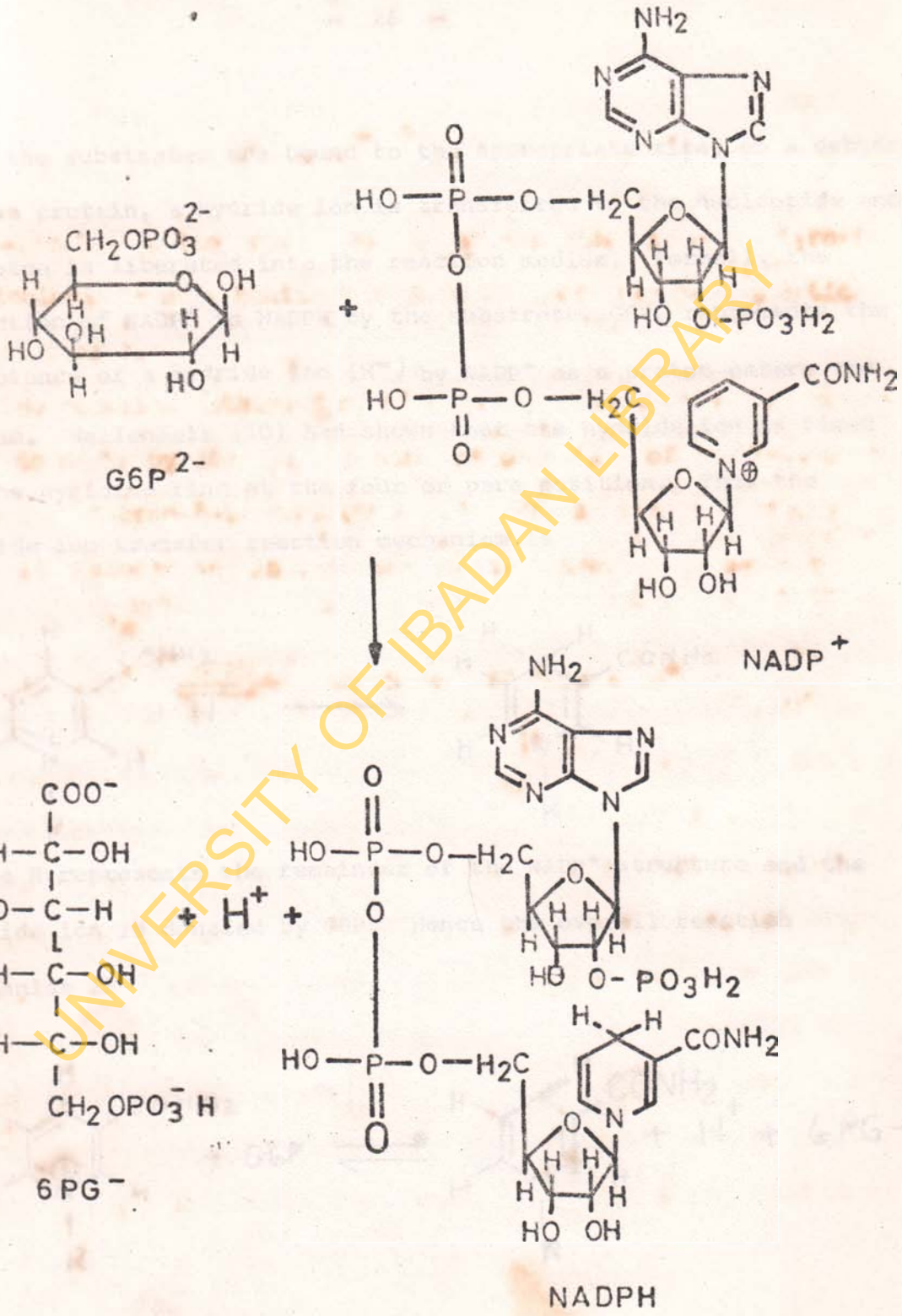
protects it from inactivation by erythrocyte stroma, which contains an active nucleotidase (5). There is some evidence that the co-enzyme is tightly bound to the enzyme (7,8). The complex relation-ship between the enzyme, its coenzyme and erythrocyte stroma may hold the key to understanding the control of G6PD in normal and pathological states. Chung et al (4) showed that each mole of enzyme as isolated from erythrocytes contains two moles of tightly bound $NADP^+$ which is reducible by G6P. This bound nucleotide deter-mines the stability and structural integrity of the catalytically active enzyme molecule. For example, it was easily found that in the crude lysate of erythrocytes, the enzyme rapidly loses activity and the loss could be prevented by the addition of $NADP^+$; and of course after inactivation of the enzyme, activity could be partially restored under suitable conditions by the addition of $NADP^+$. In view of previous reports, it seemed most probable that loss of enzymatic activity in crude preparation was due to destruction of $NADP^+$, particularly of the enzyme-bound nucleotide. The most obvious inference to be drawn is that on removal of this bound coenzyme, the apoenzyme dissociates into two catalytically inactive subunits, each approximately one-half the molecular weight of the native enzyme. Chung et al (3) found that the enzyme has at least two subunits.

The N-terminal amino acids were tentatively identified as tyrosine and alanine. These subunits may therefore possibly be unidentical peptide chains.

The reaction catalysed by G6PD is the reduction of the coenzyme $NADP^+$ to NADPH by G6P and a concurrent oxidation of the latter to 6-phospho glucono-δ-lactone (6PG-δ-L). The latter is a highly unstable compound and is instantaneously converted to 6-phospho-gluconate (9).

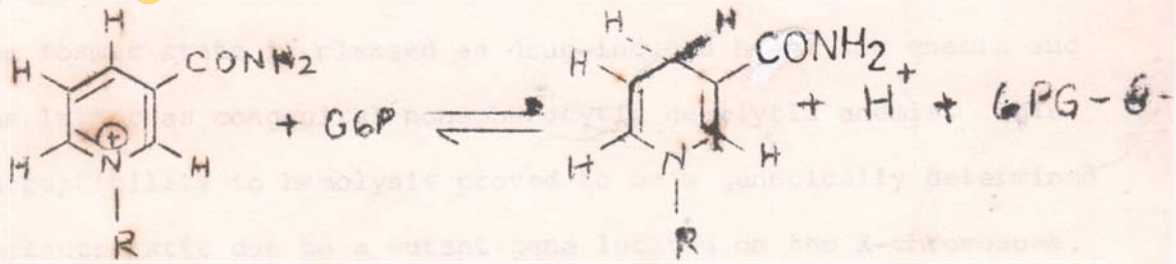
$$G6P + NADP^+ \xrightleftharpoons{G6PD} NADPH + 6PD\text{-}\delta\text{-}L + H^+$$

$$6PD\text{-}\delta\text{-}L \xrightarrow{H_2O} 6PG$$



When the substrates are bound to the appropriate sites on a dehydrogenase protein, a hydride ion is transferred to the nucleotide and a proton is liberated into the reaction medium. Formally the reduction of $NADP^+$ to NADPH by the substrate, G6P, represents the acceptance of a hydride ion (H^-) by $NADP^+$ as a proton enters the medium. Wallenfels (10) had shown that the hydride ion is fixed to the pyridine ring at the four or para position. Thus the hydride ion transfer reaction mechanism is

where R represents the remainder of the $NADP^+$ structure and the hydride ion is donated by G6P. Hence the overall reaction mechanism is



The interactions of the enzyme with both the substrate, G6P and the coenzyme, $NADP^+$ had been studied in considerable detail (11-18).

1.1.2 Genetic Variants and differentiation method

In G6PD, several types of heterogeneity are recognizable. First there is genetic variation seen in human erythrocytes with drug sensitive hemolytic anemias (19,20). Many of these anemias are associated with decreased erythrocyte G6PD activity and the presence of electrophoretically abnormal enzymes (21-29). Another type of molecular polymorphism, that associated with a difference in sub-cellular localisation, has been reported for G6PD (30).

The erythrocyte enzyme, G6PD, has been a useful tool for the study of genetic variations in man. Individuals whose red cells are deficient in this enzyme are susceptible to acute hemolytic anemia following the administration of certain drugs or the ingestion of certain chemicals or foods. In some instances, a chronic hemolytic condition is observed in the absence of exogenous agent. The former state is classed as drug-induced hemolytic anemia and the latter as congenital nonspherocytic hemolytic anemia. This susceptibility to hemolysis proved to be a genetically determined characteristic due to a mutant gene located on the X-chromosome.

The observed low level of G6PD catalytic activity in these pathological conditions may be attributed to either a quantitative difference in enzyme (G6PD) concentration (31) or to a qualitative abnormality of the enzyme in different patients (32).

The occurrence of favism more often in males than females was noted years ago (33). Investigations of primaquine sensitivity in Negroes (34) led to the suggestion that its transmission was probably by a gene of partial dominance located on the X-chromosome (35). Studies were therefore carried out on persons with G6PD deficiency and colour blindness and results have strongly suggested that the genes for both are closely located on the X-chromosome (35). Men are accordingly normal (XY) or else exhibit full expression of the enzyme deficiency and are referred to as hemizygous ($\bar{X}Y$). Women only exhibit full expression of the enzyme deficiency when a mutant gene is inherited from each parent, in which event they are homozygous ($\bar{\bar{X}}\bar{\bar{X}}$). Women inheriting a normal gene and a mutant gene from each of the parents are heterozygous ($\bar{X}X$) and exhibit remarkable variation in expression of the defect. Kirkman et al (28) showed the existence of three electrophoretic patterns of G6PD in erythrocytes of Negroes : a slow G6PD, identical in migration to that encountered in Caucasians, a

slightly faster G6PD and a broad band that seemed to represent a mixture of both the slow and fast enzymes, all at alkaline pH. These are designated B, A and AB respectively according to the nomenclature of Boyer et al (27). Primaquine sensitive Negroes have erythrocyte G6PD which migrated at the same rate as the fast G6PD of Negroes with normal activity of the enzyme. Quantitative assay for G6PD permitted a separate designation for the phenotype of sensitive Negroes (A^- by the nomenclature of Boyer et al (27)). The hemolysate from sensitive subjects could be identified readily on starch gel electrophoresis as developed by Smithies in 1955 (36) by their faint, fast enzymic band and relatively intense hemoglobin band. Thus there seems to be three common alleles in Negroes, one giving rise to B^+, one to A^+ with normal enzyme activity, and one to A^- with deficient enzyme activity.

On the basis of electrophoretic mobility on starch gel, several common G6PD phenotypes have been identified (27, 36, 37). The naming of these variants created a lot of confusion before 1966 when a study group convened by the World Health Organisation in Geneva recommended the following (38): The most common and ubiquitous variant of G6PD to be called B^+, should be used as reference with which all the other variants can be compared and can

for convenience be called the "normal" type. The variant with normal activity and electrophoretically faster than B^+ which represents about 20% (39) 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% (39) of their population, which has the same electrophoretic mobility like A^+ but is associated with enzyme deficiency, should be called A^-. The fact that women, can be either homozygous or heterozygous makes it imperative that for comparative studies of the variant enzymes, male subjects, which are necessarily hemizygous, should be chosen. Hence all studies in this thesis are on G6PD from male subjects.

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 (2). A rough correlation seems to exist between G6PD deficiency and the presence of Hemoglobin S (HbS) (21). The distribution of the two has been related to the distribution of high incidence areas of falciparum malaria (21,22). The heterozygous deficient females but not the hemizygous deficient males appear to be relatively resistant against Plasmodium falciparum malaria (22). The variant A^- conferred a distinct advantage to heterozygous females

with respect to infection by Plasmodium falciparum (40). Thus the Plasmodium prefers red blood cells that have sufficiently high level of G6PD, whether by virtue of their genetic structure or because of their young age as the parasites tend to invade the youngest cells in G6PD deficient female subjects (21). 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 (21). The occurrence 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 (2).

The presence of many variants of G6PD suggests that they are allelic and that the differences represent alterations in the sequence of amino acids in one of the polypeptide chains that make up the G6PD molecule. Since there are as many potential mutational sites in the amino acid sequence of protein molecules as there are amino acids, many different mutations can theoretically exist. It is hoped that future work will clarify the relationship between amino acid substitution, enzyme (G6PD) activity and drug

sensitivity. Amino acid substitutions have been shown in two G6PD variants by Yoshida (41,42) by the comparison of peptide mapping of the A^+ and B^+ types and of the B^+ and Hektoen types. He reported that A^+ contains aspartic acid in place of asparagine in B^+ and Hektoen contains Tyrosine in place of Histidine in B^+. The problem arises of whether the alteration of structural and functional parameters introduced by the single mutation leading to amino acid changes in the protein could be related to the changes in the reactivity of the enzyme and hence to changes in the vivo operation of the enzyme. Some of the known genetically determined variants of G6PD are characterized by a low catalytic activity and several cases such G6PD deficiency is associated with an increased susceptibility of the erythrocytes to exogenous hemolyzing agents, which can clinically result in hemolytic episodes.

1.1.3. Deficient and non-deficient G6PD Variants.

Investigations from different parts of the world over the last two decades have revealed a high degree of prevalence and genetic heterogeneity of abnormalities in glucose 6-phosphate dehydrogenase (19,32,44-48). This heterogeneity was strongly on clinical grounds and enzyme level determinations especially in deficient

enzymes in Negroes and Caucasians. The common variant in Negroes with low G6PD activity is G6PD A^- while the common mutant in Mediterranean populations is G6PD Mediterranean (29,32). Kirkman et al (29) had demonstrated kinetic differences between G6PD Mediterranean and G6PD A^- variant and Ramot (44) had also shown differences in some other Mediterranean mutants of the Jewish population in Israel.

A number of minimal criteria for the identification of new variants had been suggested by WHO Standardisation Committee (45) due to the high rate of increase of new identified variants. These criteria include:

(1) Enzyme activity in hemolysate.

(2) Electrophoretic mobility of enzyme.

(3) Michaelis constant, K_m, for G6P.

(4) Affinity for deoxyglucose 6-phosphate.

(5) Inactivation of the enzyme at elevated temperatures.

Since then a number of other criteria has been added to the above for the complete identification of new enzyme variants. These include pH optimum, K_m for $NADP^+$, Ki for NADPH, affinity for deamino $NADP^+$ and column chromatography (46,49). Using primarily these standardised criteria, several distinct types of enzyme

abnormality have been found among cases of congenital nonspherocytic hemolytic disease and G6PD deficiency. In some cases, the small amount of G6PD present appeared to be normal kinetically, but had marked decrease in stability to heat. These types of enzyme variants will call for other criteria before a complete identification could be made.

G6PD enzyme variants could be divided into three groups namely:

(a) The common and uncommon G6PD variants having normal activity or very mild enzyme deficiency and therefore are not associated with any clinical manifestations e.g. G6PD B^+ and G6PD A^+. These are basically non-deficient variants.

(b) The common and uncommon variants having enzyme deficiency but require exogenous agents, such as drugs, foods, infections or fava beans for hemolysis to occur e.g. G6PD A^- and G6PD Union. These variants have lower Km for $NADP^+$ and higher Ki for NADPH than for the normal variant.

(c) Variants with very low activities with congenital nonspherocytic hemolytic anemia even in the absence of exogenous agents e.g. G6PD Alhambra (47) and G6PD Tripler (48). These are variants with higher K_m for $NADP^+$ and lower Ki for NADPH.

Clinical problems are usually present in individuals with G6PD variants that have an enzyme activity of 0-30% of normal in their hemolysates. But some variants associated with very severe enzyme deficiency such as G6PD Union and G6PD Markham (19,20) have no hemolytic problem while other variants with less severe enzyme deficiency such as G6PD Alhambra and G6PD Tripler (47,48) are associated with chronic hemolytic disease even in the absence of exogenous agents. The kinetic characteristics (affinities for substrate and coenzymes) of these variant enzymes reported in literature cannot explain the reason for the different hemolytic manifestations of these variant subjects. Thus it looks as if the degree of enzyme deficiency does not correlate well with the clinical severity of the disease in G6PD deficient subjects.

Yoshida (39) had shown that the molar intracellular concentration of $NADP^+$ and NADPH are about 2μM and 50μM respectively. Therefore in the presence of low concentrations of $NADP^+$ and

relatively high concentrations of NADPH as in the red cells, the hemolytic variants such as Alhambra (47), Manchester (46) and Tripler (48) can hardly function because they are strongly inhibited by NADPH. Thus the hemolytic variant enzymes are strongly inhibited by physiologic concentration of NADPH because of their high Km for $NADP^+$ and low Ki for NADPH. Thus these variant enzymes cannot generate sufficient amount of NADPH in erythrocytes to maintain an adequate concentration of reduced glutathione which is necessary for the maintenance of sulfhydryl groups within the red cell (39). The nonhemolytic variants are far less sensitive to the inhibition by NADPH because of their low Km for $NADP^+$ and high Ki for NADPH. The physiologic activity of these nonhemolytic variant enzymes is estimated to be about 30% of the activity of the normal G6PD, and this activity is adequate to maintain the red cells unhemolysed at least in the absence of exogenous agents.

Hence from the foregoing, the best parameters to characterize enzyme deficiency in G6PD is the affinity constants for the oxidised and reduced coenzyme apart from the enzyme activity in the hemolysate Since these constants are known for the normal G6PD, higher Km for $NADP^+$ and lower K_i for NADPH indicate HEMOLYTIC DEFICIENT G6PD VARIANTS while lower Km for $NADP^+$ and higher K_i for NADPH indicate

NON-HEMOLYTIC DEFICIENT G6PD VARIANTS. So generally, it could be rationalized that the relative magnitudes of the affinity constants for both oxidised and reduced coenzymes compared to those for the normal non-deficient G6PD indicate G6PD DEFICIENCY.

1.1.4. Interaction of Glutathione Reductase.

The hexose monophosphate shunt pathway in red cells has a particular importance in generating NADPH. Glutathione Reductase (E.C. 1.6.4.2) in the red cell requires NADPH to maintain glutathione in the reduced state.

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH$$

Reduced glutathione had been said to be necessary for the maintenance of sulfhydryl groups within the erythrocyte (39). These sulfhydryl groups have been shown to be important in the catalytic activity of human erythrocyte G6PD (50). Hence low level of NADPH will impair the functioning of glutathione reductase in maintaining glutathione in the reduced state. This has the resultant implication of non-maintenance of the sulfhydryl groups in the erythrocyte G6PD and hence low activity of the G6PD. Thus severe genetic deficiency of G6PD is frequently associated with a low concentration of reduced glutathione and with hemolytic anemia.

Under normal conditions, about 10% of G6P is channelled through the hexose phosphate Shunt (2) thereby providing NADPH which is specifically involved in reducing oxidised glutathione (GSSG) via glutathione reductase and hence in maintaining a number of structural and functional proteins including G6PD in an active state. GSH is also effective, by means of glutathione peroxidase, as a detoxifying agent, and this function is achieved through reduction of hydrogen peroxide that may be formed within the erythrocyte especially in the presence of some hemolyzing agents (43), even under physiological conditions. Therefore the NADPH-producing enzymes, G6PD and 6-PgD, are ultimately concerned by way of the glutathione reductase system, with the protection of the erythrocyte against those structural damages produced by specific drugs or by some metabolites thereof or, more generally by any oxidative agents possibly being responsible for the hemolysis.

1.2.1. Structure of G6PD

The primary structure of glucose-6-phosphate dehydrogenase is unknown. Among about 100 variants of G6PD already reported (19,44, 47-49,51) only two variants, the common Negro variant A^+ (41) and G6PD Hektoen (42) have been elucidated at the molecular level.

Single amino acid substitutions, caused by single step base substitution in a structural gene, have been found in both cases. The exact nature of the structural change has only been demonstrated for the above two G6PD variants - from asparagine in the B^+ type to aspartic acid in the A^+ variant (41) and from Histidine in the B^+ to Tyrosine in the G6PD Hektoen (42). Thus a general study of the variants of G6PD associated with altered enzyme specificity must provide critical informations concerning the structure and function of the dehydrogenase.

But in view of the unknown primary structure of G6PD and its similarity to Glyceraldehyde 3-phosphate dehydrogenase in terms of order of substrates' binding, broad specificity as regards the pyridine nucleotides and wide and abundant occurrence in nature, a review of some of the basic structural details of glyceraldehyde 3-phosphate dehydrogenase and the nature of its binding sites might aid our understanding of the essential groups most especially at the active sites in the structure - reactivity correlation in G6PD. The three dimensional structure of glyceraldehyde 3-phosphate dehydrogenase had been determined by both X-ray diffraction studies and amino acid analysis (52,53). It is known that thiol groups (54) are essential for the catalytic activity of the enzyme. Chemical

evidence indicates that the active enzyme molecule comprises four identical protein chains (55,56) while X-ray diffraction analysis of the lobster enzyme - NAD complex (52) suggests that these four chains could be related in pairs to form a tetrameric molecule analogous in structure to the hemoglobin molecule (57) where two identical pairs of subunits are related to one another by a two-fold symmetry axis.

Cys-149 had been postulated (53) to be the sole site of reaction of iodoacetic acid with native glyceraldehyde 3-phosphate dehydrogenase, in the presence or absence of NAD. On the other hand, p-chloro mercuribenzoate does not readily react with Cys-149 in the presence of NAD (53), presumably due to steric competition at active site between reagents of this type and the bound coenzyme. The classical experiments of Velick (58) revealed the important role of reactive sulfhydryl groups in the catlytic activity of glyceraldehyde 3-phosphate dehydrogenase. The involvement of histidyl residues and sulfhydryl groups was shown in the results of pH-dependence of Michaelis constant and of maximum velocity by Keleti et al (59). In glyceraldehyde 3-phosphate dehydrogenase, the Zn ion and some sulfhydryl groups have been assumed to participate in the binding of coenzyme (120). The zinc ion was

postulated by Harris (53) to be bound to the protein through histidyl and cysteinyl residues. Friedrich et al (60) had also showed that histidine residue is essential for glyceraldehyde 3-phosphate dehhydrogenase reaction by the inactivating modification of the histidine residue.

Glyceraldehyde 3-phosphate dehydrogenase crystallises with firmly bound oxidised coenzyme (58) and therefore NAD is a constant component of the active centre. The effect of the NAD bound to the enzyme upon the physical properties and catalytic activity of the glyceraldehyde 3-phosphate dehydrogenase was rationalized by Velick et al (62) as suggestive of the bound coenzyme stabilizing the physical configuration of the enzyme molecule in solution. Both the oxidised and reduced $NADP^+$ had been shown to stabilize the physical configuration of G6PD by Cancedda et al (8) and Bonsignore et al (30).

Glyceraldehyde 3-phosphate dehydrogenase had been shown to have four non-identical binding sites and to be a tetramer of identical monomers (55,56). The differences in the NAD binding sites may therefore not be due to the amino acid sequence of the monomers (63).

Luzzatto (14) had postulated the existence of equivalent pairs of binding sites for $NADP^+$ in G6PD and the functional enzyme had

been shown to be a tetramer or dimer of identical subunits depending on the pH and ionic strength of the buffer medium (76), therefore G6PD and Glyceraldehyde 3-phosphate dehydrogenase may have similar structures. Thus we may expect a similarity in the binding kinetics of the pyridine nucleotide to the two enzymes and the chemical nature and reactivity of the catalytic binding sites in the two enzymes. The fact that the coenzyme, $NADP^+$, stabilizes the physical structure of G6PD and the fact that this enzyme possesses "structural" $NADP^+$ (13) makes one to want to predict a similarity between the primary structure and the chemical nature of the active centres in G6PD and G3PD.

1.3.1. Purification

Human erythrocyte glucose 6-phosphate dehydrogenase has been isolated to varying degrees of purity. Several methods of purification which include column chromatography with Diethylaminoethyl cellulose, calcium phosphate gel, carboxylmethyl cellulose, diethylaminoethyl sephadex and carboxymethyl sephadex have been reported (3,24,64-67). These enzyme preparations have been obtained in varying degrees of homogeneity and yield. For example, the preparation of Chung et al (3) which was reported to be 80% pure by

ultracentrifugal and electrophoretic measurements and of a specific activity of 113 enzyme units per milligram of protein was reported by Yoshida (64) to contain impurities of smaller molecular weights (One unit of G6PD is that amount which catalyzes the formation of 1μ mole of NADPH per minute at pH 8.0 and room temperature). The purity of the enzyme therefore was said to be about 65% since it was presumed that they measured the activity of partially inactive enzyme. Yoshida's preparation (64) gave a specific activity of 750 units per milligram of protein. Care was taken to avoid inactivation of the enzyme by proteolytic enzymes (e.g. plasmin) by the addition of ε-amino-N-caproic acid in the process of purification and to improve the yield of the enzyme preparation (64). Here an overall yield of about 50% (64) was obtained but after about 20 steps of purification. When abnormal and unstable enzyme variants are to be purified from small amounts of blood such as procedure is obviously not feasible. The same remarks can be applied to the methods established by Cohen et al (66), Chung et al (3) and Bonsignore et al (67). Considering this lengthy method of purification, Rattazzi (65) came up with a shorter and simpler one though from a smaller volume of blood. This procedure consists of three main steps but the final specific elution by the substrate

G6P which yields a highly labile enzyme species due to the catalytic reduction of the apoenzyme-bound $NADP^+$ which stabilizes the quaternary structure of G6PD called for a better method. The preparation by Cohen et al (66), although leading to a simplification of the procedure, still involved some steps which, according to Bonsignore et al (67) are not easily reproducible from one preparation to another. Bonsignore et al (67) thus described a method for purifying G6PD from human erythrocytes which represents a modification of the two procedures developed by Cohen et al (66) and by Rattazzi (65). They claimed to obtain stable and homogeneous enzyme preparation by this method. Here the specific elution from the final column was by the coenzyme $NADP^+$ which has been shown by many workers and us in parallel to have a lower Km (therefore a higher affinity for the enzyme) than the substrate G6P. They also detected two discrete catalytically active forms of the enzyme i.e. tetramers and dimers and a third catalytically inactive monomers by their procedure (67). The purification of G6PD A^- by Babalola et al (24) is essentially a combination of all the above methods with minor modifications peculair to the A^- variant. The preparation by Kahn et al (68) was eluted specifically by high concentration of the coenzyme $NADP^+$ which explains

the stability of the enzyme prepared by these workers. The total yield here (68) is between 80 and 90%. The higher yield, the simplicity of the method and above all, the stability of the enzyme seem to represent a major advantage of the method of Kahn et al (68) over that described by Rattazzi (65) and the other workers described above.

All purification procedures described so far are highly sophisticated and time-consuming. This consideration prompted De Flora et al (69) to develop a new method of purification, which is entirely 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 to selectively 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 decreases

the enzyme's affinity for the immobilized ligand) which results i the dissociation of the enzyme-immobilized ligand complex after unbound substances have been washed. De Flora et al (69) claimed to experience some difficulties (70) especially poor reproduciblity of the affinity chromatography step in the purification of G6PD. They later rationalised these difficulties in terms of marked modification in $NADP^+$ structure occurring during the carbodiimide-directed coupling of the dinucleotide to the matrix (70). They therefore started to use a more convenient affinity adsorbent, N^6-(6-aminohexyl) adenosine 2'5'-bisphosphate as synthesized by Morelli et al (71) and coupled to BrCN-activated agarose. The use of this matrix-bound effector allowed the isolation of G6PD in good yield and in a short time of about two days. This method is applicable for 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. This method was simplified further by the same workers (72) to involve a direct application of the hemolysate to the column of the N^6-(6-aminohexyl) adenosine 2',5'-bisphosphate and elution by a buffer of pH 7.85 containing $NADP^+$ of concentration 200μM. This procedure will elute comparable amounts of G6PD

and a new protein, which because of absence of information about its biological function, they designated FX (72). This eluent was then processed through dialysis and chromatography on CM-sephadex to obtain a complete separation of the two proteins. This procedure gave a final recovery of 60-70% and also has been successfully applied to the small-scale preparation of two genetic variantsof G6PD associated with enzyme deficiency (G6PD Seattle-like and G6PD Mediterranean) (72). Thus with this method we are moving nearer a stage where we can quickly purify the deficient enzyme variants for physicochemical analysis as these variants are very labile due to their rapid rate of inactivation. We have tried to apply the same procedure to the purification of G6PD A^-, but our effort was not very successful as this enzyme variant did not bind well to the affinity adsorbent (unpublished data). Yoshida (73) however had purified this enzyme variant by first of all converting the enzyme into the NADPH-bound form before applying it onto the agarose-bound $NADP^+$ column. This enzyme was then specifically eluted from the column by $NADP^+$ in the elution buffer. The enzyme did not however bind to the adsorbent when it was in the $NADP^+$ bound form, neither did the author obtain good yield when the enzyme to be applied to the column is free of

the nucleotides. This shows that this enzyme variant (G6PD A^-) is very labile and needs a nucleotide to stabilize it before it is attached to the affinity adsorbent. The $NADP^+$-bound enzyme in Yoshida's case could not bind as the effector is also $NADP^+$ and in our case, we applied a nucleotide-free enzyme to the effector, a greater portion of the enzyme having therefore being inactivated during the purification procedure.

Thus with purification methods which can give very pure enzymes, the previous limitation of physico chemical and molecular studies is now averted. The difficulty of these studies has always been limited by inavailability of purified enzyme. A lot of discrepancies both in kinetics and molecular properties of the different variants of the enzyme had been due to various workers using enzymes of varying purities. Thus presently we can get most variants of G6PD in very pure form for physico chemical and comparative studies.

1.4.1. Molecular Aggregation States of G6PD.

Structural studies conducted on human G6PD in a high degree of purity indicate two fundamental molecular forms, an inactive monomer and an active dimer (74). Kirkman et al (28,75) assigned

a molecular weight of about 105,000 to the dimeric form. This differs from the results of Chung et al (4) who estimated a molecular weight of 190,000 for the enzyme. Most of G6PD molecules are in the dimeric form at the optimal pH (pH 8-9) (76) while the highly associated form (the tetramer) predominates at the isoelectric point (pH 6.0) (77). Omachi et al (78) and Yoshida (39) have shown that in human erythrocytes the concentration of $NADP^+$ is about 2μM. Thus within the erythrocyte, G6PD is far from being saturated with $NADP^+$. At this low $NADP^+$ concentration the dimeric state of the enzyme is favoured (79) while at higher $NADP^+$ concentration, the enzyme is in the tetrameric form (13,79). The cooperativity phenomenon observed in G6PD B^+ (13) and G6PD A^+ (14) suggests a cooperative homotropic allosteric effect (80), resulting from conformational changes of the molecule associated with the $NADP^+$-dependent dimer-tetramer equilibrium.

The active form of G6PD purified from human erythrocytes is a mixture of dimer with probably identical functional units and tetramer. These two molecular forms are in equilibrium with each other (30,76,82). The dimeric form is in two different conformational states (8,14), i.e. the medium and high stability states,

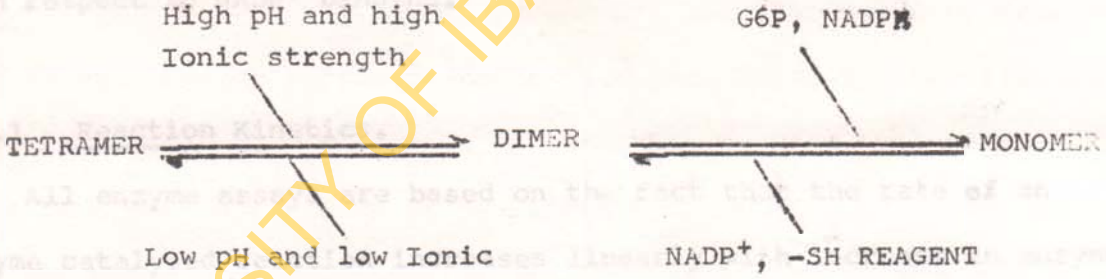
depending on the $NADP^+$ concentration. The dissociation of the dimer to inactive monomers could be effected in a variety of conditions such as treatment with a high concentration of G6P, extensive dialysis against a very low concentration of $NADP^+$, treatment with acid ammonium sulphate (4,81) or high temperature. High concentration of $NADP^+$ can restore activity in the monomers thereby bringing about dimerization. Thus two reversible disaggregation reactions of the tetramer can be followed in vitro:

TETRAMER $\rightleftarrows$ 2 DIMERS $\rightleftarrows$ 4 MONOMERS.

Studies by Wrigley et al (82) by electron microscopy demonstrated three discrete molecular forms of the enzyme and that the interconversion from one form to the other under appropriate conditions occurs within few seconds. Bonsignore et al (83) also showed that the enzyme exists in two catalytically active forms i.e. tetramers and dimers, and also monomers which are intrinsically devoid of activity. Many factors could effect the interconversion of the three enzyme forms.

The discrepancies in both the molecular weight and the subunit structure of G6PD were accounted for by the detailed ultracentrifugal studies of Cohen et al (76). These investigators showed that the enzyme has two discrete polymeric forms corresponding

to tetramers and dimers, whose equilibrium is closely related to the pH and ionic strength of the solvent, and to some other environmental parameters such as divalent ions, $NADP^+$, NADPH and sulfhydryl reagents (4,13,74,76,79). Most of the conflicting values of molecular weight reported previously could be ascribed to variations of experimental conditions resulting in different tetramer-dimer ratio. The general features of both equilibria may be summarised as follows (82):



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 (8,11,30).

Cancedda et al (8) showed that the dimeric enzyme exists in two conformational states i.e. the medium and high stability states. This finding is in consonance with the kinetic data of Luzzatto (14) who showed that the two dimeric conformational states are characterized by different affinity for $NADP^+$. Luzzatto's observation (14) of increase in interaction coefficient with increase in temperature and the very low interaction coefficient at low pH (when G6PD is predominantly a tetramer) supports the assertion that it is the dimer that is cooperative and that the tetramer is non-cooperative with respect to $NADP^+$ binding.

1.5.1 Reaction Kinetics.

All enzyme assays are based on the fact that the rate of an enzyme catalysed reaction increases linearly with increase in enzyme concentrations. But as different from non-enzymic reactions, there is no linear relationship between the rate and concentration of the substrate. Michaelis et al (84) first analyzed the deviation of enzymic reactions from non-enzymic kinetics by the study of the hydrolysis of sucrose by β-fructofuranosidase to glucose and fructose. These workers assumed that a complex was formed instantaneously between the enzyme and substrate before the

hydrolysis itself took place.

The reaction follows the following sequence:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P.$$

where E = Enzyme; S = Substrate; P = Products of the reaction and ES = Enzyme-substrate complex, k_1, k_{-1} and k_2 are reaction rate constants.

Michaelis et al (84) considered the ES complex to be in equilibrium with the free enzyme and the substrate and k_2 being so small in comparison to k_1 and k_{-1} and that the rate of break-down of the complex to the products does not affect the equilibrum concentration of ES. Therefore the kinetics derived from this model is often referred to as equilibrium kinetics. This implies that the slowest or rate-determining step is the break-down of ES to the products, and that the overall rate of the reaction, v, is proportional to the concentration of the ES complex:

$$v = \frac{dp}{dt} = k_2 [ES] \text{ ---------- (1)}$$

The equilibrium concentration of ES may be given by

$$K_s = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \text{ ---------- (2)}$$

where K_s is the dissociation constant of ES and $k_{-1} \gg k_2$, otherwise $K_m = \frac{k_{-1} + k_2}{k_1}$ when the equilibrium assumption does not hold.

From (2), $[E] = \frac{K_s.[ES]}{[S]}$ ----------(3)

But the total concentration of enzyme, $[E_t] = [E] + [ES]$ ---------(4)

$\therefore$ From (3). and (4), $[E_t] = [ES]\frac{(K_s + [S])}{[S]}$

$\therefore [ES] = \frac{[E_t][S]}{K_s + [S]}$

$\therefore$ From (1), $v = \frac{k_2 [E_t][S]}{K_s + [S]}$

When the enzyme is fully saturated with the substrates, i.e. when all the enzyme is in the form of the ES complex, v reaches a maximum value, the maximum velocity, V_{max}.

i.e. $V_{max} = k_2 [E_t]$

$\therefore v = \frac{V_{max}[S]}{K_s + [S]}$

This is the usual form of Michaelis-Menten equation. In the case of a two substrate reaction, for the derivation of this equation, the concentration of one of the substrates is kept at a very constant saturated level while the concentration of the second substrate is varied. In its reciprocal form, this equation becomes

$$1/v = 1/V_{max} + \frac{K_s}{V_{max}[S]}$$

Therefore a plot of 1/v against 1/[S] according to Lineweaver et al (85) gives a straight line of slope Ks/V_{max}, an intercept on the 1/[S] - axis of $-1/K_s$. K_s determined this way is called the Michaelis constant, designated Km. From the Michaelis-Menten theory (84), $K_m = K_s$, (where K_s is the true dissociation constant of ES), an inverse measure of the affinity of the enzyme for the substrate.

K_m is an important and useful parameter in characterizing an enzyme and its functional meaning is related to the inverse of the affinity of the enzyme for the substrate. K_m specified the quantitative dependence of the reaction rate on the substrate concentration. It has the dimensions of concentrations and is actually the substrate concentration for which the rate of the reaction is half the maximal value, V_{max}.

V_{max} is the extrapolated maximum velocity of the enzyme-catalysed reaction under the specified conditions of the reaction. It is the velocity of the reaction when the enzyme is maximally saturated with its substrates. K_m and V_{max} are the main fundamental kinetic parameters of enzyme reactions. They both vary with temperature, pH, ionic strength, type and concentration of the substrate, the presence of other molecules or ions and other

conditions for a particular enzyme.

1.5.2. Kinetics of G6P Binding to G6PD.

The kinetics of G6P binding to G6PD conforms to the Michaelis-Menten model (84) when the concentration of the coenzyme $NADP^+$ is fixed at a very saturating level (11,15,39). This kinetics of G6P binding have been widely studied for different variants of G6PD (11,15,17,18,24,66). The saturation function for all these variants is hyperbolic at all pH values and at all temperatures (11,15). This may mean that there is no interaction between the G6P binding sites and that they are identical and independent or that there is only one G6P binding site on the enzyme molecule.

1.5.3. 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 Michaelis-Menten model. Hence only a single dissociation constant of enzyme-$NADP^+$ complex has always been reported for all the G6PD variants. But Luzzatto (14) reported in 1967 that the saturation function of $NADP^+$ for the A^+ variant is sigmoid-shaped and hence does not follow the usual Michaelis-Menten equation. Luzzatto (14)

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 states, a state of low affinity and a state of high affinity for $NADP^+$. Luzzatto (14) therefore defined two dissociation constants for the binding of two molecules of $NADP^+$ by the enzyme molecule:

$$E + S \rightleftarrows ES, \quad K_{s_1} = [E][S]/[ES]$$

$$ES + S \rightleftarrows ES_2, \quad K_{s_2} = [ES][S]/[ES_2]$$

K_{s_1} = dissociation constant at low $NADP^+$ concentration;

K_{s_2} = dissociation constant at high $NADP^+$ concentration;

and that $K_{s_1} > K_{s_2}$.

This kinetics was extended to four other variants B^+, A^-, Ijebu-Ode and Ita-Bale by Afolayan et al (16) and these authors found that the four variants exhibit sigmoid kinetics. But the degree of conformity of the four variants to this kinetics 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 $NADP^+$.

Bonsignore et al (13) found two dissociation constants for G6PD under conditions at which the enzyme exists as a dimer. These are 20μM at low $NADP^+$ concentration and 12μM at high $NADP^+$ concentration. These workers also observed an interaction coefficient, n of 1.23. These findings are in consonance with those of Luzzatto (14). But the binding of $NADP^+$ to the tetrameric enzyme was found to follow a simple kinetics with a dissociation constant of 16μM and an n value of 1.02 (13). Hence binding of $NADP^+$ to G6PD depends on the state of the enzyme.

Enzymes, being proteins, possess 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 enzymes (i.e. V_{max} and Km) with pH are always interpreted in terms of ionizing groups at the enzyme's 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 enzyme active centres are derived from the studies of pH changes.

Babalola et al (11), Afolayan (15), Soldin et al (17) and Luzzatto et al (18) have studied the effects of pH changes on K_m^{G6P}, $K_m^{NADP^+}$, and V_{max} for the human erythrocyte G6PD. But the different buffers they all employed have significant effects on these parameters. The plots of log V, log K_m^{NADP} and log K_m^{G6P} against pH by Soldin et al (17) in kinetic experiments in Tris-maleat and Ammediol-HCl buffers yielded pK values of 6.6, 6.7 and 9.1 and 6.2 and 9.0 respectively. These were taken to indicate the presence of an imidazole group and a sulfhydryl group near the G6PD's active centre. Luzzatto et al (18) also indicated the presence of imidazolium group of histidine in variant B^+ but absent in variant A^+. The B^+ was also said to have a cysteine residue near its active centre (18). Evidence for the presence of sulfhydryl group and imidazolium group of histidine near the active centre had also been advanced by Babalola et al (11) in the study of G6P binding in Tris-borate and Triethylamine-borate buffers to G6PD A^+, B^+ and A^-. Luzzatto (22) 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 (11,15,17,18). But the pH dependence shows a most peculair and sharp profile in the

narrow pH range between 7.0 and 7.6. Luzzatto (22) 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 borne out by the results of gel filtration experiment as a function of pH by Babalola et al (11). These investigators observed that the pH dependence of the dimer-tetramer equilibrium corresponds closely to the narrow pH range (pH 7.2 for variants A^+ and B^+ and 7.4 for variant A^-).

