BIOCHEMICAL STUDIES OF GENETIC VARIANTS

OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

IN NIGERIA

BY

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

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CONTENTS

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

G6PD = GLUCOSE-6-PHOSPHATE DEHYDROGENASE

NADP = NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

NADPH = REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE.

NADPase = NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATASE

G6P = GLUCOSE-6-PHOSPHATE

2dG6P = 2-deoxy GLUCOSE 6-PHOSPHATE

NEM = N-METHYLMALEIMIDE

PCMB (HMB) = p-CHLOROMERCURI BENZOATE

TRIS = Tris (Hydroxymethyl) Amino Methane.

LIST OF TABLES

(1) Comparative biochemical studies have been carried out on five genetic variants of human erythrocyte glucose-6-phosphate dehydrogenase in Nigeria. These variants A, A⁻, B, Ijebu-Ode and Ita-Bale in order of decreasing electrophoretic mobility, are found with different frequencies in Nigeria. The first three variants are common (A⁻ is associated with deficiency) while the latter two are rare (below polymorphic frequency) and have been characterised for the first time.

(2) Partially purified preparations of the five enzyme types have been used in a number of kinetic studies. All the variants have approximately the same affinity for glucose-6-phosphate but when the substrate analogue 2-deoxy glucose-6-phosphate is used, Ijebu-Ode emerges as the variant with the lowest affinity for it. The effect of pH on the activities of the two rare variants (carried out according to WHO recommendation) indicate that both of them have a slightly biphasic pattern. The rate of inactivation of the two rare variants as compared with the B variant show that Ita-Bale had a minimally increased thermolability while the thermolability of Ijebu-Ode was markedly increased.

(3) A detailed analysis of how pH affects two main kinetic parameters of enzyme reactions, namely V_{max} and K_m, for glucose 6-phosphate has been carried out for all the variants. For all the variants, there is an overall steady increase of V_{max} with

SUMMARY

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pH (using tris-borate-citrate, tris-borate, tris-borate-methylamine buffers) over the entire range explored from pH 5.5 to 9.5. In addition, A^-, Ijebu-Ode and Ita-Bale have a plateau in the pH region between 7.5 and 8.2, whereas A and B have a 'peak and trough' in the same region. Characteristic patterns were obtained for each variant on plotting $\log V_{max}$, $\log K_m$ and $\log \frac{V_{max}}{K_m}$ versus pH. From the waves in the plots, the pKs corresponding to ionised imidazolium and thiol residues in free enzymes and enzyme-substrate complexes were obtained. The data also indicate the absence of thiol residue in or near the active centre of Ijebu-Ode. The K_m data obtained for type A^- have not been always consistent and a possible explanation is presented.

(4) The dependence of enzyme activity on NADP and NADPH concentration was examined for the five variants. Sigmoidal saturation kinetics of A, B and Ita-Bale variants for NADP clearly differenciate them from the near-hyperbolic kinetics of A^- and Ijebu-Ode enzyme types. Two dissociation constants (K_{s_1} and K_{s_2}), corresponding to states of low and high affinity for NADP, have be[en] obtained for each variant. An explanation about the mechanism of such interactions is also presented. The complex kinetics of interaction of the enzyme variants with NADPH has implicated this metabolite as both an activator and inhibitor, but A^- type emerges as the least activated and inhibited. The sum total of the

methylamine buffers) over the entire range explored from pH 5.5 to 9.5. In addition, A^-, Ijebu-Ode and Ita-Bale have a plateau in the pH region between 7.5 and 8.2, whereas A and B have a 'peak and trough' in the same region. Characteristic patterns were obtained for each variant on plotting $\log V_{max}$, $\log K_m$ and $\log \frac{V_{max}}{K_m}$ versus pH. From the waves in the plots, the pKs corresponding to ionised imidazolium and thiol residues in free enzymes and enzyme-substrate complexes were obtained. The data also indicate the absence of thiol residue in or near the active centre of Ijebu-Ode. The K_m data obtained for type A^- have not been always consistent and a possible explanation is presented.

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to the suggestion that G6PD is a regulatory (allosteric) protein.

(5) The five genetic variants exhibit differential interactions
with some potent inhibitors which bind to the sulphydryl groups,
like N-ethylmaleimide and p-chloromercuribenzoate. Of all the
variants, Ijebu-Ode is strickingly resistant to PCMB and totally
resistant to NEM. An explanation for the resistance of Ijebu-Ode
to these inhibitors is suggested.

(6) Enzyme inactivation as a function of temperature exhibits
distinctive profiles for all the variants and Ijebu-Ode
particularly displayed a unique picture. The implication of the
differences observed in this thermal inactivation profile studies
are also discussed.

(7) Finally, it is suggested that differences observed in the
catalytic and kinetic properties among the five variants of G6PD
are a reflection of the structural differences among them which
also implies different mutational changes in the structural
locus for red cell glucose-6-phosphate dehydrogenase.

INTRODUCTION

Since the discovery of Glucose 6-phosphate dehydrogenase (glucose-6-phosphate: NADP oxidoreductase, E.C.1.1.1.49) in yeast by Warburg & Christian (1931), a colossal amount of work in the field of biochemistry, genetics and medicine has been carried out on this enzyme which has been isolated from a variety of sources. G6PD is the enzyme catalysing the first step in the metabolism of glucose through the pentose phosphate pathway, and at the same time one of the main sources of supplies of NADPH in all cells. The significance of this enzyme in metabolism is more appreciated when considered in the light of biochemical changes associated with its deficiency in the red cells of human beings.

(1) Distribution:

G6PD is found almost ubiquitously in living organisms. Thus, it has been described in a variety of micro-organisms (Glaser & Brown, 1955; Noltmann, Gubler & Kuby, 1961; Olive & Levy, 1967) and in most tissues of higher animals (Glock & McLean, 1955; Beutler, 1965). Extensive purification and characterisation has been carried out from yeast (Noltmann, Gubler & Kuby, 1961), rat mammary gland (Levy, Raineri & Nevaldine, 1966), and human erythrocytes (Marks, Szeinberg & Banks, 1961; Kirkman & Hendrickson, 1962; Chung & Langdon 1963a, 1963b; Yoshida, 1966).

DISTRIBUTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

<u>Fig. 1.</u> World Distribution of G6PD Deficiency (from Motulsky and Campbell-Kraut, 1961). It is to be noted that the gene is not the same in all areas shown. For instance, A⁻ predominates in Africa and North America; Mediterranean predominates in Italy, Greece and the Middle East; while Canton is in the Far East.

(3) <u>Geographical distribution of G6PD deficiency and possible relationship to malaria:</u>

Most of the work on the world distribution of G6PD genetic variants have been limited to those involving more or less pronounced enzyme deficiency (see Fig. 1). The reason for this is not far fetched. The method of detection of G6PD polymorphism has consisted mainly of various quantitative assays while only a small proportion of investigations has also employed qualitative technique. Enzyme deficiency of the severe type (less than 5% of normal) is very prevalent in the Mediterranean (Motulsky & Campell-Kraut, 1961; Kirkman, Schettini & Pickard, 1964) and in the Middle East (Szeinberg, Sheba & Adam, 1958; Szeinberg, 1963; Beutler, 1965) populations. Enzyme deficiency of the mild type (about 10-20% of normal) is common in people of African ancestry (Carson <u>et al</u>. 1956; Gilles & Taylor, 1961; Boyer, Porter & Weilbacher, 1962; Kirkman & Hendrickson, 1963; Beutler 1965) and in the Far East (Motulsky & Campell-Kraut, 1961; Beutler, 1965).

The hypothesis that malaria has been a selective force favouring the spread of the gene for G6PD deficiency (Motulsky, 1960; Allison & Clyde, 1961) has been highly controversial. There is certainly a remarkable coincidence between the geographical incidence of the two conditions although Kidson & Gorman (1962) and Kruatrachue, Charoenlarp, Chongsuphajaisiddhi,

TABLE I

TYPES OF G6PD VARIANTS

No.	Variant	Population	G6PD activity (% of Normal)	Electrophoretic mobility (% of normal)	K_m G6P µM	K_m NADP µM	2AdG Utilisation	Heat stability	pH Optimum	Population Frequency
1.	Normal B	Various	100	Normal 100	50-70	2.9-4.4	4	Normal	Truncate	Usual
2.	"Baltimore"	Norwegian	100	Slow 70 (NBB) 90 (trib)	?	?	?	?	?	Rare
3.	A	Negro	88	Fast a 110	Normal	Normal	4	Normal	Truncate	Very common
4.	Baltimore Austin	Negro	75	Slow 90	68	3.1	4	Normal	Truncate	Rare
5.	Dallas Austin	Negro	72	Slow 80	68-72	3.3	4	Normal	Truncate	Rare
6.	Markham	Italian	4-60	Fast 85	Increased	Increased	?	Normal	?	Rare
7.	Kerala	Asian Indian	50	Slow 75 (NBB) 90 (trib) 23	1.5	7.4	Normal	Biphasic	Rare
8.	Tel-Hashomer	Sephardic (Yemenite) Jew	15-40	Slow 60-70	30-40	?	Normal	Normal	Slightly biphasic	Rare
9.	Columbus	Negro	35	Normal	Normal	Normal	Normal	?	?	Rare
10.	Greek	Greek	12-45	Normal	?	?	?	?	?	Common
11.	Seattle Baptist Greek	Greek	8-24	Slow 90	15-85	2.4-2.8	7-11	Normal	Slightly biphasic	Rare
12.	"Loyola"	Irish German	45	Slow 90	?	?	?	?	?	Rare
13.	A-	Negro	8-20	Fast o 110	Normal	Normal	4	Normal	Truncate	Common
14.	Canton	North Chinese	4-24	Fast 105	20-36	2.0-2.4	4-45	Slightly reduced	Biphasic	Common
15.	West Bengal	Asian Indian	9	Slow 90 (NBB) 96 (trib)	31	6.6	4	Normal	Truncate	Rare
16.	Mediterranean	Greek Sardinian Sephardic Jew (16) Asian Indian (7)	0-7	Normal a 17-86	1.2-1.6	25-37	Low	Biphasic	Common

TABLE 1 (CONTINUED)

SOME INHERITED RED CELL G6PD NON-SPHEROCYTIC HAEMOLYTIC VARIANTS

No.	Variant	Population	RBC Activity % of Normal	Electrophoretic mobility (% of normal)	K_m G6P pH	K_m NADP pH	2d-G6P utilization	Heat stability	pH Optimum	Population frequency
17	Oklahoma	West Europe	4—40	Normal	157—200	20	4	Low	Narrow	Rare
18	Chicago	West Europe	9—16	Normal	58—76	3.1—3.7	4	very low	Trimodal	Rare
19	"Green"	West Europe	0	Slow 90	?	?	?	Labile (in cold)	?	Rare
20	Ohio	Italian	2—6	Fast 110	Slightly increased	Slightly increased	Normal	very labile	?	Rare
21	"Tübingen"	Southern Germany	0.3	100	Slightly increased	Slightly increased	?	?	?	Rare
22	"Berlin"	German	0—1	100	Slightly increased	Slightly increased	0	?	?	Rare
				VARIANTS WITH HIGH ACTIVITY						
23	"Hektoen"	US White	400—500	100	?	?	?	Low	Biphasic	Rare
24	"Hartford"	Negro	200	100	31.6	6	2.5	Normal	9	Rare
25	N.C. India	Asian	2—6	Slower than normal						
				VARIANTS ACQUIRED AFTER 1966						
26	West Bengal	Asian	9	Slower than and slower than A (105)	Normal	Normal	?	Normal	?	Rare
27	"Kaplan"	African	Normal	90	19	3	10—15	Normal	8.5	Rare
28	Kabyle	Algeria	15—36	Fast 110	32.8—35.6	3.64	2.1	Normal	7 ± onto	Rare
29	Athens	Greek	15	Fast 110	38.9—42.5	4.5—4.9	1.8—2.8	Normal	Trimodal	Rare
30	Kephalonia	Greek	20	Slow 90	19.2—26.6	2—2.2	6.1	Normal	Slightly biphasic	Rare
31	Attica	Greek	90	80	60	3	4	Normal	Slightly biphasic	Rare
32	Seattle-like (perhaps identical to "Seattle")	Greek	8—11	80	90	2.7	11	Low	Slightly biphasic	Rare
33	Ijebu-Ode	Nigeria	100							
34	Ife-Ode	Nigeria	100							

The Data for Variants 1—16 were extracted from W.H.O. (1967) Wellc.p.ll

Data for Variants 25—34 are from the following references:-
25 & 26 Marrow and McGlashy (1968) Am. Hum. Genet. 31 373
27 & 28 Kaplan, Ross, Marinos and Stoeffel (1967) Enzymological Ch
29—32 Satani, Lemerial, Chan and Iurmito (unpublished)
33 & 34 Present thesis.

Harinasuta, (1962) found no evidence in support of this concept in areas with high malaria but no G6PD deficiency. Field studies aiming to establish whether the incidence and/or severity of malaria was less in G6PD deficient subjects have been conflicting (Gilles, Hendrickse, Allan, Reddy, Lindner & Gupta, 1967). However, recently, Luzzatto, Usanga & Reddy (1968) clearly demonstrated in heterozygous deficient females that deficient red cells have a much lower rate of parasitisation than normal cells. This is the first direct evidence of the selective advantage with respect to malaria afforded by the gene for G6PD deficiency in a population with endemic malaria.

(4) Qualitative variations of glucose-6-phosphate dehydrogenase:

The development of sensitive research techniques has made it possible to detect various types of G6PD exhibiting qualitative rather than quantitative differences. The starch-gel electrophoretic technique (Smithies, 1955) has proved to be an invaluable tool for detecting multiple forms not only of G6PD (Boyer et al. 1962; Shows, Tashian & Brewer, 1964) but of other enzymes as well.

On the basis of electrophoretic mobility on starch gel, several common G6PD phenotypes have been identified. The nomenclature of these variants had been confusing for some time. In 1966, the World Health Organisation convened a Study Group which recommended the following (WHO, 1967). The most common

and ubiquitous variants of G6PD, to be called B, should be used as reference with which all 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 is common among people of African ancestry, should be called A. The variant commonly found among the same people, which has the same electrophoretic mobility like A, but is associated with enzyme deficiency, should be called A^-. All other variants should be given a geographical name related to the area where they are discovered. Among these, the most widespread are the "Mediterranean" type - the variant with enzyme deficiency responsible for favism in Greece, Sardinia and the Middle East - and the Canton variant, also associated with enzyme deficiency, and found in China. All other variants are encountered sporadically (see Table I).

It is presumed, also on the basis of genetic evidence (see section 6) that G6PD variants represent the product of mutations at the structural locus for this enzyme. Accordingly, we expect that at least the majority of the variants will differ from each other by single amino acid substitutions. This has now been proved by Yoshida (1967) who has compared by peptide mapping the A and B types, and reported that A contains asparagine in place of aspartic acid in one specific tryptic peptide.

TABLE II

DISTRIBUTION OF G6PD TYPES IN NIGERIA

MALES	(n=583)	FEMALES		(n=265)
TYPE	%	TYPE	EXPECTED	FOUND
A	23.0	A/A)		
B	56.4	A/A⁻)	14.8	15.8
A⁻	20.6	B/B)		
		B/A⁻)	54.9	60.4
		A/B	26.0	20.4
		A⁻/A⁻	4.2	3.4

The possible number of genotypes obtainable from the females is
six as indicated in the table. Electrophoretically, only four
phenotypes can be recognised since it is difficult to distinguish
A/A⁻ from A/A and B/B⁻ from B/B because of the lightness in the
intensity of staining displayed by A⁻ component. The expected
frequencies of the female were calculated from the observed frequency
of the male phenotypes by assuming that the latter are equivalent to
gene frequencies and that a Hardy-Weinberg type of equilibrium
obtains in the population.

(5) <u>G6PD variants in Nigeria</u>:

It has been shown since 1960 (Gilles & Arthur 1960) that G6PD deficiency has a high incidence in Nigeria and the estimates given by various investigators for male subjects are of approximately 20% (Edozien 1964; Porter <u>et al</u>. 1964).

More recently, the incidence of variants with normal activity but different electrophoretic mobility has also been established (Luzzatto, Allan & De Flora, 1965; Luzzatto & Allan, 1968). It has thus been found that among the subjects with normal activity, both the A and B types are very common (see Table I). We are therefore dealing in this population with a three alleles polymorphism, including B, A, and A⁻. A number of sporadic variants has also been encountered, and two of these will be described in detail. None of the latter attains polymorphic frequency.

(6) <u>Genetics</u>:

Genetic studies have revealed that the gene for G6PD is located on the X-chromosome. Therefore males can only produce one type of G6PD, but females can produce either one or two. With regard to enzyme deficiency, this implies that males will be either "normal" or "deficient", but females may be either "normal" or "intermediate" or "deficient". It is known that in the females only one X-chromosome in each cell is functional

(Lyon hypothesis: Lyon 1961, 1962). As a result, normal females have the same level of G6PD like normal males and deficient females have a similar degree of enzyme deficiency as deficient males. Genetic studies have demonstrated that not only the G6PD deficiency trait, but also the electrophoretic polymorphism of G6PD are sex-linked. It has been pointed out that the G6PD type having normal electrophoretic mobility may be found in association with normal enzyme activity (B) or with extremely low enzyme activity (Mediterranean). Similarly, the common G6PD type with greater electrophoretic mobility can be found associated with normal enzyme activity (A) or with low enzyme activity (A$^-$). These data might be interpreted in terms of interplay between two genetic loci: one controlling the structure of the enzyme, the other its rate of production (see Fig. 2, Model I). Alternatively, structure and rate of production of the enzyme might be controlled by one and the same locus on the X-chromosome (Fig. 2, Model II). The two models make different predictions which can be tested. According to Model I, enzyme types B and Mediterranean should have the same structure. This was soon disproved by careful analysis of their kinetic properties which turned out to be different (Kirkman et al. 1964). Again, according to Model I, enzyme types A and A$^-$ should have the same structure. In this case, it was claimed that kinetic parameters were identical; this has now been found incorrect (chapters III - V). Furthermore,
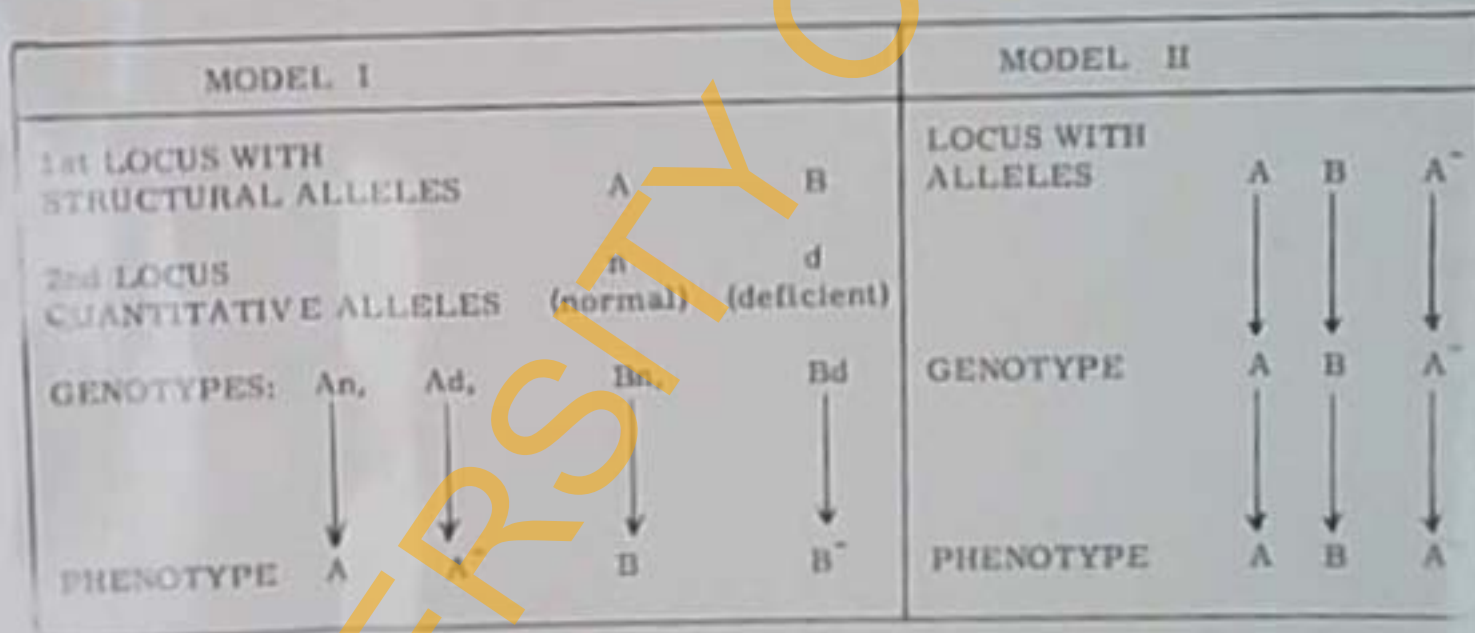
Fig. 2. Two possible models of the genetic determination of G6PD deficiency, including (I), not including (II) a non-structural locus controlling rate of synthesis of the enzyme.

sharp differences between A and A⁻ have been revealed by column

chromatography and by thermostability studies, (Luzzatto & Allan,

1965). Therefore, it appears that Model II, but not Model I, is

consistent with all available biochemical and genetic (Nance,

1964) data.

(7) <u>The mechanism of haemolysis of G6PD deficient red cells</u>:

The activity of G6PD is higher in young red cells than in

old ones (Marks, Johnson & Hirschberg, 1958). On ingesting

primaquine, it is the old red cells which are lysed first.

The mechanism of haemolysis of red cells is not completely

explained but it clearly seems to derive from a unique

susceptibility intrinsic to the red cells, rather than from

abnormal extracorpuscular factors developed in response to drug

administration (Dern, <u>et al</u>. 1954). Both Carson <u>et al</u>. (1956)

on one hand, and Szeinberg & Marks (1961) on the other hand,

demonstrated that in primaquine sensitive individuals, the red

cells can not produce enough NADPH. This seems to be the major

metabolic abnormality in G6PD deficient cells and it is therefore

most likely that drug-induced haemolysis is linked to it. The

precise mechanism, however, is unknown. Various possibilities

have been entertained. For instance, NADPH is essential for

reduction of glutathione mediated by glutathione reductase;

reduced glutathione is important in order to preserve the

structural integrity of the red cells (Fegler 1952) and lack of

reduced glutathione might therefore explain haemolysis.
NADPH is also required for lipid synthesis which might be
essential for the structure of the membrane (Beutler, 1959)
and also plays a role in peroxide detoxification mediated by
glutathione. Cohen & Hochstein (1961, 1964) have suggested
that this may be critical for red cell survival. NADPH might
also be involved in preserving in the red cell membrane sulphydryl
groups which might be essential for its integrity.

(8) <u>Phenotypic variations of G6PD Activity</u>:

Many factors such as hormones and disease states may affect
the level of enzyme activity in red cells. Thyroid hormone has
been shown to stimulate the pentose phosphate pathway of
carbohydrate metabolism in liver cells (Necheles & Beutler, 1959).
Increased G6PD activity occurs in patients with hypothyroidal
states (Pearson & Druyan, 1961; Kidson & Gajdusek, 1962). In some
disease states like viral hepatitis and diabetic acidosis, haemolytic
anaemia in G6PD deficient individuals may occur although very
rarely in the absence of drug administration (Gant & Winks, 1961;
Tarlov, Brewer, Carson & Alving, 1962; Burka, Weaver & Marks, 1966).

(9) <u>Molecular structure of G6PD</u>:

G6PD has been crystallised from yeast by Noltmann, Gubler &
Kuby (1961). These authors have shown that the enzyme has a
molecular weight of approximately 106,000. As far as the red cell

enzyme is concerned, data on the molecular weight have been controversial. Kirkman & Hendrickson (1962) and Tsutsui & Marks (1962) estimated a molecular weight of approximately 105,000 by sucrose gradient centrifugation. Chung & Langdon (1963a), on more purified preparations, obtained a value of 190,000 by the moving boundary method. More recently, Yoshida (1966) found by sedimentation equilibrum a value of 240,000 for the molecular weight of crystalline G6PD (type B). Yoshida (1966) also determined the molecular weight in the presence of 4 M guanidinium chloride and obtained a value of 43,000. On the basis of these findings, he suggested that the enzyme is made up of 6 identical sub-units. Rattazzi (1967) obtained by careful work based on the use of gel filtration on Sephadex G-200 the value of 104,000 for more crude preparations of red cell G6PD. He also obtained evidence for the presence of a small fraction (up to a few percent) of active enzyme having a molecular weight about twice that of the major component. In summary, there are two sets of values in the literature for the molecular weight of human erythrocyte G6PD; one set around 100,000 and one set around 200,000. Comparative inspection of the data reveals that the values around 200,000 (Chung & Langdon 1963a; Yoshida, 1966) were obtained at high enzyme concentration between 0.04 and 3mg./ml. whereas the values around 100,000 (Kirkman & Hendrickson, 1962; Tsutsui & Marks, 1962; Rattazzi, 1967) were obtained at very low enzyme

concentration (approximately 2µg./ml.). One rather likely explanation for these discrepancies is that the enzyme can truly exist in at least two interconvertible forms in the sense of a monomer-dimer equilibrium. If this interpretation is correct, then we might presume that at physiological enzyme concentration the molecular weight is approximately 100,000.

A related controversy regards the number of polypeptide chains making up the molecule of G6PD. The only data on N-terminal groups have been reported by Chung & Langdon (1963a), who found both N-terminal tyrosine and alanine. Yoshida (1966, 1967) on the basis of the analysis of tryptic digests and of his data on the molecular weight (see above) has discounted the evidence for two N-terminal amino acids and suggested that only one type of polypeptide chain is present; however, no data on N-terminal groups are in his paper. Clearly, more definitive data on primary structure are urgently needed.

In spite of the insufficiency of these structural data, what has been clearly established is the crucial role of one of the two substrates of G6PD, namely NADP, in stabilizing the structure of the enzyme. The first data came from the work of Carson, Schrier & Kellermeyer (1959), who found that red cell stroma could inactivate G6PD and further showed that this effect was due to the presence in the stroma of NADPase activity which would

hydrolyze NADP present in the enzyme solution and thus lead to

inactivation of G6PD. Later Marks, Szeinberg & Banks (1961)

and Kirkman (1962) showed that NADP can protect G6PD from

inactivation during incubation at $37^{\circ}C$ and that this effect is

concentration-dependent. Similar results have later been obtained

by other workers. The data on the effect of NADP on thermal

denaturation profiles of G6PD (see chapter VI) suggest that the

role of this substance is to specifically stablize the tertiary

structure of the protein. While these data on protection from

inactivation are purely qualitative, Chung & Langdon (1963b) were

able by fluorescent techniques to show that two molecules of

NADP per molecule of enzyme are strongly bound to G6PD after

extensive purification. The relationship between "structural"

NADP, "stabilizing" NADP and substrate NADP are not yet clear.

What is known is that just as the affinity of the enzyme for

substrate NADP is subject to genetic change (chapter V), so the

stabilizing effect of NADP is also modified in certain genetic

variants of the enzyme (Kirkman, Riley & Crowell, 1960). Whether

the other substrate of G6PD, glucose 6-phosphate, is also

critical for the structure of the native enzyme has not been

clearly demonstrated although Marks, Szeinberg & Banks, (1961)

suggested it protects G6PD against thermal inactivation.

(10) Kinetics of G6PD:

A great deal of data have been accumulated on kinetic parameters for different genetic variants of G6PD. Most of these have naturally been concerned with the natural substrates, glucose 6-phosphate and NADP. However, considerable interest has also come from the study of some substrate analogues, such as 2-deoxy-glucose 6-phosphate, the acetyl pyridine derivative of NADP and thionicotinamide NADP. Most of the data are tabulated in Table III and will be discussed later (see chapers III & IV). It has become clear that considerable variations occur in kinetic parameters in different variants and this fact has actually been used in order to identify new variants. However, more important and less explored is the possibility that these variants, in relation to their specific kinetic characteristics, might condition distinct patterns of metabolism within the cell.

Various inhibitors of G6PD have been described. Chung & Langdon (1963a) found that p-chloromercuribenzoate (PCMB) and silver ions inhibit erythrocyte G6PD, suggesting that sulphydryl groups are essential for activity. Physiologically, a more important inhibitor is probably NADPH. Glaser & Brown (1955) had already found that NADPH behaved as competitive inhibitor of NADP with respect to yeast G6PD. Later Kirkman (1962) confirmed this finding for the red cell enzyme. Recently, the characteristics of this NADPH inhibition have been re-investigated in more detail

COMPARISON DATA ON MICHAELIS CONSTANTS OF DIFFERENT TYPES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

VARIANTS	BUFFER	pH	Mg++	Range of substrate concentration	K_m for G6P	K_m for NADP	K_i for ?	Authors (reference)
(I) Human Red Cell (A+B)+ and A⁻	Tris-Phosphate-Glycine	8	0.02M	Not stated	3.9×10^{-5} M	3×10^{-5} M	4.7×10^{-5} M	Kirkman (1959)
B and Mediterranean	Tris-Phosphate-Glycine	8	0.02M	Not stated	3.9×10^{-5} M	2.1×10^{-5} M	6.9×10^{-5} M	Kirkman, Riley, Crowell (1960)
Mediterranean with CNSHA	Tris-Phosphate-Glycine	8	0.02M	Not stated	2×10^{-4} M	1.1×10^{-5} M	4.1 times greater than B	Kirkman, Riley, Crowell (1960)
(A+B)+	Glycylglycine	7.6	0.02M	Not stated	3.5×10^{-5} M	4.2×10^{-5} M	Not given	Marks, Szeinberg and Banks (1961)
A⁻	"	7.6	0.02M		6×10^{-5} M	6.5×10^{-5} M	" "	
Mediterranean	Tris-Phosphate-Glycine	8	0.04M	0.027—1.8mM for G6P; Range for NADP not stated	$1.5—1.6 \times 10^{-5}$ M	$1.2—1.6 \times 10^{-5}$ M	3.4×10^{-5}	Kirkman, Schettini and Pickard (1964)
Markdari	Tris-Phosphate-Glycine	8	0.02M	Not stated	3.5×10^{-5} M	4×10^{-5} M	Not stated	Marks, Banks and Gross (1962)
Chicago	Tris-Phosphate-Glycine	8	0.02M	0.027—1.8mM for G6P; range for NADP	$5.5—7.6 \times 10^{-5}$ M	$3—3.7 \times 10^{-5}$ M	Less than 4×10^{-5}	Kirkman, Rosenthal, Simon, Schrum and Brinson (1964)
Seattle	Tris-Phosphate-Glycine	8	0.02M	Not stated	$1.5—2.5 \times 10^{-5}$ M	$2.4—2.8 \times 10^{-5}$ M	Higher than normal	Kirkman, Simon, Pickard (1965)
Canton (Chinese)	Tris-Phosphate-Glycine	8	0.02M	0.027—1.8mM for G6P; range for NADP not stated	$2.1—5.6 \times 10^{-5}$ M	$3.2—4 \times 10^{-5}$ M	Much higher than normal	McCurdy, Kirkman, Naiman, Zinn and Pickard (1966)
Oklahoma with CNSHA	Tris-Phosphate-Glycine	8	amount not stated	6.30—33.3 $\times 10^{-4}$ mM for G6P; 6.66—33.3 $\times 10^{-6}$ mM for NADP	$4.3—6.3 \times 10^{-5}$ M	12×10^{-6} M	Not stated	Kirkman and Riley (1964)
(A+B)+	Tris-HCl	8.1	None	0.3—3 $\times 10^{-4}$ mM for G6P; 1.25—5 $\times 10^{-5}$ mM for NADP	6.3×10^{-5} M	$4.3—5.4 \times 10^{-5}$ M	Not stated	Yoshida and Hoagland (1965)
(II) Other sources Rat Mammary Gland	Tris-Phosphate-Glycine	8.6	None	1—600 $\times 10^{-6}$ mM for G6P; 1—500 $\times 10^{-5}$ mM for NADP	3.0×10^{-5} M	8.7×10^{-5} M	None	Levy (1963)
Human Placenta	Tris-Phosphate-Glycine	7	5.00—mM	Not stated	1.75×10^{-6} M	3.1×10^{-5} M	None	Hals and Gordon (1961)

* Pool of "normal" enzyme from subjects of African ancestry, therefore presumed to be mixture of A and B.

(Luzzatto, 1967). It has become apparent that the inhibition is not of the straightforward competitive type. The inhibition is sharpest in the range of physiological concentrations of NADPH and in this range small changes in the concentration of the inhibitor can produce marked changes in enzyme activity. It is therefore possible that we are dealing with a case of product inhibition, having regulatory function, not unlike what has been found in micro organisms (Gerhart & Pardee, 1962, 1963). The inhibitory effect of NADPH has been found to have different intensity and characteristics for different genetic variants (see chapter V).

Among the <u>activators</u> of G6PD, Mg^{++} has been the most thoroughly investigated. It is well recognised that this ion is not a required co-factor, in the sense that the enzyme is active even in its absence. It has been established for the yeast enzyme (Mangiarotti, Garre, Acquarone & Silengo (1966) as well as for the erythrocyte enzyme (Luzzatto, unpublished observations) that the activating effect bears not only on the maximal velocity but also involves a change in the affinity of the enzyme for both substrates, G6PD and NADP. It has been further established for the yeast enzyme that most monovalent cations have an inhibitory action, and most inorganic anions are activators. These results have been partly reproduced with erythrocyte G6PD and must be kept in mind when buffers are selected

for studying the kinetics of this enzyme (see chapter II).

No metabolite is known to activate red cell G6PD; the search for

such a substance might be profitable in the sense of another

possible candidate for metabolic regulation.

On the basis of the above, it is to be expected that the

dependence of G6PD activity on pH will be markedly affected by

the exact experimental conditions and especially by the choice

of buffers and by the ionic composition of the medium. Thus

Kirkman in 1959, using a triple buffer consisting of tris,

phosphate and glycine demonstrated that red cell G6PD both of

normal and of the A^- type had a broad pH optimum between 6 and 10

in the presence of Mg^{++}. Later in 1960, Kirkman, Riley & Crowell

using the same mixed buffer found a pH optimum of 9 for the normal

and primaquine sensitive enzyme. These and later data are

tabulated in Table I. It is apparent from the table that the

dependence of the reaction rate on pH is again considerably

different for different variants. It should be pointed out that

many of the data tabulated do not represent a dependence of

maximal velocity on pH, since it is not certain that in every

case buffers employed did not affect reaction rate nor that at

every pH saturation of the enzyme was achieved (see chapters II

and IV).

This work had been undertaken with a view to investigating some enzymic properties of five genetic variants of erythrocyte glucose-6-phosphate dehydrogenase found in Nigeria: these include the three common variants, A, A⁻ and B, and two new variants fully characterized (see chapter III).

The comparative biochemical studies have included the following:-

(a) Analysis of the variation with pH of the fundamental parameters of enzyme reactions, Vmax and Km (chapter IV

(b) Kinetic analysis of the interaction of G6PD with the substrate, NADP, and the reaction product, NADPH, (chapter V).

(c) Thermostability experiments (chapter VI), and

(d) The action of certain inhibitors (chapter VI).

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CHAPTER II

EXPERIMENTAL TECHNIQUES

(1) <u>Reagents</u>: All reagents used were the purest types which were obtainable from different manufacturers and which were considered suitable for research work. NaOH, HCl, Na_2HPO_4, H_3BO_3, Citric acid, Folin's reagent, and p-Chloromercuribezoic acid (PCMB) were all of 'Analar' grade from the British Drug Houses Ltd.; G6P and NADP were from Boerhringer Ltd.; Tris and NaCl were of 'Certified' grade obtained from General Biochemicals Ltd.; NADPH, 2dG6P, Diethylaminoethyl ethyl (DEAE) - Cellulose (Mesh: medium, Capacity: 1.0 mEq/gm), Carboxymethyl (CM) - Cellulose, (Mesh: medium, capacity 0.62 mg./gm.), DEAE-Sephadex (particle size: 40-120 u, capacity: 3.5 $\pm$ 0.5 mEq/gm) were all obtained from the Sigma Chemical Company Ltd.; triethylamine and β-mercaptoethanol were obtained from L. Light & Co. Ltd.; N-ethylmaleimide (NEM) was obtained from Calbiochem. Ltd.

<u>Glucose 6-Phosphate Dehydrogenase (G6PD) "Typing"</u>: Glucose 6-phosphate dehydrogenase "typing" was performed by starch gel electrophoresis in Tris-Borate-EDTA buffer, pH 8.6 according to the method of Porter <u>et al.</u> (1964) with the following modifications:

   (1) The concentration of the NADP in the gel was 10 μM.

   (2) 10 μM NADP was also added to the cathodic buffer reservoir.

(3) The electric field was 15 v/cm., the current 1.5 mA/cm and the duration of the run 6 hours.

After staining the gels, A, A⁻, B and the two new variants, designated, Ijebu-Ode and Ita-Bale respectively, and which are slower than B could be easily recognised by visual inspection.

(2) <u>Preparation of materials for column chromatography.</u>

(a) <u>Calcium phosphate gel</u>: Calcium phosphate gel-hydroxyapatite was prepared by the method of Tiselius, Hjerten and Levin (1956) with slight modifications. A brushite precipitate was prepared by allowing 2 liters of each of $0.5 M$ Na_2HPO_4 respectively to run at an equal rate of flow (about 115 drops/min.) from two separating funnels into a large glass beaker under stirring. The pricipitate was decanted and washed four times by decantation with ion free water, each time up to about 4 liters of volume. Water was again added to 4 liters total volume and then 100 mls. of freshly prepared 40%, (by weight) NaOH was also added. The mixture was boiled under stirring for 1½ hours. The precipitate, again decanted when all but the most highly dispersed material had settled, was washed six times, by decantation, with water. 0.01 M Sodium phosphate buffer (pH 6.8) was then added, and the solution was heated until it was about to boil. After a brief moment, with the fine suspension still unsettled, and then decanted, the same phosphate buffer was added but now the solution was boiled for 10 minutes. The procedure was repeated twice each time for 20 minutes boiling in the 0.01 M

phosphate buffer solution pH 6.8. The hydroxyapatite thus
obtained was then stored in the cold.

(b) Ion Exchangers.

Precycling: The weighed exchanger was stirred into 15 vols.
(i.e. vol. of liquid/dry weight exchanger) of first treatment
acid or alkali and left for at least 30 minutes. The supernatant
liquor was decanted and the resin was washed in a funnel until
effluent was at "intermediate pH". The exchanger was then stirred
into 15 vols. of second treatment acid or alkali and left for a
further 30 minutes. The supernatant was decanted and the resin
was washed in a funnel until the effluent was near neutral.

	First treatment	'Intermediate' pH	Second treatment
DEAE - Cellulose)			
DEAE - Sephadex)	0.5N HCl	4	0.5N NaOH
CM - Cellulose	0.5N NaOH	8	0.5N NCl

Equilibration: The exchanger was stirred into a volume of
appropriate buffer and then left for 20 minutes. The supernant
was decanted. The procedure was repeated 8 times. By then, the
resin is usually equilibrated and this was checked by filtering and
testing the pH of the effluent. After reaching equilibration, the
exchanger was stored in the buffer in the cold several days
before use.

(3) <u>Source of red cells</u>: All enzyme preparations were from the blood of male blood donors at the Blood Bank of the University College Hospital, Ibadan. In all cases, expired blood was used, which had been stored with standard acid-citrate-dextrose at 4°C for 3 to 5 weeks. It has been shown that no significant loss of activity occurs under such storage conditions.

(4) <u>Partial Purification of Enzymes</u>: Blood samples containing the five enzyme variants were processed following essentially the method of Yoshida (1966), with some modifications, up to one of the various ammonium sulphate precipitation steps. Four liters of blood of each enzyme type was thus partially purified. The non-availability of large volumes of Ijebu-Ode and Ita-Bale beyond a pint of blood each, put a limit to the extent of purification to the first ammonium sulphate precipitation step.

All procedures were carried out in the cold or in ice bath. The buffer used throughout was Na_2HPO_4–KH_2PO_4, α contain 10^{-4}M β-Mercaptoethanol, 10^{-4}M EDTA and 2×10^{-6}M NADP. Protein assay was by the method of Lowry (1951) with Human Gamma Globulin as standard.

(a) <u>Preparation of haemolysates</u>: 2 liters of blood at a time were centrifuged in a refrigerated MSE centrifuge at 1700 g for 30 minutes and the supernatant plasma was removed by a pipette attached to a water pump. The red cells were washed four times with 4 volumes of 0.15M NaCl containing 10^{-4}M EDTA, and then lysed via 10^{-4}M β-mercaptoethanol and 12.5 ml. of toluene per 500ml . After a vigorous shaking for some minutes, the haemolysate was spun in

a refrigerated International Equipment Corporation (IEC)
centrifuge at 11,000 g for 30 minutes and the supernatant
collected by a pipette attached to an aspirator bottle in ice.
The precipitated debris was suspended in equal volume of water
and centrifuged at the same speed and for the same length of
time. The extract was combined with the first haemolysate
making a total of about 2 liters.

(b) <u>DEAE-Cellulose Column</u>: DEAE-Cellulose (100 gm)
pre-recycled and then equilibrated with 0.005 M phosphate buffer,
pH 6.45, was placed on a porous glass filter funnel, 21 cm in
diameter. A filter paper was placed on top of the resin to serve
as antidisturbance disc. The haemolysate from the previous step
was then poured on the resin. It was washed with 2 liters of
0.005 M phosphate buffer, pH 6.45. The essence of this washing
was to remove most of the haemoglobin and 6-phosphosphogluconate
dehydrogenase while glucose-6-phosphate dehydrogenase was left
behind in the resin. G6PD was eluted with 2 liters of 0.1 M
phosphate pH 5.8 containing 0.5 M NaCl. To the effluent,
ϵ-amino-N-caproic acid (final concentration of 10^{-3}M) was added
to prevent the hydrolysis of the enzyme by contaminating
proteolytic enzymes. The pH of the effluent was adjusted to 6.2
with 0.5 M Na_2HPO_4 and after this, solid ammonium sulphate
(350 gm. per liter) was added. After being kept in the cold
overnight, the precipitates were collected by centrifugation on
the IEC (11,000 g for 30 minutes). The process of washing of the
red cells was done in one day while lysing and extraction on the

DEAE-Cellulose column was done on the following day.