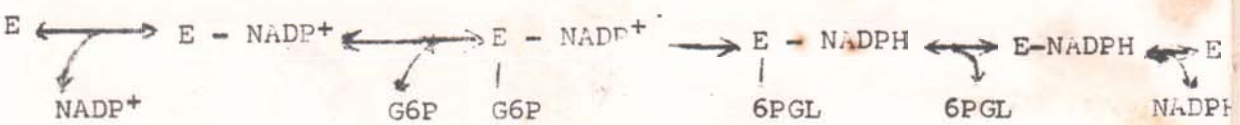
1.5.4. Kinetic mechanism of G6PD Action.

The combination of initial velocity, product inhibition and alternate substrate binding studies of purified G6PD from human erythrocytes and blood platelets gives results which are consistent with the postulate that the mechanism of substrate and coenzyme binding to this enzyme is sequential (17,39,86,87). The mechanism would appear from product inhibition data alone to be either a compulsory sequential order mechanism or a rapid equilibrium random order mechanism with a dead-end enzyme-G6P-NADPH ternary complex. Results from dead-end inhibition studies using an analogue of G6P, glucosamine 6-phosphate, help to rule out the latter mechanism (87). The initial velocity experiments on G6PD indicate

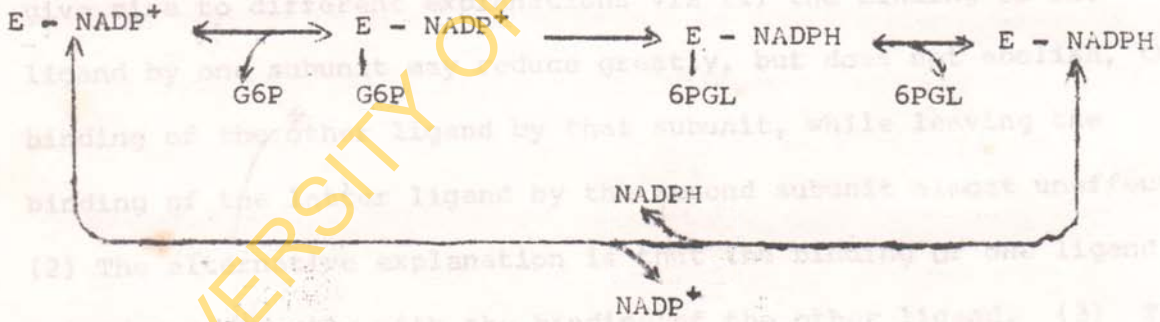
that the mechanism is sequential, thus excluding a ping-pong mechanism. Product inhibition studies using the first product to be released 6-phosphoglucono-δ-lactone, might also rule out a Theorell-Chance mechanism. However, 6-phosphoglucono-δ-lactone is highly unstable in aqueous solution (half-life 1.5 minutes) (9). But an independent observation by Yoshida (39) that G6P cannot associate with either G6PD without bound $NADP^+$ or with G6PD-NADPH complex, unequivocally shows that the association of G6P and $NADP^+$ with the enzyme should occur in a compulsory sequence.

1.5.5. Order of Substrates' Binding to G6PD.

Product inhibition by NADPH has been shown to be competitive with $NADP^+$ and noncompetitive with G6P (17,39,86,87). These data are consistent with an ordered release of products, with 6-phosphogluconate the first to dissociate and NADPH, the last. In summary, results from initial velocity studies, product inhibition and dead-end inhibition kinetics are consistent with an ordered sequential mechanism in which the coenzyme adds first and is released last and the substrate adds last and is released first. Thus we have the following order or reaction sequence:



But becuase of the low dissociation constants for the G6PD-$NADP^+$ and G6PD-NADPH complexes and the fact that G6PD is stabilized by bound $NADP^+$ (13,39,77), practically all the reacting enzyme will be associated with $NADP^+$. Therefore the reaction may proceed without any free enzyme being produced, by way of direct substitution of NADPH by $NADP^+$. Thus the order of the binding and release of the substrates will be as follows:



1.6.1. Cooperativity

The origin of cooperativity lies in the nearest-neighbour interactions of the subunits of an allosteric protein, whereby a conformational change accompanying the binding of a ligand by a subunit results in an induced conformational change in adjacent subunits, which enhances the binding of ligand by the latter. When two or more ligands are bound by a system of this kind, a theoretical description becomes complex since both the conformations of the subunits and their mutual interactions may be affected differently by the binding of the two ligands (80,87,88). This binding might give rise to different explanations viz (1) the binding of one ligand by one subunit may reduce greatly, but does not abolish, the binding of the other ligand by that subunit, while leaving the binding of the latter ligand by the second subunit almost unaffected. (2) The alternative explanation is that the binding of one ligand interferes directly with the binding of the other ligand. (3) The third alternative is that the two binding sites are in proximity and that the two ligands mutually interfere through electrostatic interaction (89).

The concerted two-state allosteric model proposed by Monod et al (80) is extended to expressions representing the initial velocity

as a function of substrate concentration for two-substrate enzymes for which an ordered sequential substrate binding is the mechanism (86). 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 (90). 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 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 (91), which is limited to the dimer case and to some limiting cases of the kinetic behaviour which arises from interaction between the substrates; that of Kirtley et al (92), which is an extension of the sequential model of Koshland et al (88) for allosteric proteins; and that of Sumi et al (93) which is limited to enzymes with a ping-pong mechanism (94).

There are at least two reasons why the kinetic behaviour of a two-substrate allosteric enzymes has 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. Second, while a general treatment is desirable, the mathematical expressions for such a 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 binding sites or between substrates at the same or different sites.

The equation derived by Monod et al (80) 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 catalyzed by an allosteric enzyme (95).

$$\frac{V_o}{nE_o} = \frac{k\alpha\,(1+\alpha)^{n-1} + k'L\,c\alpha\,(1+c\alpha)^{n-1}}{(1+\alpha)^n + L(1+c\alpha)^n}$$

In this equation, V_o is the initial velocity, n is the number of substrate binding sites per mole of enzyme; E_o is the total enzyme concentration; L is equal to $[E']/[E]$ and is 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 KA/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 the above equation assumes the existence of rapid, reversible equilibrium between two conformational states of an enzyme which has 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 rapid equilibrium (80,95). For two substrate allosteric enzymes, kinetic equations cannot be derived from binding expressions by simply including rate constants. The allosteric model asserts that indirect interactions between distinct specific binding sites, called allosteric effects, are responsible for the performance of the regulatory functions of the proteins. It is assumed in this model that these interactions are mediated by some kind of molecular transition, allosteric transitions, which is induced or stabilized in the protein when it binds an allosteric ligand.

Because the model of Monod et al (80) 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 cases depending on which of the conformational states has the greater affinity for the substrate. It is assumed for simplicity in enzyme kinetics discussion that the substrates' Michaelis constants are independent of the concentration of the other substrate and equal to the thermodyanic dissociation constants. There is evidence that K_m for the binding of $NADP^+$ in G6PD can be identified with a dissociation constant (13,14,17) and K_m for G6P is assumed to be a dissociation constant by Babalola et al (11). When the conformational states have different affinities for only one of the substrates, the kinetic behaviour obtained is that which can be predicted from analogy to the single substrate case. In addition, the kinetic behaviour is independent of the concentration of the nonvaried substrate (90). This is the result to be expected from a two-substrate enzyme in which the concentration of one substrate does not affect the kinetics of the other and in which the kinetic behaviour for one substrate is positive cooperativity and that of the other is hyperbolic. This is the case in G6PD enzyme in which

the kinetic behaviour for G6P is always hyperbolic (11,15) and for $NADP^+$ under some conditions to be discussed in this thesis favours positive cooperativity (13,14).

The Michaelis-Menten model (84) has had a great impact on the development of enzyme chemistry. However, the kinetic properties of many enzymes cannot be accounted for by this model. An important group consists of the allosteric dehydrogenase enzymes, which often display sigmoidal plots of the reaction velocity, V, versus substrate concentration for the coenzyme rather than the hyperbolic plots predicted by the Michaelis-Menten equation. This kinetic behaviour is reminiscent of situations in hemoglobin where oxygen binding curve is sigmoidal (96). In allosteric enzymes, one active site in an enzyme molecule can affect another active site in the same molecule. A possible outcome of this interaction across subunits is that the binding of substrate becomes cooperative, which would give the sigmoidal plot. In addition the activity of allosteric enzymes may be altered by regulatory molecules that are bound to sites other than the catalytic sites, just as oxygen-binding in hemoglobin is affected by DPG, H^+ and CO_2. The dependence of the reaction rate of Glucose-6-phosphate dehydrogenase on $NADP^+$ concentration, under certain experimental conditions, does not

conform to the Michaelis-Menten equation. Luzzatto (14) 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. He then determined the two dissociation constants for the enzyme-$NADP^+$ complex, at low and high $NADP^+$ concentrations respectively. 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. In the simplest case where there are two or any number of equivalent pairs of binding sites, the expression for the reaction velocity as a function of substrate concentrations was simply and readily derived. The velocity v, as a function of the substrate concentration [S] in its reciprocal form was given as:

$$\frac{V_{max}}{v} = 1 + \frac{K_{s_2}}{[S]} + \frac{K_{s_1}K_{s_2}}{[S]^2}$$ where V_{max} is the maximum velocity and K_{s_1} and K_{s_2} are the dissociation constants at the enzyme states for low and high affinities for $NADP^+$ respectively. These constants could be got from this equation by inserting two pairs of values of $\frac{V_{max}}{v}$ and [S] rewritten twice and solving the equation simultaneously.

For many proteins, it is convenient to express the ligand-binding properties by the Hill equation (97)

$$y = K[S]^{nH}/1 + K[S]^{nH}$$

in which y, the "fractions saturation" is the fraction of the total number of binding sites occupied by ligand, [S] is the free ligand concentration, and K and n_H are the dissociation constant of the protein-ligand complex, and Hill coefficient respectively. This two constant equation can be rearranged into the form of a straight line as follows:

$$\log {}^{y}/_{(1-y)} = \log K + n_H \log [S].$$

The resulting plot of $\log {}^{y}/_{(1-y)}$ against log [S] is known as the Hill plot and the degree of cooperation among substrate molecules can be conveniently ascertained from the slope n_H of the plot which is often called the Hill or Interaction coefficient. This coefficient is used extensively as a measure of homotropic interactions between substrates in multisubunit enzymes. Whereas Michaelian rate curves are characterized by a Hill coefficient of one, the sigmoid kinetics of regulatory enzymes generally yield Hill numbers larger than unity. In kinetic data, ${}^{v}/_{V_{max}}$ is always assumed to be proportional to y, the "fractional saturation".

1.6.2. Allosteric Effectors.

Allostery implies a special type of inhibition or activation and allosteric effectors therefore are the substances which are not substrate analogues and which are apparently not 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 miscontrued in literature but it can affect the degree of substrate interaction and therefore cooperativity. The effector therefore must act by inducing a conformational change which alters the activity at the catalytic site. The study of the interaction of G6PD with ions has recently variously been reported (13,14,66,82,83). 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. Among the workers who had studied the effects of some ions on G6PD are Kuby et al (98) who studied the effect of $EDTA^{3-}$ and $NADP^{+}$ on macromolecular association phenomenon in Brewer's Yeast G6PD; Cohen et al (66) who studied the influence of monovalent and divalent cations on human erythrocyte G6PD activity and Luzzatto (14) who studied the influence of buffer ions (Tris-HCl) and $MgSO_4$ on the coefficient of interaction of $NADP^{+}$ binding to human erythrocyte

enzyme. The general effect of buffer ions on the degree of cooperativity and the influence of specific ions on the coefficient of interaction of $NADP^+$ binding to human erythrocyte G6PD variants will be discussed in more details as it forms a substantial part of the work reported in this thesis.

1.7.1. Aim of the present work.

The knowledge of the primary structure of some proteins such as lysozyme (99) has yielded insight into the mechanism of substrate binding and therefore of enzyme structure. For some other proteins such as the quasi-enzyme, hemoglobin, a combination of structural, chemical and functional data had led to an understanding of important properties of the molecule, such as the cooperativity of oxygen binding and Bohr effect (96). Further, a 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. This approach has not been explored deeply in the case of G6PD variants except in the work of Babalola et al (11) where the kinetics of G6P binding to three polymorphic variants of G6PD was reported. In view of

previous data that genetically different types of human erythrocyte G6PD confer different metabolic properties on the erythrocytes (25,29,39,79), and that the different variants are due to amino acid substitution at the structural locus (11,41,42), the present work is to investigate whether a comparative analysis of kinetic and thermodynamic parameters of $NADP^+$ binding to the different genetic variants of the enzyme will help to elucidate the differences in their molecular structures and catalytic functions.

The comparative kinetic and thermodynamic studies of $NADP^+$ binding reported in this thesis were carried out on highly purified G6PD B^+, A^+ and A^- from the erythrocytes of human male subjects. In view of the discrepancies in kinetic and physico-chemical results of G6PD enzyme variants and the rationalization of these in terms of impure enzyme preparations used, it was thought necessary to compare the available methods of purification in order to see their limitations and advantages. For this purpose, it is important to prepare the three enzyme variants by both conventional multistep and affinity chromatography methods and then compare the results with the above criteria of choice of the better method in mind.

The kinetic studies which were carried out in Tris-borate and Triethylamine-borate buffers is an attempt to correlate kinetic

data with catalytic function of the enzyme variants in order to explain reactivity differences in terms of structural differences. The reactivity differences in G6P binding by Babalola et al (11) had been explained in terms of structural differences, a similar study for $NADP^+$ binding is necessary in view of the fact that it is known that $NADP^+$ binding is more sensitive to small differences in the structure of G6PD molecules (16). For this purpose it is necessary to study the dependence of $K_m^{NADP^+}$ and V_{max} on pH and temperature. From these studies, the enthalpy change of dissociation of G6PD-$NADP^+$ complex and the activation energy of the G6PD catalysed reaction under conditions of saturation of the enzyme with the two substrates could be got and studied in terms of their dependence on pH. These studies should show the differences in structure of the G6PD variants in better perspective just like electrophoretic studies (26,27,36).

The binding of $NADP^+$ to G6PD variants had been reported to be cooperative at higher ionic strength (14-16,25), and two dissociation constants had been reported for G6PD-$NADP^+$ complex (14-16,25), it was considered of importance to investigate further the variation of these dissociation constants for the B^+ enzyme variant at different pH values (5.85-9.5) and at four temperatures. With

these experimental data, one will gain an insight into the influence of ionic strength on molecular forms of the enzyme involved in the interaction with $NADP^+$ in terms of the variation of n, the Hill's coefficient with increase in pH and temperature.

All these studies will then show that single amino acid substition at the structural locus does not affect only the primary structure but also the quaternary structure of the enzyme variants.

CHAPTER TWO

EXPERIMENTAL

2.1.1. Reagents

Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) and Glucose 6-phosphate (G6P) were from Biochemica Boehringer Mannherm Germany and from Sigma Chemical Co. St. Louis, Mo, U.S.A.

ε-aminocaproic acid, Diethylaminoethyl (DEAE) Sephadex A50, Carboxymethyl (CM) Sephadex C50, Sephadex G25, G100 and G200 are all products of Pharmacia Fine Chemicals Inc. Uppsala, Sweden. DEAE cellulose was a Whatman product from W and R Baston Ltd, England and EDTA was from Scientific Co, U.S.A. Ammonium sulphate, special enzyme grade was from Schwarz/Mann, Orangeburg, New York.

The affinity adsorbent, N^6-(6-aminohexyl)-2'5'ADP, was from two sources. The first adsorbent used in this work was synthesized in the Laboratories of the Institute of Biological Chemistry, University of Genoa, Italy and was a kind gift to us by Professor A. De Flora. The second resin was from Pharmacia Fine Chemicals Inc., Uppsala, Sweden.

TRIS, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4, NaCl, KCl, β-mercaptoethanol, Boric acid, Trimethylamine, NaOH, HCl, KOH and all other reagents used in this work were all of analytical (AnalaR) grade from the British Drug Houses Ltd.

This commercial TRIS fluoresces and therefore was purified by dissolving it in hot water and left till it crystallized and then washed many times with ethanol and thoroughly dried till it did no more fluoresce. It is this purified TRIS that was used for the buffer preparations in this work.

2.2.1. Instruments.

Optical density measurements were made on a set-up that consisted of a Gilford Spectrophotometer with a constant temperature cell compartment and an automatic multiple absorbance recorder model 2000.

PYE Dynacap pH meter Type M65 was used in measurement of pH. Agla micrometer syringes were used for the measurements of small volumes of reagents while Lambda pipettes were used for the measurements of enzyme volumes.

Other instruments include a refrigerated MSE High Speed 18 Centrifuge, Fraction Collector, Quartz Micro and Macro cuvettes.

high precision thermometer and dialysis tubings.

Turner recording spectrofluorometer model 110 modified by us with thermostatic compartment. Turner Model 430 with a recorder was also used for certain parts of the work.

2.3.1. Identification of Glucose-6-Phosphate Dehydrogenase.

Identification of glucose-6-phosphate dehydrogenase was performed on starch gel electrophoresis in Tris-borate - EDTA buffer, pH 8.6 according to the methods of Smithies and Boyer et al (27, 36) with the following modifications:

(a) The concentration of $NADP^+$ in the gel was 12.5μM.

(b) The voltage was 12 volts/cm, the current was 0.5 mA/cm and the duration of the run was 15 hours.

The various types of hemoglobin were identified after electrophoresis. The gel was then stained under incubation with a staining solution which is composed of glucose-6-phosphate, Nitro Blue Tetrazolium, $NADP^+$, Tris, Phenazine methosulphate and magnesium sulphate at a pH of 8.6. After staining the gel, G6PD A^+, B^+ and A^- could be easily recognised by visual inspection (27).

2.4.1. Purification of the Enzyme G6PD Variants.

It was necessary to have, ready for use and stored at 4°C for days, all the materials needed for the purification of the enzyme before embacking on the process. Unless otherwise stated all buffers used in storing the resins that had been precycled and ready for use and those that were used in washing of columns and elution in the purification processes were 1 mM in ε-amino caproic acid to prevent hydrolysis of G6PD by contaminating proteolytic enzymes such as plasmin and protease;
0.2% in β-mercapto ethanol to stabilise the S-H groups in G6PD; 1mM in EDTA; and 20μM in NADP except in affinity chromatography column. All glass columns used were aligned with the aid of a spirit level.

2.4.2. Materials for purification.

Water: Water used for any work reported here was ion-free. It was obtained by deionising distilled water by a mixed bed ion exchange resin.

EDTA-Saline: a 0.85% NaCl solution which was 1mM in EDTA.

Phosphate Buffers: 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 solutions were prepared and then mixed until the required pH was obtained,

using the PYE Dynacap pH meter Type M65. The solution would then be diluted to the required concentration according to the stage in enzyme purification and also the resin in use.

DEAE Cellulose:

(i) The weighed quantity of the cellulose was stirred in excess water, allowed to settle and the supernatant decanted. This washing was repeated 4 or 5 times to remove all colouring and fine materials.

(ii) Excess 0.5M HCl was added with stirring and then left for at least 30 minutes.

(iii) The supernatant was decanted and the resin was washed with water until pH of supernatant was 4.

(iv) Excess 0.5M NaOH was then added to the resin with stirring and left for another 30 minutes.

(v) The supernatant was decanted and the resin washed with water until pH of supernatant was 8.

(vi) The washing was continued with 5mM phosphate buffer, pH 6.4 until pH of supernatant was 6.4.

(vii) The resin was stored in excess 5mM phosphate buffer, pH 6.4 at $4^{o}C$, ready for use.

Sephadex G100 and G25

The G100 sephadex resin was used to separate the enzyme G6P from a mixture of many other proteins. This use depends on differential elution of proteins according to their molecular weights. Using the same principle, the G25 sephadex resin was used to remove small ions (those from NaCl, KCl, $(NH_4)_2SO_4$ and G6P) from the enzyme protein at two different stages in the purification process.

The weighed sephadex was washed several times with excess water until all the fine resin granules were removed. The G100 sephadex resin and half of the G25 sephadex resin were then washed (separately) with 50mM phosphate buffer, pH 7.0 until the pH of supernatant was 7.0. They were then stored in 50mM phosphate buffer, pH 7.0. The other half of the G25 resin was washed with 5mM phosphate buffer pH 6.0 until the pH of the supernatant was 6.0. This was then stored in the same buffer of pH 6.0.

The two batches of G25 sephadex resin were first stored at room temperature (22 $\pm$ 1°C) for at least 12 hours before packing them into glass columns of 60 cm length and 5 cm in diameter. The two columns were then transferred to the cold room (4°C), ready for use.

The G100 sephadex resin was first kept at 4°C. 145 cm long and 7.5 cm in diameter glass column was aligned in the cold room stoppered air-tight with a rubber bung through which a large wide-mouthed 2-litre glass funnel was connected. A mechanical stirrer was positioned so that it could stir the resin slurry when poured into the funnel. The column was first filled with the buffer and the slurry resin poured into the funnel with stirring. The column was packed to a height of about 130 cm and the funnel and stirrer removed; the coloumn was then ready for use.

DEAE Sephadex A50 and CM-Sephadex C50

The preparations of these two resins were similar to those of sephadex G100 and G25. The only difference was the medium buffer. DEAE sephadex A50 was washed with, and stored in 50mM phosphate buffer, pH 6.9 while CM-sephadex C50 was washed with, and stored in 5mM phosphate buffer, pH 6.0. They were first left at room temperature for at least 12 hours after which the A50 sephadex was packed into a 50 cm X 3.5 cm glass column while the C50 sephadex was packed into a 40 cm x 3.0 cm glass column. They were then transferred to the cold room.

Another very short column (4 cm x 1 cm) of DEAE-sephadex A50 was also set up at 4°C. The buffer pH here was 7.0 and

$NADP^+$ concentration was 40µM.

2.4.3. Assays during Purification

Enzyme Assay: The reaction G6P + $NADP^+$ → 6-Phosphogluconate + NADPH + H^+ catalyzed by the enzyme G6PD was readily followed by taking advantage of the optical property of NADPH: light absorption at 340mµ with molar extinction coefficient of 6.22×10^3. The unit of enzyme activity is the amount of enzyme required to reduce 1µmole of $NADP^+$ to NADPH per minute at 25°C. The increase in absorbance of each assay mixture at 340mµ per minute was measured, using the Gilford spectrophotometer, model 2000. From the ΔOD_{340}/minute obtained, the enzyme activity in units per ml was calculated.

The assays were carried out at 22 ± 1°C (room temperature) in Quartz micro cuvettes with light path of 1 cm. They were carried out essentially according to the World Health Organization (WHO) recommendations (38).

The assay mixture was

0.1M TRIS-HCl buffer, pH 8.0.

0.1M Mg^{2+}

4mM G6P and 0.33 mM NADP.

The first three items were mixed to produce the stock solution, the so-called World Health Organization (WHO) mixture which could be stored at - 20°C. Between 0.01 and 0.10 ml of the enzyme solution was added according to its concentration in a final volume of 0.3 ml. $NADP^+$ was added last and water was added to make up the volume. Typical assay cuvette contained

0.18 ml WHO mixture
0.1 ml enzyme
0.09 ml distilled-deionized water
0.02 ml of 5 mM NADP.
0.30 ml

The cuvette was immediately covered with a piece of parafilm, the latter was pressed to the mouth of the cuvette and then inverted 2 or 3 times for proper mixing. The Gilford 2000 spectrophotometer was immediately started to record the change in optical density at 340 mμ per minute.

2.4.4. Protein Determination.

The estimation of protein in the fractions of the effluent at each purification stage was carried out by reading the optical absorption at 280 mμ. The buffer used at that purification stage the blank.

To determine the absolute or quantitative protein concentration

of the pool obtained at the end of each purification stage: the relationship:

1 mg of protein/ml = optical density reading at 280 mμ was used.

2.4.5. Purification Process - Conventional Method.

All blood units were collected from the Blood Bank of the University College Hospital, Ibadan. Expired male blood (blood that had been stored with standard acid-citrate-dextrose at 4°C for 3 to 5 weeks) was used. All procedures were carried out at 4°C or in ice bath.

1. Preparation of Haemolysate.

This was carried out according to the method of Ratazzi (65) except that toluene was not included in the haemolysing buffer.

For two variants (variants A^+ and B^+) prepared, the starting material was about 3 litres of blood. This was centrifuged in a refrigerated MSE centrifuge at 13 - 15,000 revolutions per minute for 30 minutes. The supernatant plasma was decanted. Excess EDTA-saline solution was added, gently mixed and then spun at the same speed for 30 minutes. The supernatant was decanted. This washing with EDTA-saline solution was done 3 times. The supernatant was usually clear after the third washing.

2 volumes of 5 mM Phosphate buffer pH 6.4 were mixed with 1 volume of the packed red cells. The mixture was well shaken for a few minutes and then centrifuged at 13 - 15,00 RPM for 60 minutes. The "fatty cake" floating was carefully removed and the supernatant decanted into a flask immersed in ice. This is the haemolysate. Enzyme assay and protein determination were carried out on a sample of it. Enzyme assay was better carried out on a 1:20 dilution of the haemolysate, using 10 µM NADP as the solvent.

2. DEAE cellulose.

This was carried out according to Chung et al (3) but with some modifications. The DEAE cellulose that had been stored in 5 mM phosphate buffer pH 6.4 and kept at 4°C was drained. It was added in small portions to the haemolysate with gentle stirring. After 25 minutes the last portion of the resin was added and the supernatant tested for enzyme (G6PD) activity. It was usually found to be nil. This is kept in the cold room (4°C) for about 15 hours and then washed with washing buffer. Elution buffer was added in 100 ml portions and fractions collected and assayed for the crude enzyme. 36 - 40% saturated weight of ammonium sulphate was added (i.e. 220 gm $(NH_4)_2SO_4$ to 600 ml of the crude enzyme) and kept for 15 hours to precipitate the

enzyme protein. The solution was centrifuged for 30 minutes at 15,000 RPM. The supernatant was carefully decanted. Small volumes of 50 mM phosphate buffer pH 7.0 were added to the centrifuge cups. With the aid of a glass rod the enzyme precipitate adhering to the walls of the centrifuge cups was meticulously dissolved in the 50 mM phosphate buffer, pH 7.0. The solution was centrifuged again (at 15,000 RPM for 30 min.) and the supernatant, now very rich in G6PD, was decanted into a small container immersed in ice. Any undissolved residue was similarly treated with more 50 mM phosphate buffer, pH 7.0. The two extracts were combined so that the pool did not exceed 90 ml. Enzyme activity and protein content of the pool were determined.

3. G100 Sephadex.

The pool from the previous step was applied to the Sephadex G100 column, and allowed to sink totally before introducing the 50 mM phosphate buffer, pH 7.0. Fraction collector was set up and fractions collected in tubes 1-180. Flow rate was about 90 ml/hour. Enzyme activity was assayed at 340 nm and estimation of protein content done at 280 nm and those tubes with high enzyme activity but of very low protein content were pooled. G6PD activity and protein content of the pool were determined.

4. DEAE-Sephadex A50.

Purification on DEAE-Sephadex A50 was carried out according to Luzzatto et al (100). The pool obtained from the G100 column was applied to the DEAE-Sephadex column at a flow rate of 60 ml/hour. The anion exchanger with the bound enzyme was washed with 50 mM phosphate buffer pH 6.9, which was 0.05M in NaCl. Activity of the enzyme was determined in the wash-off and there was no activity. Elution of the enzyme was begun with linear gradient of NaCl which consisted of 1 litre of 0.3M NaCl in 0.05M phosphate buffer, pH 6.9 and 500 ml of 0.1M NaCl in the same buffer. Each solution contained 15μM $NADP^+$. Enzyme assays and OD_{280} measurements were carried out on the fractions of the effluent from the DEAE-Sephadex A50 column. Fractions with high enzyme activity but very low protein content were pooled. G6PD activity and protein content of the pool were measured. (If the volume of this pool is more than 100 ml, one might set up a concentrating device).

5. Dialysis.

The pool from the previous step was dialysed against 20 mM phosphate buffer, pH 6.0 to desalt it. With about 100 ml of the pool, three changes of a 2-litre dialysis buffer were required which was replaced after every 2-3 hours. The dialysis buffer

contained 10μM $NADP^+$, EDTA, ε-amino caproic acid (EACA) and mercaptoethanol. Enzyme solution was then assayed and protein content determined at 280 nm.

6. CM-Sephadex C50-Sephadex G25 combined stage.

For this stage Ratazzi's method (65) was followed, though with some modifications. The desalted enzyme pool was then passed through CM-sephadex C50 column packed with 5 mM phosphate buffer, pH 6.0. (The enzyme was supposed to bind at this condition). Gradient of 20 mM phosphate buffer pH 6.0 to 5 mM phosphate buffer of the same pH was then set up and passed through the column. This washed off all the impurities.

2 mM Glucose-6-phosphate and 0.025 NaCl in 20 mM phosphate buffer pH 6.00 was then introduced to wash off the bound enzyme; the wash-off was immediately passed through Sephadex G25 column packed with 50 mM phosphate buffer pH 7.00. (The CM-Sephadex C50 and Sephadex G25 columns were connected). After connecting the two columns for 3 hours, bleeding of the connecting tubing was begun to test for G6PD activity in the effluent coming from the CM column to the G25 column.

As soon as no more G6PD activity was detected in this effluent the two columns were disconnected. The 50 mM phosphate buffer,

pH 7.0 was then used to elute the enzyme from the G25 sephadex column. Flow rate was 45 ml/hour and fractions of about 6 ml per tube were collected. G6PD activity and OD_{280} of the fractions were measured. The final purified G6PD was stored in the cold room at $4^{o}C$.

2.4.6. Purification Process - Affinity Chromatography Method.

Conventional procedures for the purification of human erythrocyte G6PD depend on differences in the physicochemical properties of the various proteins present in the hemolysate. These differences are generally not unique and most of the available separation procedures of a preparative nature are not sufficiently discriminative to permit facile separation of molecules whose physicochemical differences are subtle. Therefore purification using this conventional procedure, is often laborious and incomplete. In contrast, affinity chromatography is a "functional" purification approach that exploits the most unique property of enzymes, their biological function. The tertiary structure of enzymes is generally believed to be highly selective for maintaining the integrity of their active centres. The function of the active centres involves two separate chemical processes: those of recognition and of

catalysis of selected small molecules such as substrates and co-enzymes. It is the former property, that of recognition, which forms the theoretical basis upon which the principles of affinity chromatography have been developed.

Purification Process - Affinity Chromatography Method.

1. Preparation of Hemolysate.

This was carried out essentially according to the method of De Flora et al (70) but with certain modifications. A bag of male blood was used for each purification process.

The erythrocytes, after removing the plasma, was mixed with 0.15M KCl, and then centrifuged at 13 - 15,000 revolutions per minute for 30 minutes. The supernatant solution was decanted and the same process repeated until the supernatant solution is very clear. Four volumes of water were then mixed with a volume of the packed cells. The mixture was shaken very well for a few minutes and then centrifuged at 13 - 15,000 RPM for about 40 minutes. The hemolysate was carefully poured into a flask immersed in ice leaving the "fatty cake". Enzyme assay and protein determination were carried out on 1:20 dilution of the hemolysate.

The following chemicals were then added to the prepared hemolysate.

(a) 0.2% β-mercaptoethanol.

(b) 1 mM EDTA.

(c) 2M sodium phosphate buffer pH 6.0 (2.5% of the final volume of hemolysate).

(d) 1M Potassium acetate buffer pH 5.8 (2.5% of the final volume of hemolysate).

(e) 2M acetic acid - The mixture is titrated with this acetic acid solution until its pH is 6.1.

2. N^6-(6-aminohexyl)-2'5' ADP

The above hemolysate is then loaded onto the affinity adsorbent column (10 x 1.5 cm) which was previously equilibrated with buffer A and then washed successively with:

(a) 50 ml of Buffer A.

(b) 50 ml of Buffer B.

(c) Buffer C till the absorbance at 280 mμ is about zero.

Elution was then carried out with 80% of Buffer C in water which is 0.2 mM in $NADP^+$.

Buffer A: 0.1M Potassium acetate + 0.1M Potassium phosphate pH 6.1.

Buffer B: 0.1M Potassium acetate + 0.1M Potassium Phosphate pH 7.85.

Buffer C: 0.1M Potassium chloride + 0.1M Potassium Phosphate pH 7.85.

All solutions contain 0.2% β-mercaptoethanol and 1 mM EDTA The eluate as collected from the affinity column contains G6PD and FX (72). To get rid of the FX, the eluate is dialyzed against two changes of 3 litres of 50 mM sodium acetate buffer pH 6.0 for about 8 hours each.

3. CM-Sephadex C-50.

This was first equilibrated with the 50 mM sodium acetate buffer pH 6.0. The dialyzed eluate was then applied onto the CM Sephadex C-50 column (8 x 1.4 cm). This was then successively washed with 50 mM sodium acetate buffer pH 6.0, 20 mM sodium phosphate buffer pH 6.0 which is 25 mM in NaCl and 20 mM sodium phosphate buffer pH 6.0 which is 50 mM in NaCl.

Final elution of pure G6PD was obtained with 20 mM phosphate buffer pH 6.0 which is 50 mM in NaCl containing 0.2 mM $NADP^+$.

This method of purification is used to get pure forms of the three genetic variants of the enzyme; A^+, B^+ and A^-.

2.5.1. Preparation of Buffers.

Data from previous work by Luzzatto et al (18) and Babalola et al (11) have indicated 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, Tris and triethylamine had no effect on either of these two parameters and these reagents were therefore used to prepare all buffers from pH 5.85 to 10.00. Regarding ionic strength; there is evidence from other investigators (66) that this can affect substrate binding. Furthermore, it is known that the molecular aggregation state of G6PD is affected by ionic strength (66,76). It was therefore necessary to ensure that determinations at all pH values were carried out at the same ionic strength, which was 0.01. For this purpose, buffers were prepared by mixing suitable amounts of stock solutions of boric acid, Tris and Triethylamine according to Babalola et al (11).

Further, since ionic strength determines the molecular aggregation state of G6PD (66) and since it has been inferred by Cancedda et al (8) that the molecular form of G6PD in Luzzatto's

work (14) is the dimer, different ions were put into the above buffers to get the ionic strength Luzzatto employed in his work. The buffers were brought up to this ionic strength by using the following compounds, K_2SO_4, $MgCl_2$, $MgSO_4$ and KCl to mimic other worker's conditions for the controversy on cooperativity (14,101). The buffers (pH 5.85 to 10.00) were brought up to the high ionic strength by adding K_2SO_4. These were the buffers used in the kinetic experiments from which $K_{s_1}^{NADP^+}$ and $Ks_2^{NADP^+}$ were got.

2.5.2. Kinetic Determinations

All reaction velocity measurements were carried out in Turner recording Spectrofluorometer model 110 modified by us with a thermostatic compartment. The composition of the reaction mixture was as follows; Buffer : 3 ml; 0.06M G6P : 0.3 ml; enzyme solution : 0.01M, water and the substrate, $NADP^+$, were added in different volumes to have a final mixture volume of 4 ml. The concentration of the enzyme solution was adjusted by dilution with 0.05M Tris-borate pH 8.0 so as to give a V_{max} of approximately 0.06 A_{340}/min at the particular pH and temperature in use. The reaction mixture was always allowed to reach temperature equilibrium in the water bath attached to the instrument before enzyme solution was added from a

Lambda pipette. Mixing was effected by the use of a small plastic sheet. The reaction tube was covered by the sheet and quickly inverted three times and recording started immediately. The pH of the solution was measured with a PYE Dynacap pH-meter Type M65 at the appropriate temperature. The pH values recorded are the ones thus measured, not the nominal pH values of the buffers. It was found that the pH values thus measured were always a little less than the nominal pH values of the buffer (about 0.1 - 0.2 pH units).

All enzyme preparations were dialyzed for 4 hours before use against two changes of 250 ml 0.05M Tris-borate buffer, pH 8.0 containing 0.1 mM EDTA and 10μM $NADP^+$.

2.5.3. Measurement of $K_m^{NADP^+}$, $Ks_1^{NADP^+}$, $Ks_2^{NADP^+}$, n and V_{max}.

These kinetic constants for the G6PD variants were determined as described by Luzzatto et al (18), Afolayan (15) and Babalola et al (11) but with some modifications. $NADP^+$ binding to G6PD at a saturating concentration of 4.5 mM of G6P and at an ionic strength of 0.01 has been found to conform to Michaelis-Menten mechanism (84). From the Lineweaver-Burk equation, $1/v = 1/V_{max} + \frac{K_m}{V_{max}[s]}$, a plot of $1/v$ against $1/[s]$ yields a straight line. From this plot, the intercept on the 1/v-axis is $1/V_{max}$ and an intercept on the $1/[s]$-axis

is $-1/Km$. Hence these two kinetic parameters could be obtained from these plots at all pH values and at all temperatures. About ten concentrations of $NADP^+$ were used and all were found to be on a good straight line (Fig 3).

The following table is a typical set of readings for the determination of $K_m^{NADP^+}$ and V_{max}:

Table 1 G6PD A^+ Temperature 40^oC pH 6.86

Ionic strength = 0.01

$[NADP^+] \times 10^6 M$	$v \times 10^9$ (moles NADPH min^{-1})	$1/v \times 10^{-9}$	$\frac{1}{[NADP^+]} \times 10^{-6}$
125.0	4.31	0.23	0.008
100.0	4.31	0.23	0.010
75.0	3.99	0.25	0.013
50.0	3.67	0.27	0.020
37.5	3.47	0.29	0.027
25.0	2.76	0.36	0.040
12.5	2.25	0.44	0.080
10.0	1.86	0.54	0.100
5.0	1.22	0.82	0.200

The G6P solutions were found to be highly acidic and they reduce the pH of the buffer solutions at the different pH values at the end

of the run. We therefore decided to prepare the G6P solutions in the buffers at each pH.

At a higher ionic strength, G6PD shows cooperativity with regards to $NADP^+$ binding (14) and so in contrast to the conditions at low ionic strength where there is only one K_m^{NADP} and n, the interaction coefficient is always about one, there are two dissociation constants, $K_{s_1}^{NADP^+}$ and $K_{s_2}^{NADP^+}$ and n is consistently higher than 1 at all pH values and at all temperatures.

These dissociation constants were determined according to Luzzatto (44) by calculations employing the following equation:

$$\frac{V_{max}}{v} = 1 + \frac{K_{s_2}}{[s]} + \frac{K_{s_1} K_{s_2}}{[s]^2}$$

and n, the interaction coefficient is got from the slope of the Hill's plot (97) according to the equation:

$$\log \frac{v}{V_{max} - v} = \log K + n \log [s].$$

Table (2) is a typical set of data for the determination of n.

Variant A^+, Temperature 40°C pH 6.86, Ionic strength = 0.01

V_{max} = 4.90×10^{-9} moles NADPH min^{-1}.

$[NADP^+] \times 10^6 M$	$v \times 10^9$	v/V_{max}	$\log[NADP^+]$	$\log v/V_{max}$ v.
125.0	4.31	0.88	2.097	0.87
100.0	4.31	0.88	2.000	0.87
75.0	3.99	0.82	1.875	0.66
50.0	3.67	0.75	1.699	0.48
37.5	3.47	0.71	1.574	0.39
25.0	2.76	0.57	1.398	0.12
12.5	2.25	0.46	1.097	-0.07
10.0	1.86	0.38	1.000	-0.22
5.0	1.22	0.25	0.699	-0.48

2.5.4. Calculation of activation Energy, E_A, and enthalpy change, ΔH, of G6PD- catalyzed Reactions.

The activation energy, E_A, as a function of pH and for the three variants A^+, B^+ and A^- was obtained from the respective V_{max} values measured at different temperatures and pH values. Similarly, ΔH, the enthalpy change of the reaction, was calculated from the

$K_m^{NADP^+}$ values. In both cases, Vant Hoff's isochore was applied:

$$\frac{d \ln V_{max}}{dT} = \frac{E_A}{RT^2}$$

$$\therefore \ln V_{max} = -\frac{E_A}{RT} + \text{constant.}$$

$$\text{and } d \ln \frac{K_m^{NADP^+}}{dT} = \frac{\Delta H}{RT^2}$$

$$\therefore \ln K_m^{NADP^+} = -\frac{\Delta H}{RT} + \text{constant.}$$

Plots of $\log K_m^{NADP^+}$ against pH at different temperatures were obtained. Plots of log Km at constant pH (obtained by linear interpolation between adjacent experimental points) against 1/T are linear, indicating that within experimental error, we may assume that $\Delta C_p = \left(\frac{d\Delta H}{dT}\right)_p = 0$ over the temperature range (20 - 40°C) of determination of these kinetic parameters and hence that we can calculate ΔH from the slopes of these plots (102).

$$\therefore E_A = -2.303\ R \times \text{slope of a plot of } \log_{10} V_{max} \text{ against } 1/T$$

$$\text{and } \Delta H = -2.303\ R \times \text{slope of a plot of } \log_{10} K_m^{NADP^+} \text{ against } 1/T$$

where R = gas constant = 1.987 cals $mole^{-1}\ {}^oC^{-1}$.

The slopes of the best straight lines were used in calculating the different ΔH's at the different pH values. A smilar procedure was used in calculating E_A and for ΔH in the case of $Ks_1^{NADP^+}$ and $Ks_2^{NADP^+}$.

CHAPTER THREE

EXPERIMENTAL RESULTS

3.1.1. Purification of the A^+, B^+ and A^- Genetic Variants of G6PD using both the Conventional Multistep and Affinity Chromatography Methods.

The patterns of elution and separation from the other proteins of one of the variants (G6PD B^+) from the various chromatographic columns employed for the conventional method of purification are shown in figure 1. Variants A^+ and B^+ behaved similarly in all the steps of the purification but variant A^- because of its inherent lability and deficiency is not purified by this long multistep method. The recovery from the CM-sephadex for the two variants was very good (about 85% of what was applied). This is in consonance with Rattazzis finding (65). The two variants were effectively eluted by 2mM G6P as a single peak from this column.

Table 3 summarizes the purification of A^+ and B^+ variants by the conventional multistep method. The tables show that final specific activities of both preparations are above 150 units/mg protein.

Figure 1: Purification of Glucose 6-phosphate Dehydrogenase by the Conventional method. Elution profile of G6PD Variant B^+ from the three chromatographic columns.

●—●—● Protien o-o-o-o Enzyme activity

(a) From Sephadex G100 column.

(b) From DEAE Sephadex A50 column.

(c) From CM Sephadex C50 column.

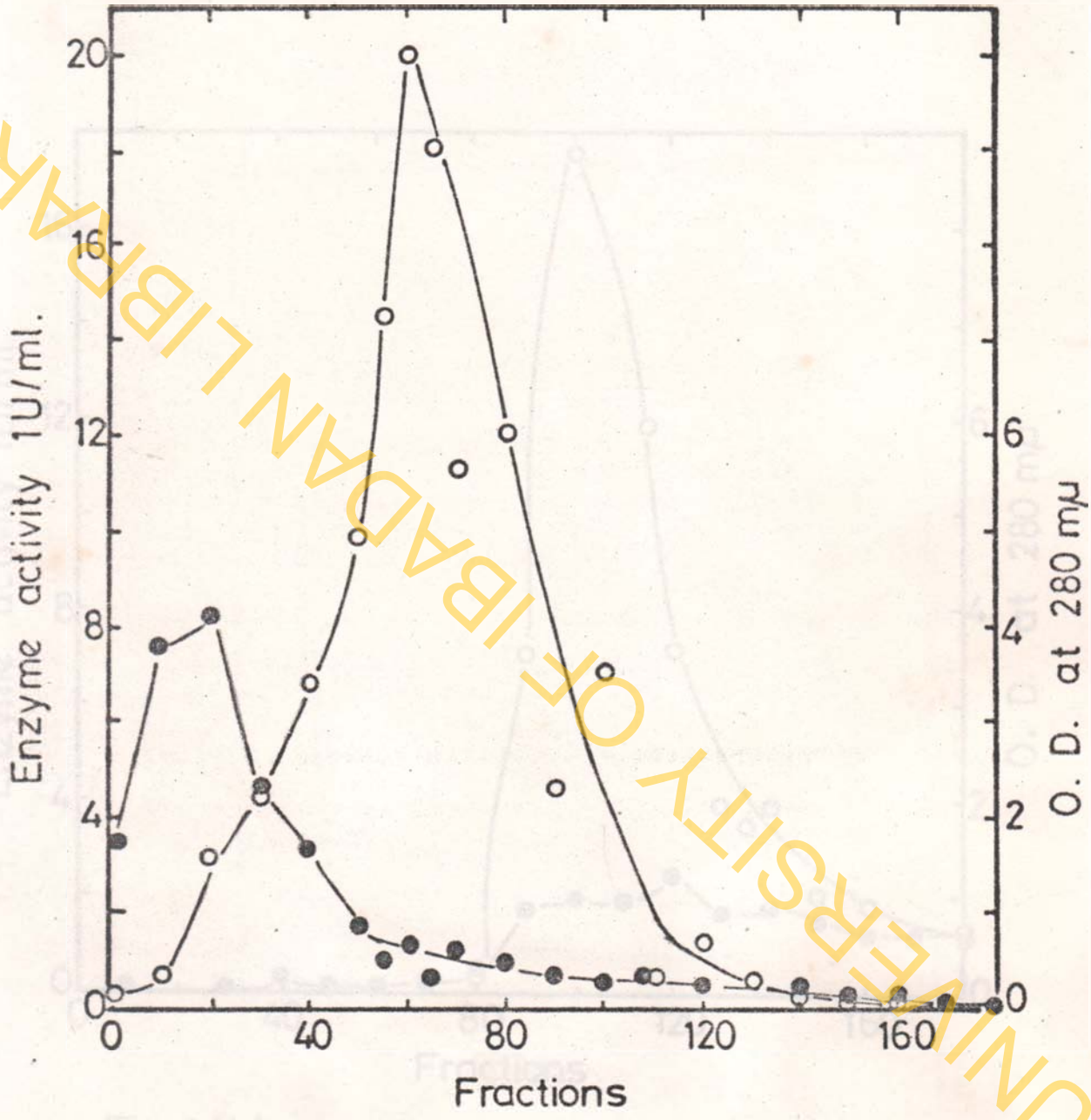


Fig. 1 (a)

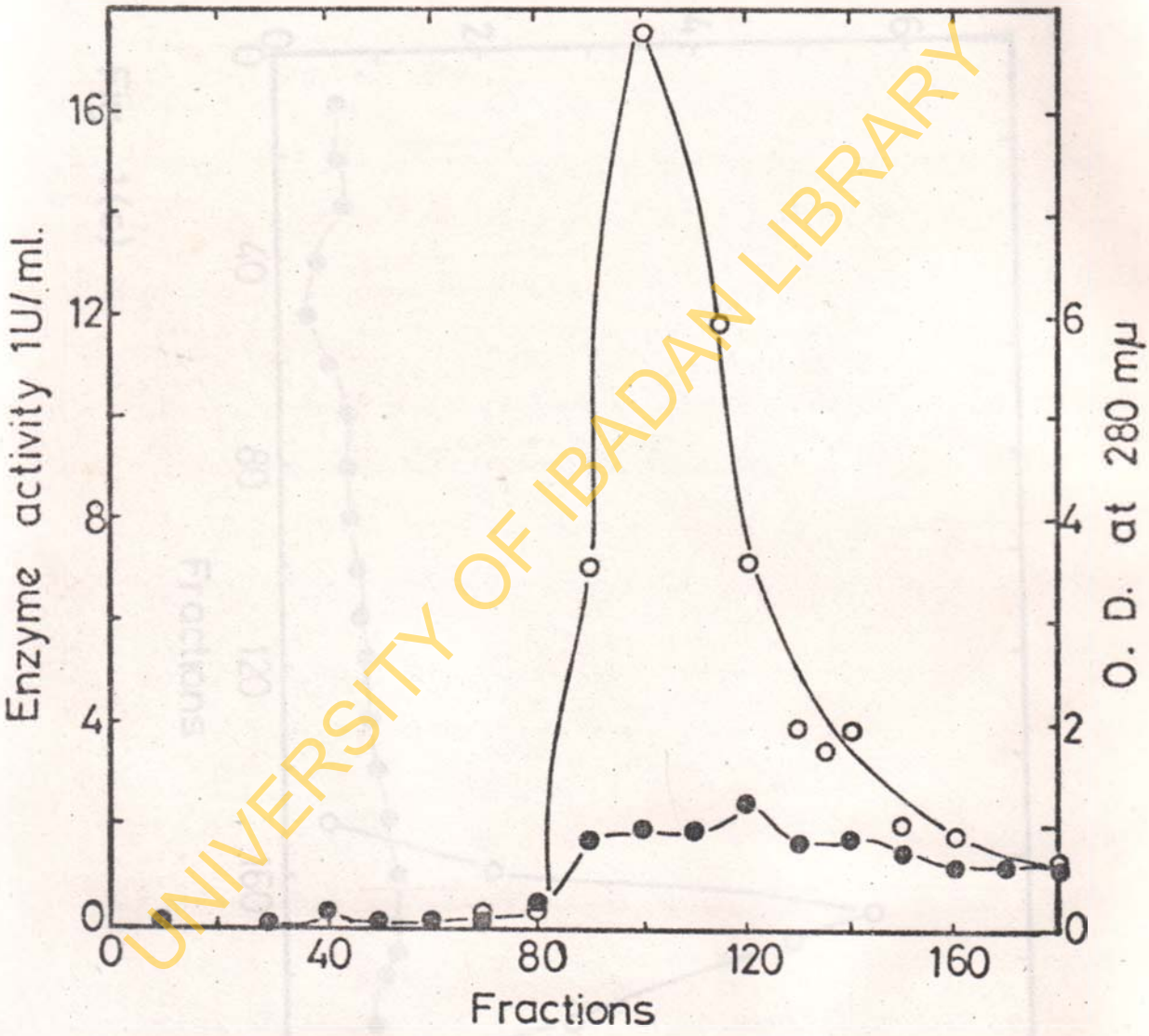


Fig. 1 (b)

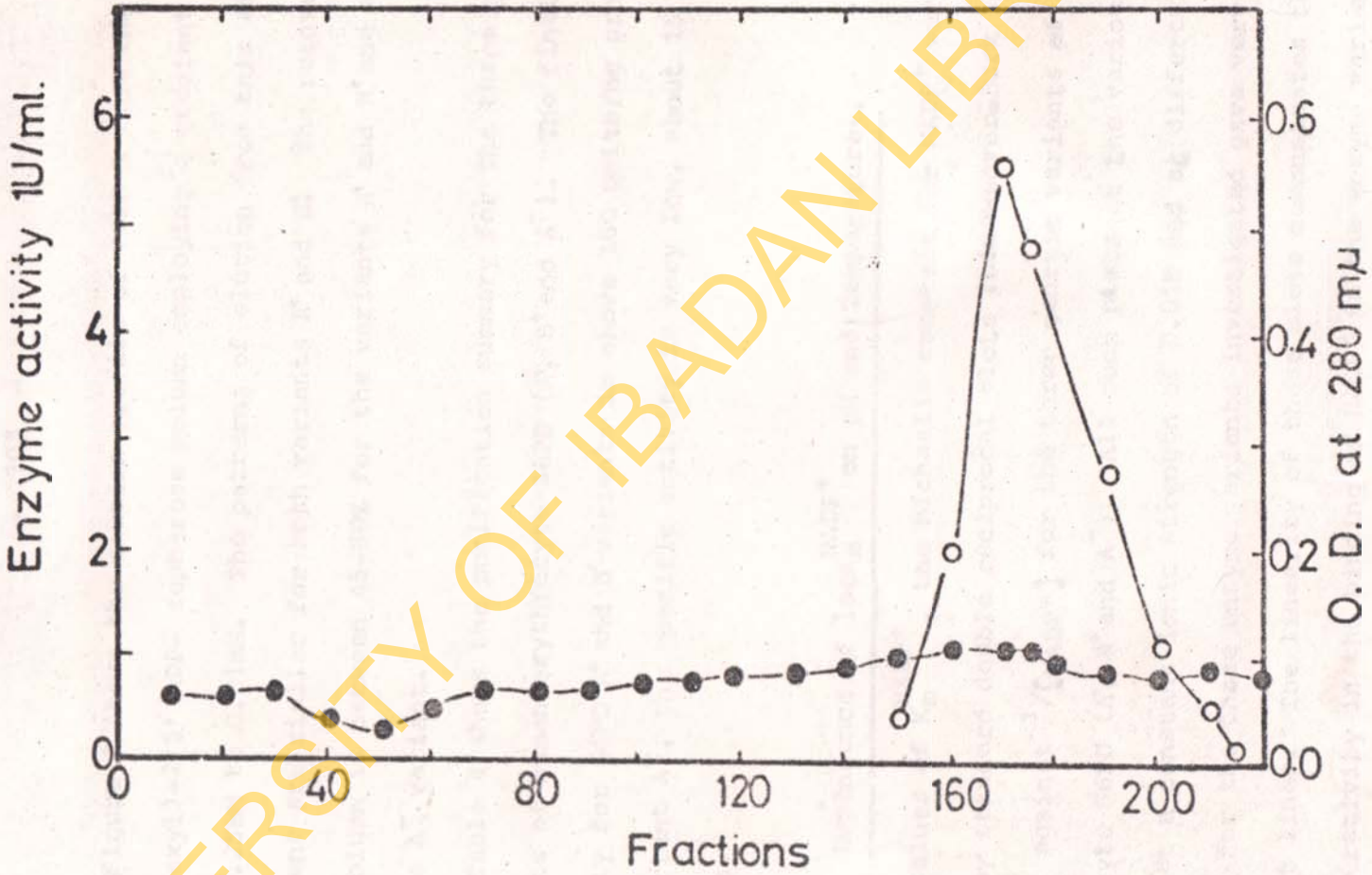


Fig. 1(c)

Figure 2 shows the elution profile of G6PD B from the N^6- (6-aminohexyl)-2'5' ADP- sepharose column employing a gradient of $NADP^+$ from 0.25mM to 0.01mM. The patterns of elution from this affinity adsorbent are similar for both variants A^+ and B^+. The recovery from this column is between 45-80% for the variants A^+ and B^+ and about 25% for the A^- variant.

Table 4 shows the purification summary for the three genetic variants of human erythrocyte G6PD (A^+, B^+ and A^-). The final specific activity for G6PD A^+ and B^+ variants is above 160 units/mg protein while for variant A^-, the specific activity is very low, about 15 units/mg protein.

3.2.1. Dependence of $\log K_m^{NADP^+}$ on pH and Temperature.

Values of $K_m^{NADP^+}$, the Michaelis constant for $NADP^+$ were determined by standard double reciprocal plots (Lineweaver-Burk method 85) of $1/v$ against $1/[NADP^+]$ for the three genetic variants of human erthrocyte G6PD (A^+, B^+ and A^-). All such plots at the various pH values at a constant ionic strength of 0.01M and at different temperatures for the three enzyme variants investigated gave very good straight lines. The linearity of these plots demonstrates the absence of cooperativity in the binding of $NADP^+$ to the enzyme variants at

Figure 2: Purification of Glucose 6-phosphate Dehydrogenase by the Affinity Chromatography method Elution Profile of G6PD Variant B^+ from two chromatographic columns.

●—●—● Protein o - ● - ● - o Enzyme Activity

(a) From Sepharose-2'5' ADP column; 10 ml/fraction

(b) From CM Sephadex C50 column; 2 ml/fraction.

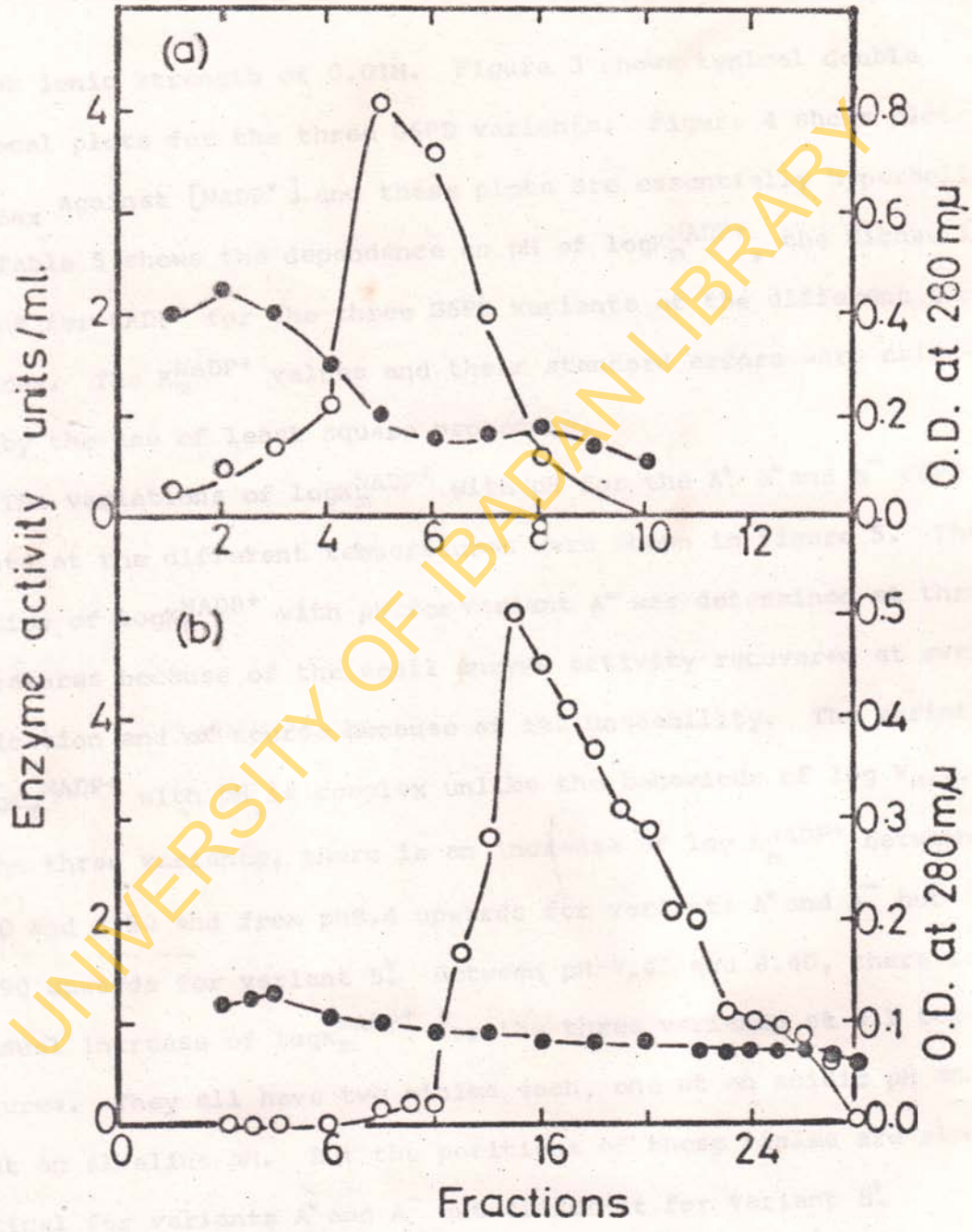


Fig. 2

this low ionic strength of 0.01M. Figure 3 shows typical double reciprocal plots for the three G6PD variants. Figure 4 shows plots of V/V_{max} against $[NADP^+]$ and these plots are essentially hyperbolic.

Table 5 shows the dependence on pH of $\log K_m^{NADP+}$, the Michaelis constant for $NADP^+$ for the three G6PD variants at the different temperatures. The K_m^{NADP+} values and their standard errors were calculated by the use of least square procedure.

The variations of $\log K_m^{NADP+}$ with pH for the A^+, B^+ and A^- G6PD variants at the different temperatures were shown in figure 5. The variation of $\log K_m^{NADP+}$ with pH for variant A^- was determined at three temperatures because of the small enzyme activity recovered at every purification and of course because of its unstability. The variation of $\log K_m^{NADP+}$ with pH is complex unlike the behaviour of log V_{max}. For the three variants, there is an increase of log K_m^{NADP+} between pH6.00 and 6.50 and from pH8.4 upwards for variants A^+ and A^- but from pH 8.90 upwards for variant B^+. Between pH 7.40 and 8.40, there is a gradual increase of $\log K_m^{NADP+}$ for the three variants at all temperatures. They all have two minima each, one at an acidic pH and one at an alkaline pH. But the positions of these minima are almost identical for variants A^+ and A^- but different for variant B^+.

K_m^{NADP+} has been interpreted as the dissociation constant for

Figure 3: Lineweaver and Burk double reciprocal plots of $1/v$ against $1/[NADP^+]$

o - o - o For G6PD A^+.

Δ - Δ - Δ For G6PD B^+.

• — • — • For G6PD A^-.

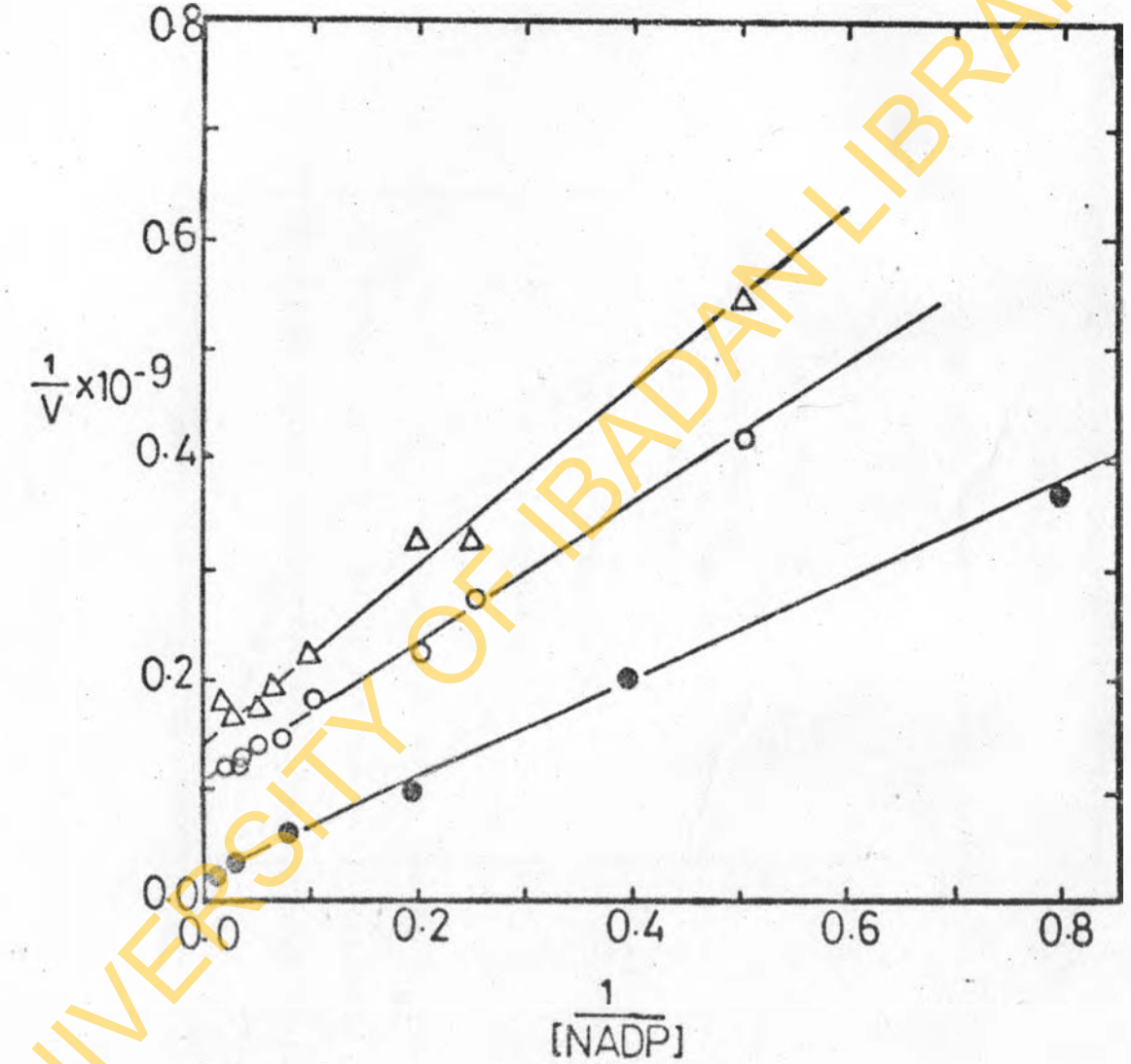


Fig. 3

Figure 4: Plots of the normalized reaction velocity against $NADP^+$ concentration at the low ionic strength of 0.01 (conductivity 1.12 mmho) and at 34°C.

o - o - o At acidic pH.

* - * - * At alkaline pH.

(a) For G6PD A^+.

(b) For G6PD B^+.

(c) For G6PD A^-.

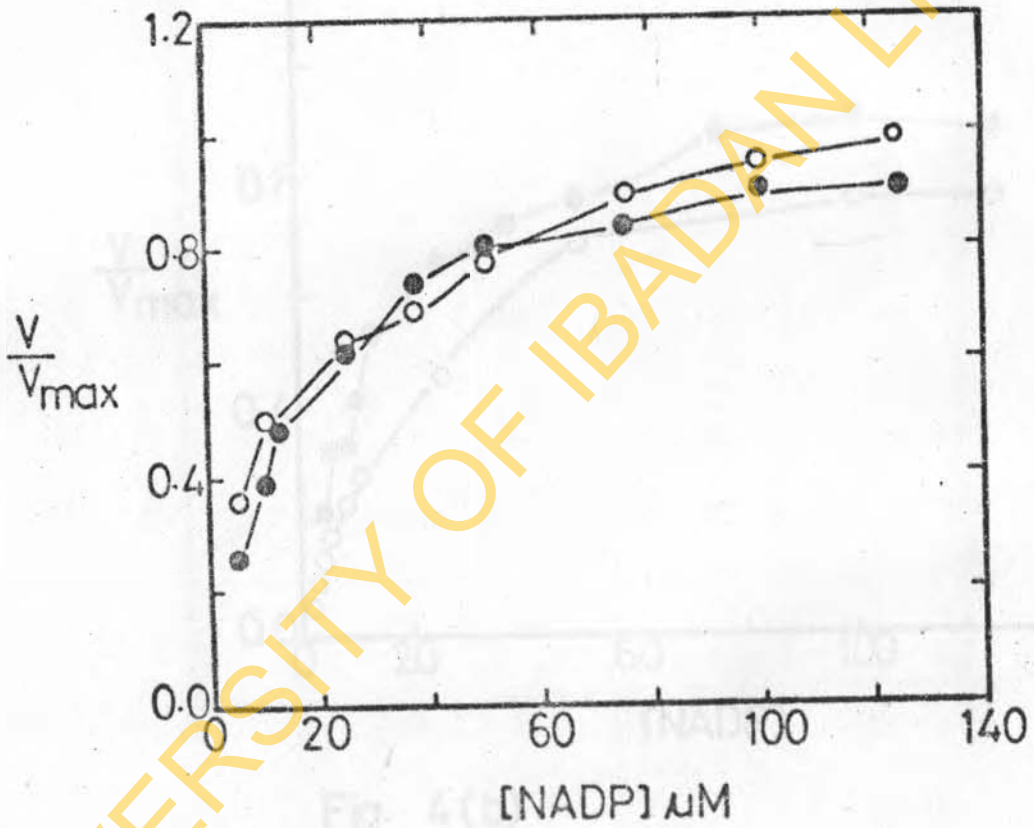


Fig. 4 (a)

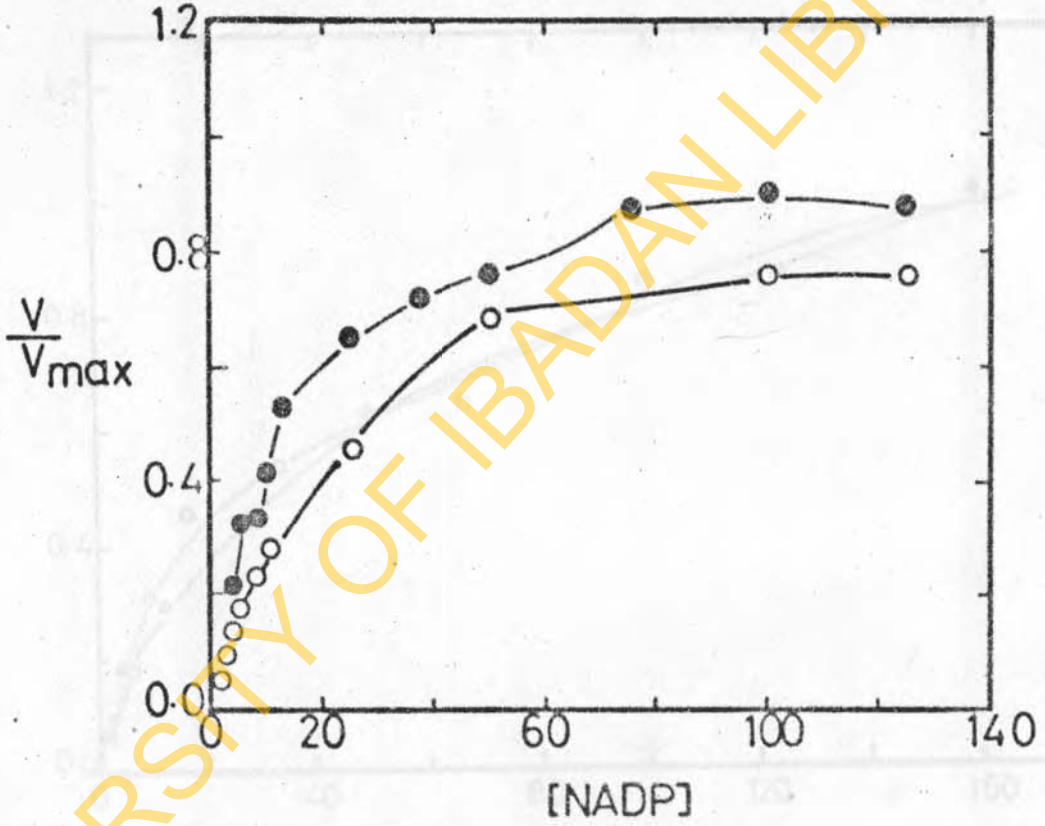


Fig. 4 (b)

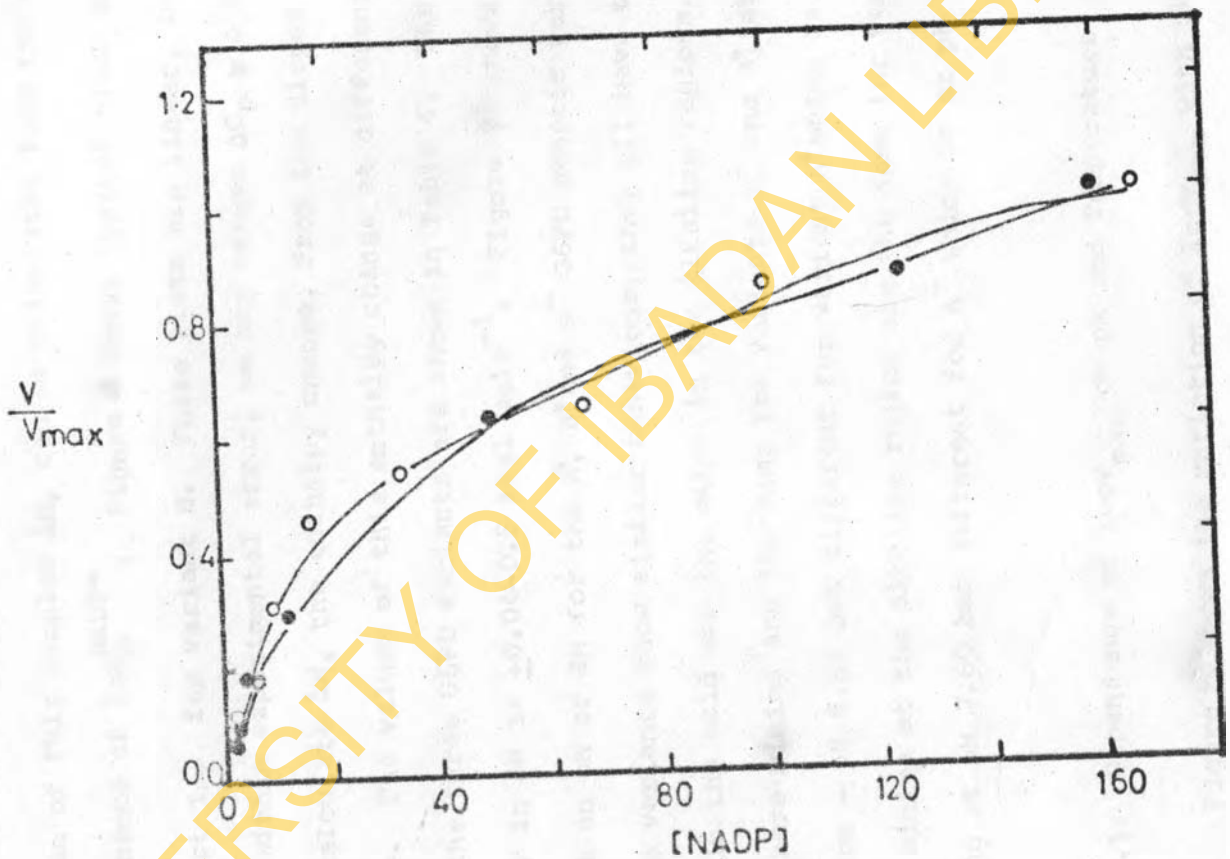


Fig. 4 (c)

the G6PD-$NADP^+$ complex (13, 14), the enthalpy charge for the dissociation of this complex ΔH, can be calculated from the temperature dependence of $\log K_m^{NADP^+}$. Figure 8 shows typical plots of $\log K_m^{NADP^+}$ against $1/T$ for variant B. These plots are linear, indicating that within experimental error, we may assume $\Delta C_p = o$ and hence we can calculate ΔH, the enthalpy change, from the slopes of these plots. The values of this enthalpy change at different pH values for the three G6PD variants are shown in Table 6. The standard error in ΔH is ± 0.04-0.5 kcal $mole^{-1}$. Figure 9 shows the dependence on pH of ΔH for the A^+, B^+ and A^- G6PD genetic variants. The three variants show similar behaviour, they all have two minima, one in the acid and the other in the alkaline regions. The positions of these minima are the same for variants A^- and B^+, at the acidic region - pH 6.60 but different for variant A^+ which is at pH 6.80. The minima at the alkaline region are the same for variants A^+ and B^+ being at pH 7.60 but different for A^- which is at pH 7.40.

3.3.1. Dependence of $\log V_{max}$ on pH and Temperature

Figure 12 shows the variation of $\log V_{max}$ with pH for the three G6PD enzyme variants (A^+, B^+ and A^-) at the different temperatures.

Table 7 shows the values of log V_{max} at different pH values and temperatures for each of the A^+, B^+ and A^- genetic variants of human erythrocyte G6PD.

Between pH 5.80 and 7.00, there is a systematic increase in $\log V_{max}$ for the three enzymes at all temperatures. Above pH 7.0, $\log V_{max}$ increases very gradually to pH 8.0 beyond which an almost plateau is observed.

Activation energies, E_A, were calculated from the slopes of Arrhenius plots of values of $\log V_{max}$ at constant pH against $1/T$. Typical plots are shown in Figure 13 for the B^+ G6PD enzyme. The three enzyme variants show very similar dependence of E_A on pH, there being a gradual fall of the activation energy from about 16 kcals $mole^{-1}$ to about 8 kcal $mole^{-1}$ in the pH region between 6.2 and 9.0 as shown in Figure 14. The values of E_A at different pH values for the three enzyme variants are shown in Table 8. The standard errors in E_A for the three enzyme variants range between $\pm$0.08 and $\pm$0.7 kcal $mole^{-1}$.

3.3.2. Ionization of the Groups influencing the Dependence of V_{max} on pH.

Assuming the existence of two species of the enzyme-substrate complex corresponding to the acid and alkaline forms which are in

equilibrium and represented by the equation.

$$H^+ES \rightleftharpoons ES + H^+$$

where H^+ES corresponds to the acidic form and ES, the enzyme-substrate complex in the basic form, one can write an equilibrium constant expression relating the pH dependence of V_{max} according to the following equation:

$$K_a = \frac{[ES]\,[H^+]}{[H^+ES]}$$

where K_a is the ionization constant of the ionizable group on the enzyme-substrate complex, and $[H^+ES]$ and $[ES]$ are the concentrations of the acid and basic forms of the enzyme-substrate complex.

Rewriting the expression,

$$\frac{K_a}{[H^+]} = \frac{[ES]}{[H^+ES]}$$

$$\text{or } 1 + \frac{[H^+]}{K_a} = \frac{[H^+ES]}{[ES]} + 1 = \frac{[H^+ES] + [ES]}{[ES]}$$

$$= \frac{[E_T]}{[ES]}$$

where $[E_T] = [H^+ES] + [ES]$

$$\therefore \frac{K_a}{K_a + [H^+]} = \frac{[ES]}{[E_T]}$$

$$\therefore \quad [ES] = \frac{K_a \ [E_T]}{K_a + [H^+]}$$

where [ES] is the concentration of the active enzyme-substrate complex.

Multiplying both sides of the equation by the kinetic rate constant k, where $V_{max} = k\,[ES]$ we have

$$V_{max} = \frac{k\ K_a\ [E_T]}{K_a + [H^+]}$$

In an alkaline medium when the ionizable group is in its conjugate base form, the whole enzyme, E_T is in the form of ES complex and the value of V_{max} under this condition is V_{alk},

$$\text{i.e.} \quad V_{alk} = k\,[ES]_{alk} = k\,[E_T]$$

$$\therefore \quad V_{max} = \frac{V_{alk} \cdot K_a}{K_a + [H^+]}$$

$$\frac{V_{alk}}{V_{max}} = 1 + \frac{[H^+]}{K_a}$$

$$\therefore \ 1/V_{max} = 1/V_{alk} + \frac{[H^+]}{K_a \cdot V_{alk}}$$

$\therefore$ A plot of $1/V_{max}$ against $[H^+]$ (figure 6) should be linear with a slope of $1/K_a.V_{alk}$ and an intercept on the $1/V_{max}$ - axis of $1/V_{alk}$. From the ratio of the intercept to the slope we obtained K_a for the three enzymesat four temperatures. pKa values for the three enzyme variants at the four temperatures of investigation are shown in Table 9. From the plot of pKa against $1/T$(Fig. 7) we obtained the enthalpy of ionization of the groups responsible for the increase in enzyme activity as the pH increases. ΔH_a^o for the three G6PD variants are shown in Table 10.

3.4.1 Dependence of $\log K_{s_1}^{NADP^+}$ and $\log K_{s_2}^{NADP^+}$ on pH and Temperature for B^+G6PD Enzyme

The values of $K_{s_1}^{NADP^+}$ and $K_{s_2}^{NADP^+}$, the dissociation constants for the G6PD-$NADP^+$ compex at low and high affinity for $NADP^+$ respectively were determined from the following equation according to Luzzatto (14):

$$\frac{V_{max}}{v} = 1 + \frac{K_{s_2}}{[S]} + \frac{K_{s1}\,K_{s_2}}{[S]^2}$$

The double reciprocal plots of $1/v$ against $1/[NADP^+]$ according

to Lineweaver-Burk method (85) are not linear at this high ionic strength unlike the case with low ionic strength (see figure 3). Figure 15 shows plots of v/V_{max} against $[NADP^+]$ for variants A and B and these are sigmoid shaped showing the presence of cooperativity in the binding of $NADP^+$ to the enzyme variants at this high ionic strength.

Table 11 shows the dependence on pH of log $K_{s_1}^{NADP^+}$ and log $K_{s_2}^{NADP^+}$ for G6PD variant B^+ and at four temperatures. The variations of log $K_{s_1}^{NADP^+}$ and log $K_{s_2}^{NADP^+}$ with pH for the B^+ variant enzyme at the different temperatures are shown in Figure 16. The variation of log $K_{s_2}^{NADP^+}$ with pH is similar to that of log $K_m^{NADP^+}$ but log $K_{s_1}^{NADP^+}$ gave a different pattern. It increases between pH 6.00 and 6.70, then decreases to a minimum at all temperatures at pH 7.00 and a gentle increase between pH 7.00 and pH 7.60

The ΔH for the two complexes between G6PD and $NADP^+$ at low and high affinity for $NADP^+$ respectively were calculated using the same procedure as that used for the calculation in the case of $K_m^{NADP^+}$. Figure 17 shows the dependence on pH of the two ΔH's for G6PD B^+. The ΔH profile from $K_{s_2}^{NADP^+}$ is almost similar to that for $K_m^{NADP^+}$ while that for $K_{s_1}^{NADP^+}$ looks almost like the inverted picture of the latter.

The ΔH profile for $K_{s_2}^{NADP^+}$ shows two minima, one each at neutral and alkaline pH regions -- one at pH 7.0 and the other at around pH 8.40. The ΔH profile for $K_{s_1}^{NADP^+}$ instead, shows two maxima, one each at neutral pH of 7.0 and the other at alkaline pH of 7.89.

Table 12 shows the values of ΔH's (the two enthalpies) at different pH values for variant B*.