The whole procedure up to this point was repeated for another 2 liters of blood for G6PD types A, A⁻, and B. The ammonium sulphate precipitates were then pooled for each enzyme type and stored in the deep freeze. Both Ijebu-Ode and Ita-Bale with a pint of blood each were purified through this step only.

(c) CM-Cellulose Column: The precipitate obtained from the DEAE-Cellulose effluent was suspended in a small volume of 0.005M phosphate buffer (pH 5.8), dialysed against the same buffer for 3 hours at a time, changed two times and was centrifuged at 11,000 g for 20 minutes. The supernatant was then placed on a column (5 x 35 cm.) of CM-Cellulose precycled and buffered with the same 0.005 M phosphate buffer pH 5.8. The elution of the enzyme with increasing concentration of NaCl was done according to Yoshida (1966) (see Fig. 4). The flow rate was 2 ml. per minute. The enzyme was precipitated from the major fractions with ammonium sulphate (360 gm. per liter). Variants B, A and A⁻ enzyme types were purified through this step.
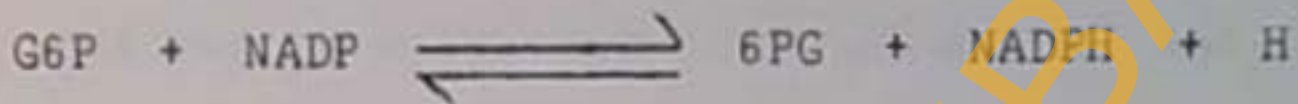
(d) Calcium Phosphate Gel: The precipitate from the CM-Cellulose column effluent was obtained by centrifuging at 11,000 g for 30 minutes and was suspended in 0.01 M phosphate buffer (pH 6.8) and dialysed against the same buffer, for 3 hours in the first instance and then changed twice again each time for the same period. The supernatant obtained by centrifuging at 11,000 g for 20 minutes, was placed on a calcium phosphate

gel column (3 x 15 cm.) buffered with 0.01 M phosphate buffer pH 6.8. The elution was carried out with increasing phosphate concentration. The flow rate was 18 ml. per hour. The bulk of the enzyme fractions was precipitated with ammonium sulphate (350 g per liter). Only A and A⁻ were purified through this step.

(e) DEAE - Sephadex: The precipitate obtained from the calcium phosphate gel effluent was suspended in 0.02 M phosphate buffer (pH 6.4) and dialysed against the same buffer. The dialysed solution (10 ml., for A type only) was placed on DEAE-Sephadex column (1.5 x 35 cm) buffered with 0.02 M phosphate buffer (pH 6.4) and was eluted with increasing concentrations of NaCl. The gradient was produced by adding 0.02 M phosphate buffer (pH 6.4) containing 0.25 M NaCl to a mixing chamber which contained 400 ml. of 0.02 M phosphate buffer (pH 6.4) containing 0.05 M NaCl (fixed initial volume of buffer). This procedure removed most of the yellow material and the enzyme fraction became almost colourless. G6PD was precipitated with ammonium sulphate (350 gm. per liter). An example of purification flow-sheet is shown in Table IV and the data on the purification of all variants employed are shown in Table V.

(5) Enzyme Assays: The assays (essentially according to Kornberg & Horecker (1955) but with different composition, see below), were carried out at 24 ± 2°C room temperature in silica micro cuvettes with light path of 1 cm. The reaction

rates were followed on the basis of increase in absorbance at 340 mµ (caused by NADPH production) as a function of time using either a spectrophotometer UNICAM SP 500 or a Gilford automatic multiple absorbance recorder, Model 2000. The NADPH is produced in the reaction catalysed G6PD between G6P and NADP according to equation,

$$G6P + NADP \rightleftharpoons 6PG + NADPH + H$$

The unit of activity is the amount of enzyme required to produce a unit change in optical density per minute. This is different from the international definition (following the recommendations of the International Enzymes Commission) which is the amount of enzyme required to produce 1 µmole of NADPH at 340 mµ per minute. The typical assay mixture, in a micro cuvette, contained 0.03 M Tris Borate buffer, pH 8.
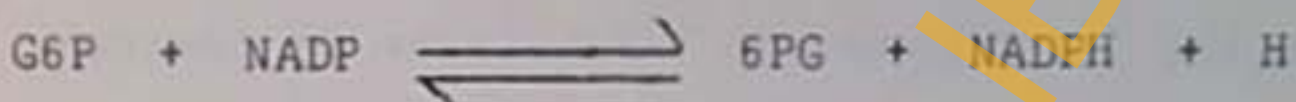
$$4 \text{ mM Glucose 6-Phosphate}$$
$$0.3 \text{ mM NADP (final concentrations).}$$

Enzyme was added last and final volume was 0.3 ml. The cuvette was quickly covered with a piece of parafilm, inverted two or three times for proper mixing and optical density measurements were taken at suitable intervals.

(6) <u>Maximal Velocity as a function of pH</u>: In order to obtain the true relationship between V_{max} and pH, it was necessary to ensure that three conditions be satisfied:

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(6) Maximal Velocity as a function of pH: In order to obtain the true relationship between V_{max} and pH, it was necessary to ensure that three conditions.

(1) The complete incubation mixture must retain at each pH and throughout the reaction time, the pH of the buffer used in its preparation.

(2) The concentration of each substrate must saturate the enzyme at each pH.

(3) The ions used in order to adjust the buffer to the desired pH values must not affect the enzyme velocity in any other way than by changing the hydrogen ion concentration.

(1) When the amount of buffer added to the reaction mixture is, for instance, such as to yield a final concentration of about 0.03 M and each one of the reaction mixture has a pH of 7.5, it is found that the pH of the complete reaction mixture deviates from the pH of the buffer. For instance, when the pH of the buffer was 8.8, the pH of the mixture was 8.2, when the pH of the buffer was 9, the pH of the mixture was 8.7. Furthermore, in the course of the reaction acid is produced, and the pH in the first 3 minutes may drop further by 0.1 - 0.2 pH units. However, if the final concentration of buffer is about 0.3 M, and the pH of the G6P solution is pre-adjusted to the same pH of the buffer, no appreciable pH change is observed. Thus, fourteen different solutions of G6P were prepared, adjusted to the desired pH values between 5.5 and 9.5 by addition of 0.5 M boric acid or 1 M Tris as required. In order to measure enzyme velocity, at each pH

point, the buffer and the G6P solution having the pH were employed. At each point, the pH was checked immediately after preparing the complete reaction mixture and three minutes afterwards: in no case was the deviation from the nominal pH more than 0.05 pH units.

(2) Substrate saturation of enzyme was checked at the extreme pH values 5.5 and 9.5 by varying concentration of one substrate at a time and keeping the other constant, until there was no change in the maximal velocity obtained. It was found that in excess of G6P (12 mM), 95% saturation was reached at 0.33 mM NADP. In excess of NADP (0.5 mM), 95% saturation was reached at 10 mM G6P. These substrate concentrations were therefore used throughout the whole pH range.

(3) The work done with yeast G6PD has shown that several anions activate and several cations inhibit the enzyme (Mangiarotti et al., 1965; Mangiarotti & Garre, 1965), while Tris and Boric acid were found to have no such effect. In the light of this, Tris-borate buffer was chosen for these experiments and used in the pH range from 7 to 8.5. With this buffer the activity of the erythrocyte enzyme was the same when the buffer concentration was varied over a 31-fold range (from 8 to 250 mM Tris and 9 to 280 mM borate at a constant pH of 8.1). As for the pH points below 7.0, citric acid was added to the Tris-borate buffer.

In order to insure that citrate had no effect on the enzyme activity, a portion of a solution of Tris-borate, pH 7.0 was brought to pH 5.5 with citric acid and then back to pH 7.0 with Tris. The reaction velocity was then measured in the remaining portion of the Tris-borate buffer and in the Tris-borate-citrate buffer both at pH 7.0, and found to be the same. Citric acid up 0.0015 M this had no effect on the activity. For the pH points above 8.5 triethylamine was added to the Tris-borate buffer and a similar experiment was performed in order to insure that this base up to 0.025 M had no effect on enzyme activity. Thus the buffers used were as follows:-

pH 7.0 - 8.5 Tris-borate, prepared by adding 1M Tris to 0.5 M boric acid until the desired pH is obtained.

pH 5.5 - 6.5 Tris-borate-citrate, prepared by adjusting the pH of a Tris-borate solution pH 7.0 with 0.5 M citric acid.

pH 8.8 - 9.5 Tris-borate-triethylamine, prepared by adjusting the pH of a Tris-borate solution pH 8.5 with 0.2 M triethylamine.

(7) Michaelis constants:

The Michaelis-Menten constant (K_m) is defined as being equivalent to substrate concentration at half maximal velocity (Dixon & Webb, 1964); it is a measure of the affinity of the enzyme for the substrate. (and is derived

The initial rate of the reaction is assumed to be proportional to the concentration of the enzyme-substrate complex.

(a) $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E + P$
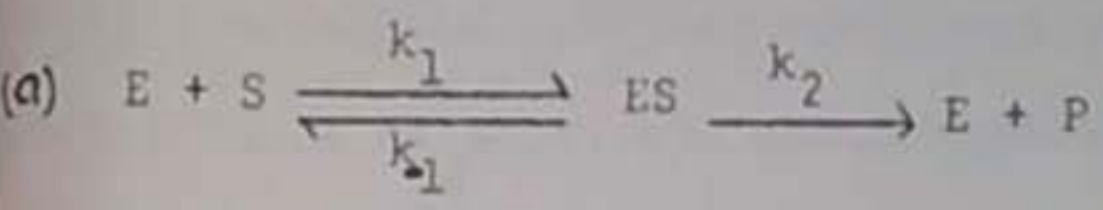
E = Enzyme
S = Substrate

(b) $K_s = \dfrac{(e-p)s}{p}$

ES = Enzyme substrate complex.

P = Product of the reaction.

v = Initial velocity

If re-arranged

(c) $p = \dfrac{es}{K_s + s}$

k = velocity constant

e = Total enzyme concentration.

But

(d) $v = kp$

K_s = Dissociation constant of the enzyme – substrate complex.

(e) $\therefore v = \dfrac{kes}{K_s + s}$

s = Free substrate concentration.

p = enzyme-substrate complex concentration

When the substrate becomes large in comparison with K_s, V will be equal to ke and this is maximal velocity (V_{max}) (i.e. the enzyme is saturated with the substrate).

In this respect equation (e) becomes

(f) $v = \dfrac{V_{max}\, s}{K_s + s} = \dfrac{V_{max}}{1 + \dfrac{K_s}{s}}$

(g) $\dfrac{1}{v} = \dfrac{K_s}{V_{max}} \cdot \dfrac{1}{s} + \dfrac{1}{V_{max}}$

the K_m is equal to K_s only when the rate constant k_2 in equation (a) is negligible. ~~and a is equal to K_s in equation (f)~~.

In the present work, K_m was determined by the Lineweaker-Burk (1934) method of plotting reciprocals of velocity($\frac{1}{v}$) against reciprocals of substrate concentration ($\frac{1}{s}$). If the enzyme follows Michaelis-Menten kinetics, a straight line is obtained, which cuts the abscissa at a point where $\frac{1}{s} = \frac{-1}{K_m}$, and cuts the ordinate at a point where $1/v = \frac{1}{V_{max}}$ (equation g). The determination of the K_m for G6P and 2dG6P was carried out for each enzyme type. This investigation was carried out further as a function of pH with respect to G6P only.

(a) The experimental conditions for determining the K_m for for G6P were as follows: Enzyme velocity measurements were carried out using 0.05 M Tris-Borate buffer, pH 7.8, 0.5 mM NADP final concentration with variable concentration of the other substrate. In order to determine the K_m for G6P, the reaction rate was measured at eight different concentrations ranging from 0.015 to 6 mM.

For 2dG6P, the reaction velocity was measured at four different concentration, ranging from 6 to 48 mM.

(b) The determination of the K_m for G6P at different pH values 5, 6, 7, 8, 9 was carried out at 0.33 mM NADP (final concentration) and varying concentration of G6P from 0.003 to 6 mM.

The buffers used were of the same composition with those used for the maximal velocity (V_{max}) measurements. The Gilford multiple absorbance recorder (model 2000) with automatic blank compensation was used and was programmed to take readings every 15 seconds. This determination of K_m for G6P as a function of pH was carried out with all the five variants.

(c) In the case of NADP, the changes in the reaction velocity as a function of concentration were found not to reflect simple Michaelis kinetics (Luzzatto, 1967). No K_m values were therefore obtained; the dissociation constants involved are discussed in chapter V. The reaction mixtures used for these enzyme velocity measurements contained 0.05 M Tris-borate buffer (pH 8), 4 mM G6P and variable amounts of NADP between 4 uM in a final volume of 0.3 ml. The reaction mixtures were usually prepared in micro cuvettes and allowed to equilibrate for about 3 minutes at room temperature in the Gilford multiple absorbance recorder. Because of the very high affinity of the enzyme for the co-enzyme, and since the concentration of NADP was limiting, there must be little or no time lost between addition of enzyme and the recordings. Reactions were usually started by adding the enzyme last; and this was done by applying the desired amount of each enzyme type on a small piece of plastic sheet (cut to fit loosely in the cuvette) which was inserted into the cuvette. The plastic sheet was then gently moved up and down thrice in order to effect thorough mixing and was then removed quickly. This particular step required great care to avoid development of bubbles inside

cuvette. As in the K_m of G6P versus pH experiments, reactions were followed in the Gilford multiple absorbance recorder at full scale settings ranging from 0.25 to 1.00 absorbance units. The time interval between addition of enzyme and the starting of the recording was less than 10 seconds. At low NADP concentrations (below 20 μM), only one cuvette at a time was used. At higher NADP concentrations, 2 cuvettes at a time were used.

(d) The same procedure was employed in investigating the effect of NADPH on the reaction rates of various enzyme types at different NADP concentrations (16 μM - 480 μM). The concentration of NADPH varied between 10 μm and 320 μM. In carrying out the experiment, at one fixed NADP concentration, the effect of NADPH 10 to 320 μM in final concentrations, was investigated. The same step was repeated at another fixed NADP concentration. The reactions were carried out with 0.05 M Tris-borate buffer, pH 8, and 4 mM G6P. These two reaction kinetics experiments were carried out with A^-, B, Ijebu-Ode and Ita-Bale enzyme variants.

(8) <u>Thermal inactivation profile studies</u>:

The temperature-dependent inactivation (melting curve) phenomenon was studied with respect to all the five variants. In setting for the experiment, each enzyme was dialysed against 0.05 M KCl, 5 μM phosphate buffer, pH 6.9, containing 0.1 μM EDTA and 2 μM NADP. The final change of the buffer contained 2×10^{-9} M NADP. The following layout of the protocol was adopted for each enzyme:

	(A)	(B)	(C)	(D)
Enzyme	0.4 ml.	0.4 ml.	0.4 ml.	0.4 ml.
NADP $2 \times 10^{-3}M$	0.4 ml.	-	-	-
$2 \times 10^{-5}M$	-	0.4 ml.	-	-
$2 \times 10^{-7}M$	-	-	0.4 ml.	-
H_2O	-	-	-	0.4
	0.8 ml.	0.8 ml.	0.8 ml.	0.8 ml.

Each mixture (A - D) was distributed in 8 small test tubes; each tube holding 0.08 ml. of mixture. For each series (A - D), each tube, which was well stoppered, was incubated for 7 minutes at various temperatures - (25°, 35°, 45°, 50°, 55°, 56°, 59°, $62^{\circ}C$) indicated. Exactly after 7 minutes, each tube was immediately cooled in ice. Afterwards, reaction velocity measurements were carried out with 0.05 M Tris-borate buffer pH 7.8, 0.33 mM NADP and 4 mM G6P in final volume of 0.3 ml. This entire procedure was carried out for each enzyme type.

Effect of Sulphydryl group reagents:

These reagents specifically react with thiol groups present in proteins and in the case of enzymes, the effect may result in reduction or loss of enzymatic activity. The implication is that thiol group is present at the active site or near it, where it exerts significant influence. Red cell G6PD has not been an exception with regards to the effect of these reagents. Investigations were carried out with p-chloromercuribenzoate (PCMB) and N-ethylmaleimide (NEM) either by direct addition of

the reagent to the reaction mixture or by pre-incubation with enzyme. In the direct reaction technique, both p-chloromercuri-benzoate and N-ethylmaleimide were added to the reaction media and measurements of reaction rates started immediately. The range of concentration used was 0.5 - 3.3 mM for p-chloromercuribenzoate and N-ethylmaleimide respectively. By a more refined technique, A and A^- G6PD variants were pre-incubated with different low concentration of PCMB for 15 minutes. Afterwards, 0.1 ml. aliquot was withdrawn for assaying.

CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF TWO NEW GENETIC VARIANTS OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE:

Since the pioneering work of Pauling and his colleagues (Pauling, Itano, Singer & Wells, 1949) on the resolution of Haemoglobins A and S by electrophoresis, this technique has become a standard method for testing polymorphism of proteins. Electrophoresis has lead to the discovery of the great majority of the approximately 80 haemoglobin variants that are now known. With the development of the starch gel technique (Smithies, 1955) and of suitable staining methods, electrophoresis has also become ideally applicable to the investigation of a number of enzymes. Although it is well established that proteins which have the same electrophoretic mobility need not be identical in primary structure, for instance Haemoglobins S and D (Wintrobe, 1967), the reverse is not true: proteins which do have different electrophoretic mobility can be assumed to have different structure, and therefore electrophoresis represents the single simplest method for the screening of a large number of samples in search for new variants.

In the haematology Laboratory at University College Hospital, Ibadan, screening by starch gel electrophoresis for G6PD variants has been carried out for about 3 years. Over 3,000 blood samples have been processed and 18 samples have been encountered in which an enzyme with abnormal electrophoretic migration was present.

In the majority of the cases, it was impossible to retrace the original donor and in some cases the amount of blood available for further study was not sufficient or the enzyme was too unstable. Here a report is submitted on two enzyme variants which **were** purified and fully characterised.

Because of the considerable number of G6PD variants which are now described in the literature, the World Health Organisation convened in December 1966, a Study Group which was asked to set certain standards for the characterization of enzyme variants. The purpose was twofold: (1) To enable different investigators to compare results by the use of standardised techniques. (2) To establish which of the reported variants are indeed different and which are not. It has been decided to follow as closely as possible the recommendations contained in the WHO report The results for the two variants will therefore be presented accordingly in the present chapter. The following chapters will report a more detailed comparative analysis of the kinetics of the two variants and of the three common variants of G6PD. Since it will be shown that these two variants are unique, they will hereafter be referred to by the names proposed for them, following the recommendations of WHO - namely, Ijebu-Ode and Ita-Bale.