The dependence of log V_{max} at this high ionic strength on pH is the same as that at lower ionic strength except that the V_{max} are consistently higher at all pH's and temperatures than for the low ionic strength case. Table 13 shows the dependence of this log V_{max} on pH at the four temperatures. E_A (activation energy) is got from a plot of log V_{max} against 1/T as has been described for the lower ionic strength case. Figure 18 shows the dependence of log V_{max} on pH at the four temperatures between 20 - 40°C. Figure 19 shows the variation of E_A with pH and this is essentially similar to the E_A at the low ionic strength except that the values are less as could be seen in Table 14.

3.4.2. DEPENDENCE OF INTERACTION COEFFICIENT ON BUFFER COMPOSITION, pH AND TEMPERATURE

The values of the interattion coefficient, n, are calculated from the plot of $\log v/V_{max}$ against $\log [NADP^+]$ according to Hill equation (97). In practice only the middle portions of these plots are straight and so the n values were got by calculating the slopes of the middle portions of the plots. Figure 20(a) shows typical plots of $\log v/V_{max}$ against $[NADP^+]$ according to the following equation:

$$\log v/V_{max} = n \log [NADP^+] + \log K.$$

Table 15 shows the dependence of n, the interation coefficient for the $NADP^+$ binding to human erythrocyte G6PD variants A^+ and B^+ on buffer compositions, pH's and at the same temperature (room temperature

Table 16 shows the variation of n with different pH values and at different temperatues for variant B^+. n increases linearly with temperature at all pH values. It could be seen that n also increases systematically from acid pH to alkaline pH. It has the lowest value at the least acid pH investigated and the highest value at the highest alkaline pH considered at a constant temperature.

TABLE 3(a)

PURIFICATION OF HUMAN ERYTHROCYTE G6PD TYPE B^+

CONVENTIONAL METHOD

Purification Step	VOLUME (mls)	G6PD ACTIVITY UNITS/ml	PROTEIN Mg/ml	SPECIFIC ACTIVITY UNITS/mg Protein	PURIFICATION TIMES	TOTAL UNITS	% RECOVERY
HAEMOLYSATE	4250	4.6	80	0.065	1	18,360	100
DEAE CELLULOSE	2600	3.2				7,962	43.4
$(NH_4)_2SO_4$ PRECIPITATION AND RESUSPENSION IN BUFFER	92	64.8				6,060	33.0
SEPHADEX G100 EFFLUENT	85	65.2	2.93	22.3	343	5,542	30.2
DEAE SEPHADEX A50 EFFLUENT	535	9.8	0.22	44.5	684.6	5,243	28.6
DIALYSIS AND CONCENTRATION	200	25.5	0.65	39.0	600.0	5,100	27.8
CM SEPHADEX C50 Cum SEPHADEX G25 EFFLUENT	82	53.0	0.382	138.7	2,134	4,351	23.7
CONCENTRATION AND PASSING THROUGH SEPHADEX COLUMN	63	55.0	0.036	152.8	23,505	3,465	18.9

TABLE 3(b)

PURIFICATION OF HUMAN ERYTHROCYTE G6PD TYPE A^+.

CONVENTIONAL METHOD.

PURIFICATION STEP	VOLUME (mls)	G6PD ACTIVITY (UNITS/ML)	PROTEIN (mg/ml)	SPECIFIC ACTIVITY UNITS/mg PROTEIN	PURIFICATION TIMES	TOTAL UNITS	% RECOVERY
HAEMOLYSATE	4950	4.7	92.4	0.06	1	23,058	100
DEAE CELLULOSE EFFLUENT	2890	4.6				13,338	57.8
$(NH_4)_2SO_4$ PRECIPITATION AND RESUSPENSION IN BUFFER	90	97.2	2.80	34.6	576.7	8750	40%
SEPHADEX G100 EFFLUENT	200	40.6	.505	80.4	1340	8125	35.2
DEA SEPHADEX A50 EFFLUENT	270	18.5	0.15	123.3	2055.6	4995	21.7
DIALYSIS AND CONCENTRATION	100	48.1	0.36	135.4	2256.7	4810	20.9
CM SEPHADEX C50 CUM SEPHADEX G25 EFFLUENT	68	64.8	0.39	166.2	2769.3	4335	18.8
CONCENTRATION AND PASSING THROUGH SEPHADEX COLUMN	55	54.0	0.03	181.5	30,250	3005	15.9

TABLE 4(a)

PURIFICATION OF HUMAN ERYTHROCYTE G6PD TYPE

A^+ AFFINITY CHROMATOGRAPHY METHOD

PURIFICATION STEP	Volume (ml)	Activity Units/mg	Protein mg/ml	Specific Activity Units/mg Protein	Total Units	% Recovery
HEMOLYSATE	450	2.55	28.09	0.091	1147.5	100
2'5' ADP SEPHAROSE ELUENT	97	5.79	0.41	14.12	561.4	48.9
CM-SEPHADEX C50 ELUENT	24	9.39	0.053	177.2	225.4	19.64

TABLE 4(b)

PURIFICATION OF HUMAN ERYTHROCYTE G6PD TYPE B* AFFINITY CHROMATOGRAPHY METHOD

PURIFICATION STEP	Volume (ml)	Activity Units/ml	Protein mg/ml	Specific Activity Units/mg Protein	Total Units	% Recovery
HAEMOLYSATE	400	1.7	6.35	0.27	680	100
2'5' ADP SEPHAROSE ELUENT	75	7.0	0.085	82.4	524	77.1
CM-SEPHADEX C50 ELUENT	19	9.4	0.057	165.2	178.6	26.3

TABLE 4(c)

PURIFICATION OF HUMAN ERYTHROCYTE G6PD TYPE A⁻

AFFINITY CHROMATOGRAPHY METHOD

PURIFICATION STEP	Volume (ml)	Activity Units/ml	Protein mg/ml	Specific Activity Units/mg Protein	Total Units	% Recovery
$(NH_4)_2SO_4$ PRECIPITATION AND RESUSPENSION IN BUFFER	43	2.2	5.45	0.403	94.6	100
2'5' ADP SEPHAROSE EFFLUENT	15.8	1.48	0.155	9.55	23.4	24.8
CM-SEPHADEX C50 EFFLUENT	5.7	1.22	0.073	16.71	7.0	7.4

TABLE 5

DEPENDENCE ON pH OF log $K_m^{NADP^+}$ FOR THE G6PD A^+, B^+ AND A^-.

(a) Temperature : 20°C.

pH	- log $K_m^{NADP^+}$		
	A^+	B^+	A^-
5.95	5.09 ± 0.01	5.27 ± 0.01	5.15 ± 0.01
6.60	4.88 ± 0.01	5.03 ± 0.01	4.96 ± 0.01
6.80	4.91 ± 0.02	5.07 ± 0.01	5.03 ± 0.02
7.05	5.06 ± 0.01	5.23 ± 0.03	4.94 ± 0.02
7.55	5.07 ± 0.01	5.05 ± 0.01	4.74 ± 0.01
8.04	4.99 ± 0.01	5.06 ± 0.02	5.45 ± 0.02
8.40	5.05 ± 0.01	5.08 ± 0.01	5.52 ± 0.01
8.86	4.98 ± 0.02	5.17 ± 0.01	4.88 ± 0.02

TABLE 5

(b) Temperature : 27°C.

pH	$-\log K_m^{NADP^+}$		
	A^+	B^+	A^-
6.00	4.95 ± 0.02	5.07 ± 0.01	5.10 ± 0.03
6.60	4.82 ± 0.01	5.00 ± 0.01	4.89 ± 0.01
6.80	4.85 ± 0.01	5.02 ± 0.01	4.95 ± 0.02
7.00	4.96 ± 0.02	5.11 ± 0.02	4.70 ± 0.02
7.50	4.93 ± 0.03	5.00 ± 0.01	4.48 ± 0.02
8.05	4.85 ± 0.01	4.99 ± 0.01	5.41 ± 0.01
8.50	4.93 ± 0.01	5.01 ± 0.01	5.38 ± 0.02
8.90	4.87 ± 0.01	5.12 ± 0.02	4.81 ± 0.02

TABLE 5

(c) Temperature : 34°C

pH	$-\log K_m^{NADP^+}$		
	A^+	B^+	A^-
6.00	4.80 ± 0.01	5.04 ± 0.01	5.02 ± 0.01
6.60	4.73 ± 0.02	4.93 ± 0.01	4.85 ± 0.01
6.75	4.78 ± 0.01	4.94 ± 0.02	4.88 ± 0.01
6.95	4.92 ± 0.01	5.02 ± 0.02	4.48 ± 0.01
7.45	4.86 ± 0.02	4.96 ± 0.03	4.40 ± 0.02
8.02	4.69 ± 0.03	4.93 ± 0.01	5.22 ± 0.01
8.40	4.75 +0.02	4.94 ± 0.01	5.26 ± 0.02
8.80	4.72 ± 0.01	5.00 ± 0.01	4.65 ± 0.01

TABLE 5

(d) Temperature : 40.0°C

pH	$-\log K_m^{NADP^+}$	
	A^+	B^+
6.05	4.71 ± 0.02	4.97 ± 0.02
6.52	4.67 ± 0.01	4.90 ± 0.02
6.78	4.74 ± 0.01	4.91 ± 0.01
6.95	4.82 ± 0.01	4.92 ± 0.01
7.50	4.74 ± 0.02	4.90 ± 0.01
7.95	4.58 ± 0.02	4.85 ± 0.03
8.35	4.62 ± 0.01	4.88 ± 0.01
8.70	4.55 ± 0.02	4.93 ± 0.01

TABLE 5(e)

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

	A^+	B^+	A^-
K_E	$3.09 \times 10^{-70.25}$	1.07×10^{-71}	$6.42 \times 10^{-30.4}$
K_{ES}	$9.30 \times 10^{-48.62}$	$2.26 \times 10^{-47.69}$	$6.67 \times 10^{-27.88}$
n	12	12	12
x	9.75	10	4.20
y	6.66	6.67	3.84

TABLE 6

DEPENDENCE ON pH OF ΔH FOR THE A^+, B^+ AND A^- GENETIC VARIANTS OF HUMAN ERYTHROCYTE G6PD

pH	ΔH (KCALS MOLE^{-1})		
	A^+	B^+	A^-
6.20	7.32 ± 0.39	5.25 ± 0.25	3.49 ± 0.22
6.60	4.88 ± 0.62	3.43 ± 0.06	2.86 ± 0.08
6.80	3.97 ± 0.19	4.71 ± 0.10	3.49 ± 0.17
7.00	4.58 ± 0.22	7.12 ± 0.11	4.81 ± 0.16
7.40	5.72 ± 0.04	4.94 ± 0.17	3.57 ± 0.06
7.80	6.01 ± 0.06	4.58 ± 0.11	5.67 ± 0.15
8.20	8.15 ± 0.08	5.61 ± 0.17	6.64 ± 0.12
8.60	8.84 ± 0.28	6.61 ± 0.31	8.69 ± 0.24

TABLE 7

DEPENDENCE ON pH OF log V_{max} FOR G6PD A^+ AND B^+.

(a) Temperature : 20.0°C

pH	- log V_{MAX}	
	A^+	B^+
5.95	9.06 ± 0.01	9.18 ± 0.02
6.60	8.72 ± 0.01	8.79 ± 0.03
6.80	8.68 ± 0.02	8.72 ± 0.01
7.05	8.68 ± 0.01	8.63 ± 0.01
7.55	8.51 ± 0.03	8.54 ± 0.01
8.04	8.46 ± 0.01	8.41 ± 0.02
8.30	8.52 ± 0.02	8.37 ± 0.02
8.86	8.59 ± 0.01	8.58 ± 0.01
9.35	8.70 ± 0.01	8.79 ± 0.01

TABLE 7

(b) Temperature : 27°C

	- log V_{max}	
	A^+	B^+
6.00	9.01 ± 0.01	8.84 ± 0.01
6.60	8.65 ± 0.02	8.61 ± 0.01
6.80	8.58 ± 0.01	8.56 ± 0.02
7.00	8.54 ± 0.01	8.50 ± 0.02
7.50	8.44 ± 0.01	8.36 ± 0.01
8.05	8.35 ± 0.02	8.31 ± 0.03
8.50	8.42 ± 0.01	8.25 ± 0.01
8.90	8.47 ± 0.01	8.46 ± 0.01
9.30	8.62 ± 0.01	8.71 ± 0.02

TABLE 7

(c) Temperature: 34°C

pH	- log V_{max}	
	A^+	B^+
6.00	8.74 ± 0.02	8.70 ± 0.02
6.60	8.45 ± 0.03	8.25 ± 0.01
6.75	8.35 ± 0.01	8.20 ± 0.02
6.95	8.34 ± 0.01	8.15 ± 0.02
7.45	8.30 ± 0.01	8.11 ± 0.01
8.02	8.26 ± 0.02	8.14 ± 0.01
8.40	8.28 ± 0.01	8.18 ± 0.01
8.70	8.35 ± 0.01	8.24 ± 0.01
9.20	8.48 ± 0.01	8.50 ± 0.01

TABLE 7

(d) Temperature: 40.0°C.

pH	- log V_{max}	
	A^+	B^+
6.05	8.49 ± 0.02	8.43 ± 0.02
6.52	8.35 ± 0.01	8.18 ± 0.02
6.78	8.29 ± 0.02	8.12 ± 0.03
6.95	8.26 ± 0.02	8.03 ± 0.01
7.50	8.24 ± 0.01	8.04 ± 0.01
7.95	8.21 ± 0.01	8.05 ± 0.02
8.35	8.19 ± 0.01	8.07 ± 0.01
8.70	8.23 ± 0.01	8.13 ± 0.02
9.20	8.36 ± 0.02	-

TABLE 7

(e) DEPENDENCE ON pH OF log V_{MAX} FOR G6PD A^-

pH	log V_{MAX}		
	20°C	27°C	34°C
5.80	-0.160 ± 0.02	-0.041 ± 0.02	0.090 ± 0.01
6.53	0.46 ± 0.01	0.60 ± 0.02	0.75 ± 0.01
7.00	0.55 ± 0.02	0.70 ± 0.01	0.90 ± 0.02
7.45	0.60 ± 0.03	0.76 ± 0.02	0.95 ± 0.01
8.15	0.65 ± 0.02	0.80 ± 0.01	1.01 ± 0.02
8.50	0.87 ± 0.01	0.87 ± 0.02	1.05 ± 0.01
8.80	0.76 ± 0.01	0.89 ± 0.01	1.04 ± 0.02

TABLE 8

DEPENDENCE ON pH OF E_A FOR THE A^+, B^+ AND A^- GENETIC VARIANTS OF HUMAN ERYTHROCYTE G6PD.

pH	E_A (KCALS MOLE^{-1})		
	A^+	B^+	A^-
6.20	14.19 ± 0.22	16.02 ± 0.22	12.54 ± 0.13
6.60	11.90 ± 0.39	16.34 ± 0.28	11.58 ± 0.36
7.00	12.08 ± 0.50	16.24 ± 0.50	10.87 ± 0.36
7.40	9.41 ± 0.39	14.74 ± 0.72	10.30 ± 0.18
7.80	8.31 ± 0.22	12.13 ± 0.56	9.98 ± 0.22
8.20	7.32 ± 0.08	9.31 ± 0.06	10.30 ± 0.13
8.60	7.71 ± 0.56	10.20 ± 0.72	10.07 ± 0.18
9.00	7.69 ± 0.17	9.81 ± 0.39	9.46 ± 0.27
9.20	7.82 ± 0.11	8.89 ± 0.56	-

TABLE 9

pKa OF THE IONIZABLE GROUP ON G6PD VARIANTS A^+, B^+ AND A^- ON BINDING $NADP^+$.

	pKa		
T^oK	B^+	A^+	A^-
293	6.82 ± 0.03	6.69 ± 0.03	6.90 ± 0.02
300	6.59 ± 0.03	6.44 ± 0.03	6.68 ± 0.03
307	6.38 ± 0.02	6.29 ± 0.03	6.45 ± 0.03
313	6.15 ± 0.03	6.10 ± 0.02	-

TABLE 10

THE ENTHALPIES OF IONIZATION, ΔH_a^o OF THE IONIZABLE GROUP FOR THE THREE GENETIC VARIANTS OF G6PD.

G6PD VARIANT	$-\Delta H_a^o$ (kcal $mole^{-1}$)
B^+	14.19 ± 0.29
A^+	13.27 ± 0.22
A^-	13.73 ± 0.27

TABLE 11

DEPENDENCE ON pH OF $\log K_{s_1}^{NADP^+}$ AND $\log K_{s_2}^{NADP^+}$ FOR B^+ GENETIC VARIANT OF HUMAN ERYTHROCYTE G6PD.

(a) Temperature: $20^{o}C$

pH	$-\log K_{s_1}^{NADP^+}$	$-\log K_{s_2}^{NADP^+}$
6.10	4.85 ± 0.03	5.90 ± 0.02
6.70	4.80 ± 0.04	5.58 ± 0.02
7.00	4.94 ± 0.02	5.62 ± 0.01
7.60	4.86 ± 0.04	5.60 ± 0.01
7.90	5.00 ± 0.01	5.51 ± 0.02
8.40	4.95 ± 0.02	5.41 ± 0.04
8.90	4.79 ± 0.02	5.46 ± 0.04

TABLE 11

(b) Temperature: 27.0°C

pH	$-\log K_{s_1}^{NADP^+}$	$-\log K_{s_2}^{NADP^+}$
6.10	4.74 ± 0.02	5.65 ± 0.03
6.70	4.69 ± 0.01	5.52 ± 0.01
7.00	4.77 ± 0.02	5.55 ± 0.02
7.60	4.79 ± 0.02	5.43 ± 0.06
7.90	4.82 ± 0.02	5.38 ± 0.01
8.40	4.80 ± 0.02	5.30 ± 0.02
8.90	4.71 ± 0.04	5.35 ± 0.02

TABLE 11

(c) Temperature: 34°C

pH	$-\log K_{s_1}^{NADP^+}$	$-\log K_{s_2}^{NADP^+}$
6.10	4.67 ± 0.04	5.52 ± 0.02
6.70	4.61 ± 0.01	5.46 ± 0.02
7.00	4.74 ± 0.02	5.49 ± 0.04
7.60	4.65 ± 0.02	5.24 ± 0.02
7.90	4.69 ± 0.02	5.22 ± 0.02
8.40	4.71 ± 0.06	5.20 ± 0.02
8.90	4.58 ± 0.01	5.27 ± 0.04

TABLE 11

(d) Temperature: 40.0°C

pH	$-\log K_{s_1}^{NADP^+}$	$-\log K_{s_2}^{NADP^+}$
6.10	4.63 ± 0.02	5.45 ± 0.02
6.70	4.53 ± 0.02	5.38 ± 0.01
7.00	4.64 ± 0.02	5.45 ± 0.04
7.60	4.60 ± 0.03	5.06 ± 0.02
7.90	4.63 ± 0.04	5.05 ± 0.02
8.40	4.60 ± 0.02	5.07 ± 0.02
8.90	4.51 ± 0.01	5.17 ± 0.02

TABLE 12

DEPENDENCE ON pH OF ΔH FROM $\log K_{s_1}^{NADP^+}$ AND $\log K_{s_2}^{NADP^+}$ DATA FOR GENETIC VARIANT B^+ OF HUMAN ERYTHROCYTE G6PD

pH	ΔH FOR $\log K_{s_1}^{NADP^+}$	ΔH FOR $\log K_{s_2}^{NADP^+}$
6.2	4.58 ± 0.15	9.38 ± 0.13
6.6	5.49 ± 0.08	4.81 ± 0.10
6.8	6.41 ± 0.05	4.35 ± 0.04
7.0	6.86 ± 0.19	3.70 ± 0.11
7.6	6.10 ± 0.11	11.23 ± 0.15
7.8	8.50 ± 0.10	10.53 ± 0.09
8.2	7.85 ± 0.04	8.69 ± 0.06
8.4	7.63 ± 0.08	6.86 ± 0.07
8.8	7.19 ± 0.12	6.66 ± 0.14

TABLE 13

DEPENDENCE ON pH OF log V_{max} AT HIGHER IONIC STRENGTH FOR THE HUMAN ERYTHROCYTE G6PD B^+.

pH	log V_{max}			
	20°C	27°C	34°C	40°C
6.10	- 0.20 ± 0.01	- 0.013 ± 0.01	0.17 ± 0.02	0.24 ± 0.01
6.70	0.46 ± 0.02	0.63 ± 0.01	0.79 ± 0.01	0.88 ± 0.01
7.00	0.72 ± 0.02	0.85 ± 0.02	0.92 ± 0.02	1.02 ± 0.01
7.60	0.92 ± 0.01	1.01 ± 0.02	1.13 ± 0.01	1.19 ± 0.02
7.90	0.93 ± 0.01	1.05 ± 0.01	1.14 ± 0.01	1.18 ± 0.01
8.40	0.94 ± 0.01	1.04 ± 0.02	1.15 ± 0.01	1.24 ± 0.01
8.90	1.07 ± 0.02	1.17 ± 0.01	1.23 ± 0.01	1.28 ± 0.01

TABLE 14

DEPENDENCE OF E_A ON pH AT HIGHER IONIC STRENGTH FOR HUMAN ERYTHROCYTE G6PD B$^+$

pH	E_A (KCALS MOLE^{-1})
6.10	10.90 ± 0.38
6.70	8.93 ± 0.10
7.0	6.97 ± 0.11
7.5	6.54 ± 0.14
7.9	7.02 ± 0.14
8.4	6.00 ± 0.14
8.9	6.00 ± 0.13

TABLE 15

DEPENDENCE OF n, THE INTERACTION COEFFICIENT ON BUFFER COMPOSITIONS AND pH FOR G6PD VARIANTS A^+ AND B^+

BUFFER COMPOSITION	CONDUCTIVITY (mmho)	n, INTERACTION COEFFICIENT	
		A^+	B^+
TRIS - BO_3 I = 0.01 pH 8.0	1.12	1.21 ± 0.12	1.18 ± 0.08
TRIS - BO_3 0.5M pH 8.0	2.35	1.66 ± 0.09	1.91 ± 0.13
TRIS - HCl 0.05M + 0.10M KCl pH 7.3	20.0	1.23 ± 0.04	1.27 ± 0.11
TRIS - HCl 0.05M + 0.10M KCl + 4mM $MgCl_2$ pH 7.3	20.7	1.51 ± 0.11	1.30 ± 0.06

TABLE 15 (contd.)

BUFFER COMPOSITION	CONDUCTIVITY (mmho)	n, INTERACTION COEFFICIENT	
		A^+	B^+
TRIS - BO_3 I = 0.01 + 4mM K_2SO_4 pH 7.95	2.40	1.53 ± 0.12	1.37 ± 0.11
TRIS - BO_3 I = 0.01 + 4mM $MgCl_2$ pH 7.97	2.38	1.32 ± 0.06	1.34 ± 0.09
TRIS - BO_3 I = 0.01 + 6mM $MgSO_4$ pH 7.97	2.36	1.39 ± 0.14	1.41 ± 0.15
TRIS - BO_3 I = 0.01 + 37mM $MgSO_4$ pH 7.97	6.22	1.44 ± 0.11	1.41 ± 0.07
TRIS - BO_3 I = 0.01M + 3mM $MgSO_4$ pH 5.92	2.55	1.04 ± 0.09	1.07 ± 0.10

TABLE 16

DEPENDENCE OF n, THE INTERACTION COEFFICIENT ON pH AND TEMPERATURE FOR G6PD VARIANT B*

pH	n, INTERACTION COEFFICIENT			
	20°C	27°C	34°C	40°C
6.10	1.02 ± 0.12	1.18 ± 0.11	1.20 ± 0.13	1.32 ± 0.16
6.70	1.06 ± 0.13	1.11 ± 0.08	1.17 ± 0.07	1.30 ± 0.07
7.00	0.98 ± 0.09	1.12 ± 0.06	1.23 ± 0.04	1.33 ± 0.09
7.60	1.03 ± 0.08	1.38 ± 0.10	1.53 ± 0.14	1.67 ± 0.20
7.90	1.19 ± 0.07	1.25 ± 0.09	1.47 ± 0.19	1.53 ± 0.14
8.40	1.16 ± 0.11	1.27 ± 0.08	1.33 ± 0.07	1.48 ± 0.08
8.90	1.43 ± 0.16	1.57 ± 0.11	1.79 ± 0.18	1.87 ± 0.08

TABLE 17

THERMODYNAMIC PARAMETERS FOR $NADP^+$ BINDING TO THE G6PD A^+, B^+ AND A^- GENETIC VARIANTS AT DIFFERENT pH VALUES.

G6PD A^+

pH	ΔG (Kcal $mole^{-1}$)	ΔH (Kcal $mole^{-1}$)	$-\Delta S$ (cal $mole^{-1}$)
5.95	6.83	7.32	- 1.79
6.60	6.54	4.88	5.67
7.05	6.60	4.58	3.48
7.55	6.62	5.72	3.07
8.04	6.69	6.01	2.32
8.40	6.77	8.15	- 4.71
8.86	6.68	8.84	- 7.37

TABLE 17 (contd.)

G6PD B^{+}

pH	ΔG (Kcal $mole^{-1}$)	ΔH (Kcal $mole^{-1}$)	-ΔS (cal $mole^{-1}$)
5.95	7.07	5.25	6.21
6.60	6.74	3.43	11.30
7.05	7.01	7.12	- 0.38
7.55	6.77	4.94	6.25
8.04	6.78	4.58	7.51
8.40	6.81	5.61	4.10
8.86	6.93	6.61	1.09

TABLE 17 (Contd.)

G6PD A$^-$

pH	ΔG (Kcal mole^{-1})	ΔH (Kcal mole^{-1})	-ΔS (cal mole^{-1})
5.95	6.91	3.49	11.67
6.60	6.65	2.86	12.94
7.05	6.62	4.81	6.18
7.55	6.36	3.57	9.52
8.04	7.31	5.67	5.60
8.40	7.40	6.64	2.59
8.86	6.54	8.69	- 7.34

TABLE 18

COMPENSATION TEMPERATURE, T_c FROM THE THERMODYNAMIC PARAMETERS OF $NADP^+$ AND G6P BINDING FOR G6PD A^+, B^+ AND A^- VARIANTS.

$NADP^+$ BINDING

A^+	:	$300.0 \pm 4.4^{\circ}K$
B^+	:	$307.7 \pm 6.5^{\circ}K$
A^-	:	$314.3 \pm 9.2^{\circ}K$

G6P BINDING

A^+	:	$300.3 \pm 5.6^{\circ}K$
B^+	:	$298.2 \pm 9.8^{\circ}K$
A^-	:	$312.5 \pm 7.4^{\circ}K$

CHAPTER FOUR

DISCUSSION

4.1.1. Purification of G6PD Variants.

The specific use of the biological functional property of the enzyme in the affinity chromatography method as compared to gross physical and chemical differences between the proteins to be isolated in conventional chromatographic techniques makes it possible to apply the hemolysate directly on to the affinity column. In the conventional techniques, other proteins are eliminated by the techniques of ammonium sulphate precipitation and gel filtration on Sephadex G100 column. This affinity purification approach has two advantages: (a) it shortens the period of the whole purification, (b) it prevents the loss of substantial amount of the enzyme during the additional precipitation and gel filtration steps. Thus the former method is shorter and is better applied to the deficient enzyme variant (G6PD A^-) which is highly unstable. The quantitative yields are higher for the affinity chromatography method than for the other method as could be seen in Tables 1 and 2 for variants A^+ and B^+.

Our unsuccessful attempt to purify G6PD A^- in as high a yield as we have for the other two variants may be due to the fact that we applied a free enzyme on to the column. Yoshida (73) reported the same result when he applied free G6PD A^- (free of either the reduced or oxidised dinucleotide) on to $NADP^+$-agarose column, but a high yield when he converted the enzyme to NADPH-bound G6PD. He rationalized his former results to rapid inactivation of the enzyme when it does not have a dinucleotide bound to it to stabilize it. Difficulty in purifying G6PD A^- by the conventional method had been reported by Babalola et al (24) in which there was molecular interconversion by disulfide bridge formation. This behaviour could explain the decreased stability during purification on affinity chromatography column. The stability effected by the reduced dinucleotide (73) could be due to the binding of NADPH to the sulfhydryl groups hence preventing disulfide bridge formation, a process that inactivates the enzyme. NADPH is a competitive inhibitor of $NADP^+$ as regards binding on G6PD (17, 86). Since a competitive inhibitor binds to the same binding site as the corresponding substrate, it means the sulfhydryl groups may be involved in the substrate binding process in G6PD.

The separation of another protein (FX) which binds both $NADP^+$ and NADPH nonenzymatically as similarly observed by Morelli et al (72) after the affinity chromatography step which had never been possible to identify and separate by the conventional method shows that the affinity chromatography method gives a much purer and more homogeneous enzyme. Thus the affinity chromatography technique has been used by us as a successful technique for the purification of G6PD variants because of its simplicity, shorter period of purification, higher yield and purity of the enzyme. Its added advantage also lies in the fact that it made possible the purification of enzyme variants from a single donor as compared to batch purification in conventional method from pooled blood samples from different subjects. Hence it is now possible to compare the physicochemical properties of pure G6PD enzymes from different subjects. Our observation is that the interspecies variation among the different subjects having the same G6PD type is more a reflection of the quantitative enzyme level than an interspecies structural polymorphism.

4.2.1. Effect of pH on Kinetic parameters.

We have interpreted the variation of kinetic parameters with pH in accordance with Dixon's rules (103). The variation of V_{max}

with pH shows the effect of ionization of groups on those enzyme forms present at infinite substrates' concentrations while the variation of Km with pH may be attributed to the effects of ionizations in the free substrates, the free enzyme or the enzyme-substrate complex. The slope in any region of log Km against pH according to Dixon's rule may be approximated to the change of charge occurring when the enzyme-substrates' complex break down to the enzyme and the substrate. Every change in direction of the graph corresponds to the ionization of a group in one of the reaction components; when the upper side of the bend is the concave side, it indicates a pK of the enzyme-substrate complex, while when the concave side faces downwards, it indicates a pK of either the free substrates or the free enzyme.

Inspection of the graphs of log Km against pH for the three genetic G6PD variants at all temperatures (figure 5) shows that they show similar profiles. Log Km increases to a maximum at pH values around 6.60 for variants A^+ and B^+ and 6.50 for variant A^-; decreases to a minimum around neutral pH and another minimum at around pH 8.90 for variant B^+ and 8.50 for variants A^+ and A^-. According to Dixon's rules, the bend at around pH 6.60 with the concave side facing downwards represents the pK of one of the

Figure 5: Variation of log Km^{NADP^+} with pH

▲ - ▲ - ▲ 20°C Δ - Δ - Δ 27°C

● - ● - ● 34°C o - o - o 40°C

(a) For G6PD A^+.

(b) For G6PD B^+.

(c) For G6PD A^-.

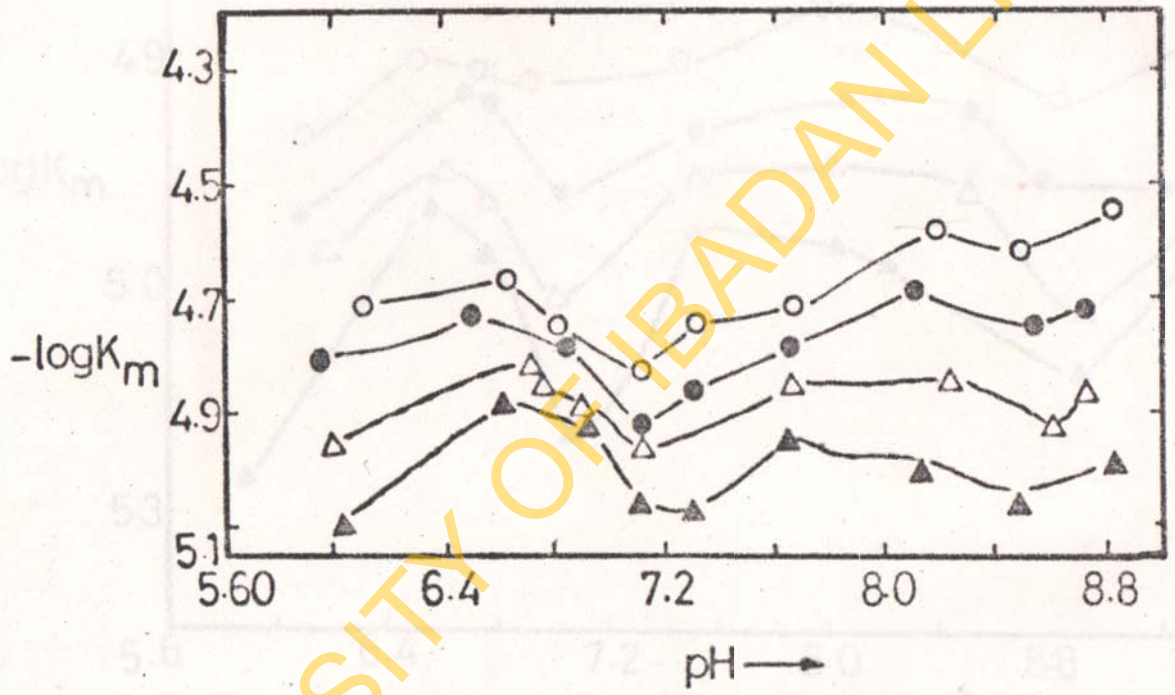


Fig. 5 (a)

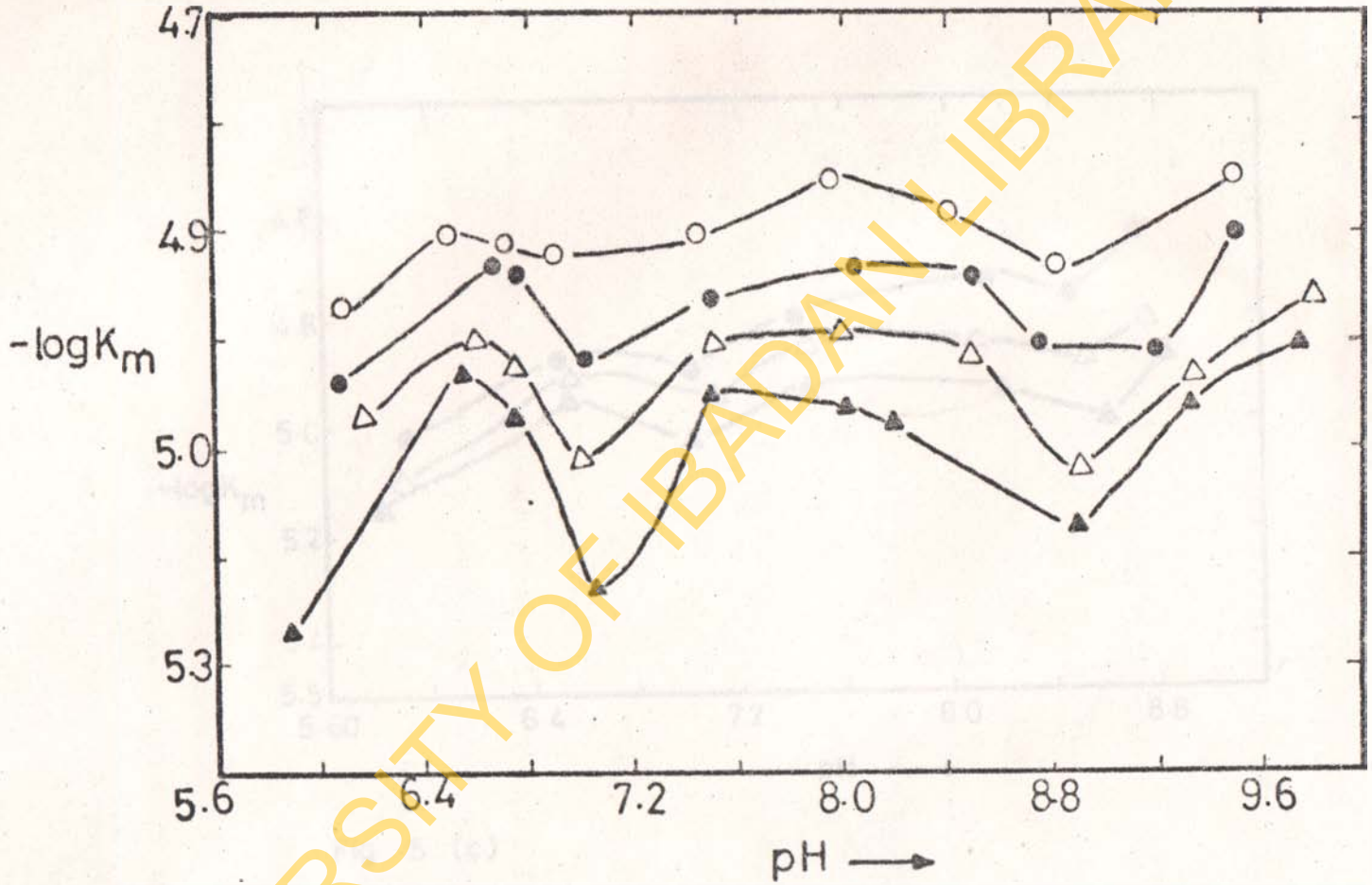


Fig. 5(b)

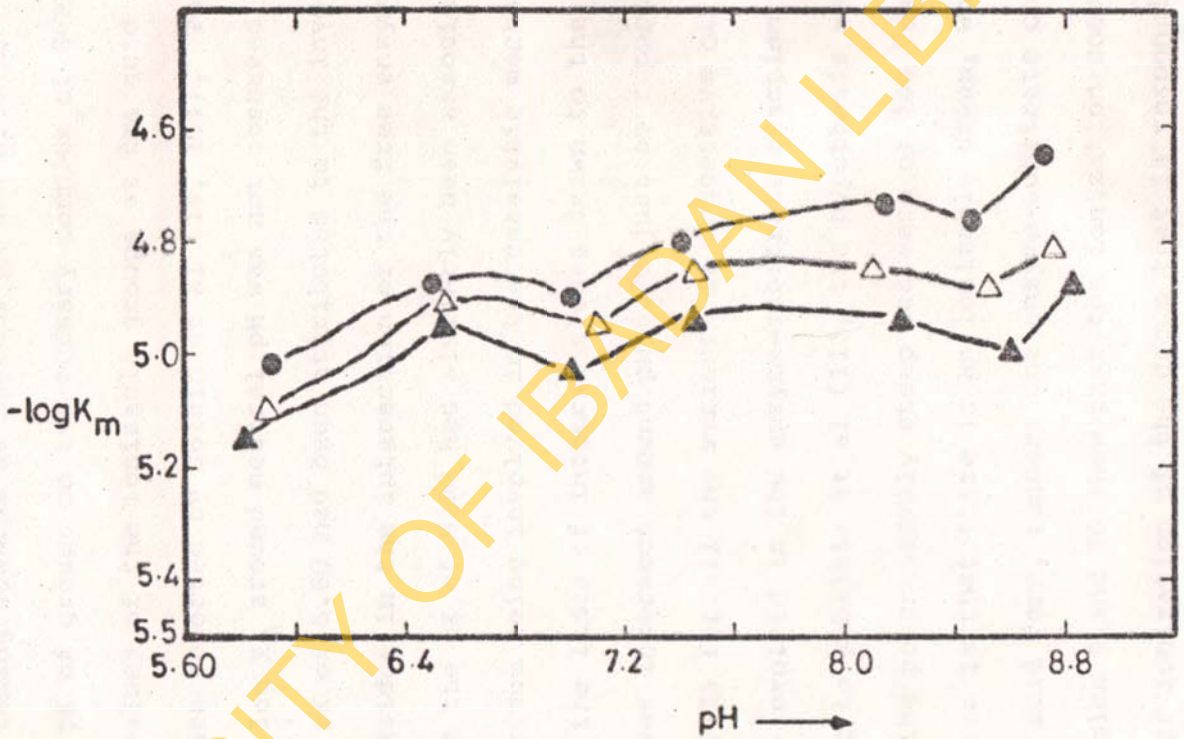


Fig. 5 (c)

substrates or of a group on the enzyme while the bends with the concave sides facing upwards at neutral pH and 8.50 to 8.90 represent the pK of groups on the ternary complex of G6PD-$NADP^+$-G6P. The pK values of the ionizable groups at the acid and alkaline regions had been reported by Soldin et al (17, 104). The abrupt change in the log K_m around neutral pH was not observed by these workers. The pK of 6.60 had been attributed to the involvement of a histidine residue in the interaction of the free enzyme with its substrates and the pK of 8.90 had similarly been associated with a cysteine residue being involved in the catalytic mechanism. The fact that the lines of integral slopes drawn to the log V_{max} versus pH curves intersect around pH 6.70 just as reported by Soldin et al (17) for all the variants is suggestive of a histidine residue being involved in the enzyme-substrate reaction mechanism.

According to Babalola et al (11), 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. Tables 9 and 10 show that the ionization constant and the enthalpy of ionization of the group that is responsible for the decrease in enzyme activity as the pH decreases from neutral

Figure 5(d): Data fit of log $K_m^{NADP^+}$ variation with pH.