Fig. 3. Electrophoretic migration of the G6PD variants on starch gel. The gel is stained for enzyme activity. The origin is at the bottom (not shown). The above picture was mounted from two separate photographs. Experimental conditions are described in chapter II. Tracks (1) A⁻ (2) A (3) (B) (4) Ijebu-ode (5) Ita-Bale.
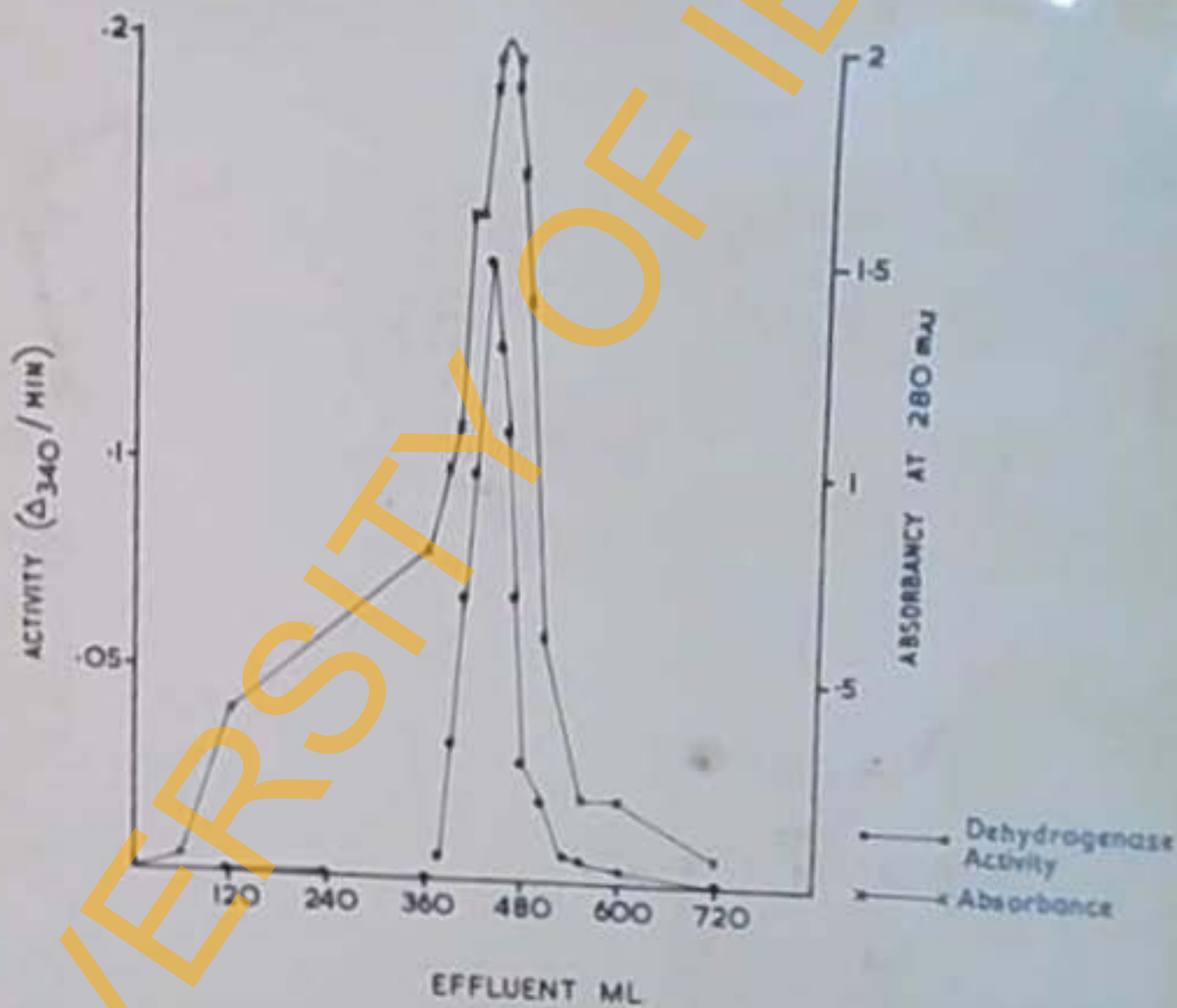
Fig. 4. Elution pattern of G6PD type A from a CM-cellulose
column. Partially purified glucose-6-phosphate
dehydrogenase type A (obtained through a DEAE-Cellulose
column) was placed on CM-cellulose (5 x 35 cm.) and
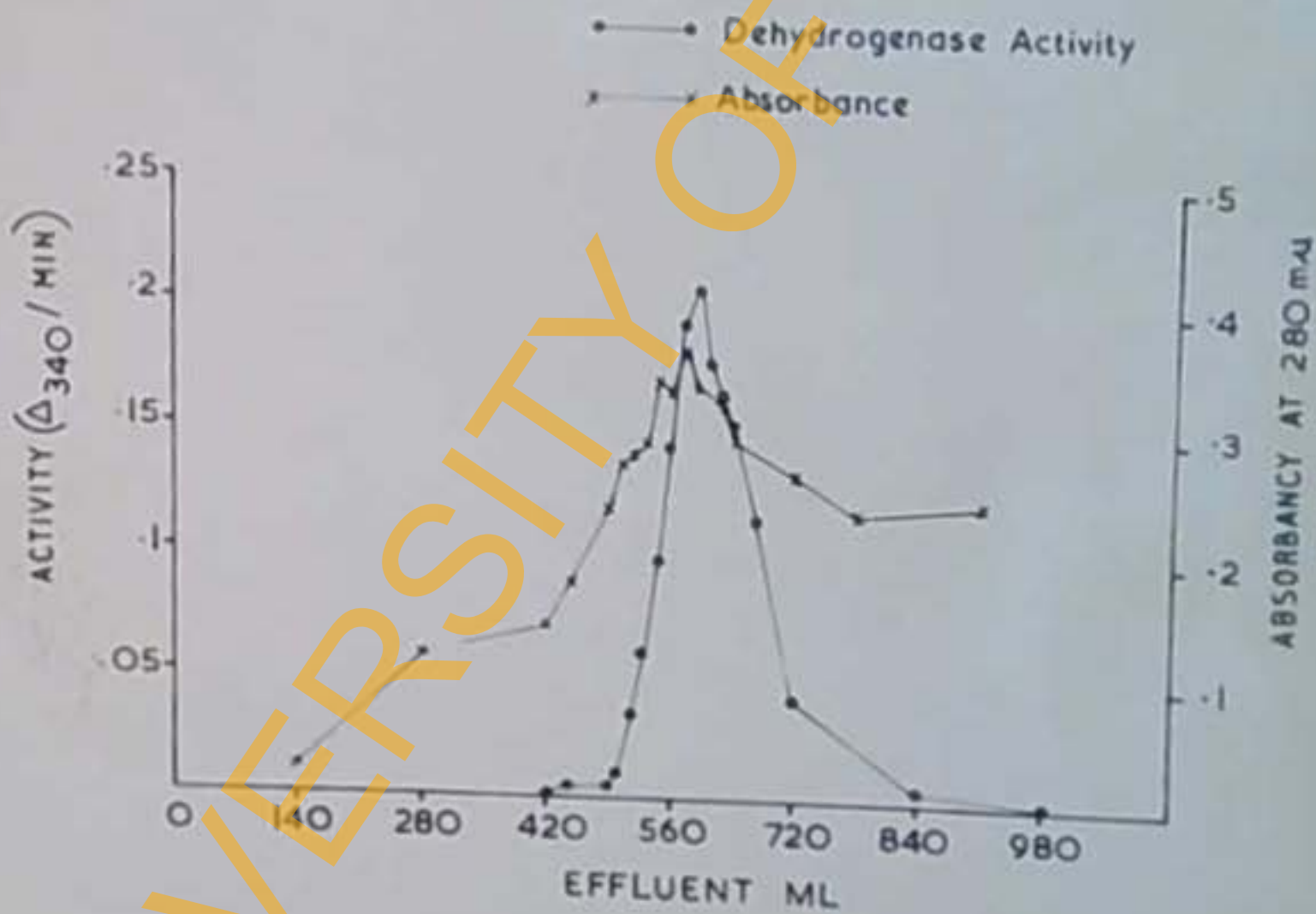eluted with increasing pH and concentration of NaCl.

Fig. 5. Elution pattern of G6PD type A from a calcium phosphate
gel column. Partially purified glucose-6-phosphate
dehydrogenase type A (peak from a CM-cellulose column)
was placed on calcium phosphate gel column (2.5 x 30 cm.)
and eluted with phosphate buffer (pH 6.8) of increasing
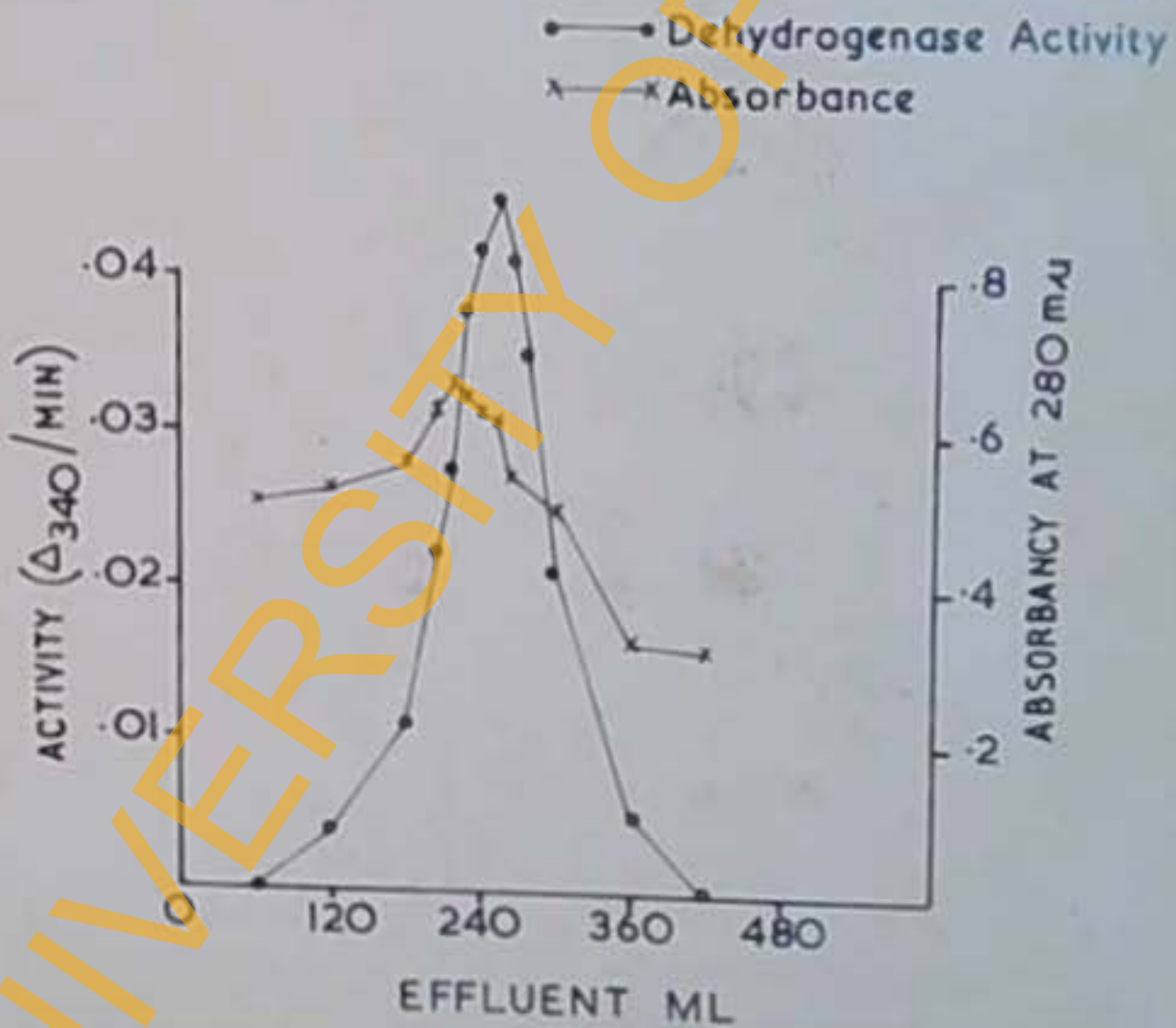concentration.

Fig. 6. Elution pattern of G6PD type A from DEAE-Sephadex column. Partially purified glucose-6-phosphate dehydrogenase (peak from calcium phosphate gel column) was placed on a DEAE-Sephadex column (1.5 x 35 cm.) and eluted with increasing concentration of NaCl.

TABLE IV

SUMMARY OF DATA ON PURIFICATION OF G6PD TYPE A

Steps of Purification	Total Volume ml.	Activity Units =umoles/ml.	Protein mg./ml.	Specific Activity Units/mg. Protein.	Purification times	Total Units	Recovery %
Haemolysate	4,000	0.2	100	0.002	1	800	100
DEAE - Cellulose Effluent	3,500	0.2	30	0.07	35	700	88
Precipitate with $(NH_4)_2SO_4$ and resuspended in buffer.	100	3	20	0.15	75	300	38
CM - Cellulose Effluent	100	2	21	0.3	150	200	25
Precipitate with $(NH_4)_2SO_4$ and resuspended in buffer.	20	7	10	0.7	350	140	18
Calcium Phosphate gel column effluent.	70	2	2	1	500	140	18
Precipitate with $(NH_4)_2SO_4$ and resuspended in buffer.	10	11	6	2	1,000	110	14
DEAE - Sephadex Column effluent	40	2	0.8	2.5	1,250	80	8
Precipitate with $(NH_4)_2SO_4$ and and resuspended in buffer.	3	9.2	0.5	18.4	9,200	28	3.5

TABLE V

PURIFICATION OF G6PD VARIANTS USED IN PRESENT WORK

	Purification times.	Specific activities units/mg. protein.
A	9,200	18.4
A⁻	80 - 300	0.10 - 0.5
B	80 - 300	0.12 - 0.6
Ijebu-Ode	100	0.14
Ita-Bale	100	0.15

R E S U L T S

(a) Electrophoresis: The electrophoretic behaviour of the two new variants is shown in Figure 3. It is seen that with the electrophoretic system employed the mobility of Ita-Bale is approximately 70% of that of B and the mobility of Ijebu-Ode is approximately 85% of that of B. Thus, both variants fall in the category of those that are electrophoretically slow. These include Baltimore-Austin, Ibadan-Austin, (see Table I).

(b) Activity in fresh haemolysates: Freshly prepared haemolysates from the donors of Ijebu-Ode and Ita-Bale have an activity of 5.82, 5.54 international units/g. of haemoglobin respectively, that is within the normal range observed in this country (6.32 ± 0.93 international units/g. of haemoglobin). Thus, the variants are not associated with enzyme deficiency. The donors of the variants did not demonstrate any clinical or haematological abnormality. The two variants therefore have to be grouped among those which would not have been detected without a systematic screening of numerous blood samples.

(c) Determination of Michaelis constant for G6P: The values for the Michaelis constant for glucose 6-phosphate and 2-deoxy glucose 6-phosphate are shown in Tables VI and VII. It is seen that whereas for Ijebu-Ode the values measured lie within the range found with the more common variants, the K_m of Ita-Bale is higher. From the point of view of demarcation of the
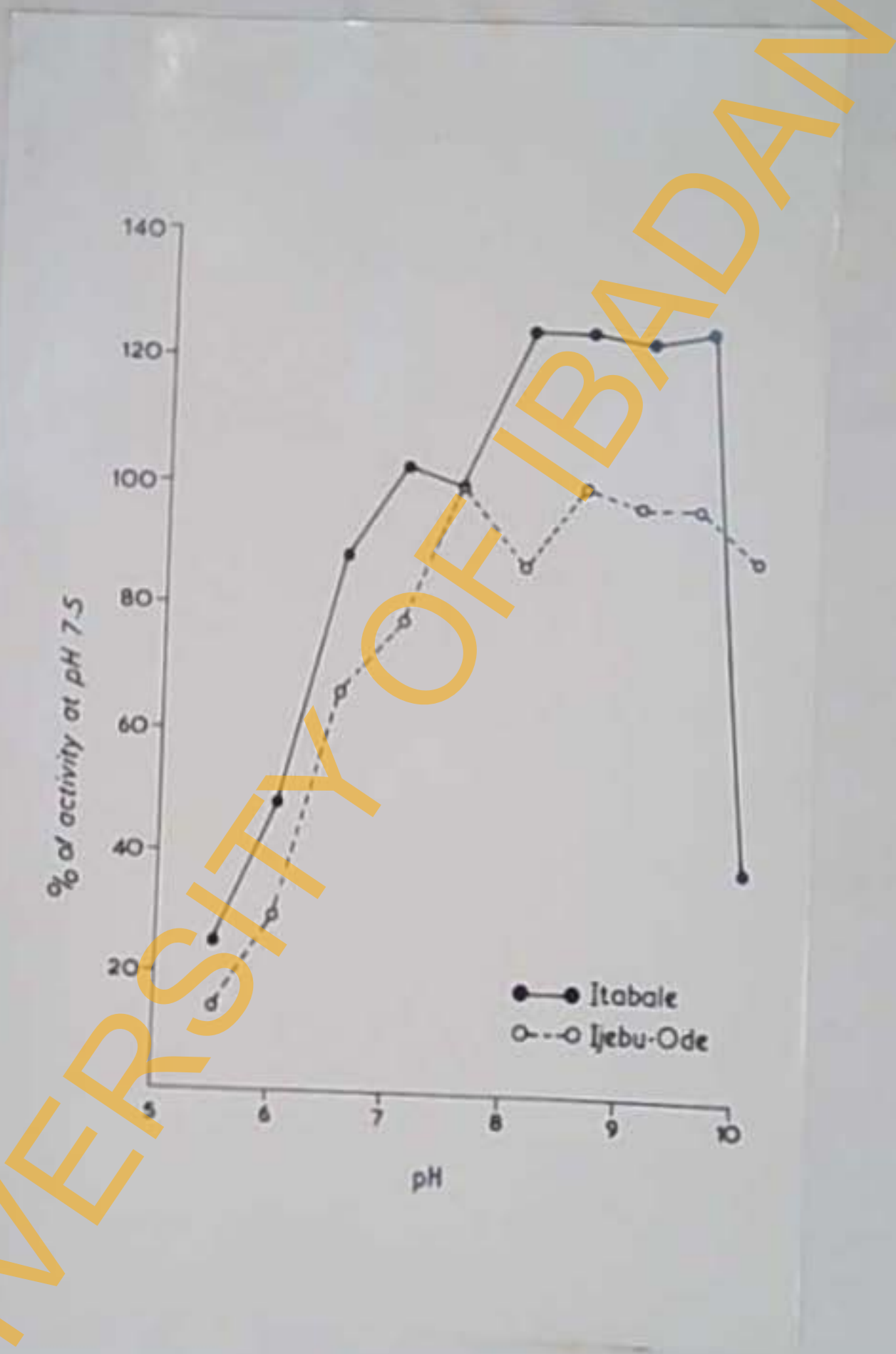
Fig. 7. Variation of velocity of the two new G6PD variants with pH. Activity determined in tris-glycine-phosphate buffer, adjusted to desired pH with NaOH or HCl according to Kirkman, Rosenthal, Simon, Carson and Brinson (1964). (J. Lab. Clin. Med. 63: 715)

new variants with respect to previously described ones, the
Michaelis constants are therefore not useful in the case of
Ijebu-Ode. In the case of Ita-Bale, the abnormally high value of
K_m sets this variant aside from another electrophoretically slow
variant, namely, Ibadan-Austin.

The Michaelis constant for the other substrate, NADP, cannot
be determined by standard methods due to the peculiar saturation
kinetics. The relevant data will be presented separately in
Chapter V.

(e) <u>Determination of the effect of pH on enzyme activity</u>:
The determination has been carried out according to the
recommendations of WHO. It must be pointed out that by the
technique recommended the curves obtained are not representative
of the dependence of V_{max} on pH. This will be fully discussed
in Chapter IV. The present data (see Figure 7) have been
included for purposes of completeness in the characterization of
the variants. It is apparent that both variants show slightly
biphasic pattern, but (the picture) is close to the so called "truncate" pattern
known for the three common variants A, B and A^- and for several
of the rare variants, whereas Ijebu-Ode is fairly similar to what
has been reported for the variants Canton and Mediterranean.

(d) <u>Time course of heat inactivation</u>: The rate of
inactivation of Ijebu-Ode and Ita-Bale was measured at $37^{o}C$ and
compared with that of the B variant at the same temperature.

Ita-Bale had a minimally increased thermolability, Ijebu-Ode
a markedly increased thermolability. The time at which 50% of
the activity was lost was 85 minutes for B, 72 minutes for
Ita-Bale and 30 minutes for Ijebu-Ode. The time-course of
inactivation of a mixture of Ita-Bale and B was - as expected -
intermediate between those of the two separate enzymes. In the
case of Ijebu-Ode, however, the mixture was less liable than
expected from the results with the two separate enzymes,
presumably because of protection of Ijebu-Ode by some unknown
impurity in the preparation of B. From the point of view of
variants identification, these results are not useful as far as
Ita-Bale is concerned, but in the case of Ijebu-Ode, the
increased thermolability set this variant apart from another
electrophoretically slow variant - namely, Baltimore-Austin.

DISCUSSION

If the two new variants here described are compared with a
comprehensive table of previously described ones (see Table I),
it appears certain that they differ from all of them. Ijebu-Ode
resembles Baltimore-Austin in some respects, but its thermostabilit
(Figure 19C), its activity/pH curve (Figure 7) and its behaviour
with respect to sulphydryl group reagents (see chapter VI) are
very distinctive, although not all of these properties have been
similarly tested for Baltimore-Austin. Ita-Bale resembles
Ibadan-Austin but has normal activity in the crude lysate, and an

abnormally high K_m for glucose 6-phosphate (Table VI), resembling in this respect the Barbieri (Marks, Banks and Gross, 1962) and Oklahoma, (Kirkman and Riley, 1961) variants (though it is clearly distinct from them by other criteria). For both Ijebu-Ode and Ita-Bale the curves of V_{max} versus pH (Figure 10) are distinctive.

In the course of an electrophoretic analysis of haemolysates from approximately 2,000 unrelated male subjects, the Ita-Bale variant was encountered only once, while a band having the electrophoretic mobility of Ijebu-Ode was observed five times. Since only one of the five has been fully characterized, it is not known whether the others were biochemically identical to Ijebu-Ode or not. Therefore one can only give upper estimates for the gene frequencies in the Western Nigerian population, and these are of less than 0.0005 for Ita-Bale and between 0.0005 and 0.0025 for Ijebu-Ode: both of these are below polymorphic frequencies (i.e below 0.01). It is noteworthy that all the subjects with such electrophoretically slow-moving variants of red cell glucose 6-phosphate dehydrogenase were clinically and haematologically normal.

CHAPTER IV

KINETIC PARAMETERS AS A FUNCTION OF pH FOR FIVE GENETIC VARIANTS OF G6PD:

The determination of pH optima has been a classical method for the characterisation of enzymes. A detailed analysis of how pH affects the two main kinetic parameters of enzymes reactions, namely V_{max} and K_m, has been done by Dixon & Webb (1958). In individual cases, where careful data on these relationships have been collected, it has been possible from plots V_{max} and K_m versus pH, to make an educated guess as to which ionizable groups in the enzyme reaction. The difficulty in this approach stems partly from the paucity of available data in the literature, but even more from the uncertainty in their interpretation. Indeed, it is often impossible to know whether any observed effect of pH bears on the ionization of the enzyme, or of the substrate(s) or sometimes even of the product. Moreover, the method of determining which amino acid(s) in the enzyme is or are involved in the reaction is based on the assumption that their pK corresponds to break points in the plots of V_{max} versus pH or K_m versus pH respectively. This method therefore depends on the knowledge of the pK values of amino acids within the protein molecule. Since it is known that pK can be affected markedly by neighbouring groups, there is a high degree of uncertainty inherent in the method.