●—●—● G6PD A^+

O - O - O G6PD A^-

▲—▲—▲ G6PD B.

The experimental points are shown with their standard errors while the curves are the data fits.

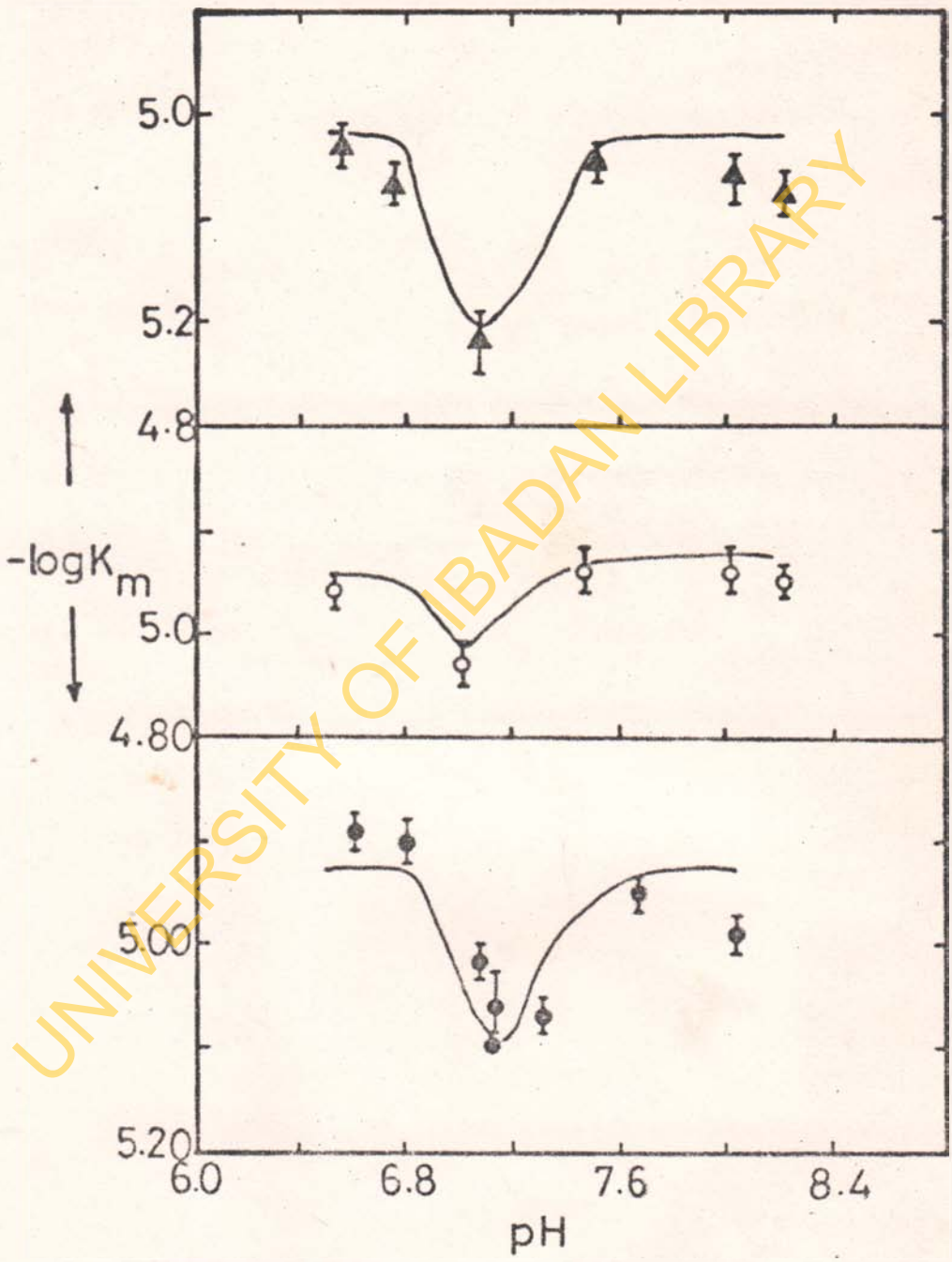


Fig. 5(d)

Figure 6: Typical plots of $1/V_{max}$ against $[H^+]$ at 27°C for G6PD Variants A^+, B^+ and A^-.

● - o - o G6PD A^+

● - ● - ● G6PD B^+

▲ - ▲ - ▲ G6PD A^-

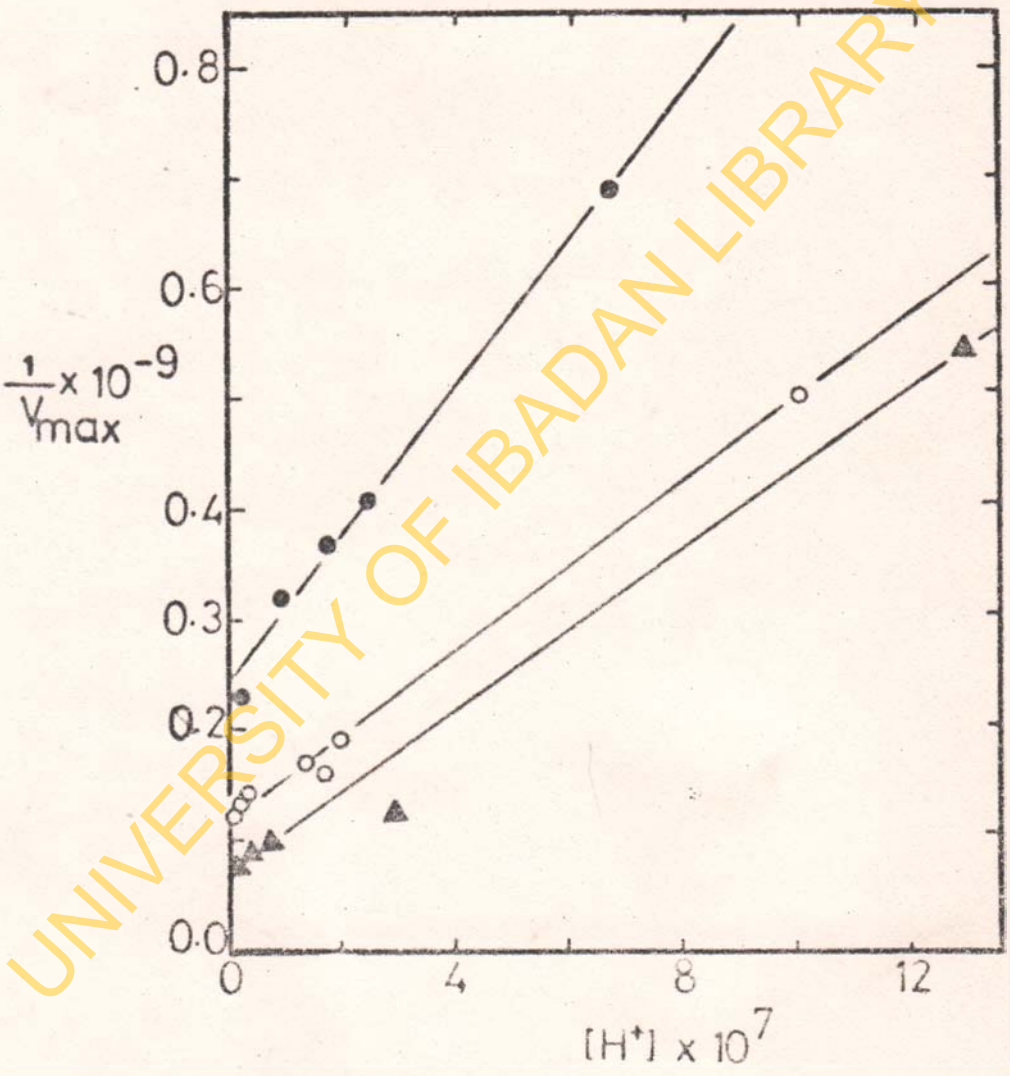


Fig. 6

Figure 17. Plots of pKa against $1/T$ for G6PD A^+, B^+ and A^-.

o - o - o G6PD A^+

Δ - Δ - Δ G6PD B^+

• — • — • G6PD A^-.

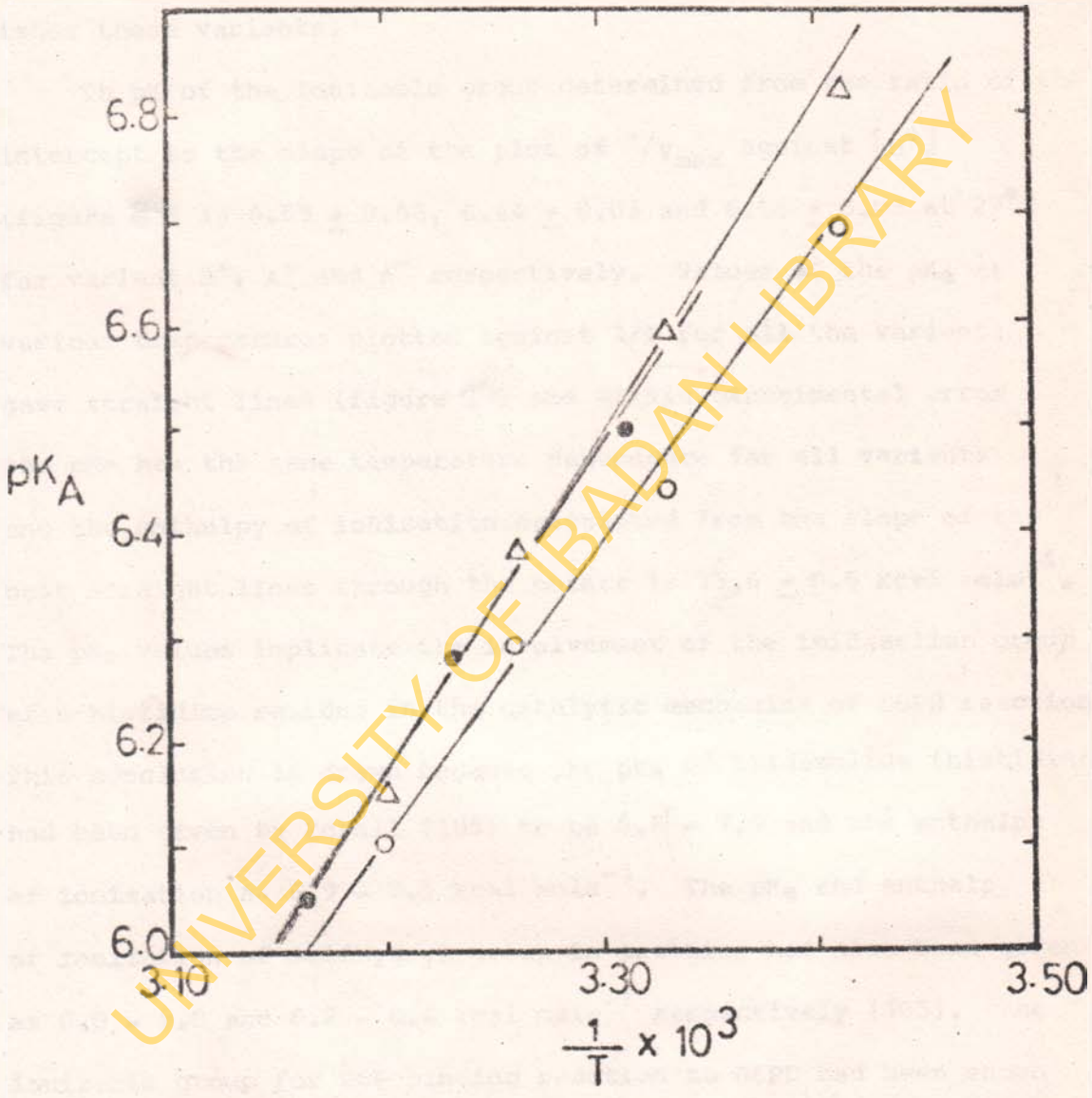


Fig. 7

pH are unaffected by the amino acid substitution which distinguishes these variants.

Th pK of the ionizable group determined from the ratio of the intercept to the slope of the plot of $1/V_{max}$ against $[H^+]$ (figure 6) is 6.59 ± 0.03, 6.44 ± 0.03 and 6.68 ± 0.03 at 27°C for variant B^+, A^+ and A^- respectively. Values of the pK_a at various temperatures plotted against 1/T for all the variants gave straight lines (figure 7) and within experimental error the pKa has the same temperature dependence for all variants and the enthalpy of ionization calculated from the slope of the best straight lines through the points is 13.5 ± 0.6 Kcal $mole^{-1}$. The pKa values implicate the involvement of the imidazolium group of a histidine residue in the catalytic mechanism of G6PD reaction. This conclusion is drawn because the pK_a of imidazolium (histidine) had been given by Edsall (105) to be 5.6 - 7.0 and the enthalpy of ionization as 6.9 - 7.5 kcal $mole^{-1}$. The pK_a and enthalpy of ionization of sulfhydryl group in proteins had also been given as 8.0 - 9.0 and 6.2 - 8.4 kcal $mole^{-1}$ respectively (105). The ionizable group for G6P binding reaction to G6PD had been shown to have a pKa of 6.65 at 34°C and enthalpy of ionization of 7.0 kcal $mole^{-1}$ for all the three G6PD variants (11).

These conclusions show that the same group is involved in the binding of G6P and $NADP^+$ but the enthalpy of ionization of the group for $NADP^+$ binding is about twice that for C6P binding. This result shows either that many more groups are involved in $NADP^+$ binding than in G6P binding or that the groups are situated in an atypical environment in the G6PD molecule that $NADP^+$, because of its larger size is able to interact more with them. $NADP^+$ had been reported to play a critical role in determining the conformations of G6PD molecule and thus the binding of $NADP^+$ is extremely sensitive to structural changes in G6PD (16). The large size of $NADP^+$ had also been shown to involve a much wider field of interaction with the polypeptide chains of G6PD (16). Thus there could be cooperative ionization of the ionizable group with groups in its micro-environment (106). Thus the binding of $NADP^+$ triggers off the ionization of many groups which are linked to the binding site and this linked ionization affects the pK_a and enthalpy of ionization of the ionizable group. In this case, the effect is to make the enthalpy of ionization twice as exothermic as an independent ionization of the group.

Evidence for the presence of sulfhydryl group and imidazolium group of histidine residue had previously been advanced by

Soldin et al (17, 104) and independently by Babalola et al (11) for the binding of both substrates to G6PD variants. Additional evidence for the presence of a sulfhydryl group near the active site come from the inactivation of the enzyme by PCMB and heavy metal ions such as mercuric ion as reported by Anstall et al (74) and Balinsky et al (107). We found no evidence to implicate the ionizations of the two substrates in the reaction mechanism. Our observation of the change from negative to positive slope at the pH value of around 8.90, may be attributed to the participation of an unionized sulfhydryl group in the binding of the substrates at this pH region. The observation by Babalola et al (24) that a second fraction of G6PD A^- as obtained on CM-Sephadex chromatography has a low affinity for $NADP^+$ in the absence of sulfhydryl producing agent such as dithioglycol is an indication that a sulfhydryl group which is already oxidized in this fraction of variant A^- is not available for $NADP^+$ binding. This observation has been explained in terms of an imposition of a novel constraint by the formed disulfide bridge on the possible conformations of the enzyme molecule for $NADP^+$ binding, thus the molecule is locked in a state of low affinity for $NADP^+$.

We can account for the pH profile of log K_m of the $NADP^+$ binding if there are ionizable groups on the protein which are linked to substrate binding in such a way that the pK of the groups would be different on the enzyme and enzyme-substrate complex. As a consequence of the changes in pK the dissociation of the enzyme - $NADP^+$ complex will result in either an uptake or release of hydrogen ions. Then we can write

$$ES + \varphi H^+ \rightleftarrows H_\varphi E + S.$$

where φ is the number of hydrogen ions released or taken up on substrate binding depending on whether φ is negative or positive respectively. According to Wyman linkage equation we would then have

$$\frac{d \log K_m^o}{dpH} = -\varphi$$

Assuming as intrinsic in the Wyman's assumption for the derivation of the linkage equation that the ratio of the activity coefficients of E and ES is independent of pH, in which case,

$$\frac{d \log K_m}{dpH} = -\varphi \qquad \text{----------} \qquad (1)$$

The abrupt variation of log K_m must therefore be accompanied by abrupt changes in φ over a narrow range of pH. Between the pH

of 7.0 and 7.2 there are large changes in the slope of log K_m versus pH for $NADP^+$ for G6PD variants A^+, B^+ and A^- giving rise to significant changes in φ of the order of 0.83, 0.52 and 0.15 respectively. The minimum in the log K_m versus pH implies that φ is zero at this pH. The position of minimum log K_m has a pH value of 7.13 ± 0.02, 7.06 ± 0.02 and 7.03 ± 0.02 in variants A^+, B^+ and A^- respectively. The rise of log K_m with pH between pH 7.2 and 7.5 implies that φ is negative in this pH range. The magnitude of the change in log K_m between either side of the pH of minimum log K_m decreases for the variant enzyme in the order G6PD $B^+ > A^+ > A^-$.

If the uptake of protons upon dissociation of the enzyme-substrate (ES) complex arises from the associated change of the pK of a single ionized group, we can then write an expression for φ in terms of the dissociation constants K_{E_1} and K_{ES_1} of the group on the enzyme and enzyme - substrate complex respectively,

$$\varphi = \frac{[H^+]}{[H^+] + K_{E_1}} - \frac{[H^+]}{[H^+] + [K_{ES_1}]}$$

For log K_m to show a minimum with pH, the pK of at least two ionizable groups must change. In this case the expression for

φ would be

$$\varphi = \left[\frac{[H^+]}{[H^+] + K_{E_1}} + \frac{[H^+]}{[H^+] + K_{E_2}} \right] - \left[\frac{[H^+]}{[H^+] + K_{ES_1}} + \frac{[H^+]}{[H^+] + K_{ES_2}} \right] \quad \text{--- (2)}$$

where K_{E_2} and K_{ES_2} are the ionization constants of the other second group on the enzyme and enzyme-substrate complex respectively.

The most significant feature of the pH dependence of log K_m of $NADP^+$ binding to G6PD is the position of the minimum in the value of log K_m observed for each variant of G6PD in the pH range 7.0 - 7.5. Similar abrupt variation of log K_m with pH has been observed for G6P binding to the same enzyme variants in the same interval of pH (11).

Substituting equation (2) into equation (1) we can obtain on integrating the resultant expression the equation describing the variation of log K_m with pH as follows

$$\log K_m = \log K_m^1 + \log \frac{([H^+] + K_{E_1})([H^+] + K_{E_2})}{([H^+] + K_{ES_1})([H^+] + K_{ES_2})}$$

where $\log K_m^1$ is the value of log K_m at the extremes of pH when $[H^+] \gg K_{E_1}$, K_{E_2}, K_{ES_1}, and K_{ES_2}. At these extremes of pH on either side of pH of minimum log K_m, φ will be zero as log K_m is invariant with pH in these regions and the difference between the values of log K_m at the high and low pH would be equal to

$pK_{ES_1} - pK_{E_1}$. Also $pK_{ES_1} - pK_{E_1}$ would be equal to $pK_{ES_2} - pK_{E_2}$ The fact that K_{E_1}, K_{ES_1} and also K_{E_2} and K_{ES_2} can vary significantly over more than two pH units would show that the minimum shown in the variation of log K_m with pH would be very broad and thus we cannot account for the sharp minimum in log K_m with pH by postulating just two independent ionizable groups thermodynamically linked to the binding of the substrate. However the pH range of the minimum value of log K_m would become sharper if we postulate the existence of groups which would ionize cooperatively. The sharpness of the variation of log K_m with pH will depend on the number of the groups and the degree of cooperativity between them.

The model with which to describe the cooperative ionization need not be as complex as either the MWC (80) two state model or the Koshland sequential model (88). We can simply assume successive linked ionization constants defined as follows for the enzyme substrate complex

$$H_nES \rightleftarrows H_{n-1}ES + H^+, \quad K_{ES_1} = \frac{[H_{n-1}ES][H^+]}{[H_nES]}$$

$$H_{n-1}ES \rightleftarrows H_{n-2}ES + H^+, \quad K_{ES_2} = \frac{[H_{n-2}ES][H^+]}{[H_{n-1}ES]}$$

$$H_{n-i+1}ES \rightleftharpoons H_{n-i}ES + H^+, \quad K_{ES_i} = \frac{[H_{n-i}ES][H^+]}{[H_{n-i+1}ES]}$$

where $[H_iES]$ is the concentration of all enzyme species with i protons on the site. K_{ES_i}, may be identified with the average ionization constant of a class of ionizing groups rather than with any specific group.

The degree of cooperativity will be large and positive if the value of K_{ES_1} --- K_{ES_i} show a stepwise increase.

We can define a similar set of ionization constants where $\bar{\gamma}_E$ is given by the above equation with ionization constant K_{E_1} --- K_{E_i} for the enzyme by replacing the ionization constants K_{ES_1} --- K_{ES_i} for the E - S complex.

If $\bar{\gamma}_{ES}$, and $\bar{\gamma}_E$ are the fraction of sites on the ES complex and enzyme respectively that are occupied by protons we can write an expression for φ in terms of $\bar{\gamma}_{ES}$ and $\bar{\gamma}_E$ as

$$\varphi = n[\bar{\gamma}_{ES} - \bar{\gamma}_E] \qquad \text{---------- (3)}$$

The variation of log K_m with pH may then be obtained by integration of equation (1) after substitution of φ from equation (3) above. With sufficiently large value of n, the sharp minimum in the pH variation of log K_m can be accounted for.

Cooperative effects in the ES complex can be described by a simple Hill's empirical equation, with two constants according to the expression.

$$\bar{Y}_{ES} = \frac{[H^+]^Y}{K_{ES} + [H^+]^Y}$$

Similarly for the Enzyme

$$\bar{Y}_E = \frac{[H^+]^X}{K_E + [H^+]^X}$$

x and y have values between 1 and n and are measures of the free energy of interaction between the groups

K_{ES} is given by $[H^+_{0.5}]^Y$

where $[H^+_{0.5}]$ is the hydrogen ion concentration when half the ionizable groups have ionized and hence may be taken as the average intrinsic ionization constants of the ionizable groups. For the enzyme we have a similar equation

$$K_E = [H^+_{0.5}]^X$$

Substituting the $\bar{Y}_E$ and $\bar{Y}_{ES}$ in the expression for φ we have

$$\varphi = n\left[\frac{[H^+]^X}{K_E + [H^+]^X} - \frac{[H^+]^Y}{K_{ES} + [H^+]^Y}\right] \quad \text{-------} \quad (4)$$

Integration of equation (1) after substituting for φ we obtain the expression

$$\log K_m = \log K'_m + \frac{n}{x} \log (K_E + [H^+]^x) - \frac{n}{y} \log (K_{ES} + [H^+]^y) \text{---} (5)$$

We can thus evaluate the value of log K_m at different pH values by an appropriate choice of six constants: K'_m, K_E, K_{ES}, n, x and y.

log K'_m may be evaluated from the flat portion of the log K_m versus pH curve. Imposing the conditions that is characteristic of the log K_m versus pH profile we can equate the value of the log K_m at the plateau part of the curve to be equal to log K'_m. Under these conditions, equation (5) could be computed to give

$$K_{ES} = K_E^{y/x} \text{---------} (6)$$

and $$K_E = [H^+_{min}]^x \text{----------} (7)$$

where $[H^+_{min}]$ is the value of the $[H^+]$ at the position of the minimum value of K_m (K_{min}). Substituting from equation (6) and (7) into equation (5), we have

$$\log K_{min} - \log K'_m = \frac{n(y - x)}{xy} \log 2 \text{----------} (8)$$

With an appropriate choice of values of n and x we can calculate a value for y and then substitute for these values in equation (5) to obtain the values of log K_m at different pH values which best

fit the experimental points. The fitted curves for G6PD A^+, B^+ and A^- are as shown in figure 5(d) and the values of K_E, K_{ES}, n, x and y that best fit the data for $NADP^+$ binding for the three variants are shown in Table 5(e).

The abrupt change in the log K_m versus pH curve leading to a minimum of log K_m in the pH region of 6.80 to 7.30 for all the variants had therefore been interpreted in terms of cooperative ionization of groups on the enzyme and enzyme-substrate complex which may be associated with a major change in the quaternary structure of the G6PD enzyme molecules. The structural change that gives rise to the cooperative ionization may be associated with a pH dependent dissociation of the tetramer enzyme into dimers more so when the pH corresponding to log $K_{(min)}$ lies in the range of pH where the enzyme undergoes a transition from tetramer to dimer (66). This pH-linked dissociation-association equilibrium itself could be the major configurational change that links substrate binding and metabolic control of the enzyme to the large positive interaction between ionizable groups. The dissociation of a tetramer composed of subunits into dimers should involve the breaking of some interchain links. We know that the genetic variation in

these enzymes is due to amino acid substitution (41, 42). If these interchain contacts involve these different amino acids, there should be different variation in log K_m versus pH profile for the different variants. Since this is not the case, it is reasonable to surmise that these amino acids are not involved in the interchain links.

As shown in table 5(e) we however observe a significant difference in the cooperative ionization of groups in the enzyme and enzyme substrate complex in G6PD B^+ and A^-. The lower value of cooperativity in G6PD A^- would suggest that less groups are involved in cooperative ionization in G6PD A^- than G6PD A^+ or B^+. The amino acid change that leads to susceptible disulfide bond in G6PD A^- and not in G6PD A^+ or B^+ may account for this phenomenon. We would therefore postulate that the amino acid change in G6PD A^- involves loss of groups that are capable of positive cooperative interaction with the other ionized groups. This may involve opposite charged group change or neutral amino acid substitution for any of the charged groups in going from G6PD A^+ or B^+ to A^-.

In the pH range 5.90 to 9.50 the maximum variation in the standard free energy of the dissociation of the enzyme - $NADP^+$

Figure 8. Typical Arrhenius plots of log $K_m^{NADP^+}$ against 1/T for the determination of Enthalpy change for $NADP^+$ binding to G6PD B.

● — ● — ● pH 8.20

x - x - x pH 8.60

Δ - Δ - Δ pH 9.00

▲ - ▲ - ▲ pH 9.20

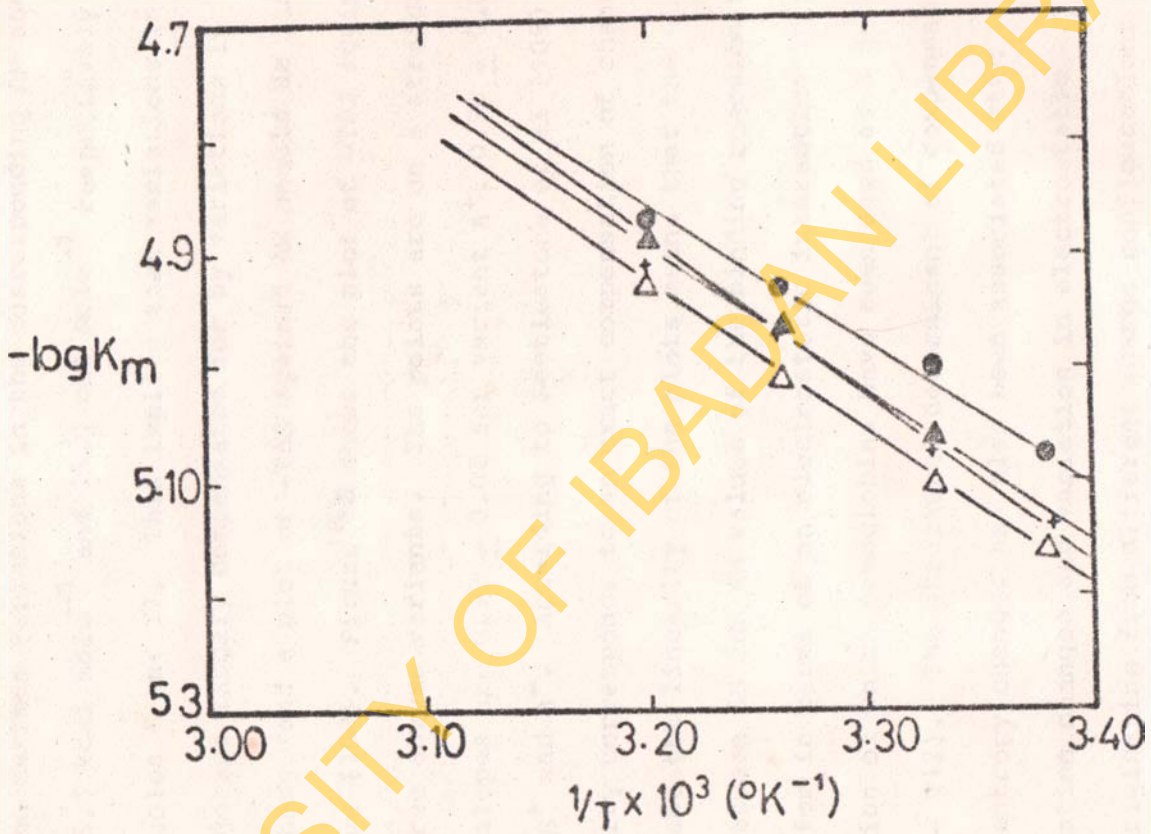


Fig. 8

complex is about 1.0 kcal mole^{-1} for the three enzyme variants. By contrast the maximum variations in the corresponding ΔH and ΔS are about 5.0 kcal mole^{-1} and 15.0 cal mole^{-1} respectively as shown in Tables 6 and 17. This implies that variations in enthalpy change are largely compensated for by variations in the entropy change and a plot of $-T\Delta S$ against ΔH should be linear if this is true (109). Figure 19 shows the plot of $-T\Delta S$ against ΔH for the three enzyme variants. The points are on a straight line and the slopes are 0.93 $\pm$ 0.02 for variant A^+, 0.92 $\pm$ 0.02 for variants B^+ and A^-. According to Beetlestone et al (109) a slope of unity corresponds to an exact compensation of changes in ΔH and ΔS and the linearity of the plots means that the differences between ΔS and ΔH values of the binding reaction may be accounted for in terms of an electrostatic interaction in the protein reaction of which hemoglobins have been used as a model example (108 - 112). The structural determinant of compensated enthalpy and entropy changes has also been associated with variable hydration changes and variation in electrostatic interaction originating from different charge configurations on each of the methemoglobins. The reaction of $NADP^+$ with a charged active site on the enzyme molecule may involve significant

Figure 9: Dependence on pH of the enthalpy change (ΔH) for the dissociation of the G6PD-$NADP^+$ complex for enzyme Variants A^+, B^+ and A^-.

● — ● — ● G6PD A^+.

▲ - ▲ - ▲ G6PD B^+.

● - o - o G6PD A^-.

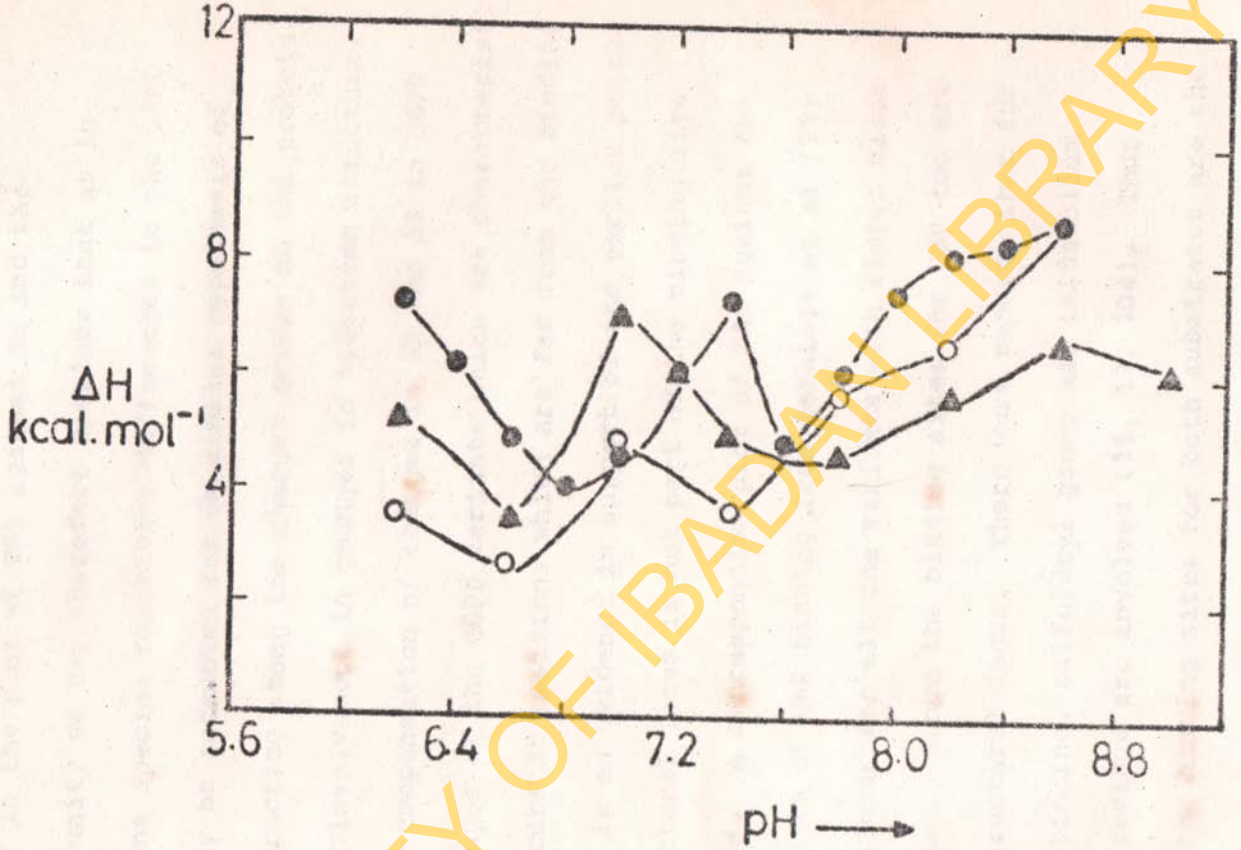


Fig. 9

pH-dependent change in the hydration structure of G6PD. Since there is linearity in the plot of $T\Delta S$ against ΔH and the slope is close to unity we may therefore conclude that as in hemoglobin reactions species reactivity differences in the G6PD enzyme variants may be accounted for on similar hypothesis of electrostatic interaction among the charged groups on the protein molecule and their involvement in changes in hydration structure. There is therefore compensation of changes in ΔH and ΔS in G6PD and the charge changes of the G6PD variants which are characteristic of each variant occurs at positions which are far from the binding centre. Thus this is an evidence in support of the earlier postulate that the structural locus is not part of the binding site but far away from it. A corresponding plot of $T\Delta S$ against ΔH was done from the data of G6P binding from Babalola et al (11) and the plots are linear for all the variants with slopes close to unity in each case. Hence the binding sites for G6P too are not close to the structural locus. There were reports that for both $NADP^+$ and G6P binding, sulfhydryl group and imidazolium group of histidine residue are involved (11, 17, 104). Thus the postulate that the binding sites for both substrates are the same may be true.

Figure 10: Plots of $T\Delta S$ against ΔH for $NADP^+$ binding.

Δ - Δ - Δ G6PD A^+

O - O - O G6PD B^+

▲ - ▲ - ▲ G6PD A^-.

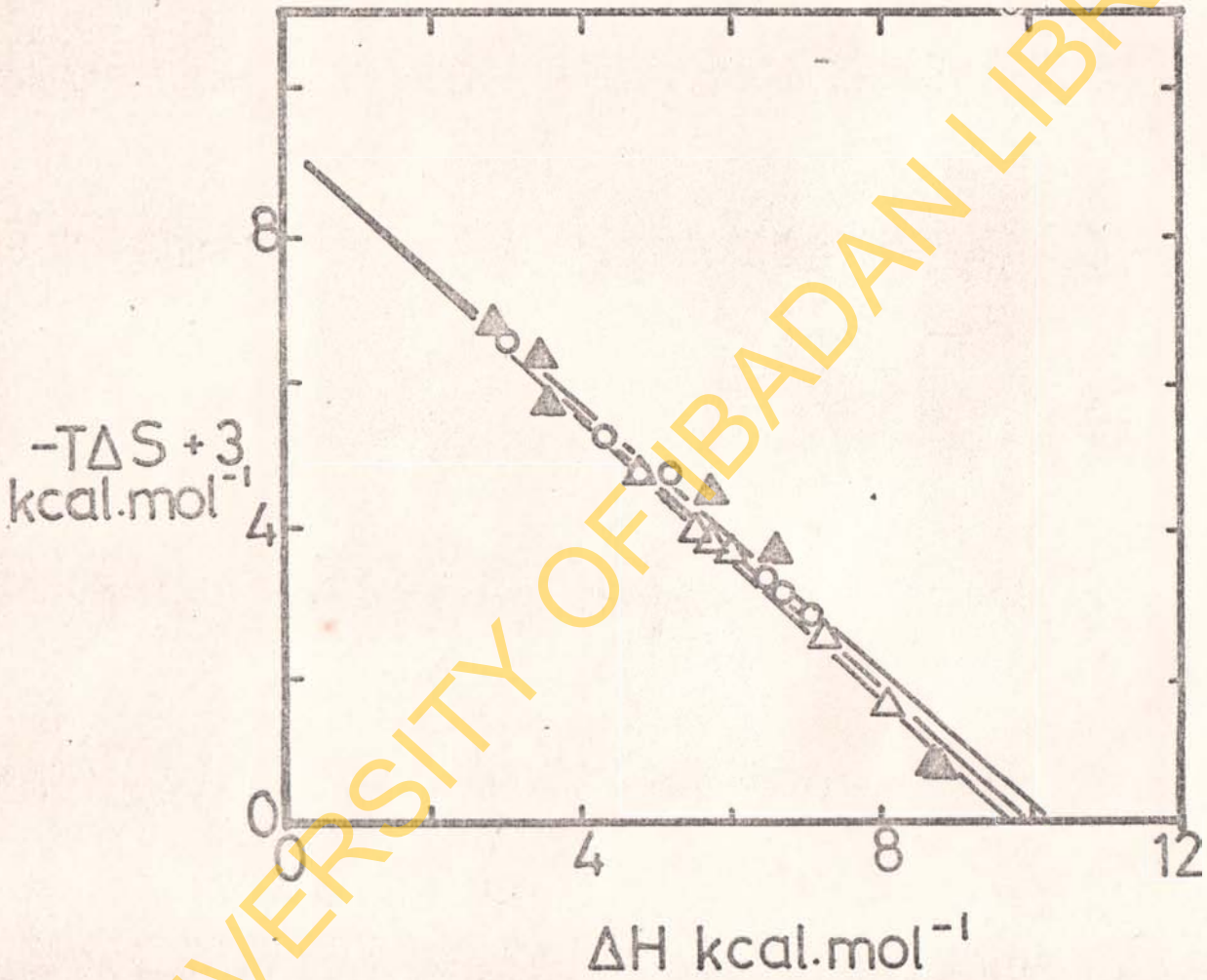


Fig. 10.

Figure 11: Plots of ΔH against ΔS for the determination of the Compensation Temperature, T_c.

$\Delta - \Delta - \Delta$ G6PD A^+

$O - O - O$ G6PD B^+

$A - A - A$ G6PD A^-.