It was considered that some of the difficulties in evaluating the functions expressing the dependence of kinetic

TABLE VIII

VARIATION OF V_{max} WITH pH

pH	A	A⁻	B	V_{max} $\triangle$ 340/min	
				Ijebu-Ode	Ita-Bale
5.5	0.0015	0.007	0.005	0.003	0.008
6	0.010	0.019	0.027	0.010	0.027
6.5	0.022	0.026	0.053	0.014	0.033
7	0.030	0.037	0.063	0.023	0.053
7.5	0.036	0.044	0.070	0.033	0.066
8	0.041	0.043	0.080	0.034	0.067
8.2	0.036	0.045	0.077	0.036	0.067
8.3	0.041	0.046	0.074	0.035	0.065
8.4	0.042	0.049	0.077	0.038	0.075
8.5	0.043	0.047	0.080	0.039	0.077
8.8	0.046	0.054	0.086	0.045	0.083
9	0.044	0.056	0.090	0.049	0.093
9.3	0.047	0.062	0.096	0.051	0.110
9.5	0.049	0.060	0.090	0.054	0.112

See Fig. 10.

parameters of pH could be overcome in a situation where several enzyme variants, presumably differing only by single amino acid substitution (see Yoshida, 1967), can be comparatively examined.

R E S U L T S

(a) <u>Dependance of V_{max} on pH</u>: The experimental findings are summarised in Table VIII and Figure 10. It is seen that for all variants examined there is an overall steady increase of V_{max} with pH over the entire range explored from pH 5.5 to 9.5. However, localized differences are noticed. Thus A⁻, Ijebu-Ode and Ita-Bale have a plateau in the pH region between 7.5 and 8.2, whereas A and B have a *appears* "peak and trough" *in addition* in the same region. *However the troughs are at the limit of resolution of the data since they are about 1-2% of the neighbouring values and the reproducibility of the measurement is of the order of 6 to 10%.*

(b) <u>Dependance of K_m for Glucose 6-Phosphate upon pH</u>: The Michaelis constants for each variant were measured at each of 5 pH values 5.0, 6.0, 7.0, 8.0 and 9.0. Determinations were carried out on at least two preparations for the common variants. One set of data for each variant is shown in Fig. 12(a) - (e). The K_m values derived are shown in Table IX and Figure 13. The values of V_{max} in Table VIII have been divided by the K_m values at the corresponding pH (see Table X) and the logarithm of the ratio is plotted in Figure 14. The data for the A⁻ variant have been omitted from Table X and Figures 13, 14 for reasons discussed below.
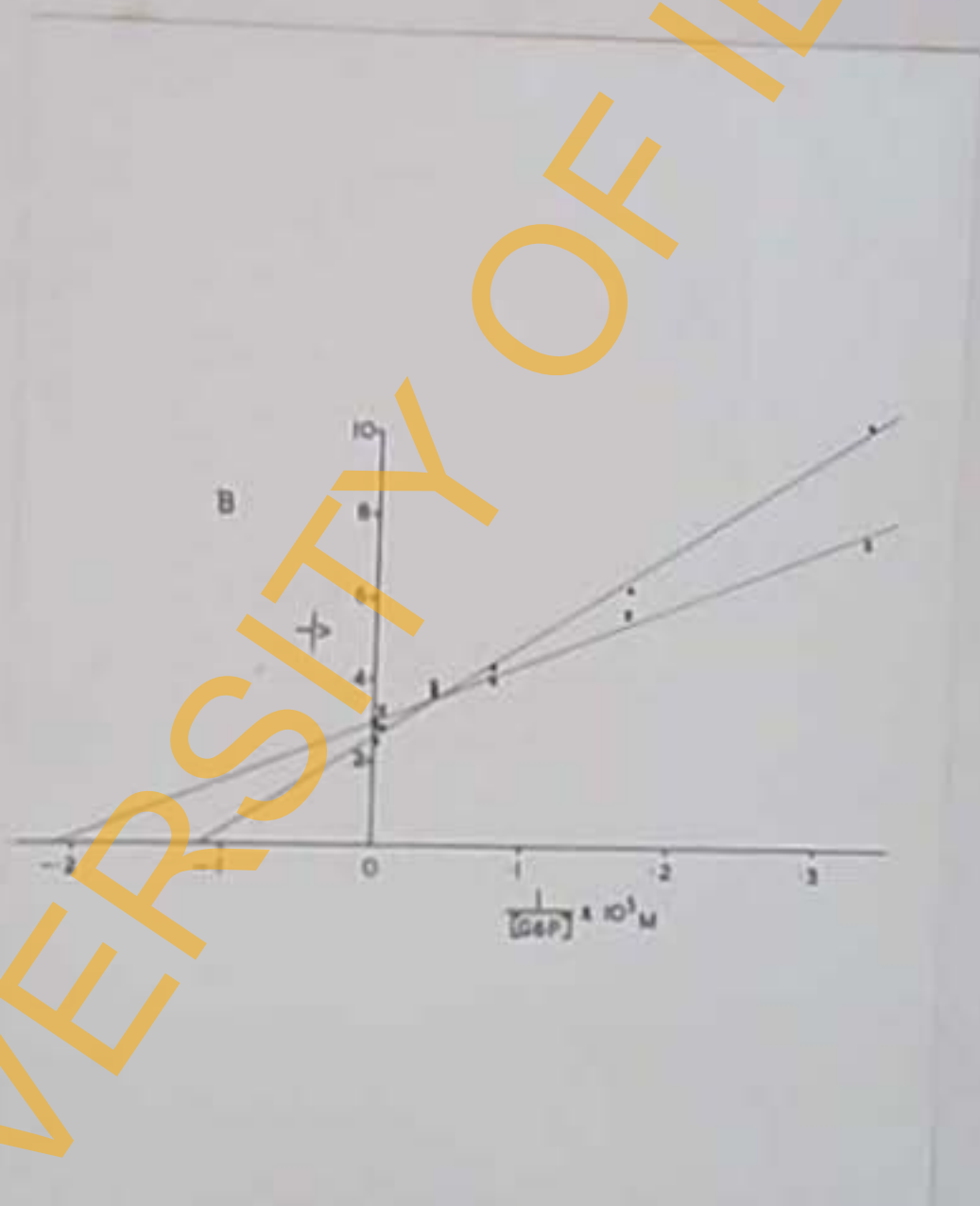
Fig. 8(a). Reciprocal plots (Lineweaver & Burk, 1934) of 1/v
and 1/G6P for determination of K_m. Buffer used
was Tris-boric acid - triethylamine, pH 7.8.
The reaction mixture contained 0.33 mM NADP (final

concentration) and variable amounts of G6P. Two different
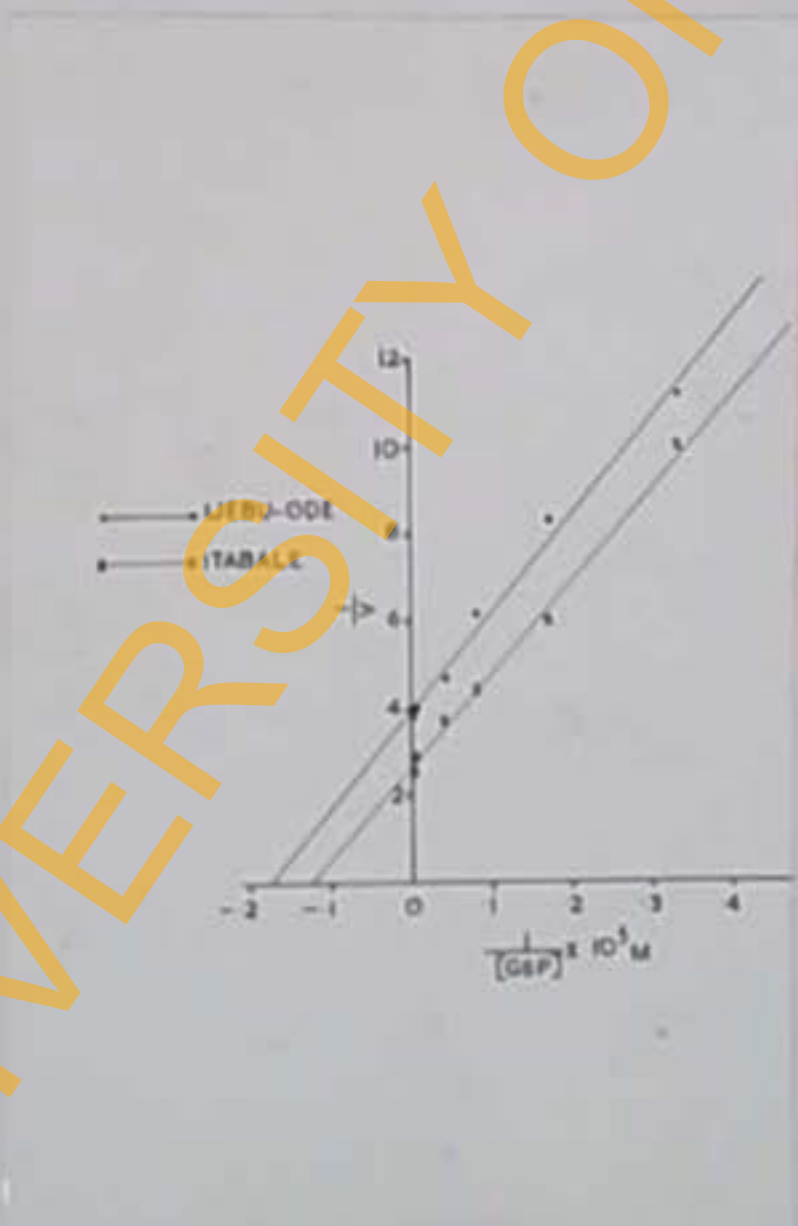preparations of A, A* and B were employed in the K_m determinations

Fig 8(b) Same legend as in Fig. 8(a). However only one
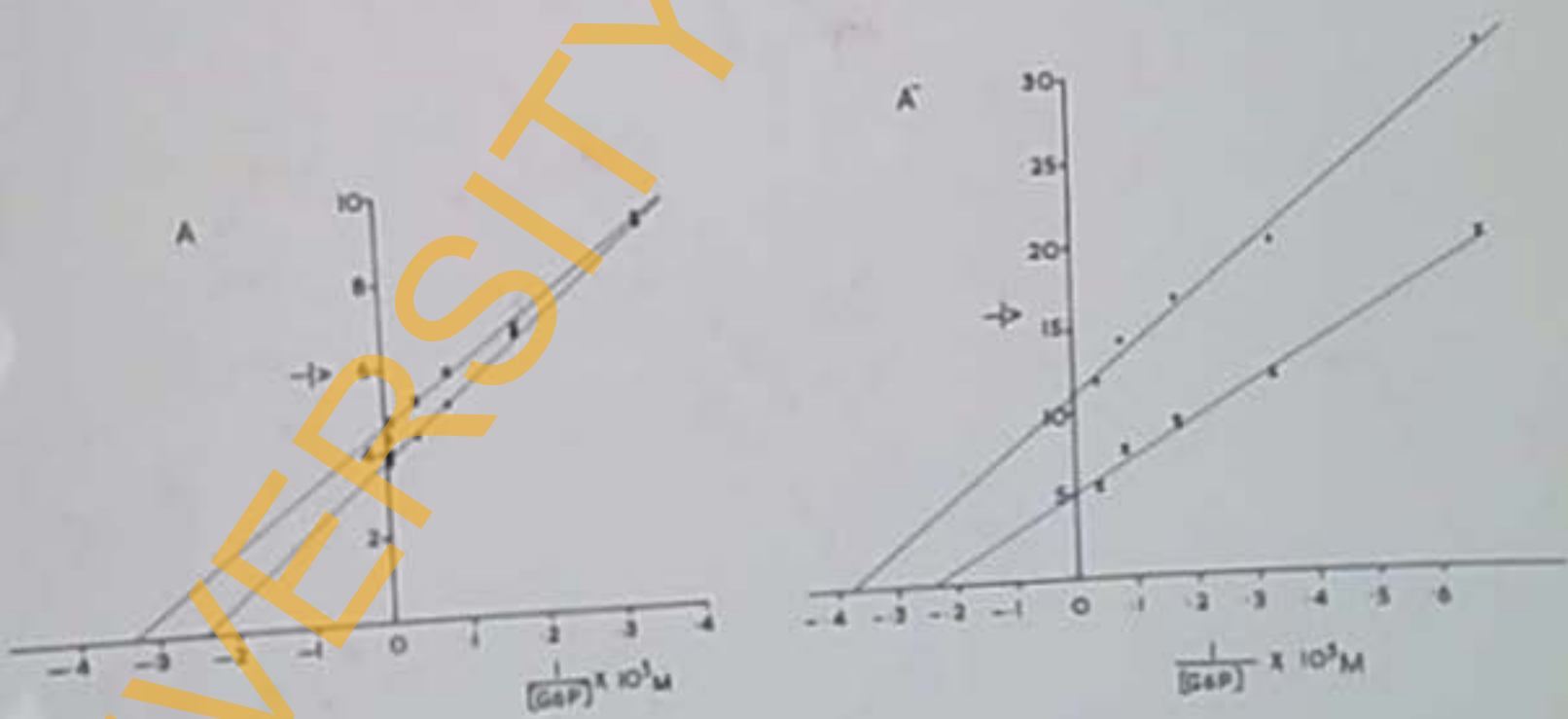
preparation of each variant was used.

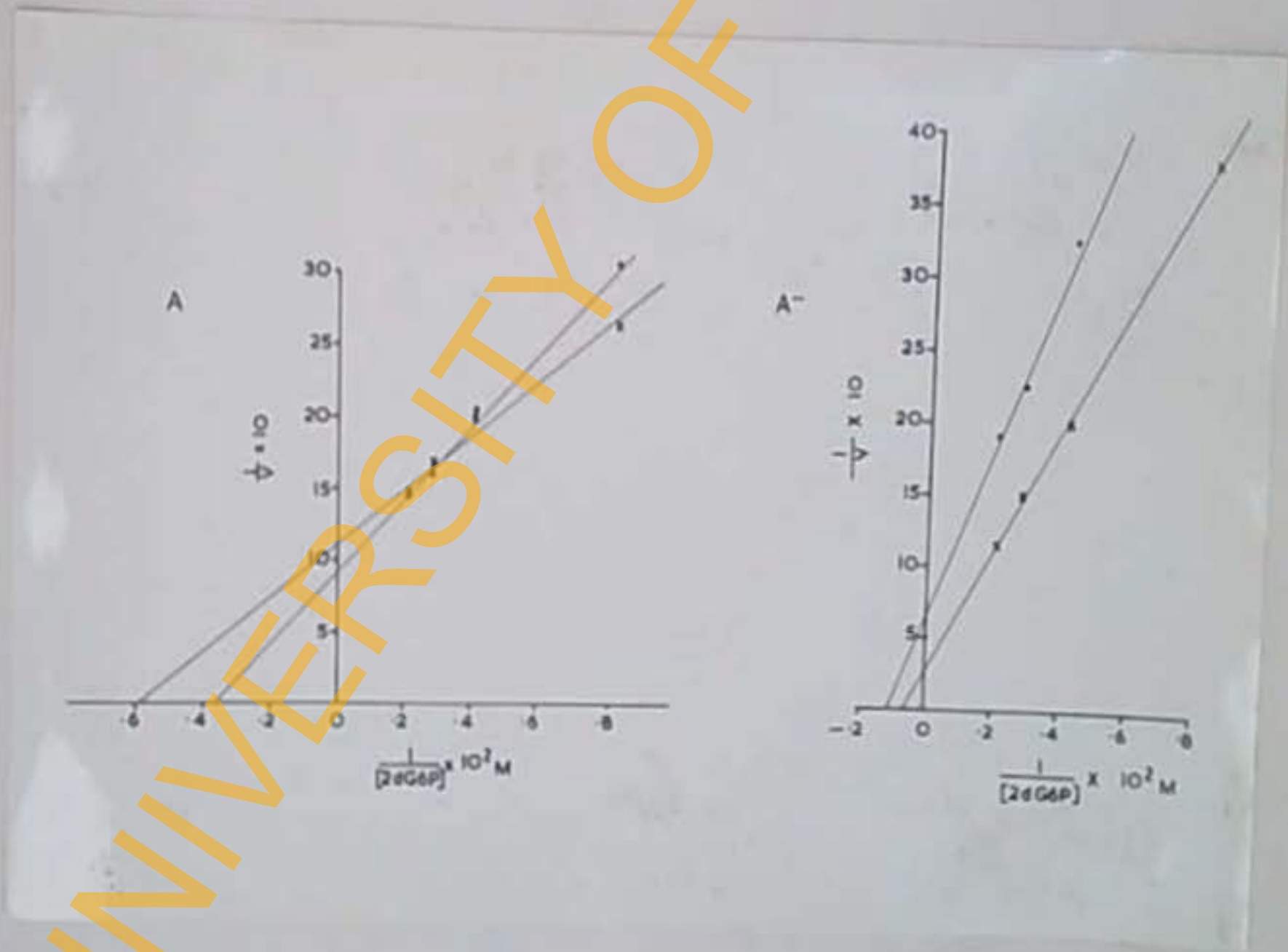Fig. 8(c). Same legend as in Fig. 8(a).

Fig. 9(a). Reciprocal plots of velocity and 2dG6P concentrations in the determination of K_m. The reaction mixture contained 0.33 mM NADP and variable amount of 2dG6P. Tris-boric acid buffer pH 7.8 was used. Two different preparations of A, A⁻ and B were used for the K_m determination.
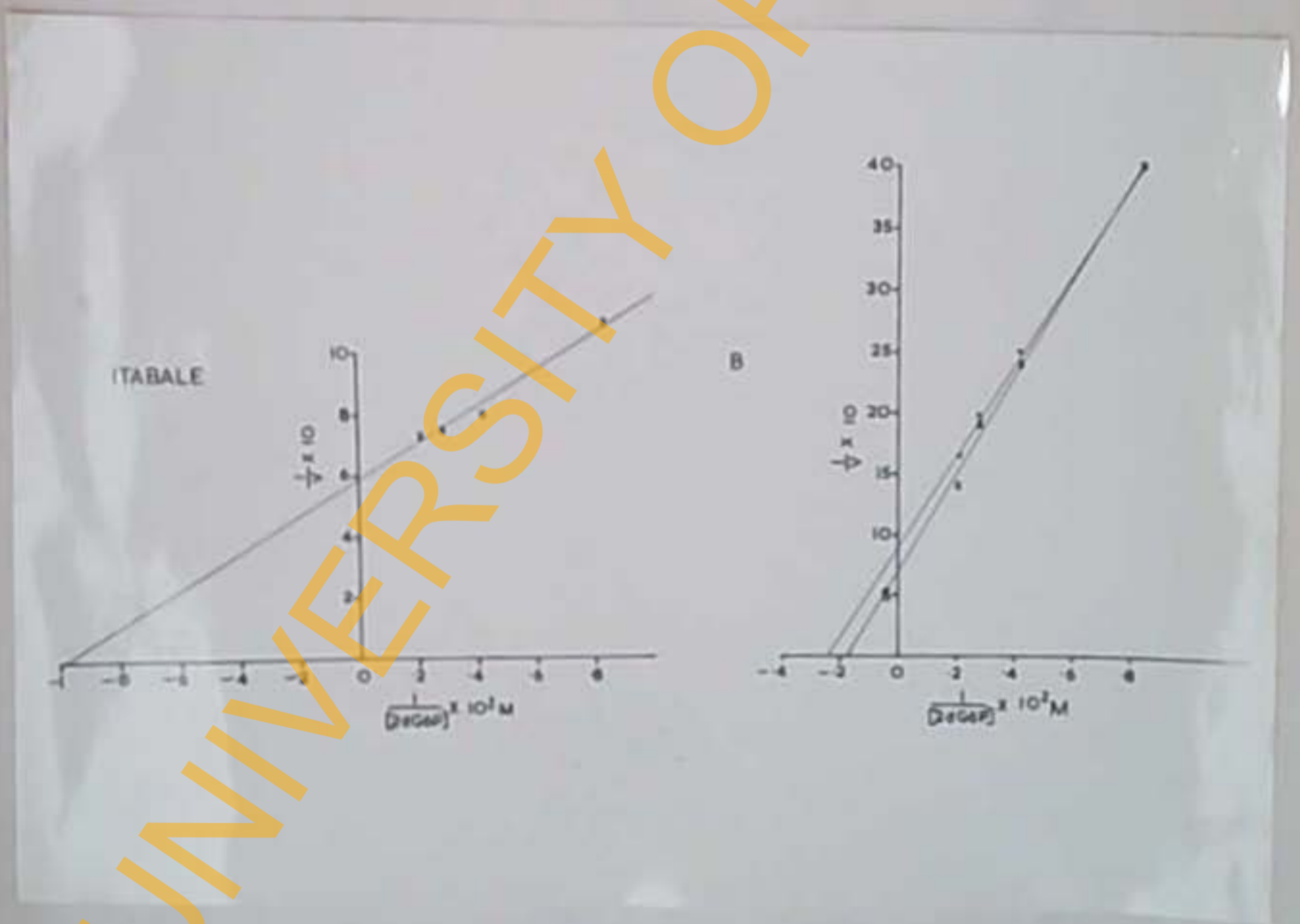
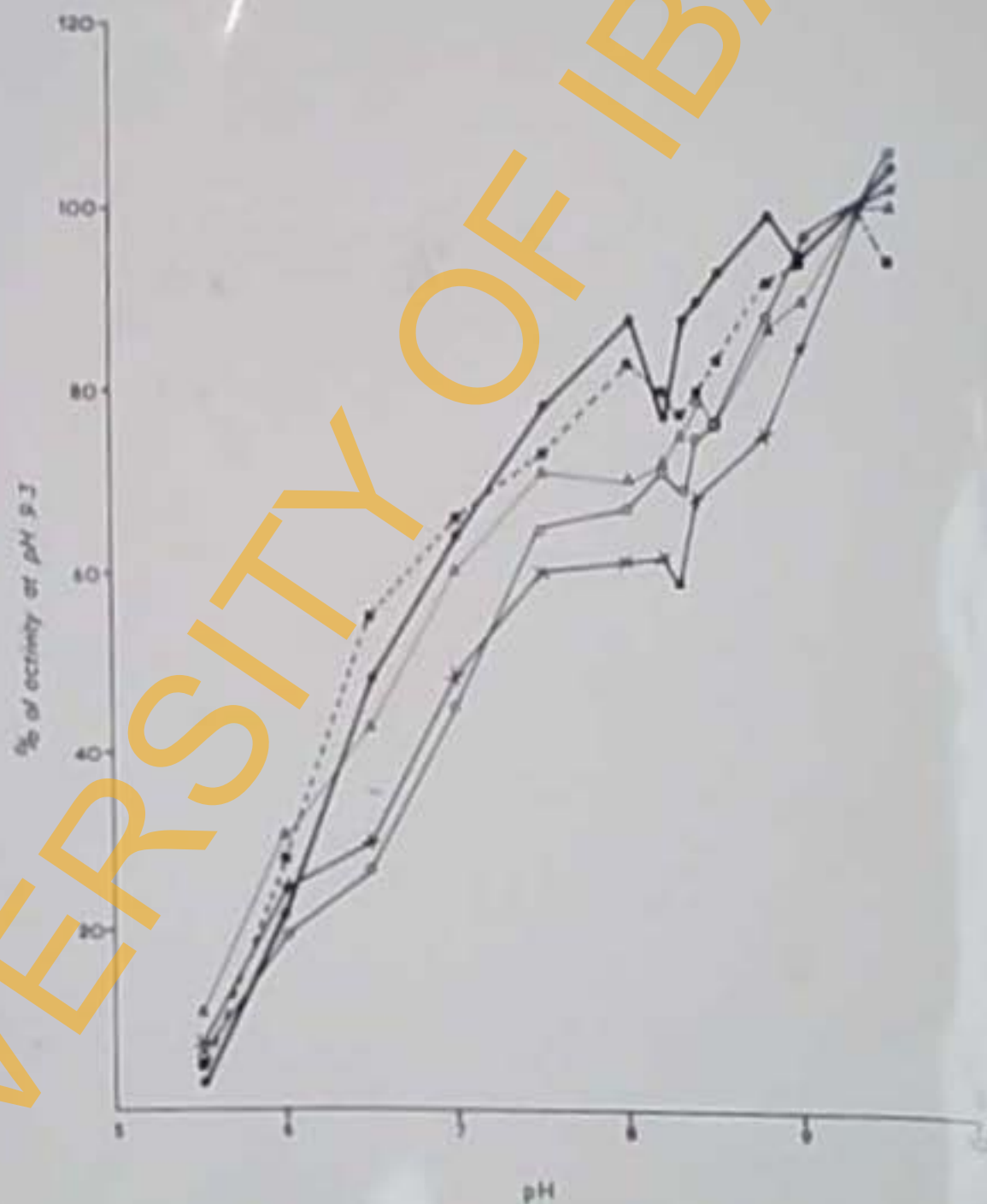Fig. 9(b). Same legend as in Fig. 9(a).