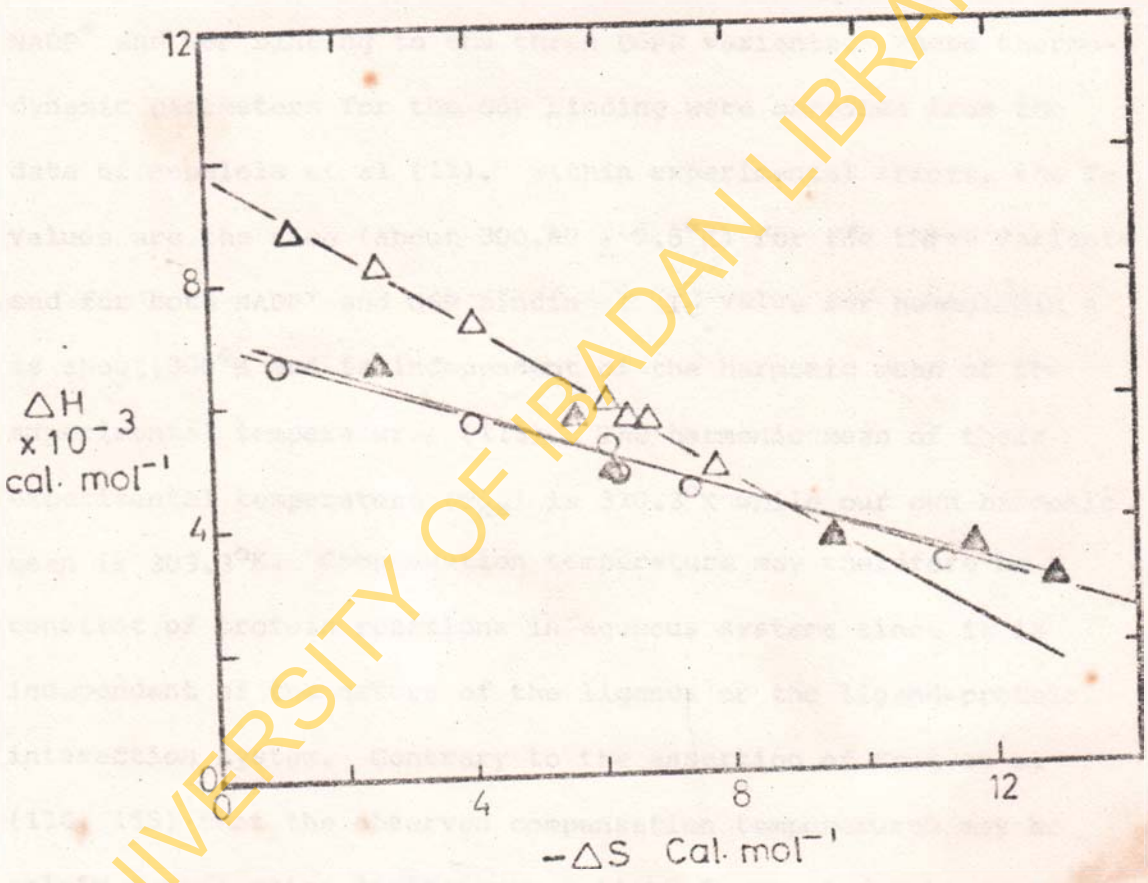


Fig. 11

Figure 11 shows the plot of ΔH against ΔS for the three enzyme variants. The slopes of the straight lines give the value of T_c, the compensation temperature (112). Table 18 shows the values of the compensation temperatures, T_c, for both $NADP^+$ and G6P binding to the three G6PD variants. These thermodynamic parameters for the G6P binding were obtained from the data of Babalola et al (11). Within experimental errors, the T_c values are the same (about 300.80 $\pm$ 9.8°K) for the three variants and for both $NADP^+$ and G6P bindings. T_c value for hemoglobin A is about 300°K and is independent of the harmonic mean of the experimental temperatures (113). The harmonic mean of their experimental temperature (T_{hm}) is 310.3°K while our own harmonic mean is 303.3°K. Compensation temperature may therefore be a constant of protein reactions in aqueous systems since it is independent of the nature of the ligands or the ligand-protein interaction system. Contrary to the assertion of Krug et al (114, 115) that the observed compensation temperatures may be solely a reflection of the propagation of experimental errors and not chemical effects, the compensation observed for G6PD reactions as in ferrihemoglobin reactions may be intimately linked to the same configurational change that has an important control on

Figure 12: Variation of log V_{max} with pH

▲ - ▲ - ▲ 20°C Δ - Δ - Δ 27°C

● - ● - ● 34°C o - o - o 40°C

(a) For G6PD A^+

(b) for G6PD B^+

(c) for G6PD A^-

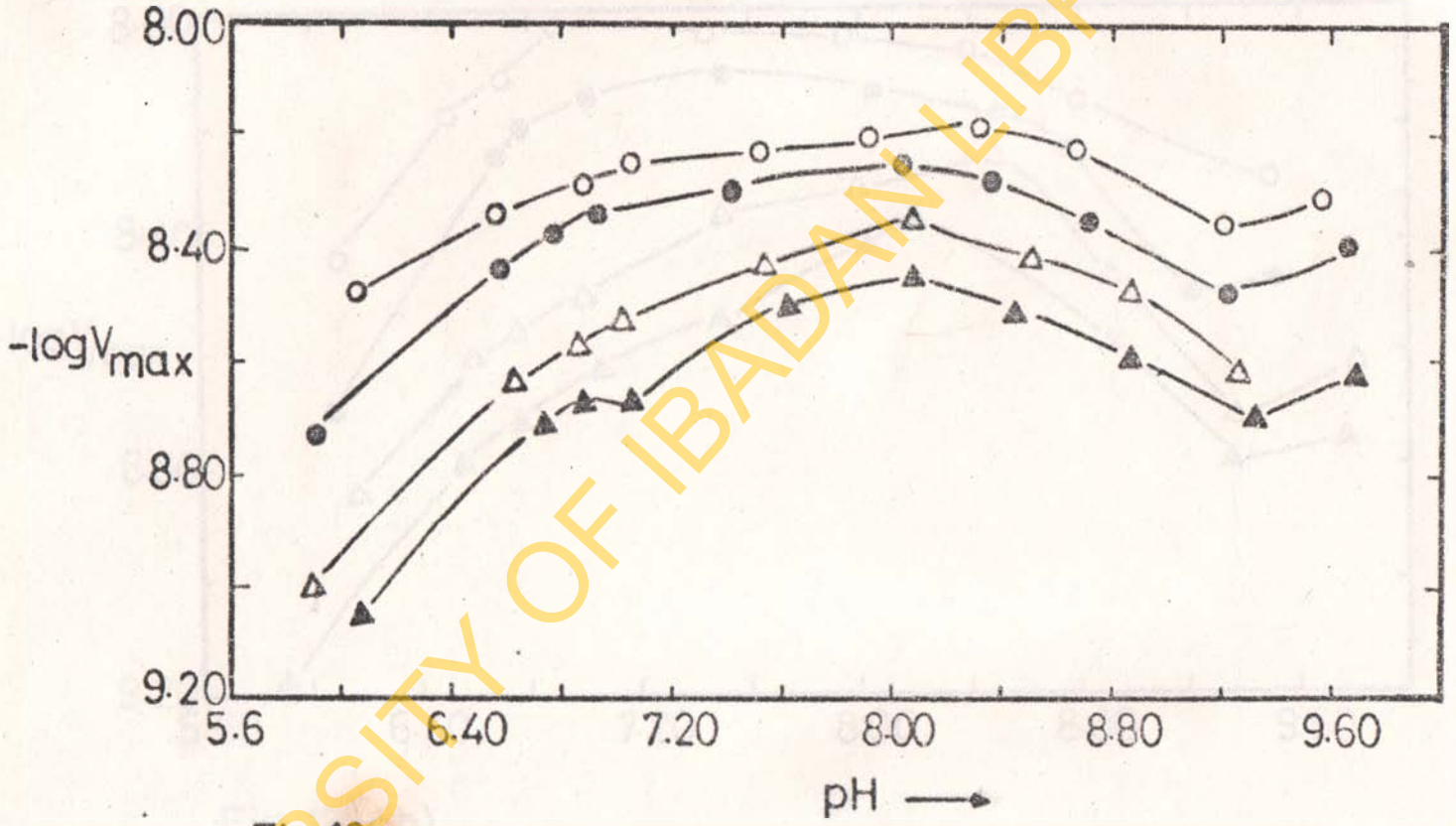


Fig. 12 (a)

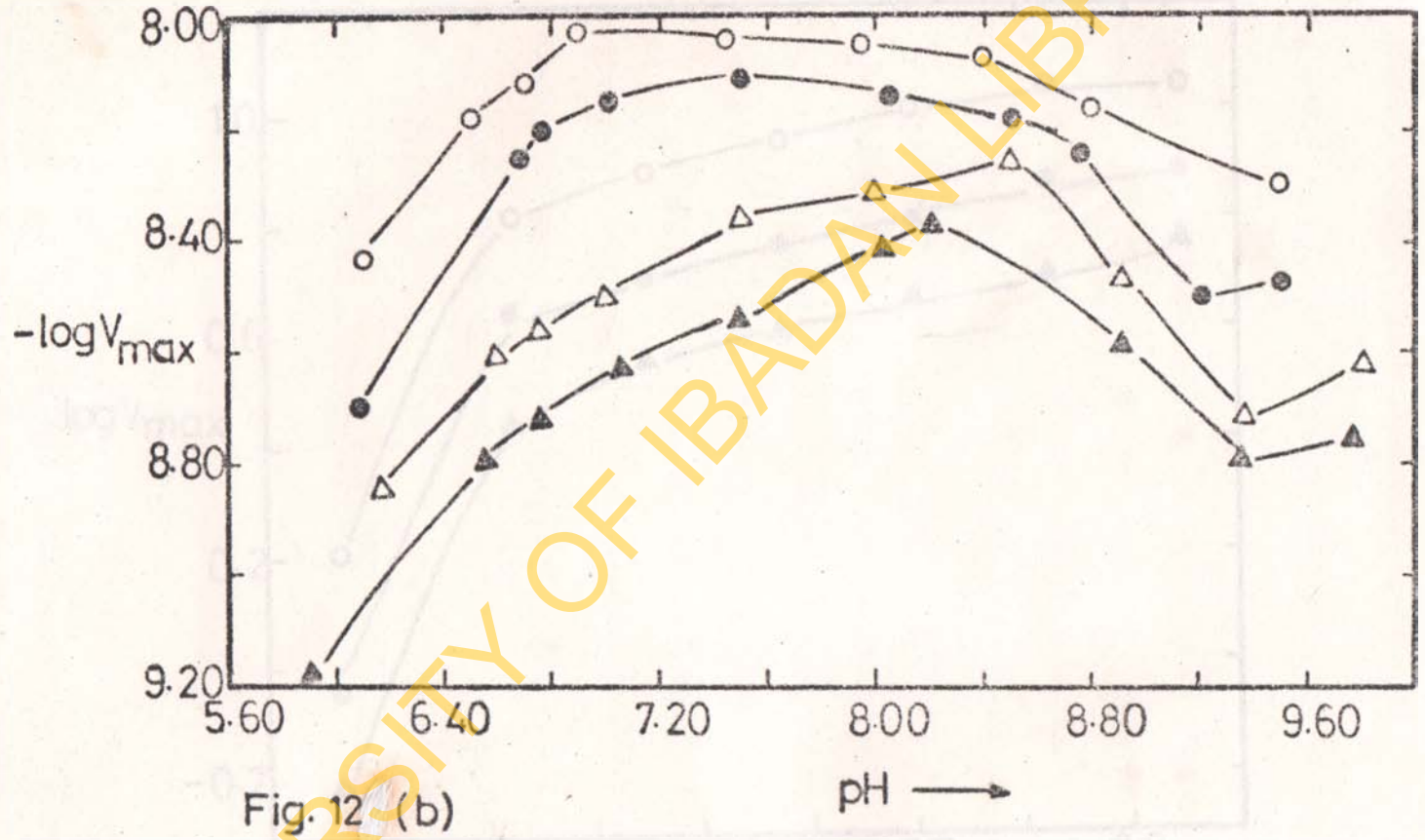


Fig. 12 (b)

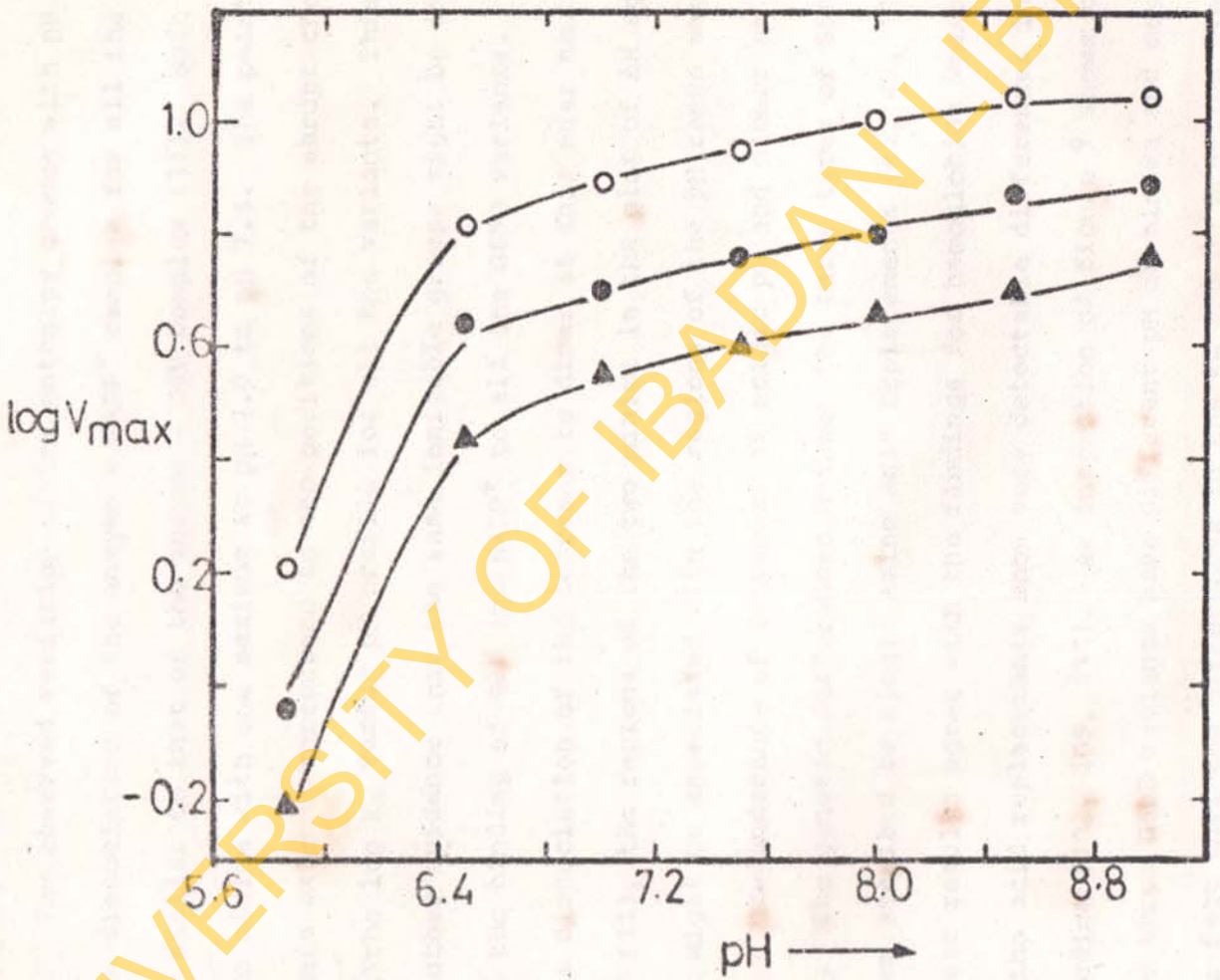


Fig. 12 (c)

the reactivity of the enzyme in vivo.

The observed variation of the enthalpy change with pH for the dissociation of the enzyme - $NADP^+$ complex for all the variants is similar to that of the enzyme - G6P complex (11). Both show two minima with one maximum at pH 7.0 to pH 7.4. The points of these extrema correspond to the positions of the abrupt changes in the log K_m versus pH profile for all the variants. This is another evidence that the same ionizable groups might be involved in the binding of G6P and $NADP^+$ to all the G6PD variants. With the dissociation of the tetramer to dimer at this near neutral pH (11), the regions of the two minima in the plot of ΔH against pH 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. The genetic differences between the three types of G6PD had been ascribed to single amino acid replacements (41, 42, 117). These results agree with the findings for hemoglobins where single amino acid replacements show very detectable differences in ligand binding (102, 109, 111). An inspection of figure 9 shows that the three G6PD variants have different pH of minima and maximum. The fact that the differences in the K_m and ΔH profiles over a pH range are not large supports the postulate that the structural

locus is not part of the binding site. The effect of the amino acid replacement even at a distantly remote part of the enzyme molecule could indirectly affect the $NADP^+$ binding site because of the cooperative nature of the entire protein structure.

Our data presented here lead to the same conclusion as with earlier findings on the structural properties of human erythrocyte G6PD variants (11, 17). The involvement of the sulfhydryl group in $NADP^+$ binding, the presence of imidazolium group of histidine as being responsible for the activation of the enzyme molecules had been reported by many workers for both G6P and $NADP^+$ bindings (11, 17, 104). The same groups had been reported by Kuby et al (118) in Brewers' Yeast G6PD. These authors ascribed the first set of protonic equilibrium to proton dissociation from an imidazolium group and the second set to proton dissociation from a lysyl residue. They had earlier on reported that a sulfhydryl group lies in close proximity to either substrate binding site(s) (98). Keleti (120) reported the involvements of sulfhydryl groups and histidyl residues in the catalytic functioning of glyceraldehyde-3-phosphate dehydrogenase. It then looks as if the involvement of these groups in the catalytic mechanism of reaction is a general characteristic of pyridine nucleotide

Figure 13 : Typical Arrhenius plots of log V_{max} against $1/T$ for the determination of activation energy of G6PD reaction for G6PD B^+.

o — o — o pH 8.20

x — x — x pH 8.60

Δ — Δ — Δ pH 9.00

o — o — o pH 9.20

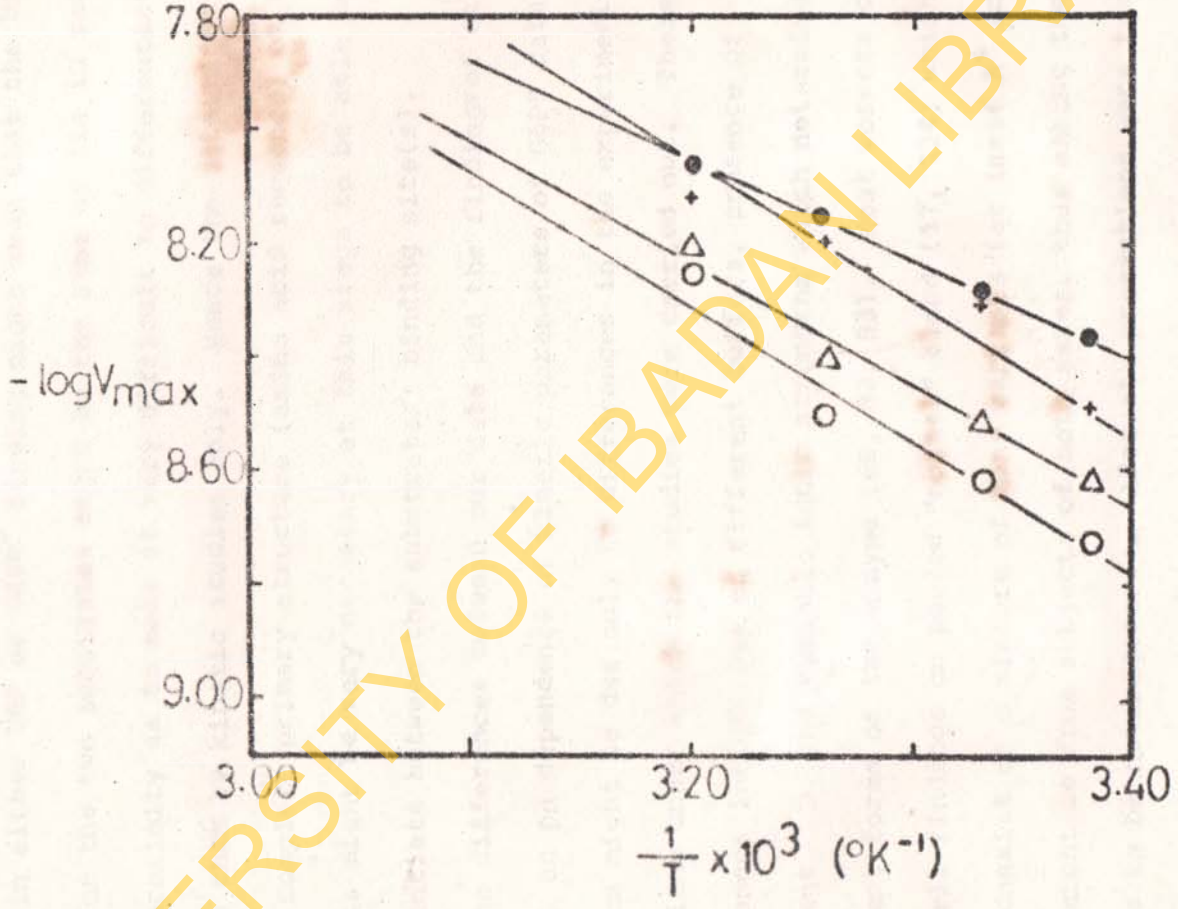


Fig. 13

dependent dehydrogenases. In G6PD, the involvement of the same groups in either G6P or $NADP^+$ binding might mean that the binding sites for the two substrates might be the same or lie in such close proximity as to make it very difficult to differentiate between them by kinetic studies only. Hence the determination of the complete primary structure (amino acid sequence) of G6PD variants might be very necessary at this stage to be able to differentiate between the substrates' binding site(s).

The differences between our data and the findings of other workers on pH dependence of kinetic parameters of G6PD catalyzed reaction might be due only to differences in the experimental conditions under which the studies were carried out. These differences include use of different buffers, presence of different [illegible] and variable ionic strengths which determine the different forms of the enzyme (66, 82, 83). Most workers carried out their findings on pooled "normal" blood (17, 104) which could consist of a mixture of many variants (at least A^+ and B^+). These might be from subjects of both sexes, thus making their enzymes to be heterogeneous. Adequate precautions were taken to eliminate the effects of such interacting conditions on the kinetic studies of G6PD variants as reported here.

Figure 14 : Variation of the activation energy (EA) with pH for G6PD variants A^+, B^+ and A^-.

● — ● — ● G6PD A^+

o - o - o G6PD B^+

+ - + - + G6PD A^-

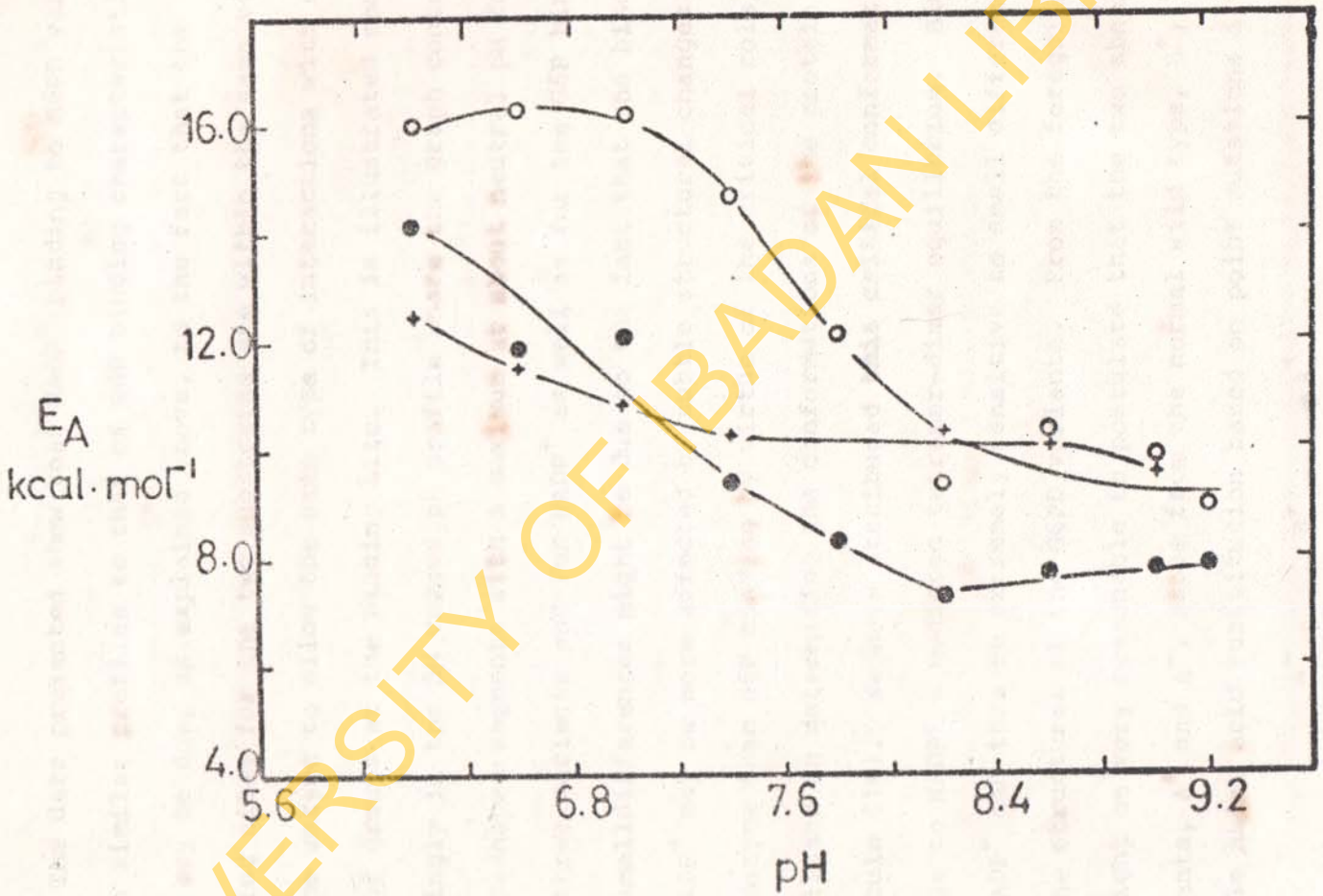


Fig. 14

The data presented show that $NADP^+$ binding to G6PD variants gives similar profiles to that of G6P binding characteristics. This may be due, as explained above, to the fact that the binding sites for the two substrates are within the same vicinity in the enzyme to allow the same type of interactions with similar set of groups at the binding site. This is illustrated convincingly in the ΔH versus pH profile where the graph contains two U-shaped segments with a maximum at about neutral pH for the three variants and for $NADP^+$ as well as for the G6P binding. The smalldifferences might be due to the fact that the binding of $NADP^+$ may be more affected by subtle structural changes in the enzyme than G6P binding by virtue of the critical role of the ligand in determining the conformations of the protein molecule (16). We have attributed this critical conformational change to $NADP^+$ - mediated tetramer-dimer equilibrium. Binding of $NADP^+$ can thus be extremely sensitive to small differences in the structures of the G6PD variants. From the foregoings, it might be very reasonable to postulate that the two abnormal variants, A^+ and A^-, arose from the normal wild type, B^+, by single amino acid substitution based on point mutations i.e.

$$A^- \longleftarrow B^+ \longrightarrow A^+$$

G6PD A^+ and Hektoen had been reported to evolve from G6PD B^+ by single amino acid substitution (41, 42), from asparagine in the B^+ type to aspartic acid in the A^+ variant and from histidine in the B^+ type to tyrosine in G6PD Hektoen. All the other variants had also been postulated to have evolved from G6PD B^+ by single amino acid substitution (117) at the structural locus. The above reasoning evolves from the fact that $NADP^+$ binding show general similar Km^{NADP^+} versus pH profile unlike in the case of G6P binding where significant differences are shown especially in G6PD A^- (11). In the $NADP^+$ binding, the same types of groups are involved in the reaction mechanism and all the variants show differences in the ΔH versus pH profile, the pH of minima being different. This is reminiscent of the situation in hemoglobins, where the ΔH versus pH profile show maximum which are characteristic (characteristic pH) of each variant (102, 111). Since most hemoglobins are known to have arisen from the normal type A, by single amino acid substitution it may be reasonable to infer that most G6PD variants also arise similarly from the normal type, B^+.

A study of the temperature and pH-dependent kinetic parameters, Km and V_{max}, of substrate binding in general is a sensitive probe

of structural changes in polymorphic enzymes. Using Dixon's generalization (103) it has been possible to analyse which ionizable groups are involved in the enzyme reaction. But the knowledge that the pK values of individual groups can be considerably affected by the presence of a neighbouring group within the protein which can interact with these ionizing groups called for caution in applying Dixon's rules to associate the pK values of the ionizable groups on the protein with specific amino acids. Studies of pH-dependence of the kinetics of enzyme competitive inhibitors and protein modification may be a way of corroborating the data from substrate kinetics. These types of inhibitors bind to the same groups on the enzyme as the substrates, the pK of these groups will be shown on the log K_i versus pH plots. The study of the kinetics of NADPH and p-OHMB binding to G6PD at different pH values and temperatures is suggested for the confirmation of the groups ionizing during the enzyme-substrate reaction. NADPH and p-OHMB had been reported to be competitive inhibitors of $NADP^+$ binding to G6PD (17, 86, 107). The study of the pH-dpendence of NADPH and p-OHMB competitive inhibitions to G6PD will therefore form the subject of the next chapter.

4.3.1. Effect of high ionic strength, pH and temperature on $NADP^+$ binding.

An inspection of Table 15 for the effect of buffer compositions on the interaction coefficient of G6PD variants A^+ and B^+ reveals different values of interaction coefficient, n, at different conditions resulting in variations of ionic strength and conductivity. Increase in ionic strength increases the degree of interaction among the $NADP^+$ binding sites. Table 16 shows the values of n at different temperatures and pH values. The interaction coefficient increases with pH at the same temperature and increases with increase in ionic strength in buffers of the same pH and same ionic composition. pH and ionic strength had been shown to be very critical parameters in the determination of molecular forms of G6PD (76, 82, 83). Hence at acidic pH and/or low ionic strength, the tetrameric enzyme is present dominantly and at alkaline pH and/or high ionic strength, the dimer is the dominant enzyme form. According to independent findings of Cancedda et al (8) and Bonsignore et al (13), the cooperative enzyme with two dissociation constants of the G6PD - $NADP^+$ complex is the dimer while the non-cooperative enzyme with a single dissociation constant is the tetramer. This result could be interpreted to

mean that when the enzyme is in the tetrameric state, the $NADP^+$ binding sites are so locked in the conformation that they are not easily available for interaction. The alternative is that the association of the dimers to form the tetramer bring some groups that interact with the binding sites near them, changing their right conformations to interact with one another but not changing their intrinsic property of binding the substrate, $NADP^+$. Omachi et al (78) reported that the dimeric enzyme is favoured in the human erythrocytes and Cancedda et al (8) and Bonsignore et al (13) showed that the cooperative enzyme is the dimer. We have found that high ionic strength borate buffers promote dimer formation most especially at high pH and thus we may infer that borate buffers may be an ideal buffer in which we can simulate the in vivo properties of G6PD. We have now established that the earlier reports about non-cooperativity in this enzyme (101) may be due to the use of buffers of low ionic strength where the enzyme exists largely as a tetramer and as with Yoshida et al (101) in an attempt to simulate physiological conditions, putting allosteric effectors such as Mg^{++} and Cl^- ions. These conditions favour tetramer formation (66, 76, 82, 83). An inspection of the plot of v/V_{max} against $NADP^+$ concentration in the paper by

Figure 15: Plots of v/V_{max} against $[NADP^+]$ in Tris-borate buffer of conductivity 2.35 mmho for G6PD variants A^+ and B^+.

○ - ○ - ○ G6PD A^+

Δ - Δ - Δ G6PD B^+

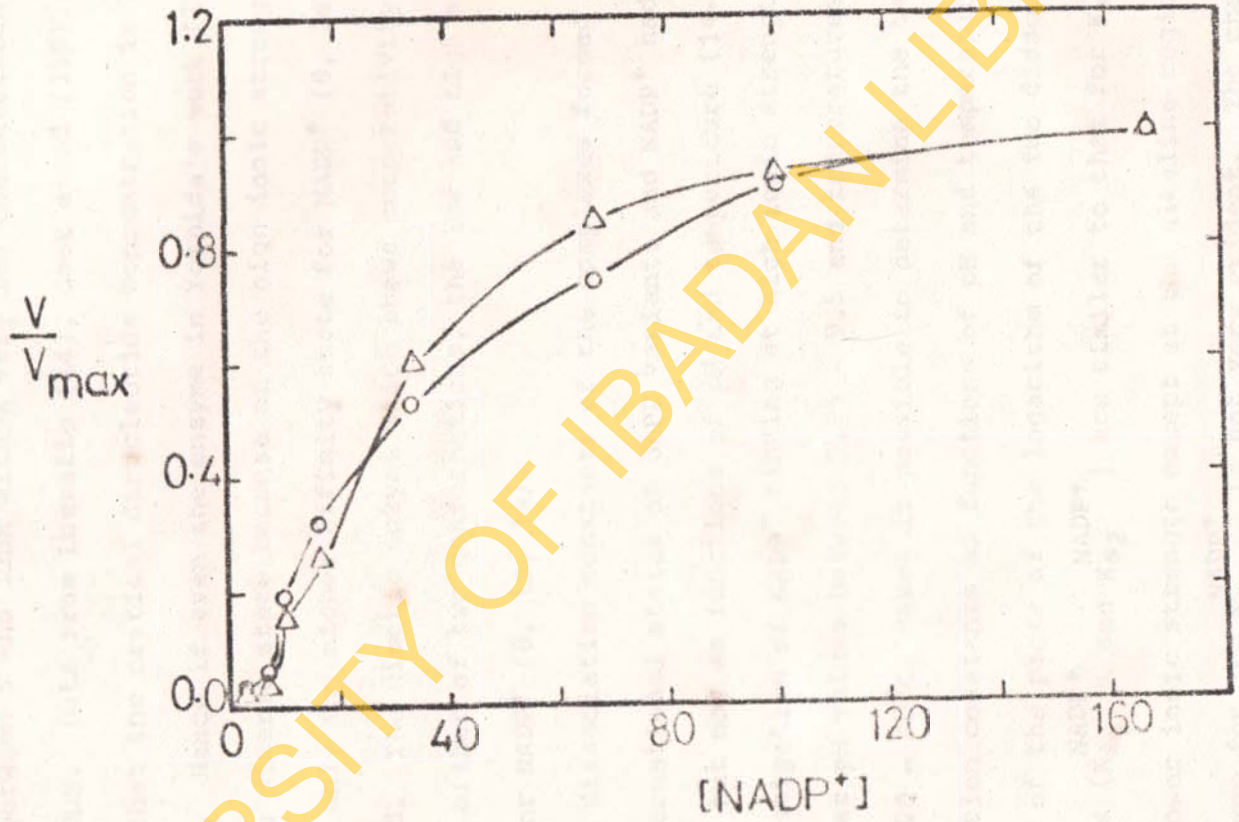


Fig. 15.

Yoshida et al (101) shows that the least $NADP^+$ concentration used is between 5 and 10μM with a very low concentration of G6P of 60μM. Data from Luzzatto (14), Cook et al (119) and us show that the critical dinucleotide concentration is lower than 5μM. Hence if even the enzyme in Yoshida's work (101) is in the dimeric state because of the high ionic strength, the data for only the higher affinity state for $NADP^+$ (8, 14) is presented. The dimeric enzyme which shows cooperativity could exist in either of two conformations, the low and high affinity states for $NADP^+$ (8, 13, 14).

The dissociation constants of the complexes formed by the two conformational states of G6PD variants and $NADP^+$ had been reported but not as functions of pH and temperature (14-16, 75). The investigation of $NADP^+$ binding at high ionic strength to G6PD B^+ at pH values between 5.85 - 9.5 and temperatures between 20 - 40°C, makes it possible to determine the two dissociation constants as functions of pH and temperature. The profiles of the plots of the logarithm of the two dissociation constants ($K_{s_1}^{NADP^+}$ and $K_{s_2}^{NADP^+}$) are similar to that for $K_m^{NADP^+}$ at the lower ionic strength except at the alkaline region where the maximum for log $K_{s_2}^{NADP^+}$ is not very evident. The change in

Figure 16 Dependence on pH of the dissociation constants at low (Ks_1) and high (Ks_2) affinity states of G6PD B^+ for $NADP^+$.

Δ - Δ - Δ 20°C Δ - Δ - Δ 27°C

● - ● - ● 34°C o - o - o 40°C

(a) log Ks_1 against pH.

(b) log Ks_2 against pH.

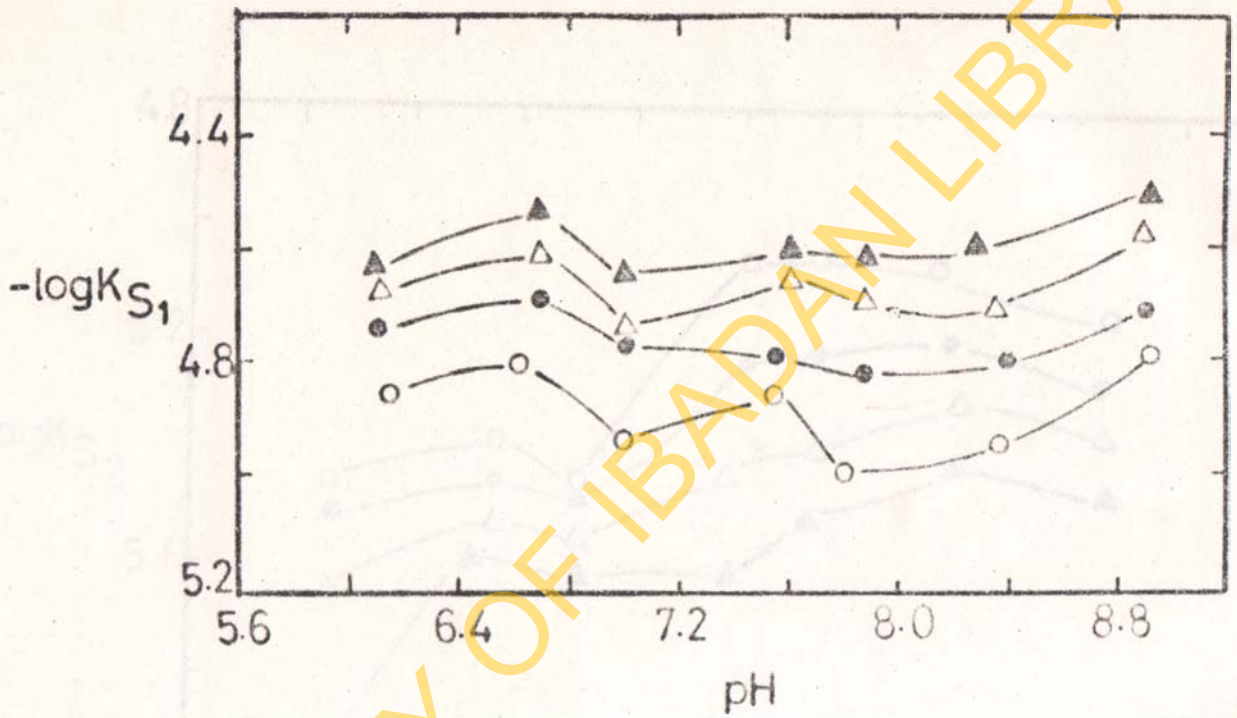


Fig. 16(a)

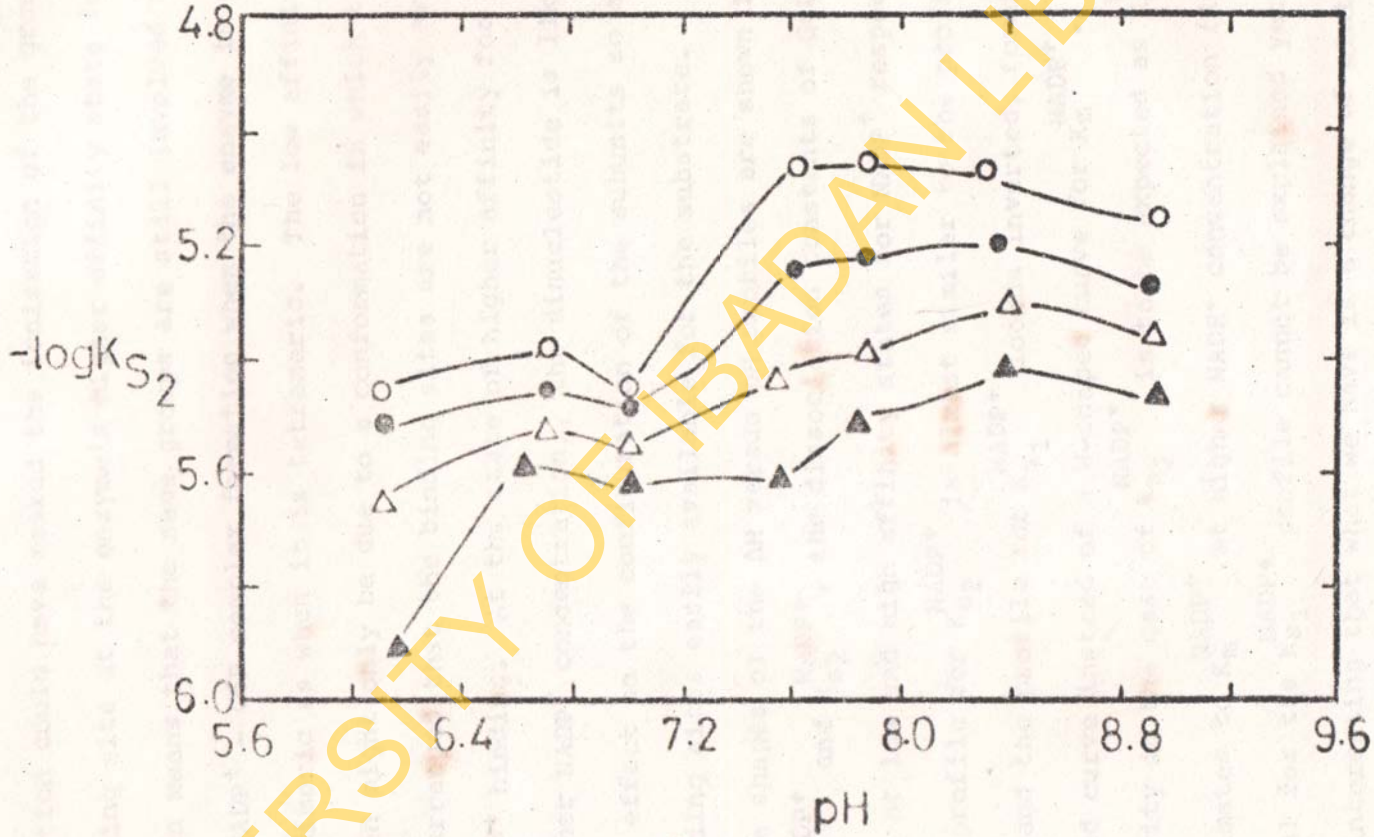


Fig. 16 (b)

conformation could have masked the ionization of the groups of the binding site at the enzyme's higher affinity state for $NADP^+$. This then means that the same groups are still involved in the G6PD - $NADP^+$ - G6P complex formation when the enzyme is predominantly dimeric as when it is tetrameric. The low affinity conformation might only be due to a conformation in which the subunits are so arranged that the binding sites are not easily available for $NADP^+$ binding. At the state of higher affinity for $NADP^+$, (at higher $NADP^+$ concentration), the dinucleotide is likely to have an effect on the configuration of the subunits so as to make the binding sites easily available for the substrate.