Fig 10. Variation of V_{max} with pH for G6PD variants. For buffers and substrate concentrations employed see Chapter II; ——• A; △——△ A⁻; □——□ B ×——× Ita-Bale- ○——○ Ijebu-Ode. Only one preparation of each of the last two variants was used and each assay was carried out twice. For other variants, each point represents average of two values from two different preparations which varied by ± 5%.

TABLE VI

K_m FOR G6P. DETERMINATION AT pH 7.8 0.05 M
TRIS-BORIC ACID BUFFER

		$\underline{K_m}$ for G6P
A	I.	0.41×10^{-4} M
	II.	0.32×10^{-4} M
A^-	I.	0.26×10^{-4} M
	II.	0.4×10^{-4} M
B	I.	0.8×10^{-4} M
	II.	0.48×10^{-4} M
Ijebu-Ode		0.6×10^{-4} M
Ita-Bale		0.9×10^{-4} M

Two different preparations of A, A^- and B G6PD variants were used for assays. Each assay was carried out twice and differences in value were not more than 10%.
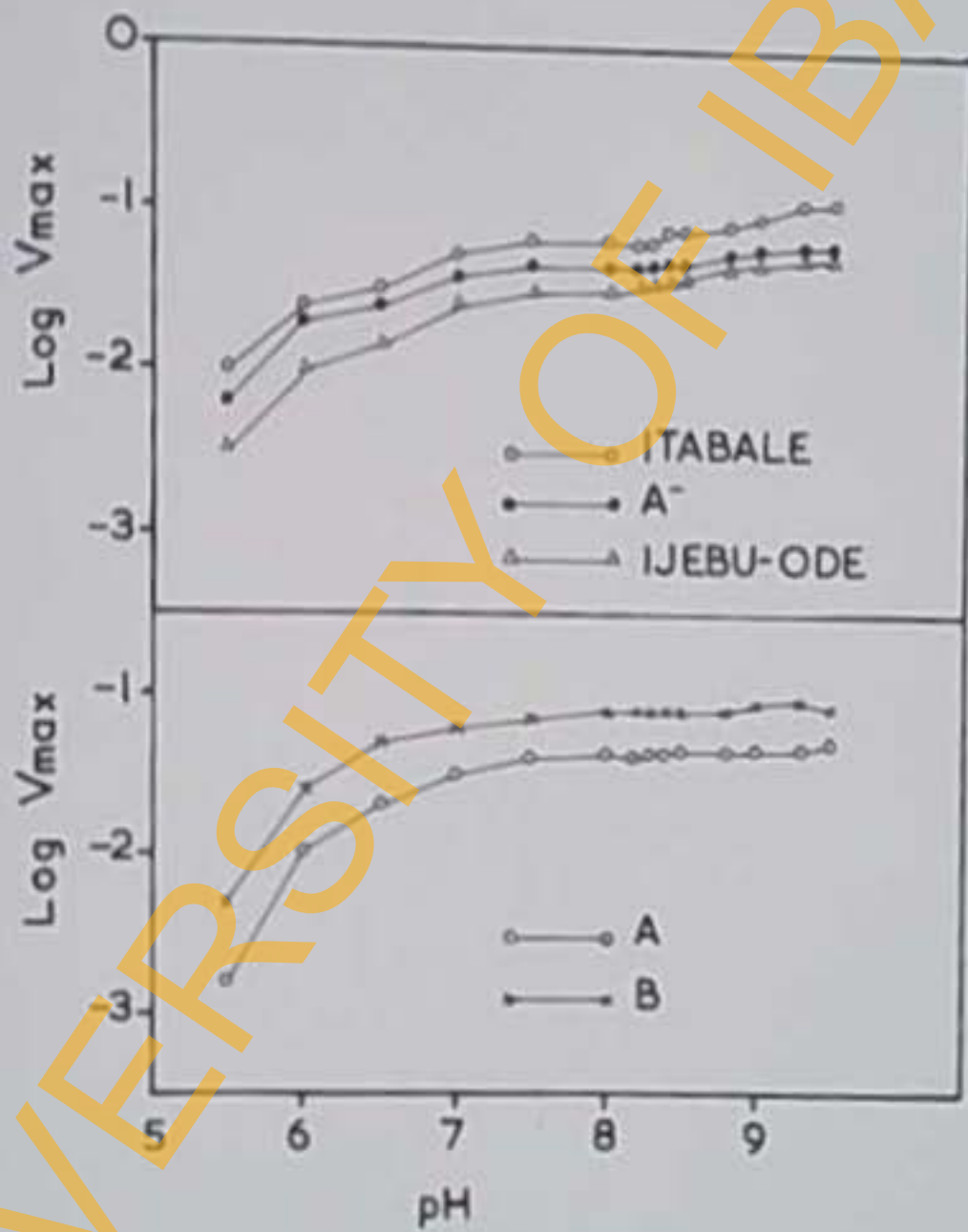
See Figs. 8 (a), (b), (c).

Fig. 11. Variation of log V_{max} with pH. Data from the same experiments of Fig. 10.

Fig. 12.   (a)   Reciprocal plots (Lineweaver & Burk, 1934) of
velocity and G6P concentrations for the determination
of K_m as a function of pH. The reaction mixture contained
0.33 mM NADP (final concentration) and variable amount
of G6P. The pH of the G6P was adjusted to appropriate
buffer pH value before being added to reaction mixture.
The buffers were as follows: pH 5, 6: Tris-boric-citrate;
pH 7,8: Tris-boric acid pH 9 Tris-boric acid -
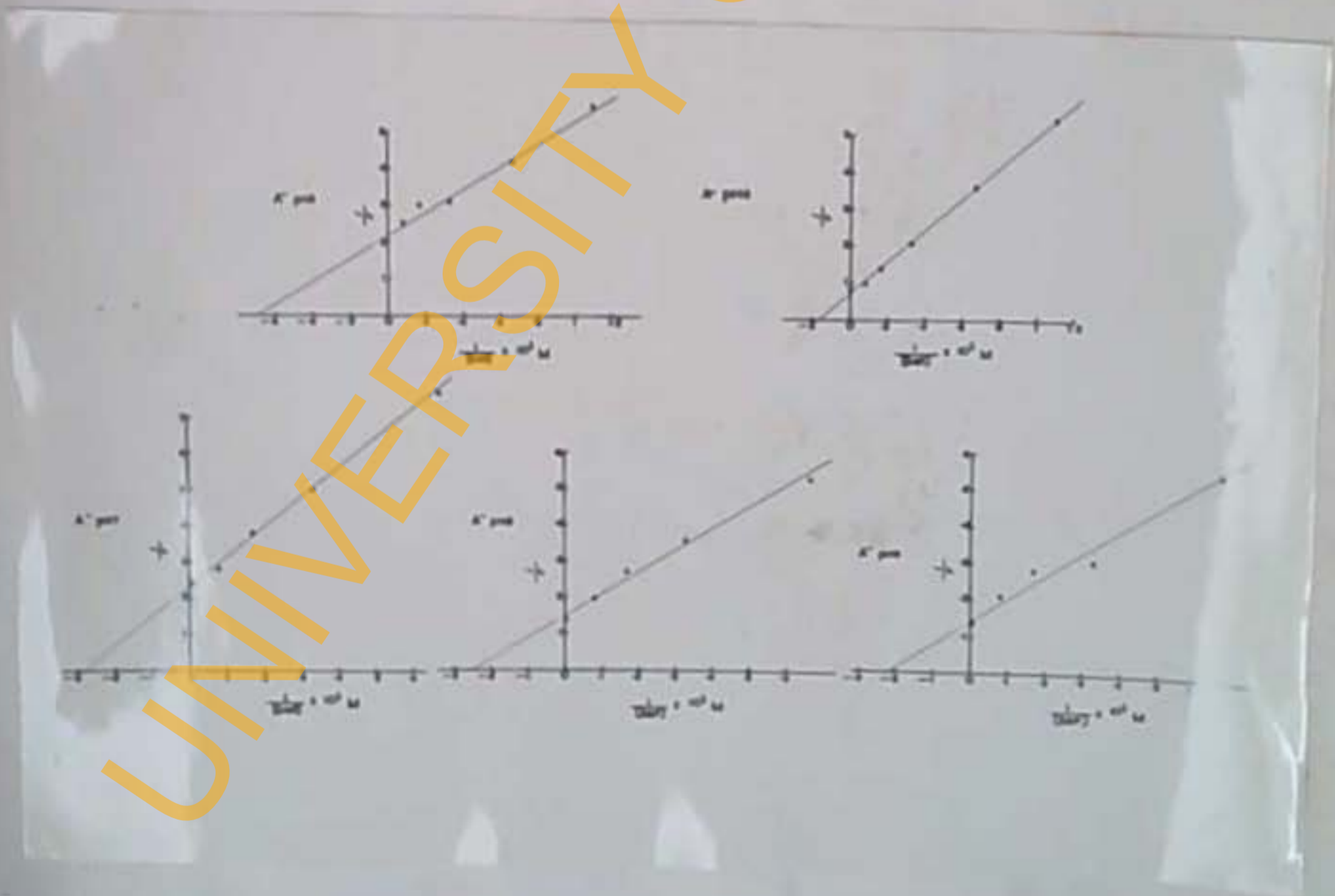triethylamine. G6PD variant A.

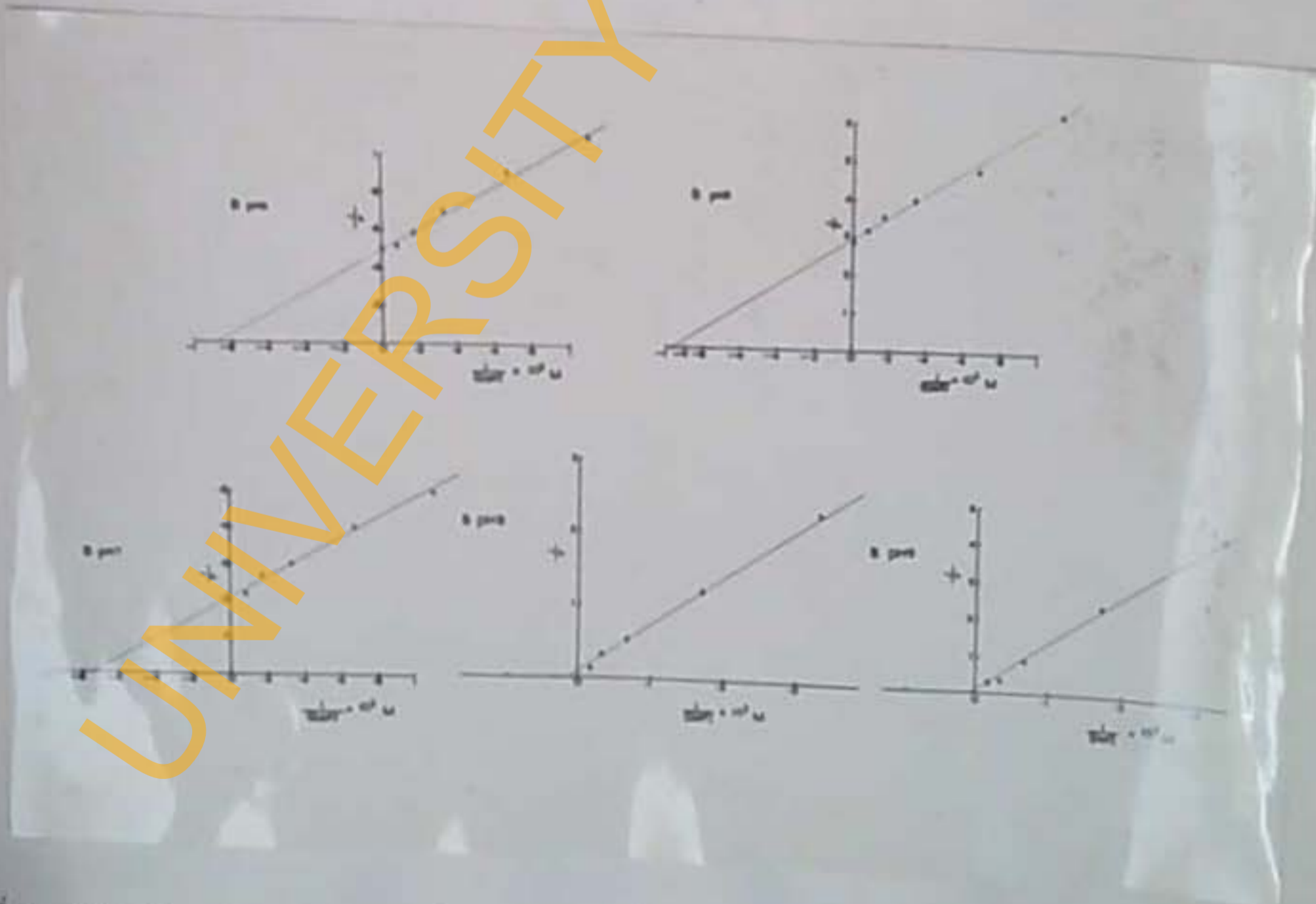Fig. 12(b). Same legend as in Fig. 12(a). G6PD variant A⁻.

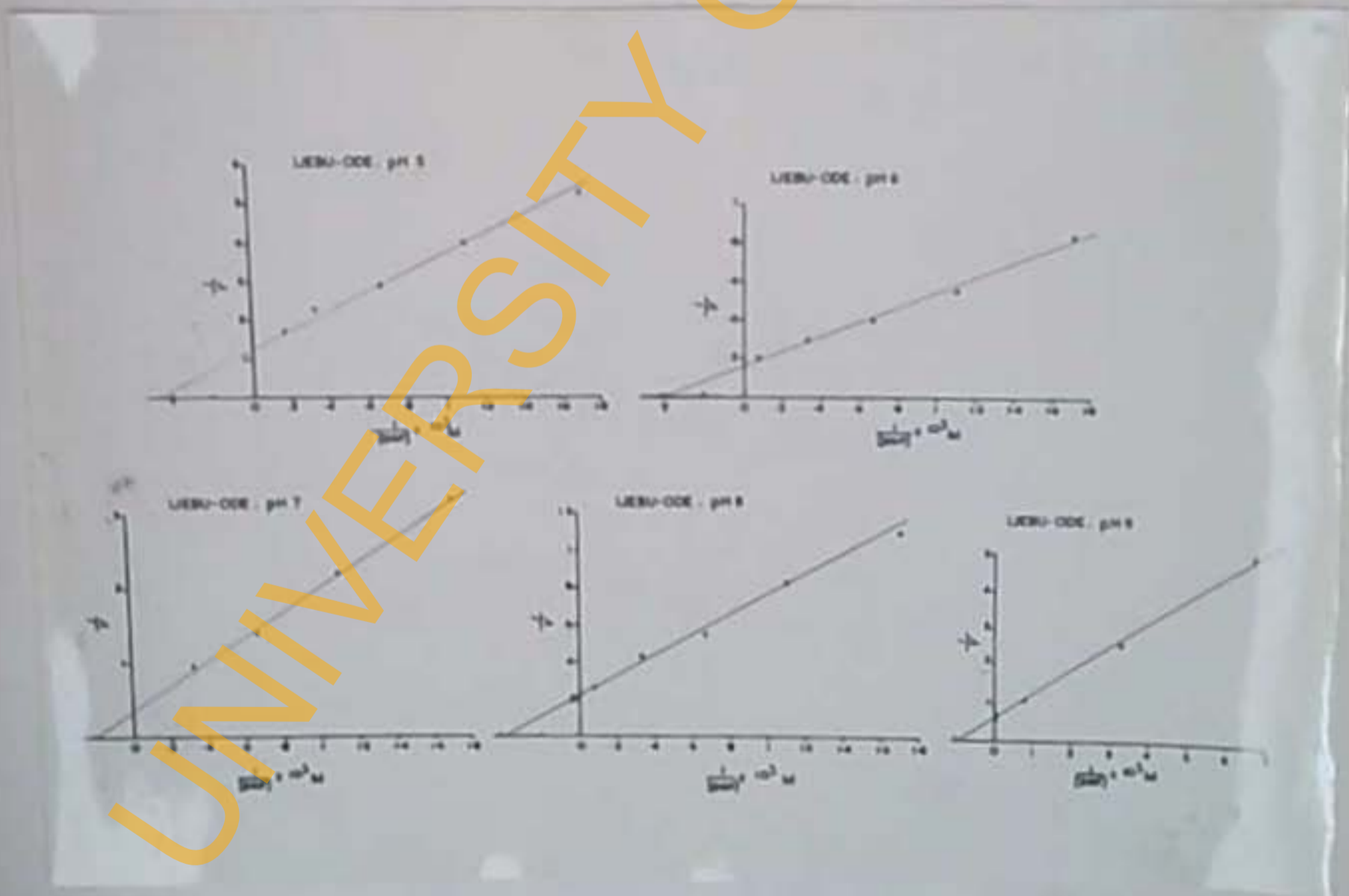Fig. 12(c). Same legend as for Fig. 12(a). G6PD variant B.

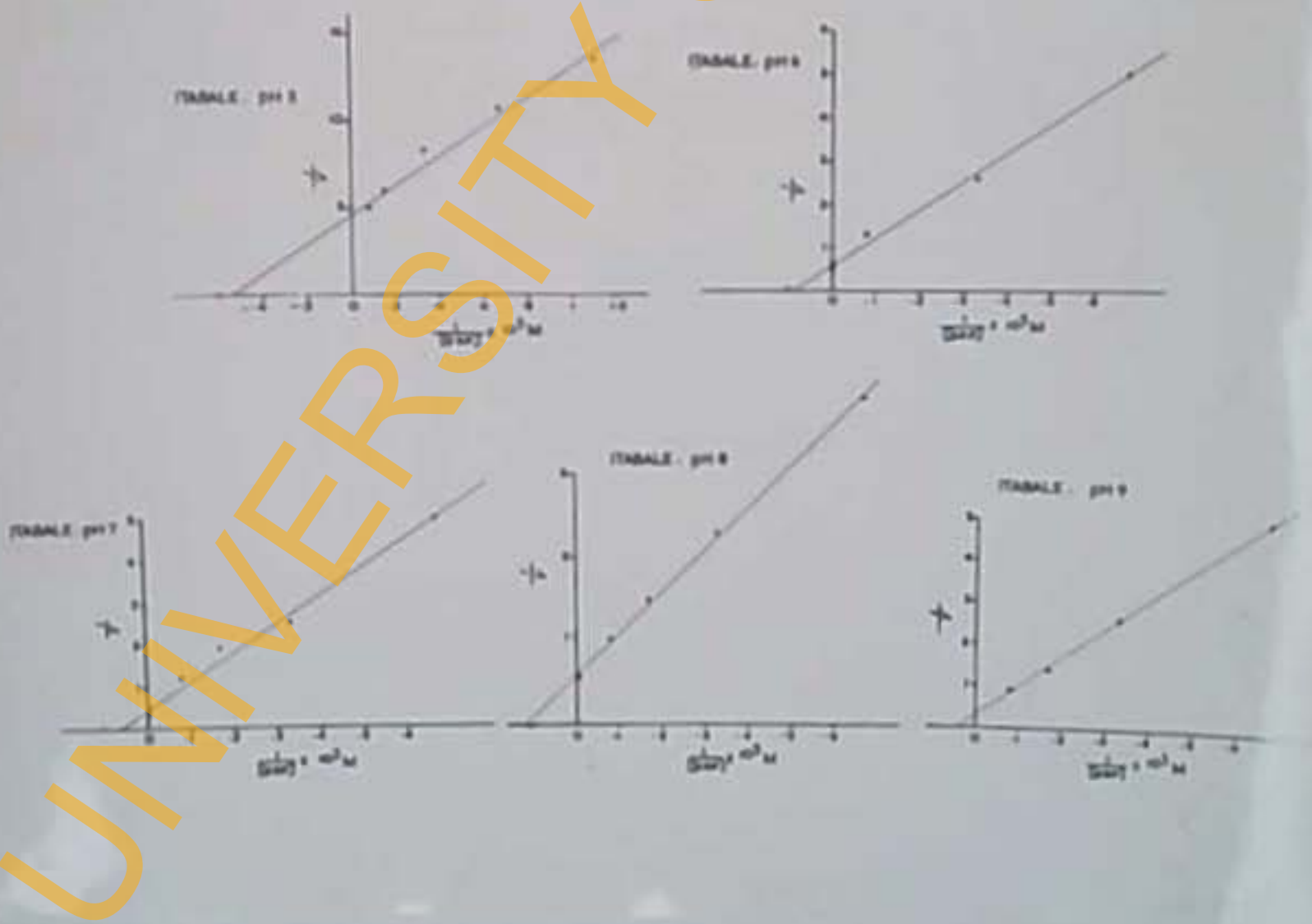Fig. 12(d).   Same legend as for Fig. 12(a).   G6PD variant
Ijebu-Ode.

Fig. 12(e). Same legend as for Fig. 12(a). G6PD Variant Ita-Bale.

TABLE IX

THE K_m FOR G6P DETERMINED AS A FUNCTION OF pH.

Variants	K_m x 10^{-4} M at pH				
	5	6	7	8	9
B	0.28	0.20	0.8	0.8	2.2
	(0.19)	(0.15)	(0.48)	(0.8)	(2.1)
	0.1	0.11	0.13	0.8	2.0
A	0.55	0.90	0.40	0.9	1.3
	(.39)	(.70)	(.37)	(1.0)	(1.35)
	0.25	0.50	0.34	1.1	1.4
A⁻	0.14	0.62	0.35	0.4	0.45
	0.18	0.62	2.5	1.5	3.1
	-	0.2	0.24	3.3	5
	0.8	0.25	0.34	0.45	2
Ijebu-Ode	0.22	0.24	0.53	0.47	1.0
Ita-Bale	0.18	1.1	1.6	1.0	2.5

See Fig. 12 (a), (b), (c), (d), (e). Different
preparations of A, A⁻ and B G6PD variants were used for assays.
Each assay was carried out twice and differences in value were
not more than 10%. The average values for types B and A are in
parentheses and these values were used subsequently in all
calculations. In the case of A⁻ the last two lines refer to
values obtained with the same preparation at two different
stages of purification (0.1 units/mg. and 0.4 units/mg.
respectively). For all the G6PD variants, Figs 12(a) - (e)
illustrate only one set of K_m values presented in this
table.
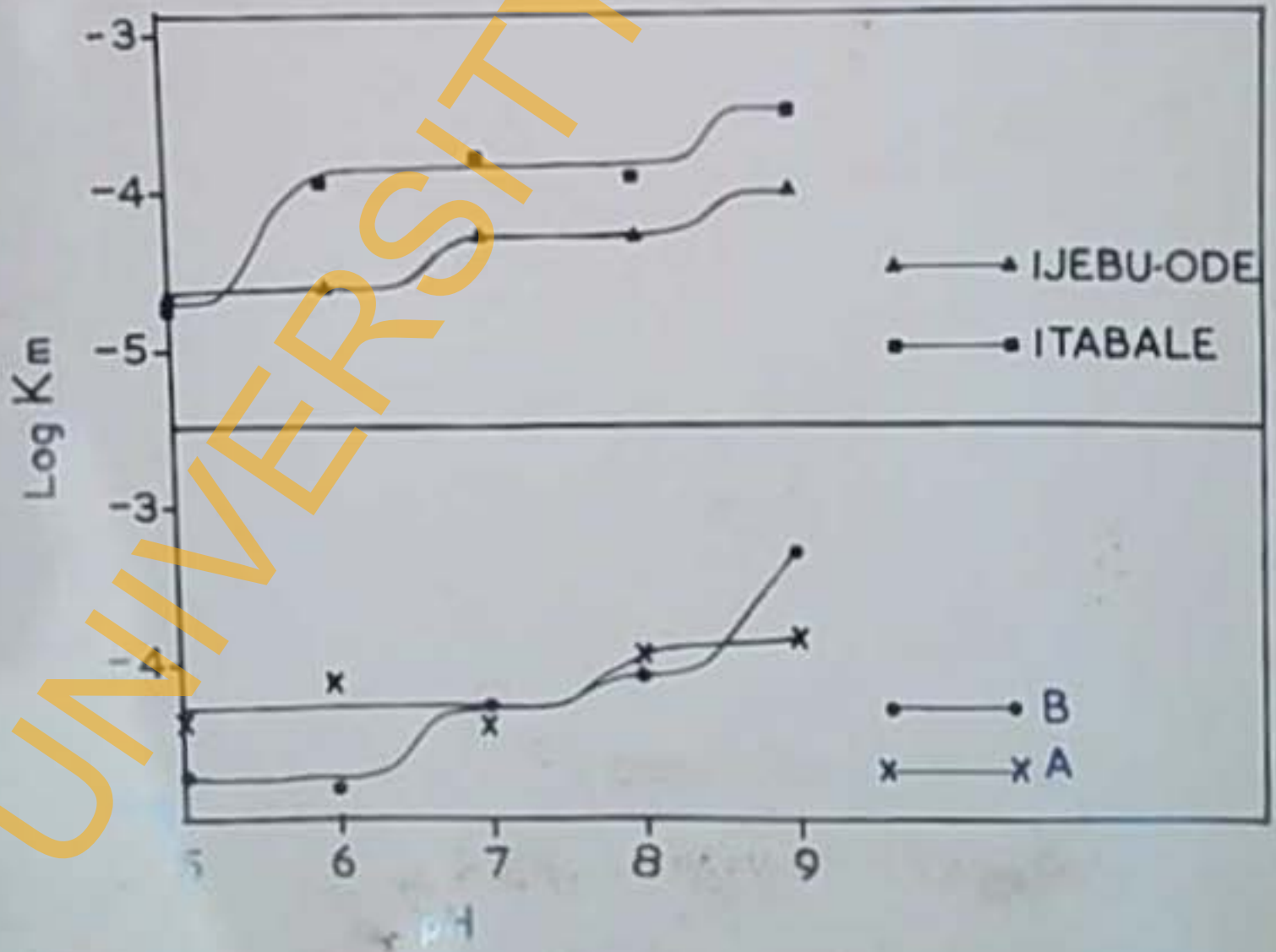
Fig. 13. Plot of log K_m for G6P versus pH. Data from the experiments of Fig. 17.

TABLE X

$\dfrac{V_{max}}{K_m}$ VALUES AT DIFFERENT pH FOR ALL THE VARIANTS

Variants	pH				
	5	6	7	8	9
A	0.85	1.6	7.9	9.6	4.9
B	1.74	12.5	34	25	5
Ijebu-Ode	3.8	22.8	35.8	6.9	1.7
Ita-Bale	1.2	1.5	1.4	2	1.3

See Fig. 14. V_{max} was calculated from the ordinate intercept in the plot of $\frac{1}{v}$ versus $\frac{1}{[G6P]}$ (See Fig. 12).
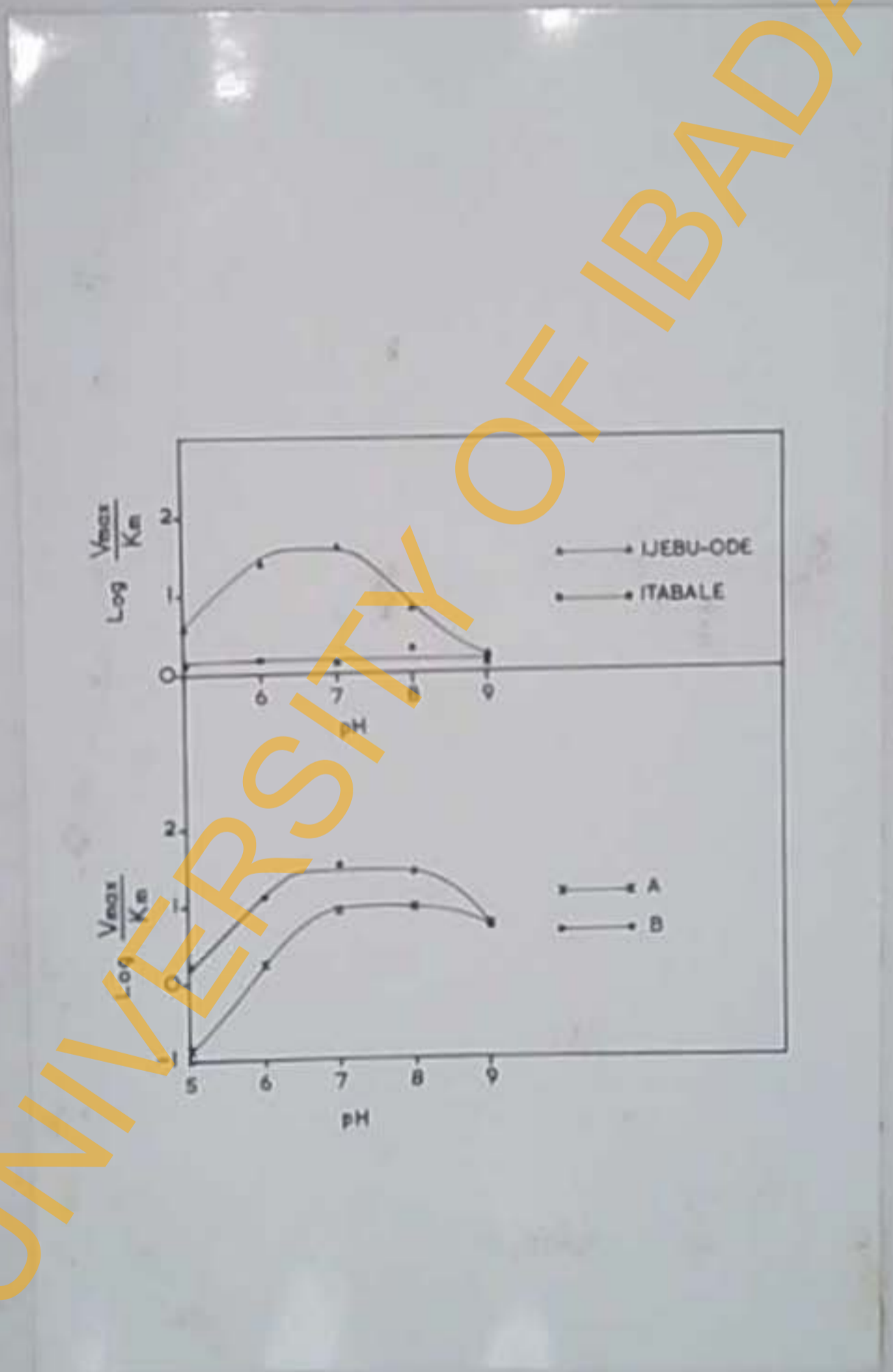
- 75 -

Fig. 14.

Plot of Log V_{max}/K_m versus pH. Data from the experiments of Fig. 12

TABLE XI

pK VALUES OF SOME GROUPS PRESENT IN PROTEINS

Group	pK (25°)
Carboxyl (α)	3.0 - 3.2
Carboxyl (aspartyl)	3.0 - 4.7
Carboxyl (glutamyl)	ca 4.4
Phenolic hydroxyl (tryrosine)	9.8 - 10.4
Sulphydryl	8.3 - 8.6
Imidazolium (histidine)	5.6 - 7.0
Ammonium (α)	7.6 - 8.4
Ammonium (α, cystine)	6.5 - 8.5
Ammonium (ϵ, lysine)	9.4 - 10.6
Guanidinium (arginine)	11.6 - 12.6

The data in the table are taken from Proteins, Amino
Acids and Peptides: Edit, COHN E.J. and EDSALL, J.T.
p. 445 (New York, Reinhold Publishing Corp, 1943).

DISCUSSION

The most thorough approach to an interpretation of the effect of pH on kinetic parameters of enzymes has been done by Dixon (1953). By referring to a model which takes into account the ionization of groups on the free enzyme, as well as on the enzyme-substrate complex, this author was able to derive general rules on how the break-points and "waves" in the plots of kinetic parameters versus pH can be used in an attempt to determine which particular amino acid is involved. The most informative is the plot of log K_m versus pH. In such a plot, according to the model proposed by Dixon, downward bends correspond to the pK of ionizable groups in the free enzyme or the free substrate. Upwards bends correspond to the pK of groups in the enzyme-substrate complex. When two such bends (one upwards and one downwards) are very near each other, producing a "wave" in the graph, one possible interpretation is that both bends are due to the presence of the same group, the difference between them representing the change in pK of the ionizable group associated with the formation of the enzyme-substrate complex. According to Dixon's model, all pK's of groups involved should be evident on the plot of log K_m versus pH. On the other hand, plots of log V_{max} versus pH might show bends only in correspondence to the pK of groups that are ionizable in the enzyme-substrate complex, and plots of log V_{max}/K_m versus pH should show bends only in correspondence to the pK of groups that are ionizable in either the free enzyme or the free substrate.

Soldin & Balinsky (1966; 1968) have made a systematic attempt to use these rules in the case of human erythrocyte G6PD, a case made more complex by the presence of two substrates, G6P and NADP. The plot of log K_m^{G6P} versus pH (compare Fig. 13 of present work) yielded pK values of 6.2 and 9.0, and the plot of log K_m^{NADP} versus pH yielded pK values of 6.7 and 9.1. These were taken to indicate the presence of an imidazole and a sulphydryl groups near the active center (Soldin & Balinsky, 1966). The plots of log V_{max} versus pH (compare Fig. 11 of present work) gave a pK around 6.6, which was taken to indicate the presence of an unprotonated imidazole group in the ternary complex enzyme-NADP-G6PD. The plots of log V_{max}/K_a^1, log V_{max}/K_b^1, and log K_a versus pH (see Florini & Vestling, 1957 for definition of symbols) yielded pK values of 7.0 and 9.0, 6.8 and 8.7, 6.3 and 9.5 respectively (Soldin & Balinsky, 1968). The pK values between 6 and 7 were attributed to the involvement in substrate(s) binding of either the phosphate group(s) in the free substrate(s) or an imidazole group in the free enzyme. The pK values around 9 were taken to reflect the presence of unionized sulphydryl group in the free enzyme participating in substrate(s) binding.

The data presented here agreed largely, but not entirely, with those of Soldin & Balinsky (1966, 1968). The differences in the findings are likely due to the following differences in the experimental design. (1) The buffers employed. Soldin & Balinsky have used Tris, which does not affect the G6PD reaction,

as well as ammediol, glycine and maleate which <u>probably</u> do not affect the reaction, but also HCl and NaOH, which certainly do affect the reaction (see Chapter II). In this work, the buffer components used (Tris, borate, citrate, triethylamine) were all shown to have no effect on the G6PD reaction. (2) The homogeneity of the enzyme. Soldin & Balinsky carried out their work on preparations obtained from pooled "normal" blood. On the basis of the distribution of variants in their population, this must have consisted of a mixture of at least two variants, namely A and B. The assumption that these have identical kinetics, has been found here not to be uniformly valid (see Fig. 13). Furthermore, it should be remembered that the rules for deriving which ionizable groups are involved in the enzyme reaction must be very cautionsly applied to individual cases (Dixon & Webb, 1964). It is well known that the pK of individual groups can be considerably affected by the presence of neighbouring groups within the protein. Edsall (1943) has given figures for the usual pK range of ionizable groups found in proteins, and these will be referred to in the following (discussions) (see Table XI). Because of these uncertainties, it is not intended here to concentrate primarily on what groups are suggested by the data presented for the normal enzyme, type B. Rather, a comparative approach might be more profitable. Attention is to be focussed on how mutational changes from B to any other enzyme type have affected the shape of the curves. The basic idea is that whatever changes in the pK's of individual groups are brought about by the presence of neighbouring groups, will be the same or very similar for all

variants examined. Further, no difference can be attributed to the free substrates, since these are always the same. Thus, any difference observed must be specifically related to the amino acid substitution that has taken place in an individual variant. Another advantage of comparing the five variants is that, as mentioned before, the downward bends in the log K_m versus pH plots are taken to indicate the pK of groups present either in the free enzyme or in the free substrate. When we compare individual variants any difference observed clearly cannot be attributed to the free substrate which is the same for all, and therefore must be due to the free enzyme. The reverse situation (one enzyme and several substrates) has been exploited by Dixon & Webb (1964) in the study of the effect of pH on K_m in the case of arylsulphatase.

In brief, inspection of Figures 11, 13, 14 brings out the following points:-

(1) <u>Comparison between A and B</u>: In the plot of log K_m versus pH, A lacks the upward bend at pH 6.3 and the downward bend at pH 6.7. These differences are consistent with the absence from this enzyme of a ionizable group with a pK around 6.4. The most likely candidate for such a group is the imidazolium of histidine.

(2) <u>Comparison between Ijebu-Ode and B</u>: A remarkable change is the absence from the plot of log K_m versus pH of Ijebu-Ode of the "wave" around pH 7.7 found in the plot

for the B enzyme. This is consistent with the altered shape of the curve of log V_{max} versus pH. The most likely altered group in this case would be a cysteine residue near the active centre in enzyme B, absent in Ijebu-Ode. It is interesting to note that the same inference has been drawn quite independently by the study of some inhibitors (see Chapter 6).

(3) <u>Comparison between Ita-Bale and B</u>: In this case one notices that the upward bends found in B at a pH of about 6.6 is shifted to pH of about 5.7. In this case, the shift from pH 6.6 to 5.7 might be interpreted in terms of the group involved still being imidazolium, but with a change in pK due to some neighbouring group.

The variability of the results of K_m for G6P with different preparations of A^- (see Table IX) and with the extent of purification can not be easily explained. One possible reason is that the rate of intracellular degradation of A^- is faster than that of A (Yoshida, 1967). In consequence, the amount of inactivated A^- increases with the age of the red cell. The inactivated enzyme may not be catalytically active but can still bind the substrate. If this is so, it would compete with the active fraction for the available substrate. Thus, a faulty K_m value might be obtained. If, in the process of purification, the amount of the inactive moiety is reduced, the apparent affinity of the active form of the enzyme for the substrate will

increase. Hence, the greater the purification, the lower the K_m value for the substrate. This hypothesis is consistent with the results in Table IX. At any rate, it is clear that, because of the variability in the results obtained, the true dependence of K_m on pH for this variant cannot be established with certainty at the present time. The way and manner by which the in vivo red cells get rid of the inactivated enzyme is not known.

In summary, the analysis of the pH-dependence of kinetic parameters has exhibited reactivity differences among variants which are the direct expression of structural modifications. It is well known from the example of haemoglobins that such reactivity differences may occur even when the structural change is not very close to the ligand binding site (see Anusiem, Beetlestone & Irvine 1966; Beetlestone & Irvine, 1964; 1968), especially if the amino acid substitutions involve differences in electrostatic charge. Therefore, it is probably unsafe, to carry any further, any attempt to infer, from such reactivity differences, the nature of the structural differences. It is possible that more information could be obtained by the study of temperature effects on K_m; these effects would be related to the ΔH of substrate binding, like K_m itself is related to the ΔG of substrate binding. However, it is clear that data on primary structure are urgently needed at this stage, the limiting factors being - for the rare variants - availability of material and difficulties of sufficiently high purification.