The shapes of the ΔH versus pH profiles are shown in figure 17 for $K_{s_1}^{NADP^+}$ and $K_{s_2}^{NADP^+}$, the dissociation constants of G6PD - $NADP^+$ complex at low and high affinity states for $NADP^+$ respectively. The ΔH profile for $K_{s_2}^{NADP^+}$ is almost similar to the profile for $K_m^{NADP^+}$ and the profile for $K_{s_1}^{NADP^+}$ lookds inverted, forming an M-shaped curve instead of a W-shaped curve for $K_m^{NADP^+}$. The similarity in the case of $K_{s_2}^{NADP^+}$ is to be expected as $K_{s_2}^{NADP^+}$ approximates to $K_m^{NADP^+}$ at higher NADP+ concentration (14). The reversal for the $K_{s_1}^{NADP^+}$ profile cannot be explained yet. But it is interesting that what we have is a change of minimum

Figure 17: Variation of ΔH with pH for the dissociation of G6PD-$NADP^+$ complex for G6PD B^{+}_{-}

● - ● - ● from log K_{s_1} against pH plot.

O - O - O from log K_{s_2} against pH plot.

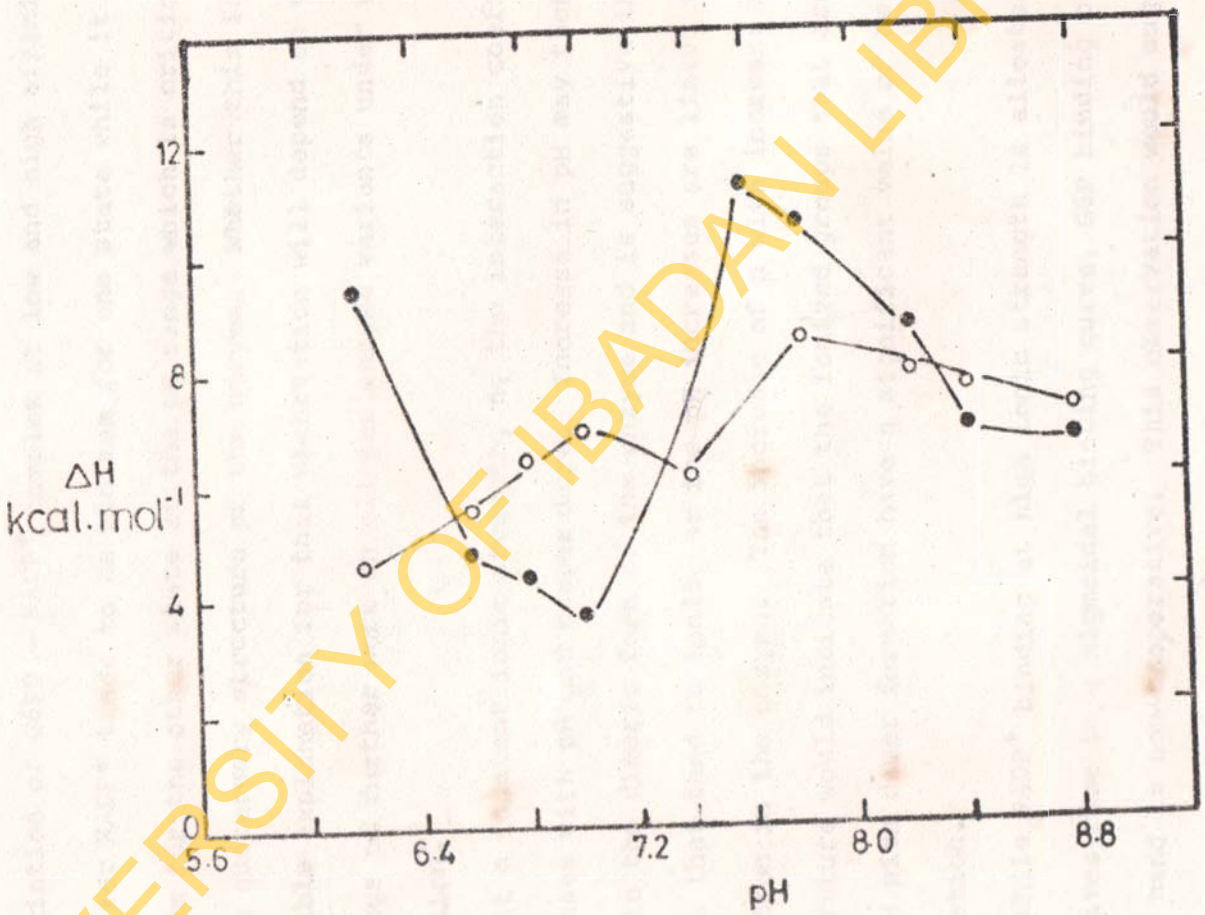


Fig. 17

to maximum at around pH values 7.2 to 7.6. Thus there could be a compensatory mechanism involved in which the enthalpy of dissociation of G6PD - $NADP^+$ complex at low and high affinity state for $NADP^+$ tends to be minimum for one state while it is maximum for the other state at the pH range which is critical to the quaternary structure of the enzyme. Whether this is a plausible explanation for this observation will depend on the findings of further work on all the enzyme variants under this condition.

At a constant ionic strength, n, the interaction coefficient increases with pH and temperature. Increase in pH may therefore promote the dimeric form of the enzyme and is suggestive that groups that tend to ionize as the pH increases are linked to dimer formation in the enzyme. The increase of n with increase in temperature would indicate that the ionized groups that are linked with dimer formation have a significant value of heat of ionization.

While $NADP^+$ binding at high ionic strength is allosteric and gives rise to a sigmoidal binding curve, G6P binding on the other hand is non-cooperative. This observation would suggest

Figure 18: Variation of log V_{max} with pH in Tris-borate buffer of conductivity 2.40 mmho for G6PD B^+.

Tris-borate buffer of conductivity 1.12 mho (I = 0.01) was brought to conductivity of 2.40 mmho with K_2SO_4.

▲ - ▲ - ▲ 20°C Δ - Δ - Δ 27°C

● - ● - ● 34°C o - o - o 40°C.

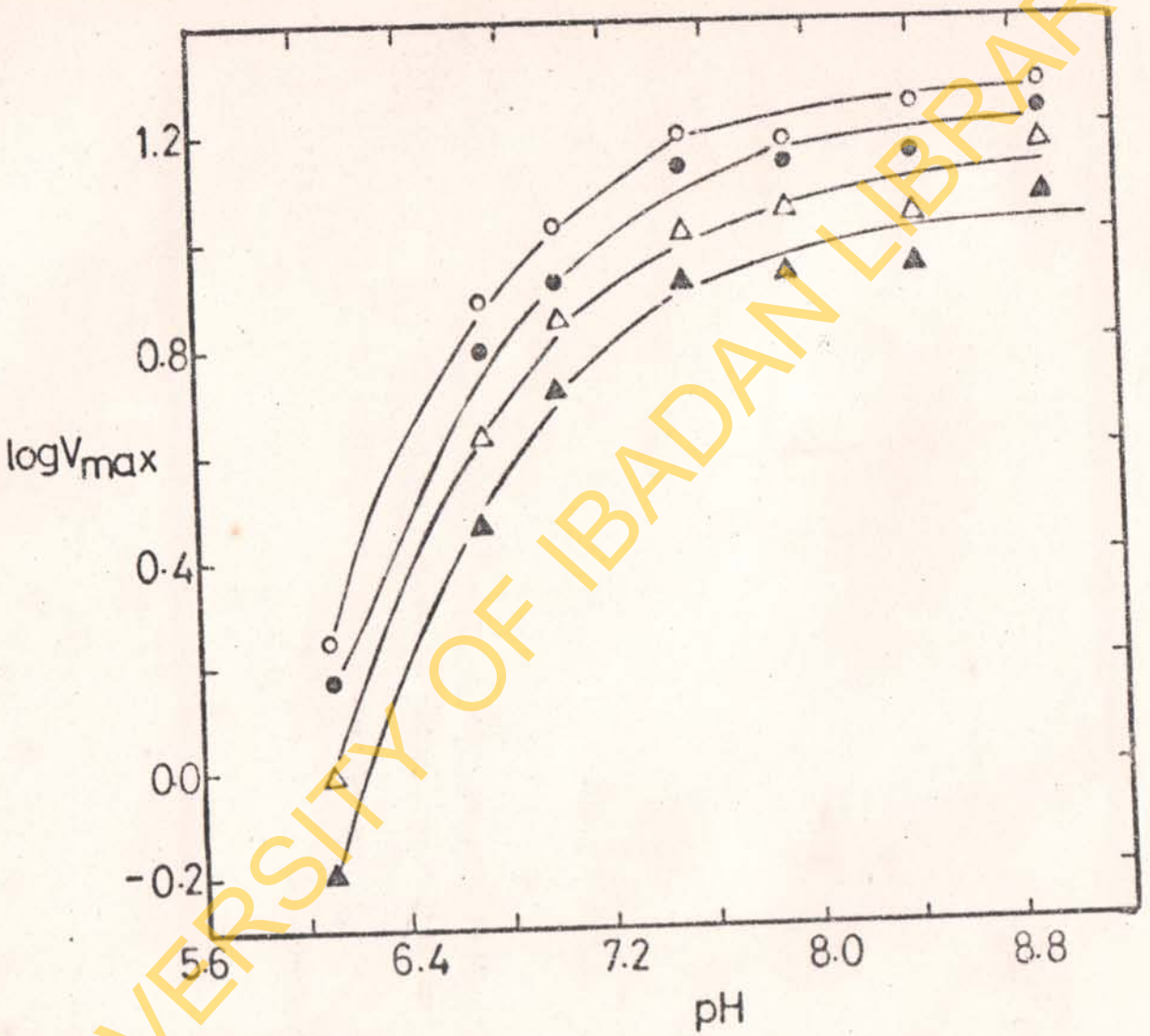


Fig. 18

Figure 19: Plot of E_A against pH for G6PD B^+ in Tris-borate buffer of conductivity 2.40 mmho.

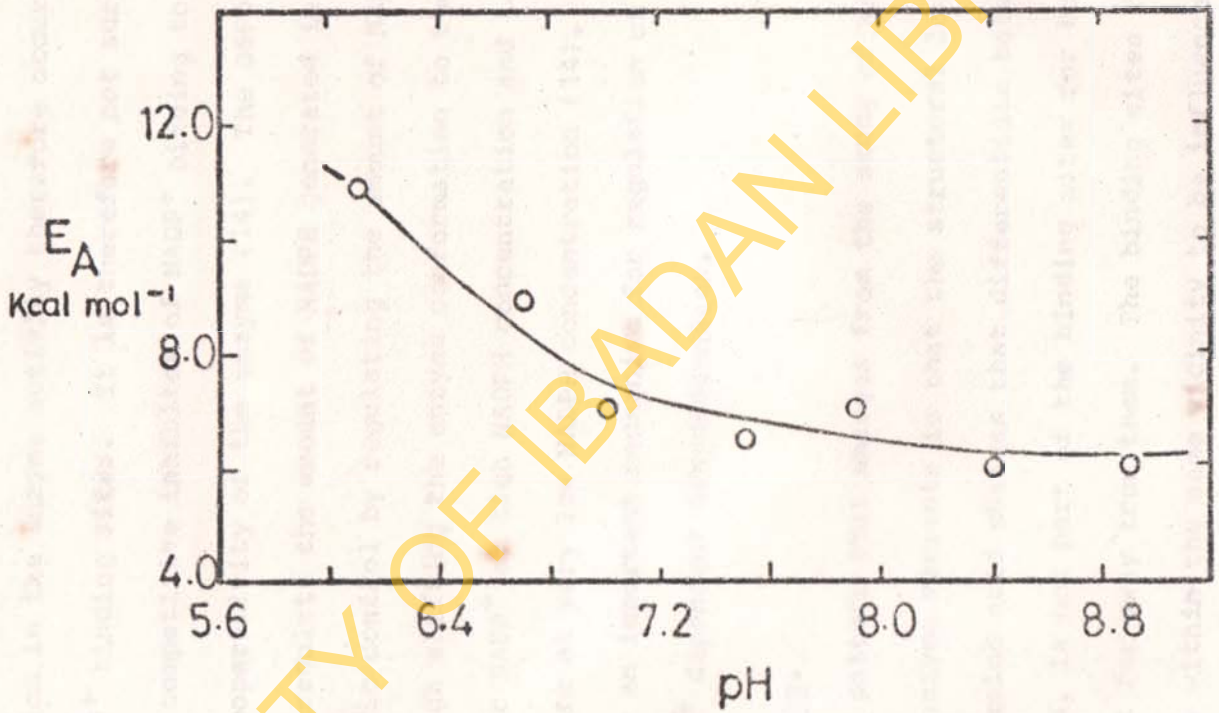


Fig. 19

that only $NADP^+$ and not G6P causes quaternary structural changes that affect the indirect interaction between the specific binding sites. Regulation in the enzyme activity therefore occurs mainly through the $NADP^+$ binding sites. It is therefore not surprising that NADPH as a competitive inhibitor of $NADP^+$ binding to G6PD decreases the cooperativity of the enzyme (14). The G6PD enzyme could therefore regulate the amount of NADPH generated in vivo through allosteric control by regulating the amount of $NADP^+$ to be used up through shifting the enzyme conformation to the low affinity form for $NADP^+$ at high NADPH concentration and to the high affinity form at very low NADPH concentration (14). This postulate may be an important mechanism for regulation of enzyme activity in $NADP^+$ dependent dehydrogenases.

4.4.1. Conclusion.

The general pattern that emerges from the study of $NADP^+$ binding to G6PD enzyme variants is that the structural locus i.e. the position of amino acid changes that differentiate between the G6PD variants, is not part of the binding sites for both G6P and $NADP^+$ but far away from them. The binding sites for $NADP^+$ and G6P are within the same vicinity to be influenced by

Figure 20a Typical Hill plots of log $v/V_{max} - v$ against log $[NADP^+]$ for the determination of interaction coefficient in Tris-borate buffer of conductivity 2.35 mmho for G6PD A^+ and B^+.

o - o - o G6PD A^+

● - ● - ● G6PD B^+

Figure 20b. Variation of n with temperature

+ - + - + at pH 6.50

● - ● - ● at pH 7.0

o - o - o at pH 8.40

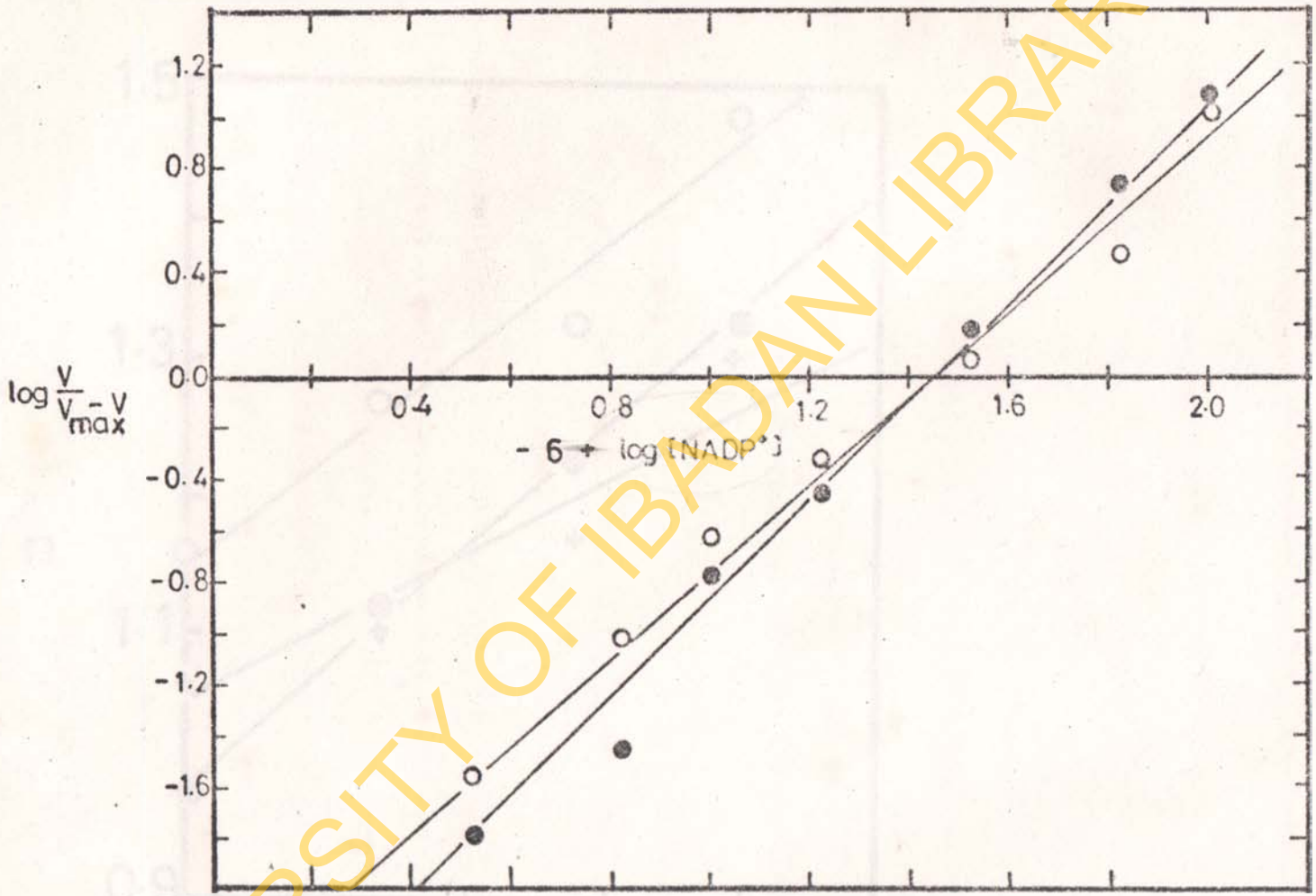


Fig. 20 (a)

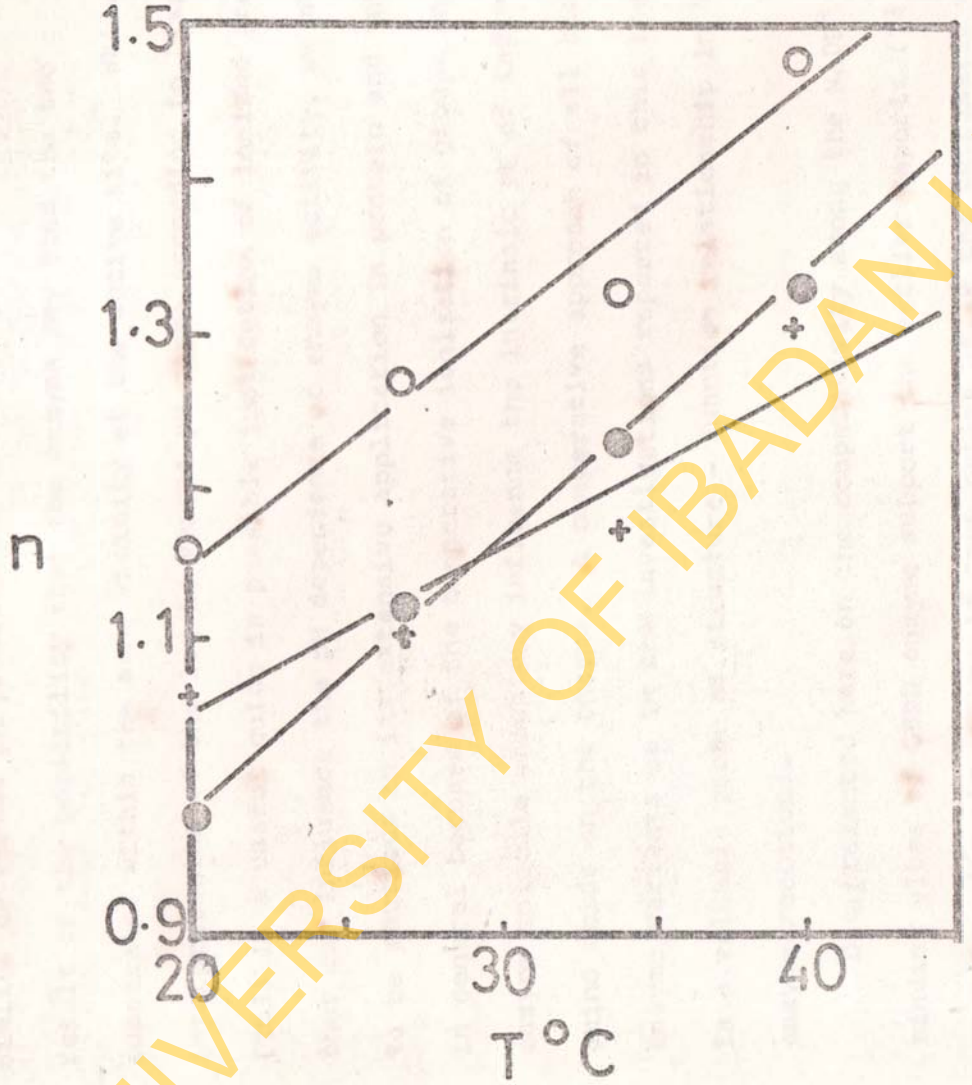


Fig. 20 (b)

the same groups at the activity site as in G6PD A^-. The very similar ΔH versus pH profiles for the two substrates is as a result of the possibility that the enzyme may bind the two substrates within the same vicinity at the active site. While the interpretation of the log Km versus pH curve according to Dixon (103) is a useful guide in possible implication of ionized groups that may influence the pH dependence of enzyme activity, we need to be cautious in its extensive application in protein and enzyme in general becuase of the cooperative ionization of groups on the enzyme molecule which may influence the intrinsic pK of individual amino acids on the enzyme. A comparative approach of all the enzyme variants as in the investigations reported in this thesis is a valuable probe of structure - function relationship in the enzyme reactions.

Data presented here on the cooperativity among the $NADP^+$ binding sites of G6PD enzyme support the earlier reports (13, 30) that the dimeric enzyme is cooperative while the tetrameric enzyme is not cooperative. This is as evident from an n value of 1.9 at 40°C and at pH 9.0 when the enzyme is predominantly a dimer, and an n value of 1.0 at pH 6.0 and at 20°C, when the enzyme is a preponderance of tetramers. The dimeric enzyme

had been reported to be the G6PD entity in the erythrocytes (78), the conditions that give cooperativity in G6PD is a simulation of the physiological conditions in vivo. From our data as reported in this thesis we have established two types of cooperative mechanism closely associated with the reactivity of human erythrocyte G6PD enzyme. The first mechanism is a cooperative interaction of ionized groups on the surface of the enzyme which influence the pH dependence of both the $NADP^+$ binding (cite loci) and the G6P binding (11) as reflected in the complex dependence of the respective K_m on hydrogen ions. There are at least 12 ionized groups which are cooperatively involved in the pH dependence of log Km in $NADP^+$ binding. This is to be compared with 8 ionized groups reported by Babalola et al (11) in G6P binding. We are therefore left to infer that at least there are about four more ionized groups that are linked to $NADP^+$ binding which may not be involved in G6P binding reaction. The second mechanism is an allosterie interaction between two $NADP^+$ binding sites in the dimeric enzyme with two intrinsic binding constants. The G6P substrate binding lacks this form of cooperative interaction. From this study the nature and form of cooperativity among $NADP^+$ binding sites in the G6PD enzyme have been established.

CHAPTER FIVE

pH-DEPENDENT EFFECTS OF INHIBITORS ON G6PD ACTIVITY

5.1.1 Introduction

Inhibition of an enzyme-catalysed reaction is a process whereby the rate of the reaction is reduced by a substance called an inhibitor. Inhibition of isolated enzymatic reactions in general have been widely studied and the overall inhibitory effect, may result in complex reaction schemes and rate equations in a way that alters the kinetic parameters of the enzymatic reactions. There are three different kinds of ways by which an inhibitor may influence the rate of simple enzymatic reactions characterised by the manner in which the concentration of the inhibitor affects the rate of reaction in respect of the substrate concentration. The degree of inhibition, i is given by the equation:

$$i = \frac{V_o - v}{V_o}$$

where V_o and v are the velocities of the uninhibited and inhibited reaction respectively. The three situation are:

(1) The degree of inhibition may depend on the substrate concentration - usually the degree is reduced as the substrate concentration is increased. The inhibitor in this case may resemble the substrate sufficiently in structure as to be bound in its stead to the active site, thus competing for the same binding site with the substrate. The inhibitor may have an intrinsic affinity for the substrate's binding site; in this case the inhibitor may not necessarily resemble the substrate in structure. This type of inhibition is said to have some COMPETITIVE character. In some enzymatic reactions, the products of the reaction can compete with the substrate thus slowing down the conversion of the substrate to the products. This is the case with the product, NADPH, in G6PD reaction, competing with $NADP^+$ for the latter's binding sites on the G6PD molecule (17, 39, 86, 87).

(2) The degree of inhibition may not be affected by the substrate concentration. The inhibitor in this case can combine with the enzyme at an unidentical site to the substrate binding site affecting the binding at the substrate binding site in an allosteric manner. The effect may be on the affinity of the enzyme for the substrate in which case K_m is increased or on the catalytic rate, in which case V_{max} is decreased.

This is NON-COMPETITIVE inhibition. This is the case with the product inhibition by NADPH when G6P is the variable substrate in G6PD reaction system (17, 86, 87).

(3) The degree of inhibition may be increased as the substrate concentration is increased. In this case, the inhibitor may combine exclusively with the enzyme-substrate complex. This is UNCOMPETITIVE inhibition. This type of inhibition is rare in simple enzymatic reaction but an example is the inhibition pattern exhibited by glucosamine 6-phosphate with respect to $NADP^+$ binding in G6PD reaction system (86)

Various inhibitors of G6PD have been described. Chung et al (4) and Anstall et al (74) had found that PCMB and heavy metal ions such as Hg^{++} and Ag^+ inhibit the activity of erythrocyte G6PD. This suggests that sulfhydryl groups are essential for the enzyme's catalytic activity because these inhibitors have intrinsic affinity for these groups. Physiologically a more important inhibitor is NADPH because of its regulatory role on the G6PD activity in the red cell (14). Glaser et al (122) had already observed that NADPH is a competitive inhibitor of $NADP^+$ in Brewers' yeast G6PD. Later, this finding was confirmed by Kirkman (123) for the human erythrocyte

G6PD enzyme. The characteristics of this NADPH inhibition was reinvestigated in more details by Luzzatto (14). The inhibition is most pronounced in the range of physiological concentrations of NADPH and in this range small changes in the concentration of NADPH can produce marked changes in enzyme activity. When the $NADP^+$ concentration is in the range where G6PD already has high affinity for it, NADPH will compete for the $NADP^+$ binding site and will this show an inhibitory action with competitive features. This is expected considering the very similar and close structural relationship between $NADP^+$ and NADPH.

The inhibitory effect of NADPH had been found to have different intensities and characteristics for different genetic variants of G6PD. This is shown by the study of the kinetics of inhibition of NADPH in G6PD A^+ by Luzzatto (14) and three other variants, B^+ A^- and Ijebu-Ode by Afolayan et al (16). The inhibition constant, Ki, an inverse measure of the affinity of the enzyme for NADPH for the four variants, A^+, B^+, A^- and Ijebu-Ode have been determined (14, 16). They show that A^- is the least sensitive to inhibitory effect of NADPH. (K_i = 210 μM for the A^- variant compared with 16 and 30 μM respectively for A^+ and B^+ variants (14, 16)). The A^-

variant thus differs in kinetic behaviour from the normal variants A^+ and B^+ in having the highest affinity for the substrate $NADP^+$ but lowest affinity for the inhibitor NADPH. (Ki = 210 uM and K_{s_2} = 1.3 uM for A^- variant compared with K_{s_2} of 13 and 12 μM and Ki of 16 and 30 μM respectively for A^+ and B^+ variants (14, 16). Though the kinetics of inhibition of NADPH and mercaptide-forming reagents such as PCMB and P-OHMB to G6PD have been studied by many workers (14, 16-18, 50, 86, 87, 107, 124), there has been no systematic study of the variation of inhibition by these substances with such factors as temperature and pH. The kinetics of competitive inhibitors studied as a function of pH and temperature has been recognized as a tool for investigating the mode of action of enzymes. Such a study might be expected to throw valuable insight on the mechanism of a particular enzymatic reaction, since it would be expected to yield informations about the thermodynamics of the process, and from the theory of Dixon (103), about the changes in electrical charge accompanying inhibition. A combination of such a study with a similar study of the kinetics of the coenzyme, $NADP^+$ binding in G6PD reactions will confirm the presence of certain groups in the catalytic process already postulated from the study of $NADP^+$ binding as a function of temperature and pH.

5.1.2 INHIBITING EFFECT OF PRIMAQUINE DIPHOSPHATE (AN ANTIMALARIAL SUBSTANCE) ON G6PD ACTIVITY

Individuals with erythrocyte G6PD deficiency have a high incidence of hemolytic anemia when given antimalarial drugs prophylactically. This observation might suggest that antimalarial drugs have inhibitory effects on G6PD activity. It will be very interesting to know if these compounds are capable of such inhibition in vitro. We have therefore carried out an inhibition experiment of primaquine diphosphate on G6PD activity on $NADP^+$ binding. Cotton et al (129) had carried out experiments on the inhibition of $NADP^+$ binding to G6DP from yeast from Sigma and tissue extracts of erythrocytes using some antimalarial substances. Their findings show that the antimalarial compounds are competitive inhibitors with respect to $NADP^+$ binding in G6PD reaction system.

EXPERIMENTAL

5.2.1 Kinetic Determinations

The reaction velocity measurements for the inhibition by NADPH p-OHMB and primaquine diphosphate were carried out in Gilford spectrophotometer with a constant temperature cell compartment and an

automatic absorbance recorder model 2000. This instrument (and not Turner recording spectrofluorometer model 110 as for the kinetic experiment for $NADP^+$ binding in other sections) was used because of the difficulty to annul to zero the fluorescence of the added NADPH and primaquine diphosphate. The composition of the reaction mixtures was as follows:

Buffer: 2ml; 0.06M G6P: 0.25ml; enzyme solution: 0.01ml; 10mM $NADP^+$: 0.03ml to 0.15ml; water and 5mM NADPH, p-OHMB or primaquine diphosphate were added in different volumes to have a final mixture volume of 3ml. All other procedures were as carried out for the kinetic experiments for the binding of $NADP^+$ to the G6PD variants.

5.2.2. Measurement of Inhibitor Constant

The inhibitor constant is determined by the method of Dixon (125) by plotting $1/v$ against $[I]$ from the equation

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} + \frac{K_m [I]}{V_{max} [S]} Ki$$

at two substrate concentrations where K_i and $[I]$ represent the inhibitor constant (i.e. the equilibrium constant of the reversible

combination of the enzyme with a competitive inhibitor) and concentration of the competitive inhibitor respectively. All other notations are as defined in chapter One. The intersection point of the two straight lines lies on the left of the ordinate (the $1/v$ - axis) and above the abscissa (the $[I]$-axis). This point lies at $[I] = -K_i$ and can therefore be read off directly. Higher concentration of the substrate, $NADP^+$ had to be used for p-OHMB inhibition than for NADPH inhibition to be able to get measurable velocities because of the protective function of $NADP^+$ against the mercaptide-forming reagent's inhibition of $NADP^+$ binding reaction (4, 50, 124).

Calculation of ΔH from the inhibitor constant for both NADPH and p-OHMB at all pH values was as discussed for the Michaelis constant for $NADP^+$

RESULTS AND DISCUSSION

5.3.1. Effect of pH on inhibitor constant of NADPH and p-OHMB

Table 19 shows the results of the inhibition kinetics of NADPH and p-OHMB at two concentration of $NADP^+$ in each case and at pH 7.5 and 34°C. A calculation of the degree of inhibition at the two $NADP^+$ concentrations for the same inhibitor concentration, shows that the degree of inhibition is reduced with increase in $NADP^+$ concentration for the two inhibitors. Thus as already concluded by many other investigators (4, 17, 86, 107, 123), both NADPH and p-OHMB are competitive inhibitors of G6PD with respect to $NADP^+$ binding. Fig 21 shows the Dixon's plot (125) for NADPH and p-OHMB inhibitions of the $NADP^+$ binding reactions to G6PD B^+ at pH 7.5 and 34°C. The plots give straight lines and they intersect on a point at the left side of the ordinate for the inhibitions by both compounds.

The inhibitor constants from these plots are as shown in Table 20 at different pH values and at three temperatures. The plots of log K_i against pH at the three temperatures are shown in Fig 22. The log K_i^{NADPH} versus pH profile is very similar to the same plot for log $K_m^{NADP^+}$ This finding is similar to the inhibition behaviour of

TABLE 19(a)

INHIBITION KINETICS OF NADPH BINDING TO HUMAN ERYTHROCYTE G6PD B^+ AT pH 7.5 AND AT 34°C.

[NADPH] μM	100μM $NADP^+$		200μM $NADP^+$	
	$V(\Delta A_{340}\ min^{-1})$	$1/_V \times 10^{-3}$	$V(\Delta A_{340}\ min^{-1}$	$1/_V \times 10^{-3}$
0	0.00577	0.173	0.00625	0.160
8.33	0.00469	0.213	0.00625	0.160
16.67	0.00438	0.229	0.00563	0.178
25.00	0.00417	0.240	0.00521	0.192
33.33	0.00391	0.256	0.00511	0.196
41.67	0.00391	0.256	0.00500	0.200
50.00	0.00310	0.323	0.00455	0.220

TABLE 19(b)

INHIBITION KINETICS OF p-HYDROXYMERCURIBENZOATE BINDING TO HUMAN ERYTHROCYTE G6PD B^+ AT pH 7.5 AND AT 34°C.

[p-OHMB] μM	0.33mM $NADP^+$		0.50mM $NADP^+$	
	$V(\Delta A_{340}\ min^{-1})$	$1/v \times 10^{-3}$	$V(\Delta A_{340} min^{-1})$	$1/v \times 10^{-3}$
0	0.00438	0.229	0.00446	0.224
16.67	0.00408	0.245	0.00441	0.227
33.33	0.00370	0.270	0.00391	0.256
50.00	0.00361	0.277	0.00375	0.267
66.67	0.00333	0.300	0.00357	0.280
100.00	0.00313	0.320	0.00341	0.293

TABLE 20(a)

DEPENDENCE ON pH OF $\log K_1^{NADPH}$ FOR G6PD B^+.

pH	$-\log K_1^{NADPH}$		
	27°C	34°C	40°C
6.14	5.19 ± 0.03	5.00 ± 0.03	4.94 ± 0.01
6.62	5.10 ± 0.02	4.90 ± 0.04	4.82 ± 0.01
7.03	5.15 ± 0.02	5.02 ± 0.01	4.90 ± 0.02
7.60	5.16 ± 0.03	4.89 ± 0.01	4.78 ± 0.02
8.68	5.30 ± 0.06	5.16 ± 0.02	4.98 ± 0.02
9.02	5.26 ± 0.03	5.00 ± 0.03	4.82 ± 0.01

TABLE 20(b)

DEPENDENCE ON pH OF $\log K_i^{P\text{-OHMB}}$ FOR G6PD B*

pH	$-\log K_i^{P\text{-OHMB}}$		
	27°C	34°C	40°C
6.02	4.80 ± 0.02	4.60 ± 0.01	4.46 ± 0.02
6.54	4.60 ± 0.05	4.50 ± 0.02	4.42 ± 0.01
7.05	4.38 ± 0.03	4.20 ± 0.01	4.10 ± 0.01
7.51	4.51 ± 0.02	4.41 ± 0.02	4.25 ± 0.02
8.50	4.46 ± 0.02	4.33 ± 0.02	4.22 ± 0.02
9.00	4.34 ± 0.02	4.22 ± 0.02	4.03 ± 0.01

TABLE 21(a)

DEPENDENCE ON pH OF ΔH OF INHIBITION BY NADPH

pH	ΔH (Kcal $mole^{-1}$)
6.20	10.53 ± 0.52
6.60	10.21 ± 0.46
7.00	9.15 ± 0.10
7.40	13.04 ± 0.46
7.80	13.73 ± 0.56
8.20	12.42 ± 0.10
8.60	10.68 ± 0.36
9.00	14.87 ± 0.26

TABLE 21(b)

DEPENDENCE ON pH OF ΔH OF INHIBITION BY p-OHMB.

pH	ΔH (Kcal mole^{-1})
6.20	9.86 ± 0.10
6.60	5.63 ± 0.10
7.00	10.07 ± 0.31
7.40	9.50 ± 0.15
7.80	9.15 ± 0.10
8.20	8.10 ± 0.15
8.60	10.21 ± 0.10
9.00	11.44 ± 0.36

TABLE 22(a)

INHIBITION OF ERYTHROCYTE G6PD B^+ BY NADPH. DEPENDENCE OF REACTION VELOCITY NORMALIZED TO THE VELOCITY OF UNINHIBITED REACTION ON INHIBITOR CONCENTRATION.

pH 7.60 $34^{\circ}C$

[NADPH] μM	100μM $NADP^+$		200μM $NADP^+$	
	$V(\Delta A_{340}\ min^{-1})$	v/V_o	$V(\Delta A_{340}\ min^{-1})$	v/V_o
0	0.00577	1.00	0.00625	1.00
8.33	0.00469	0.81	0.00625	1.00
16.67	0.00438	0.76	0.00563	0.90
25.0	0.00417	0.72	0.00521	0.83
33.33	0.00391	0.68	0.00511	0.82
41.67	0.00391	0.68	0.00500	0.80
50.00	0.00310	0.54	0.00455	0.73

TABLE 22(b)

INHIBITION OF ERYTHROCYTE G6PD B^+ BY p-HYDROXYMERCURIBENZOATE, DEPENDENCE OF REACTION VELOCITY NORMALIZED TO THE VELOCITY OF UNINHIBITED REACTION ON INHIBITOR CONCENTRATION.

pH 7.51 34°C.

[p-OHMB] μM	0.33mM $NADP^+$		0.50mM $NADP^+$	
	$V(\Delta A_{340}\ min^{-1})$	v/v_o	$V(\Delta A_{340}\ min^{-1})$	v/v_o
0	0.00438	1.00	0.00446	1.00
16.67	0.00408	0.93	0.00441	0.99
33.33	0.00370	0.85	0.00391	0.88
50.00	0.00361	0.82	0.00375	0.84
66.67	0.00333	0.76	0.00357	0.80
100.00	0.00313	0.72	0.00341	0.77

D-malate in fumarase where D-malate (a competitive inhibitor of fumarase activity) behaves in an analogous way to the natural substrate, fumarate (126). The structures of the substrate and the inhibitor are similar just like for $NADP^+$ and NADPH. The log $K_i^{p\text{-OHMB}}$ versus pH profile is a bit different from the log $K_m^{NADP^+}$ profile but it shows the important extrema at pH values of 6.60, 7.10 and 8.50. The extrema at pH values of around 6.60 and 8.50 represent the ionizations of groups on the enzyme - substrates - inhibitor complex while the extremum at pH value of around 7.10 represents the pH - linked dissociation - association equilibrium of G6PD (11).

The results are interesting in that they show that since NADPH has a structure that is very similar to that of $NADP^+$, it should bind at the binding sites of $NADP^+$ on the G6PD enzyme molecule. The interpretation of the dependence of log K_i on pH just like for log K_m is according to Dixon (103). Hence the present data show that NADPH binds to the same binding sites already postulated for $NADP^+$. p-OHMB is known to react specifically with available sulfhydryl groups in proteins. Thus these findings show that imidazolium group of histidine and sulfhydryl group are involved in the catalytic mechanism of $NADP^+$ binding to G6PD. The fact that the enzyme is not completely inactivated by p-OHMB shows that these groups are only linked to the binding

Figure 21: Dixon's plots of $1/v$ against inhibitor concentration for the determination of inhibitor constant, K_i for G6PD B^+ at pH 7.5 and at 34°C.

(a) Inhibition by NADPH at o - o - o 100μM $NADP^+$ ● - ● - ● 200μM $NADP^+$.

(b) Inhibition by p-OHMB at O - O - O 0.33mM $NADP^+$, ● - ● - ● 0.50mM $NADP^+$.

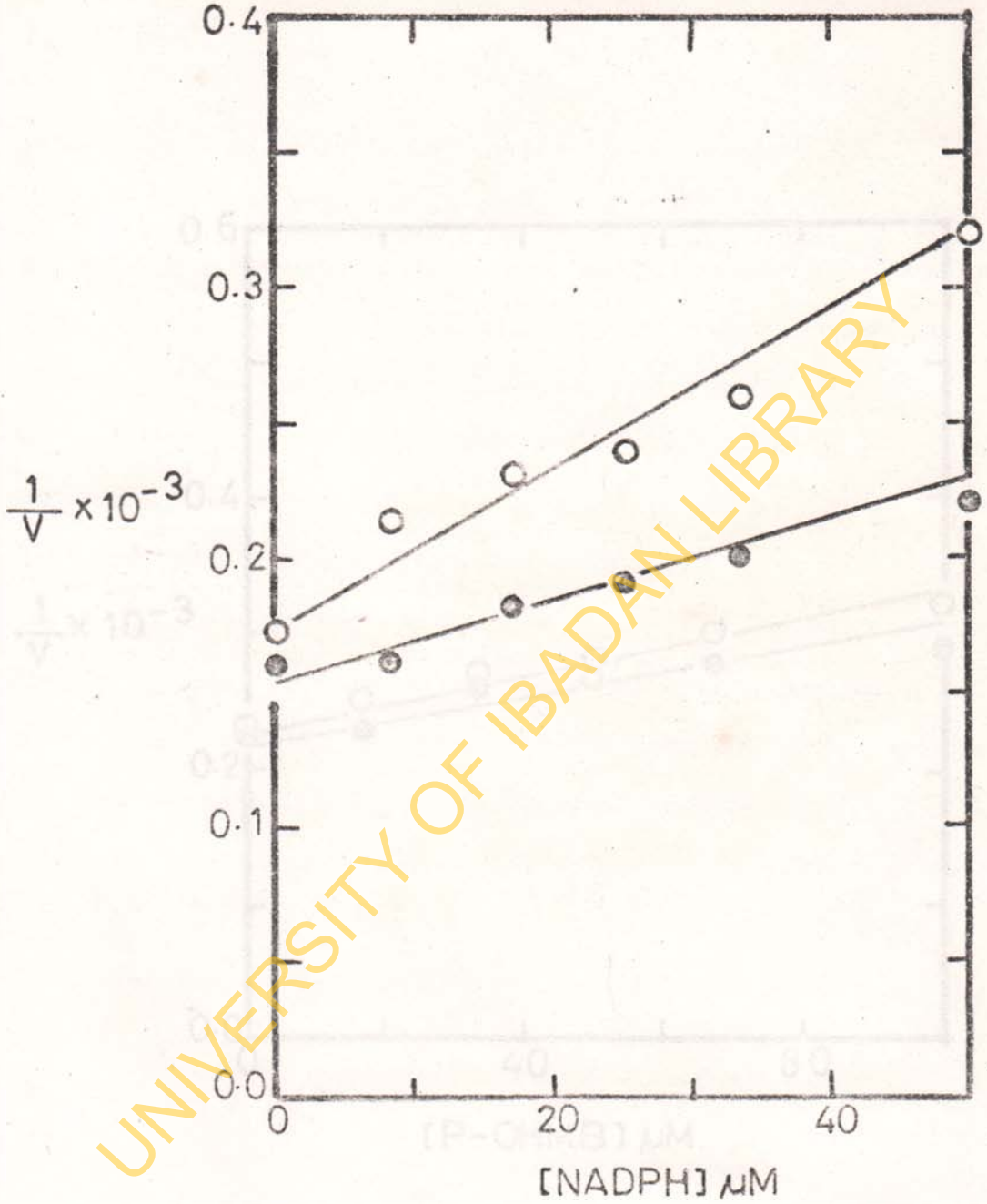


Fig. 21 (a)

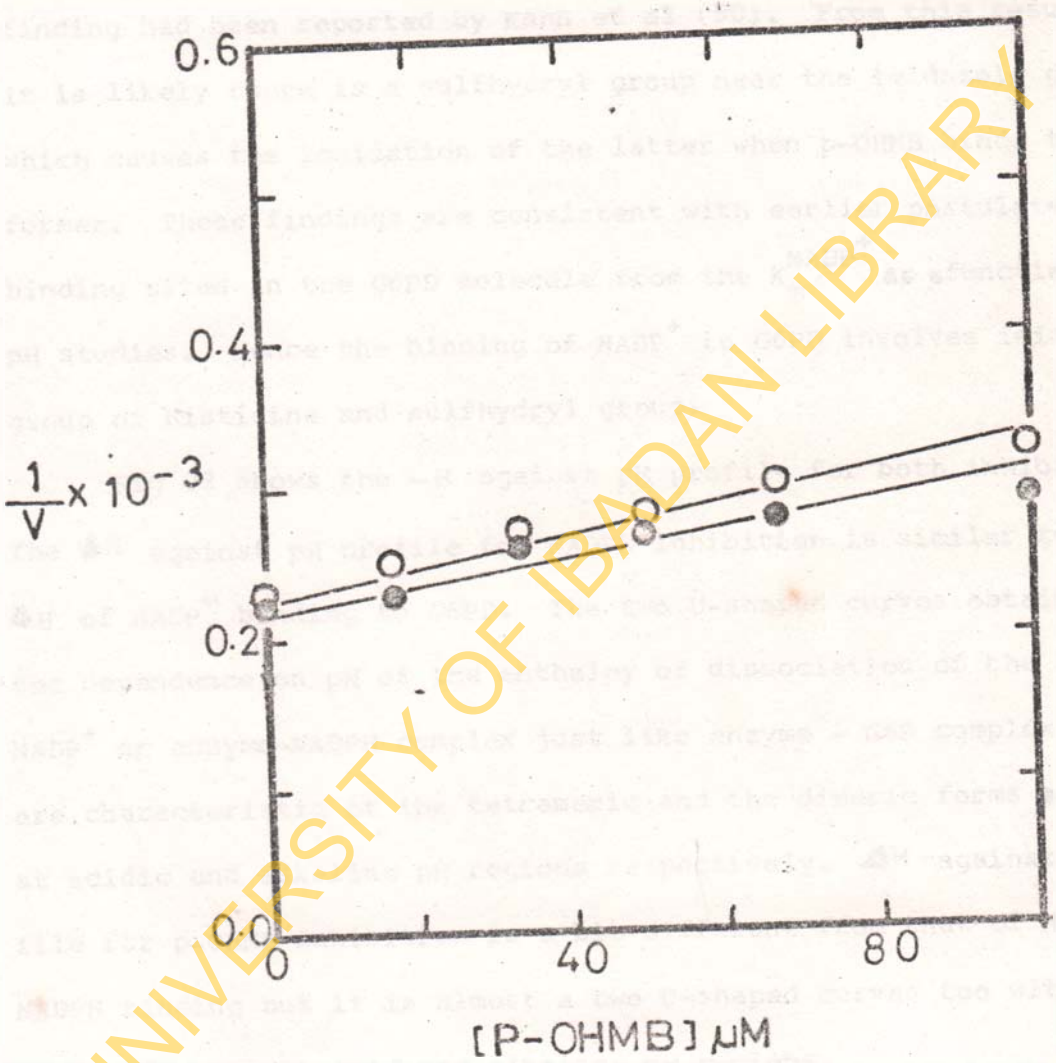


Fig. 21 (b)

sites, they are not the sole groups in the binding site. This same finding had been reported by Kahn et al (50). From this result, it is likely there is a sulfhydryl group near the imidazole group which causes the ionization of the latter when p-OHMB binds to the former. These findings are consistent with earlier postulate of two binding sites in the G6PD molecule from the $K_m^{NADP^+}$ as a function of pH studies. Hence the binding of $NADP^+$ to G6PD involves imidazolium group of histidine and sulfhydryl group.

Fig 23 shows the ΔH against pH profile for both inhibitors. The ΔH against pH profile for NADPH inhibition is similar to the ΔH of $NADP^+$ binding to G6PD. The two U-shaped curves obtained for the dependence on pH of the enthalpy of dissociation of the enzyme $NADP^+$ or enzyme-NADPH complex just like enzyme - G6P complex (127) are characteristic of the tetrameric and the dimeric forms of G6PD at acidic and alkaline pH regions respectively. ΔH against pH profile for p-OHMB inhibition is a bit different from that of $NADP^+$ or NADPH binding but it is almost a two U-shaped curves too with each U-shaped curve at acid and alkaline pH regions.

Fig 24 shows the graph of v/V_o against the inhibitor concentration for the two inhibitors at pH 7.5 and at 34°C as shown in Table 22.

Figure 22: Variation of log K_i with pH for G6PD B^+.

O - O - O 27°C Δ - Δ - Δ 34°C

▲ - ▲ - ▲ 40°C

(a) Inhibition by NADPH

(b) Inhibition by p-OHMB.

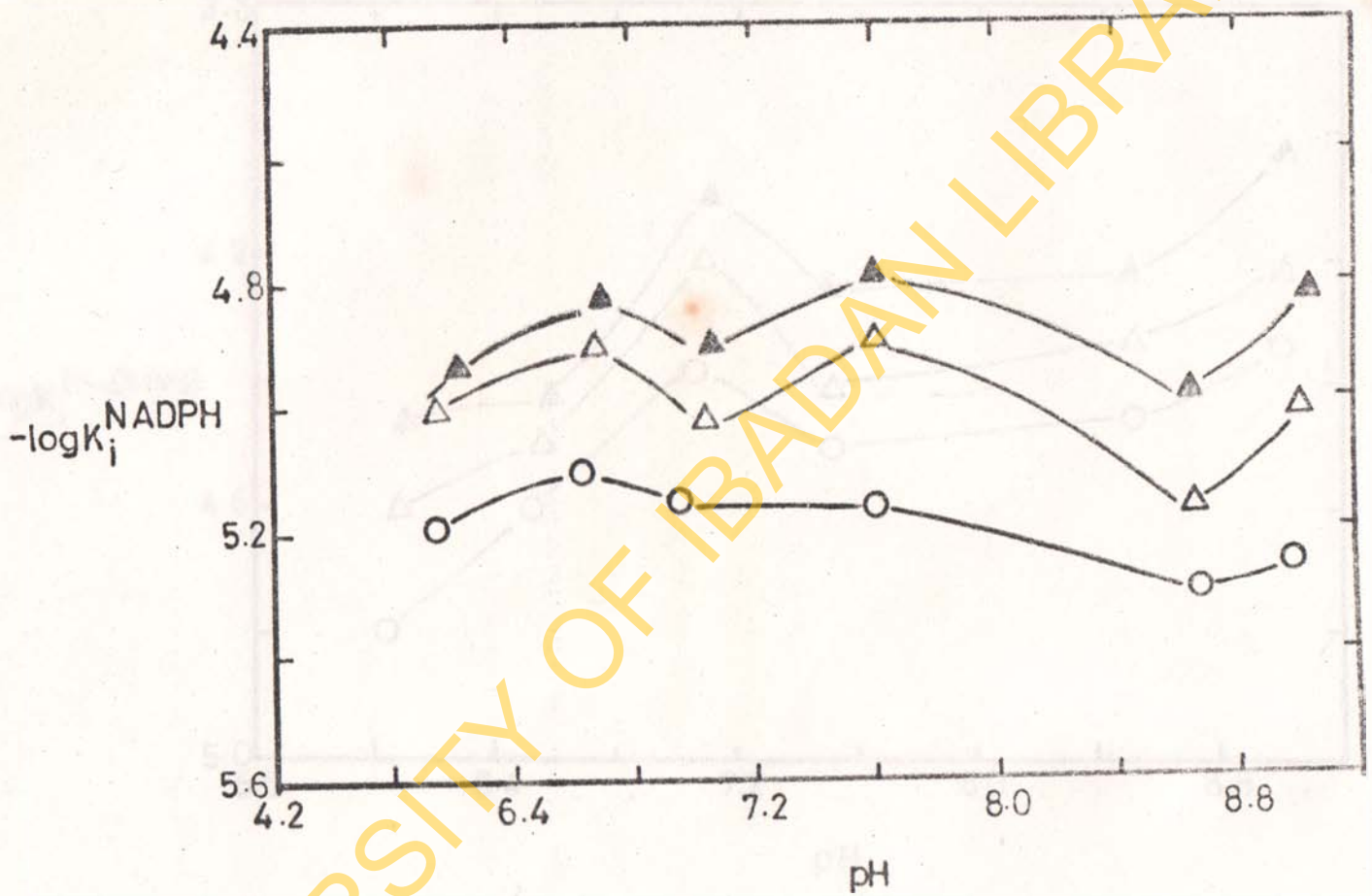


Fig. 22 (a)

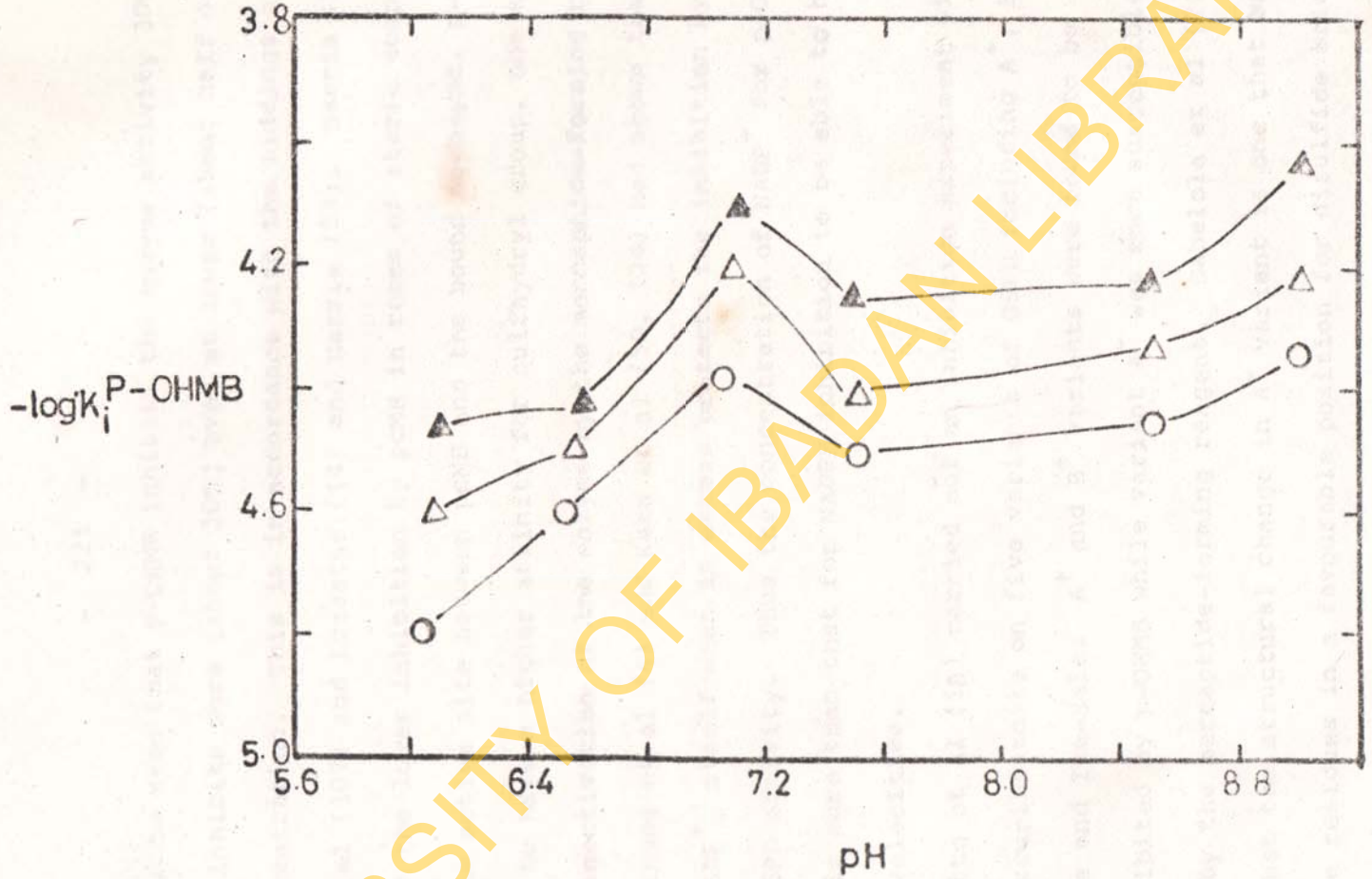


Fig. 22 (b)

Here it could be seen that p-OHMB inhibits the enzyme activity 30% while NADPH inhibits more (about 50%) even at lower (about half of p-OHMB) concentration. This is in accordance with the findings of Balinsky et al (107) and Luzzatto (14) and Harris (53). Harris (53) rationalized the lower inhibition by pcMB in terms of steric competition at the active site between pcMB and the bound coenzyme. p-OHMB is supposed to have a higher affinity for sulfhydryl group, one actually expects an inactivation of the enzyme by the mercaptide-forming reagent. But Chung et al (4) and Kahn et al (50, 124) had shown that the coenzyme, $NADP^+$, itself when in excess protects the inhibition by the p-OHMB of G6PD activity. Thus the concentration of $NADP^+$ for p-OHMB inhibition is more than that for NADPH inhibition to be able to have measurable velocities.

Luzzatto et al (18) carried out an inhibition experiment with p-hydroxymercuribenzoate on five variants of G6PD including A^+, B^+, A^-, Ijebu-Ode and Ita-Bale. A^+ and B^+ variants were found to be equally inhibited by p-OHMB while variant A^- was more susceptible to inhibition by the mercaptide-forming reagent. Babalola et al (24) suggested that the structural change in A^- variant is one that brings two cysteine residues in a favourable position for disulfide bridge formation and the inhibition by p-OHMB is rationalized as suggesting

that the structural abnormality of A^- may lie at or near a cysteine residue (18). The fact that A^- variant is more sensitive to inhibition by p-OHMB than the A^+ and B^+ variants and the fact that A^- variant has more affinity for $NADP^+$ than A^+ and B^+ variants suggest that the sulfhydryl groups are involved in the binding of $NADP^+$ to G6PD variants. This is because the A^- variant with more sulfhydryl groups near the binding site than the other two variants shows more affinites for p-OHMB, which is known to bind specifically to sulfhydryl groups, and for $NADP^+$, which from the inhibition experiments by NADPH and p-OHMB by us and other investigators (4, 17, 18, 86, 107) bind to groups on the enzyme molecule in which sulfhydryl groups are involved.

5.3.2. Conclusion: The foregoing shows the involvement of both imidazolium group of histidine and sulfhydryl group in the binding reactions of $NADP^+$ to G6PD variants. A combination of both substrate binding kinetics of $NADP^+$ and inhibition kinetics of NADPH and p-OHMB shows the importance of these groups in the catalytic activity of human erythrocyte G6PD. The strict relationship between catalytic activity and inactivation of a cysteine residue by p-OHMB suggests that the sulfhydryl group is

Figure 23: Variation of ΔH with pH for the dissociation of G6PD-inhibitor complex.

(a) For inhibition by NADPH.

(b) For inhibition by p-OHMB.

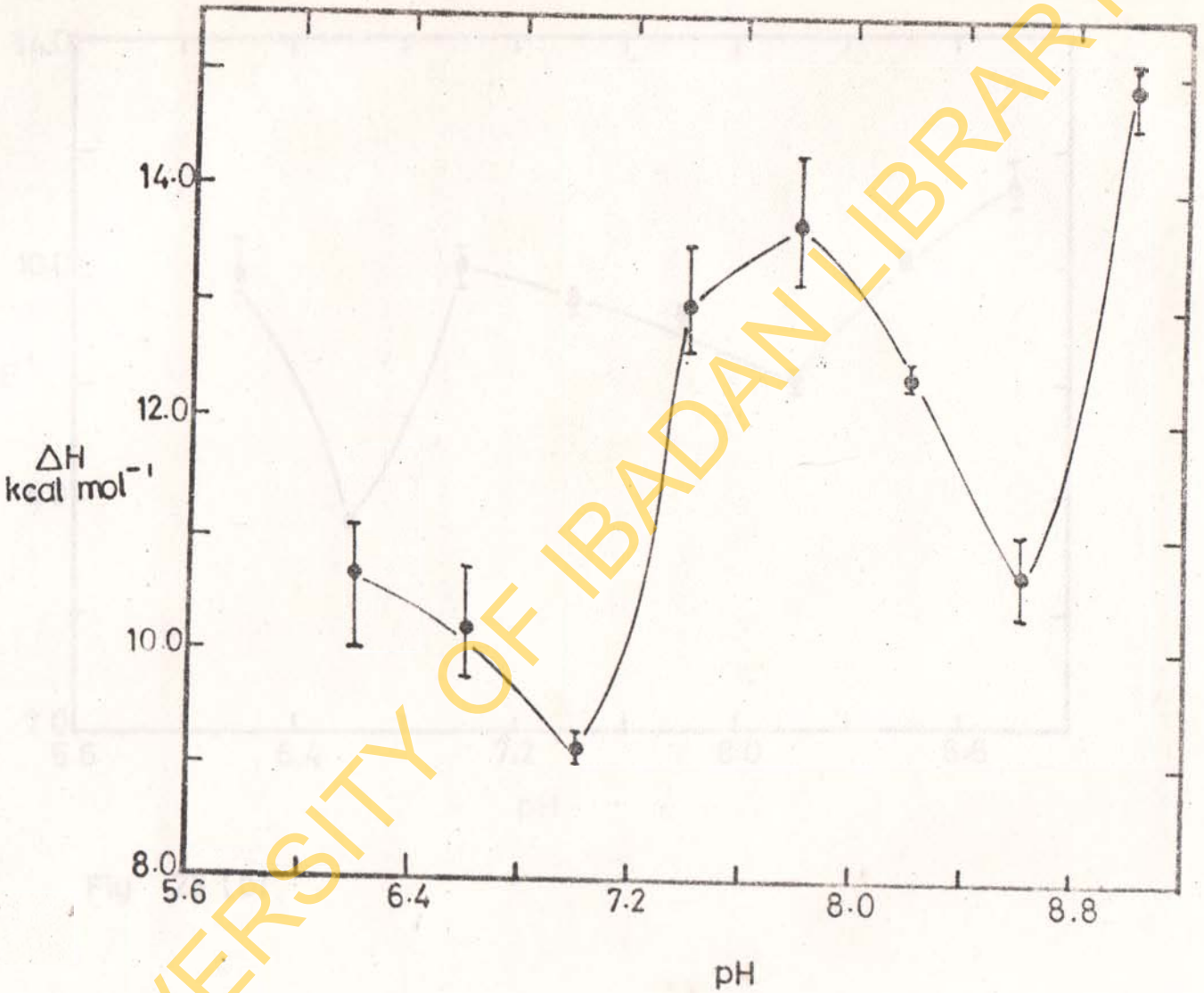


Fig. 23 (a)

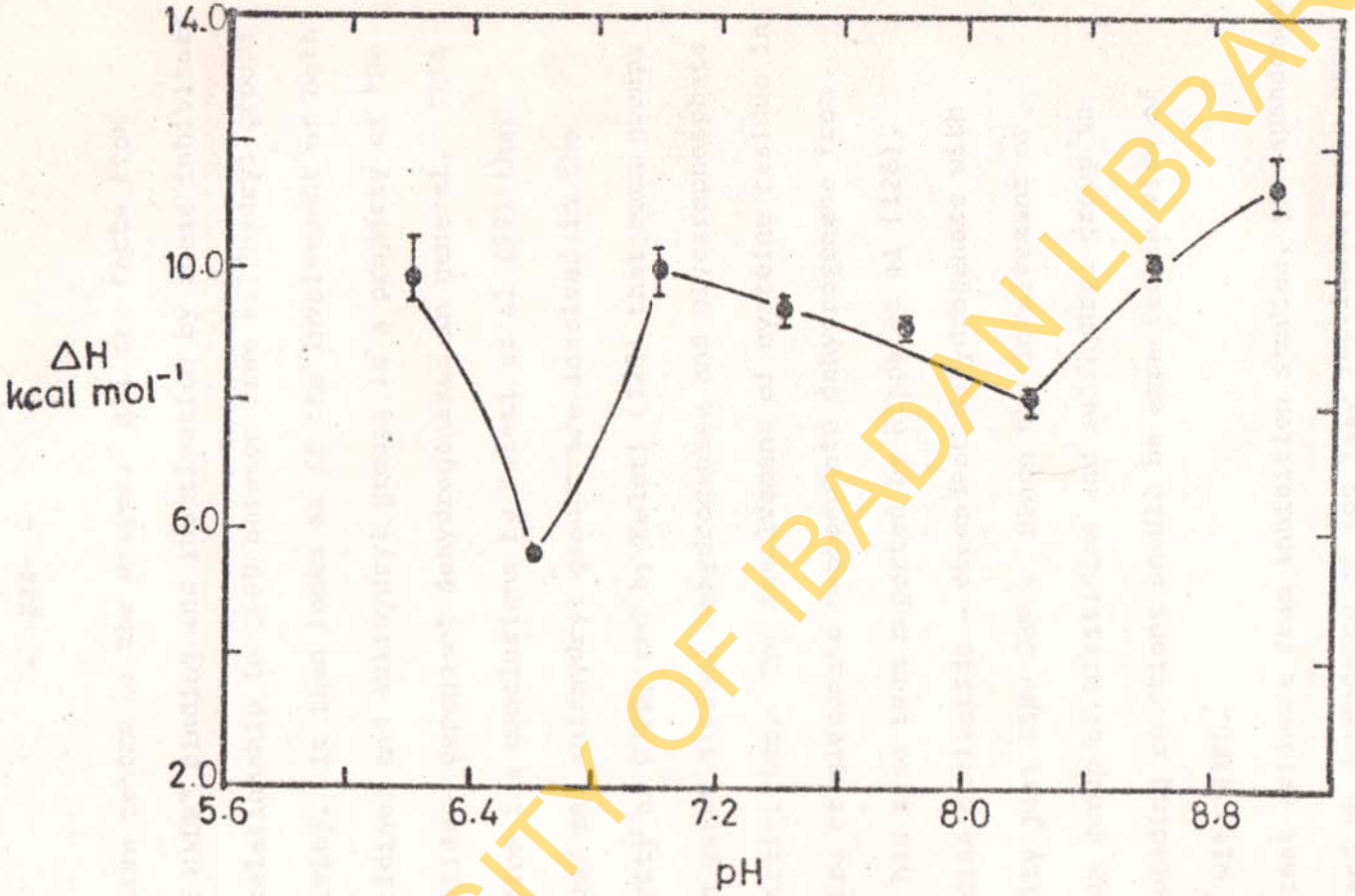


Fig. 23 (b)

part of the active centre of the enzyme. All the facts from the kinetics of $NADP^+$ binding and inactivation by these inhibitors point out the relationship in G6PD between free sulfhydryl groups and enzyme activity. It then looks as if the involvement of both imidazole (histidine) and sulfhydryl groups is a property of the pyridine nucleotide - dependent dehydrogenases in general. This fact is borne from the conclusions by Keleti et al (59) that histidyl residues and sulfhydryl groups are involved in the catalytic activity of G3PD, and by Keleti (120) that both groups are involved in yeast alcohol dehydrogenase and glycerophosphate dehydrogenase activities. The involvement of cysteine residue in the active centre of gluconate 6-phosphate dehydrogenase from Candida utilis has also been reported by Rippa et al (128). G6PD is a pyridine nucleotide - dependent dehydrogenase with broad specificity just like G3PD. Hence the involvement of both imidazolium group of hisitidine and sulfhydryl group in G6PD in $NADP^+$ binding reactions should be expected in view of its similarity with G3PD.

The fact that evidence from inhibition studies, pH dependence of $\log K_m^{NADP^+}$ and pH dependence of $\log V_{max}$ implicates both groups as being involved in the catalytic mechanism of $NADP^+$ binding

Figure 24: Plot of the reaction velocity (v) normalized to the velocity of the uninhibited reaction (V_o) at pH 7.5 and at 34°C.

(a) Inhibition by NADPH with o - o - o 100μM $NADP^+$ and ● - ● - ● 200μM $NADP^+$

(b) Inhibition by p-OHMB with o - o - o 0.33mM $NADP^+$ and ● - ● - ● 0.50mM $NADP^+$.

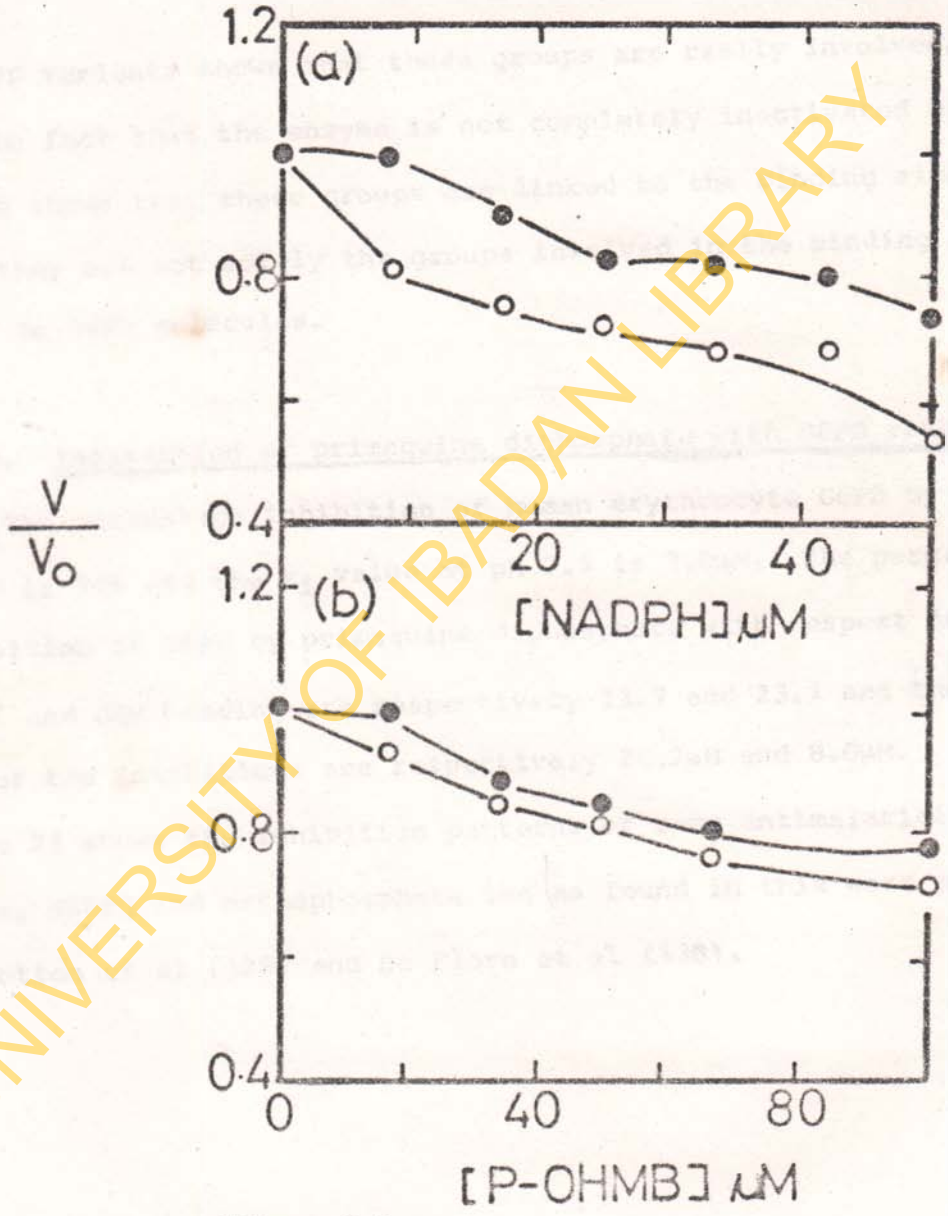


Fig. 24

to G6PD variants shows that these groups are really involved. But the fact that the enzyme is not completely inactivated by p-OHMB shows that these groups are linked to the binding site, that they are not solely the groups involved in the binding of $NADP^+$ to G6PD molecules.

5.3.3. Interaction of primaquine diphosphate with G6PD reaction.

The percentage inhibition of human erythrocyte G6PD by NADPH is 54% and the K_i value at pH 7.5 is 7.0μM. The percentage inhibition of G6PD by primaquine diphosphate with respect to $NADP^+$ and G6P binding are respectively 13.7 and 23.1 and the K_i for the inhibitions are respectively 24.2μM and 8.0μM. Table 23 shows the inhibition patterns of some antimalarial drugs, NADPH and orthophosphate ion as found in this work and by Cotton et al (129) and De Flora et al (130).

TABLE 23

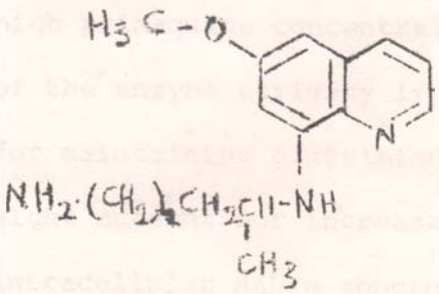
Inhibitor	% inhibition at 100μM $NADP^+$ and 50μM inhibitor	K_i(NADP)	K_i(G6P)	Reference
Primaquine	28.1	1.2×10^{-3}	-	129
Paludrine	30.0	1.1×10^{-4}	-	129
Mepacrine	17.2	1.8×10^{-3}	-	129
Chloroquine	47.5	5.14×10^{-4}	-	129
Daraprime	36.4	8×10^{-5}	-	129
Primaquine Diphosphate	13.7	24.2×10^{-6}	8×10^{-6}	This work
NADPH	54.0	7.0×10^{-6}	-	This work
Orthophosphate	-	6.0×10^{-3}-3.3×10^{-2}	-	130

The inhibition by primaquine is competitive with respect to $NADP^+$ binding and non-competitive with respect to G6P binding but the K_i for G6P binding is less than that for $NADP^+$ binding. This suggests that the inhibition of G6P binding by primaquine diphosphate is more than that for $NADP^+$ binding. Hence binding of the primaquine diphosphate at an

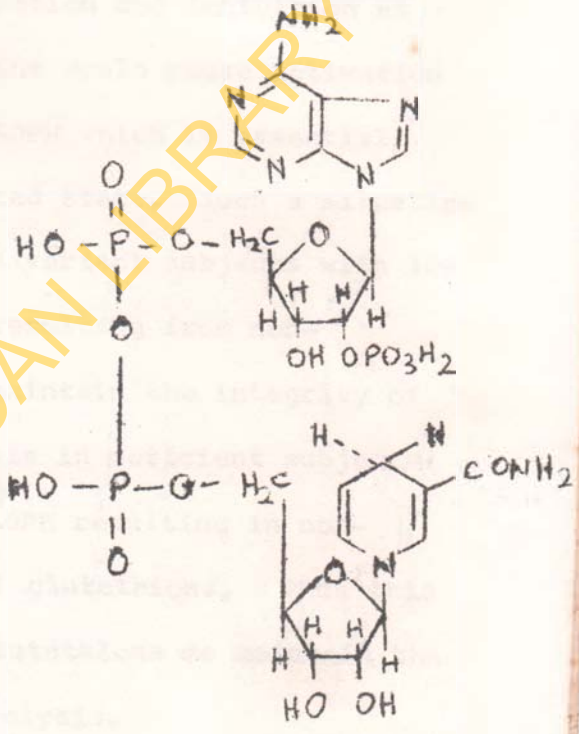
allosteric site to the binding site of G6P is a likely mechanism to explain this finding. The allosteric binding of the inhibitor is such that the conformational change in the G6PD molecule makes it more difficult for the substrate, G6P to bind to its binding site.

The inhibition of $NADP^+$ binding by both NADPH and primaquine diphosphate are competitive (17, 86, 129). Our data show that the Ki for NADPH is less than that for primaquine diphosphate. This observation might be due to the fact that $NADP^+$ and NADPH have similar structures and that primaquine diphosphate binds to the same site but because of its smaller structure, could not inhibit the binding of $NADP^+$ as effectively as NADPH.

Structures of Primaquine and NADPH.



PRIMAQUINE



NADPH.

Inhibition of G6PD activity especially by the antimalarial drugs will have far reaching effects on the metabolism of the cell considering the fact that G6PD plays a major role in the hexose monophosphate pathway shunt which is the sole producer of NADPH. The resulting hemolytic episode may be the consequence of interaction with G6PD reaction by antimalarial drugs in subjects with G6PD deficiency. Primaquine by our finding has a

complex inhibition pattern for $NADP^+$ binding. There is some form of activation at low primaquine concentration and inhibition at high primaquine concentration. Primaquine would cause activation of the enzyme activity if it oxidises NADPH which is essential for maintaining glutathione in the reduced state. Such a situation might account for increased hemolysis in variant subjects with low intracellular NADPH concentration (39) resulting from non-availability of reduced glutathione to maintain the integrity of the red cells. The mechanism of hemolysis in deficient subjects might then be an oxidation of the low NADPH resulting in non-availability of NADPH to reduce oxidised glutathione. Thus this results in non-availability of reduced glutathione to maintain the integrity of the erythrocytes, hence hemolysis.

$$GSSG + H^+ + NADPH \rightarrow NADP^+ + 2GSH$$

The question then is whether individuals in the Nigerian or African populations who experience skin irritations as a side effect of antimalarial therapy have a skin G6PD deficiency or erythrocyte G6PD deficiency or both. It will be a very useful finding to investigate this problem in respect of the G6PD A^- variant subjects and the many antimalarial drugs.

SUMMARY

The Michaelis constant, K_m and the maximum velocity, V_{max} for the binding of Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) to human erythrocyte G6PD variants A^+, B^+ and A^- in Tris-borate and Triethylamine-borate buffers were determined as a function of pH and temperature. The log V_{max} versus pH curves for each of the G6PD enzymes increases monotonically between pH 5.90 and 7.0 and becomes constant from about pH 7.5 upwards. These curves, and their temperature - dependences, are compatible with the presence of a single ionizable group which, in its conjugate acid form, renders the enzyme-substrate complex inactive. The pK of this group is 6.59 at 27°C and its enthalpy of ionization is 13.5 kcal $mole^{-1}$. The log K_m versus pH curves show a broad plateau between pH 6.7 and 8.2, interrupted by a sharp minimum at pH 7.1 for all the enzyme variants. An explanation of this unusual behaviour in terms of cooperative ionization of groups on the enzyme and enzyme-substrate complex which may be linked to the association-dissociation behaviour of the enzyme is proposed. The imidazolium group of histidine and sulfhydryl

group may contribute significantly to the observed pH dependent behaviour of both V_{max} and $K_m^{NADP^+}$. For each G6PD variant, the plot of ΔH of $NADP^+$ binding against pH gives the shape of a two U-shaped curves intersecting at a point of maximum ΔH. The position of intersection of the two U-shaped curves varies for the three G6PD variants. The amino acid changes in the different genetic variants of the human erythrocyte enzyme therefore confer varied reactivity in terms of differences in pH of maximum ΔH for the different enzymes. Each U-shaped region of the ΔH versus pH curve coincided with the pH of stability of the tetramer and dimer forms of the enzyme. The tetramer of the enzyme being more stable at the more acidic pH.

The G6PD enzyme variants show no cooperative interactions among the $NADP^+$ binding sites in Tris-borate and Triethylamine-borate buffers of low ionic strength but are cooperative in buffers of higher ionic strength. This observation in the variation of cooperative interactions with pH and ionic strength is interpreted in terms of high populations of tetramers and dimers at the low and high ionic strengths respectively.

The inhibitor constants, K_i for NADPH and p-OHMB with respect to $NADP^+$ binding to G6PD B^+ were determined as a function

of pH and temperature. The enthalpy associated with the inhibition varies with pH in a similar way as the ΔH of the $NADP^+$ binding indicating that imidazolium group of histidine and sulfhydryl group also influence the pH dependence of NADPH and p-OHMB inhibitions in the same way as does the $NADP^+$ binding thermodynamics.

Interaction of primaquine as a typical antimalarial drug with G6PD activity may result in hemolytic episode in deficient enzyme subjects through the impaired generation of NADPH which is essential for maintaining sufficient level of reduced glutathione.

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