CHAPTER V

KINETIC ANALYSIS OF THE INTERACTION OF FIVE G6PD VARIANTS WITH NADP AND NADPH:

(a) <u>Reaction rate as a function of NADP concentration</u>: It has been previously found with erythrocyte G6PD type A that the saturation function for NADP is sigmoid shaped and that it does not follow simple Michaelis-Menten kinetics (Luzzatto, 1967). The analysis has now been extended to the other G6PD variants and the result are shown in Table XII and in Figures 15 (a) and (c). Striking differences were observed among different variants. Thus, whereas the saturation curves for B and Ita-eale are quite similar to those previously reported for A, the curves for A^- and Ijebu-Ode are quite different and appear to be near-hyperbolic.

The sigmoid shape of the curves has been previously interpreted as being the expression of the existence of multiple binding sites for NADP and of the transition of the enzyme from a state of low affinity for NADP to a state of high affinity for NADP as the concentration of the latter is increased. A simple model was presented which makes it possible, from the analysis of the curves, to calculate the two dissociation constants (K_{s_1}, K_{s_2}) which refer to the binding of NADP in the state of low affinity and high affinity respectively (Luzzatto, 1967). The same treatment of the data has been carried out for the four variants illustrated here, and it has again been found that the theoretical saturation curves derived from the use of the two

G6PD Variants	
B	C
A⁻	C
Ijebu-Ode	C
Ita-Bale	C

See Fig

concentration) glucose-6-phosphate, NADP as indicated in the abscissa. The plots of the reaction velocities were normalised to V_{max}. The theoretical plots were obtained as indicated in the text. The above plots are for variants B and A⁻. See Table XII

TABLE XII

SATURATION FUNCTION OF G6PD VARIANTS WITH NADP

G6PD Variants	[NADP] μM										
	480	160	48	24	20	16	12	10	8	6	4
B	0.06	0.056	0.051	0.035	0.032	0.020	0.015	0.0127	0.009	0.006	0.003
A⁻	0.054	0.053	0.053	0.048	0.048	0.045	0.044	.036	.030	.025	.017
Ijebu-Ode	0.038	0.035	0.033	0.03	0.029	0.027	0.018	0.015	0.013	0.009	0.008
Ita-Bale	0.0625	0.058	0.05	0.033	0.027	0.019	0.015	0.01	0.003	0.0025	0.002

See Figs. 15 (a), (b). Reaction rates are expressed in ΔA_{340}/Min.
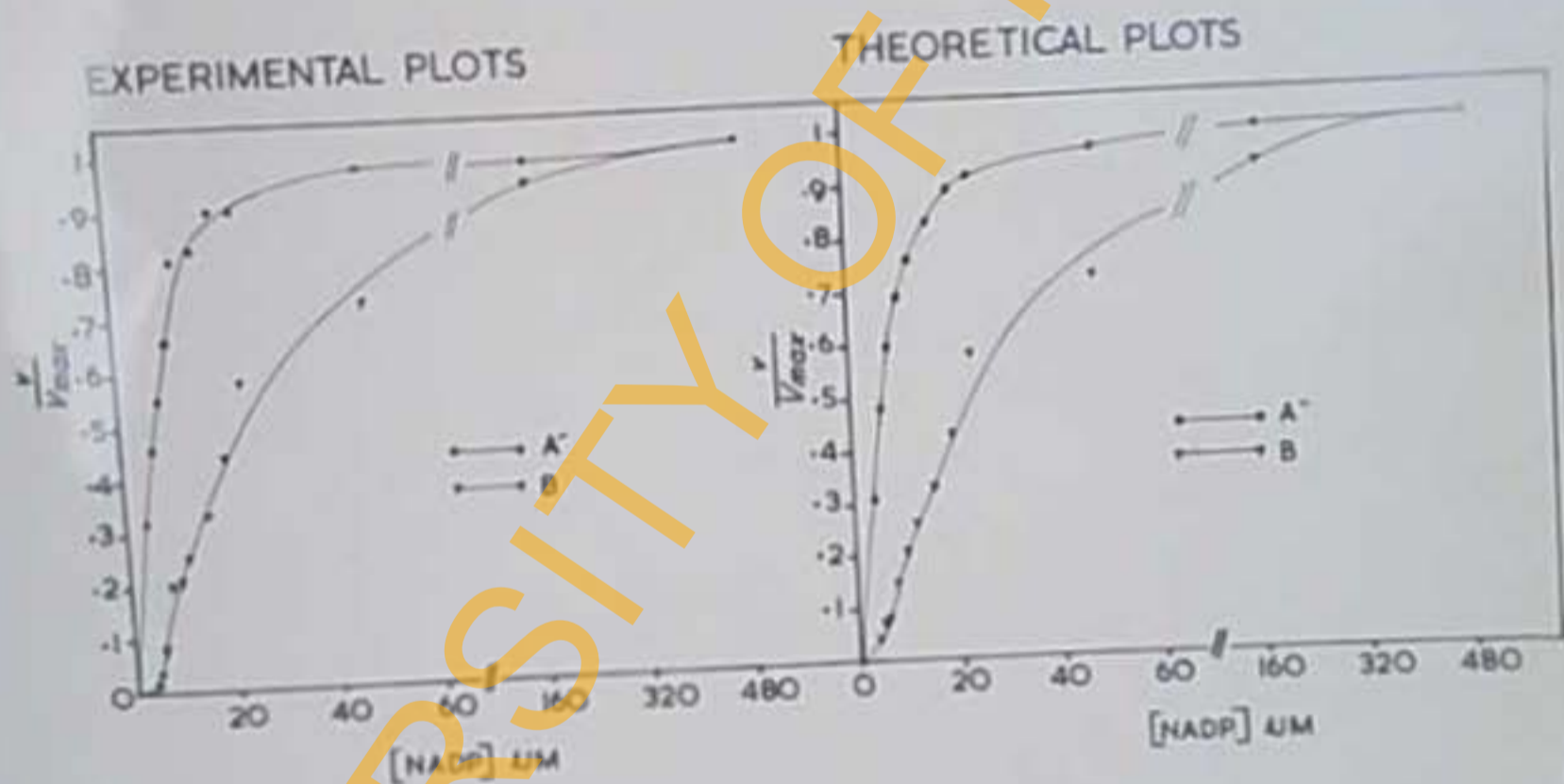
EXPERIMENTAL PLOTS   THEORETICAL PLOTS

Fig. 15(a). Saturation function of red cell glucose-6-phosphate
dehydrogenase with respect to NADP. The reaction
mixture contained 0.05M (final concentration)
Tris-boric acid buffer (pH 8.0) 4 mM (final
concentration) glucose-6-phosphate, NADP as indicated
in the abscissa. The plots of the reaction velocities
were normalised to V_{max}. The theoretical plots were
obtained as indicated in the text. The above plots
are for variants B and N. See Table XII

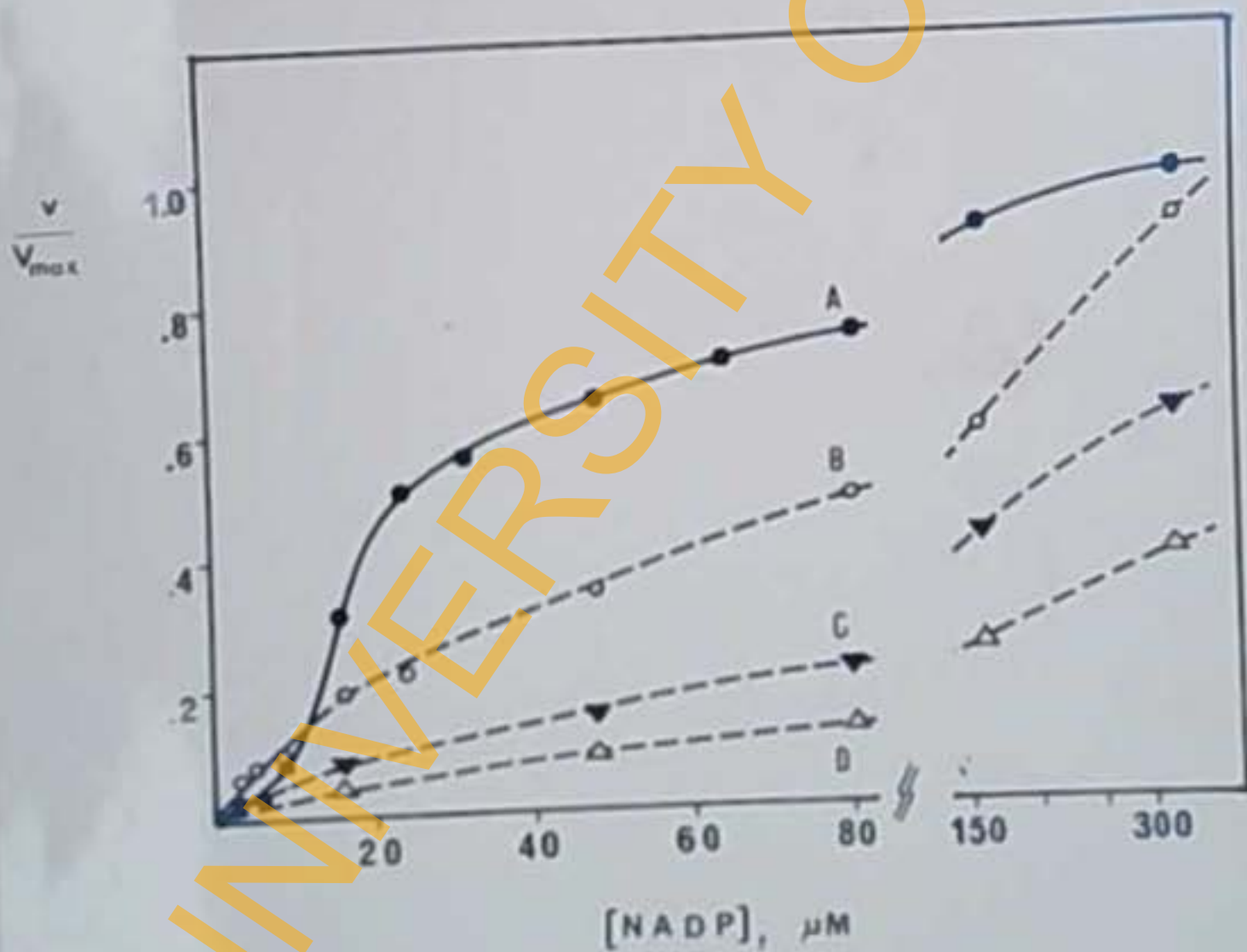**Fig 15(b)** Legend see Fig. 15(a). The above picture is for variant A (see Luzzatto, 1967a). Only curve A in the above picture is relevant.
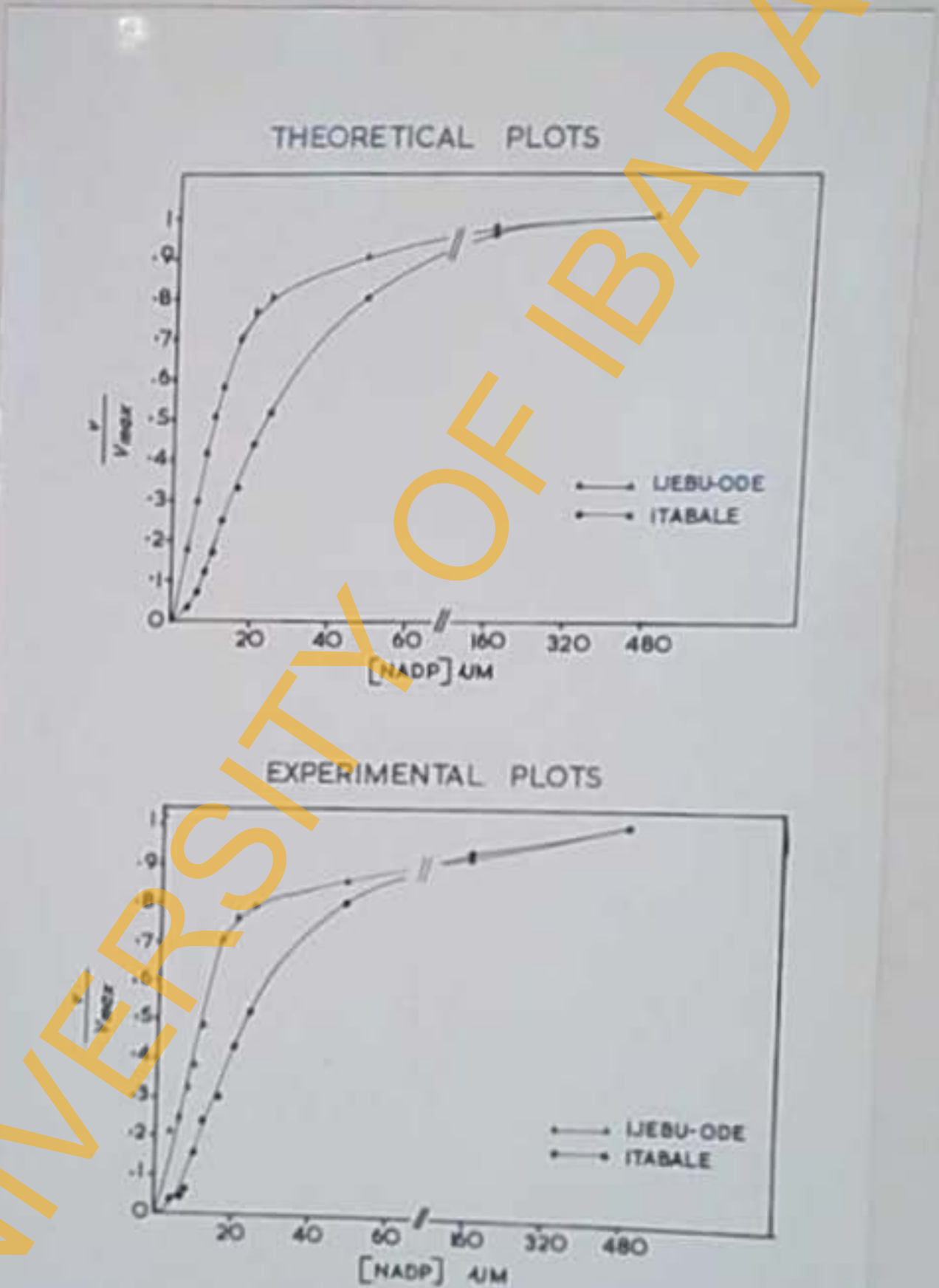
THEORETICAL PLOTS

EXPERIMENTAL PLOTS

Fig. 15(c). Legend see Fig. 15(a). Variants Ijebu-Ode and Ita-Bale.

TABLE XIII

KINETIC PARAMETERS OF INTERACTION BETWEEN G6PD AND NADP

G6PD Variants	K_{s_1} μM	K_{s_2}	n
A^-	23	1.3	1.78
B	21	12	1.58
Ijebu-Ode	22	3	1.40
Ita-Bale	170	2.7	2

The two dissociation constants K_{s_1}, K_{s_2} were calculated as done by Luzzatto (1967). The n, interaction coefficient values, are from the Hill plots of Fig. 16.
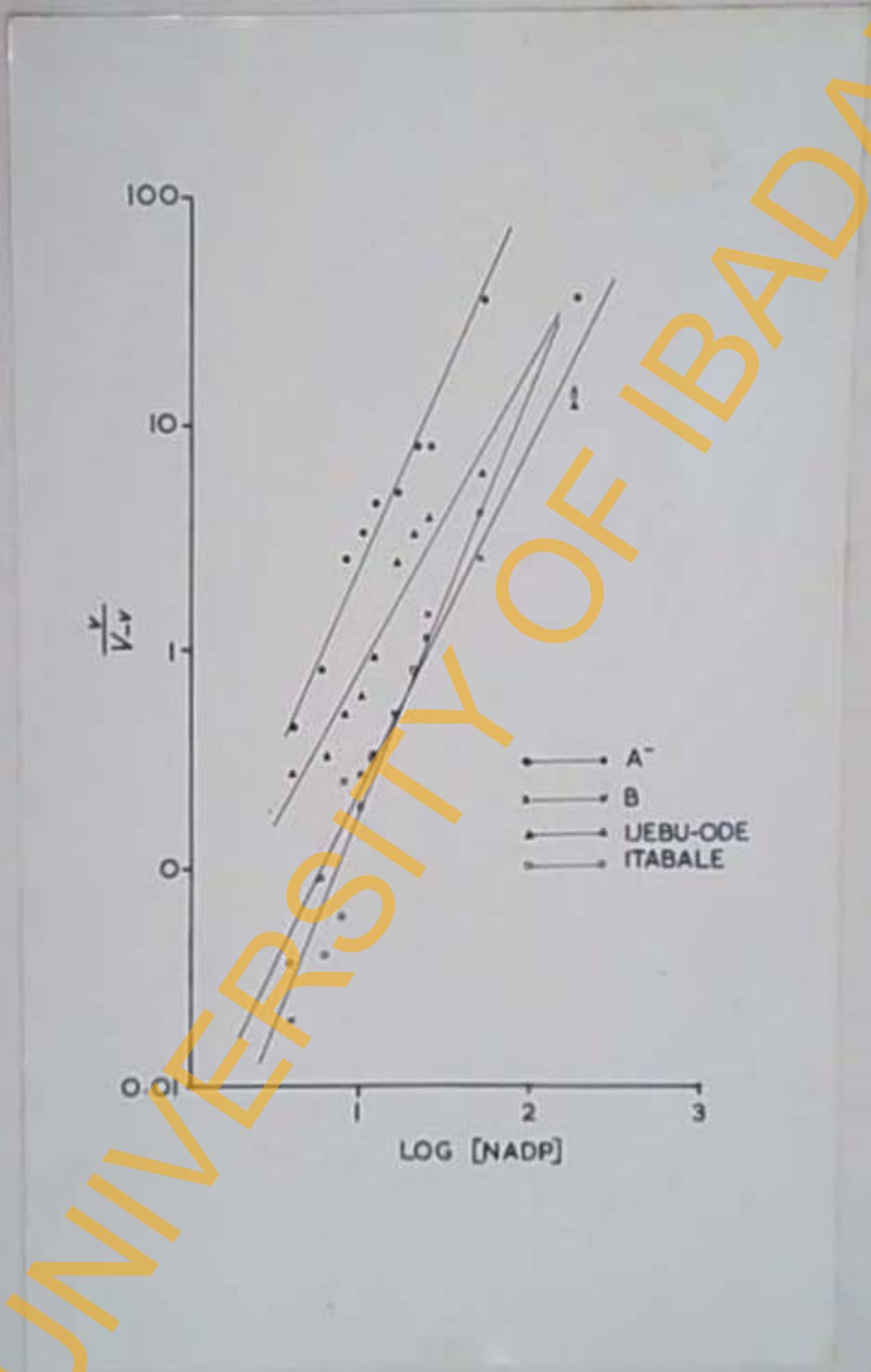
Fig. 16. Hill's plot (see Monod, Changeux & Jacob, 1963) of the NADP saturation function data (see Fig. 15). The values of the interaction coefficient **n** are in Table XII.

constants K_{s_1}, K_{s_2} are superimposable to the experimental curves (compare the two portions of Figures 15 (a) and (c)). The use of Hill's equation represents a convenient measure of sub-unit interactions, in the binding of a ligand to a protein (see Monod, Changeux & Jacob, 1963). The values obtained from plots of log $v/$ ($V_{max} - v$) against log NADP (Fig. 16) have been calculated. These values, along with those of the dissociation constants K_{s_1} and K_{s_2} are shown in Table XIII.

(b) <u>Reaction rate as a function of NADPH concentration:</u>
NADPH had already been observed to behave as an inhibitor of G6PD, in both yeast and red cell enzyme. The inhibition was considered to be of the competitive type (Glaser & Brown, 1955; Chung & Langdon, 1963(b)), which is not surprising considering the very close structural relationship between NADP and NADPH. However, more detailed analysis of this inhibition has shown that the kinetics is more complex (Luzzatto, 1967). It has therefore been decided to investigate this matter in full in the other variants. The only reliable way of doing this was to study the inhibition as a function of the concentrations of both NADP and NADPH. Thus, a family of curves has been obtained (see Figure 17(a), (b) and (c)) expressing the extent of inhibition as a function of NADPH concentration in the presence of different amounts of NADP. It is seen at a glance that these curves are not consistent with any simple type of inhibition, whether competitive or non-competitive or uncompetitive(see Dixon & Webb, 1964). Plots of v against v/s (Figure 18 (a), (b) and (c))

TABLE XIV

INTERACTION OF G6PD VARIANTS WITH NADPH

G6PD Variants	NADP µM	[NADPH] µM						
		0	10	20	40	80	160	320
B	480	0.056	0.064	0.064	0.064	0.059	0.054	0.054
	160	0.056	0.054	0.054	0.054	0.0525	0.0525	0.041
	48	0.041	0.036	0.03	0.03	0.03	0.03	0.026
	16	0.019	0.015	0.013	0.0105	0.0088	0.0075	0.0075
A⁻	480	0.06	0.06	0.06	0.06	0.64	0.057	0.057
	160	0.06	0.059	0.057	0.056	0.051	0.050	0.0425
	48	0.042	0.042	0.0443	0.0387	0.038	0.0331	0.025
	16	0.023	0.021	0.021	0.021	0.015	0.015	0.014
Ijebu-Ode	480	0.038	0.41	0.035	0.034	0.033	0.033	0.029
	160	0.035	0.036	0.0325	0.0325	0.031	0.024	0.022
	48	0.0325	0.0275	0.024	0.022	0.022	0.022	0.022
	16	0.027	0.029	0.023	0.023	0.0175	0.0175	0.014

Reaction rates are expressed in ΔA340/Min.
This experiment could not unfortunately be carried out with the
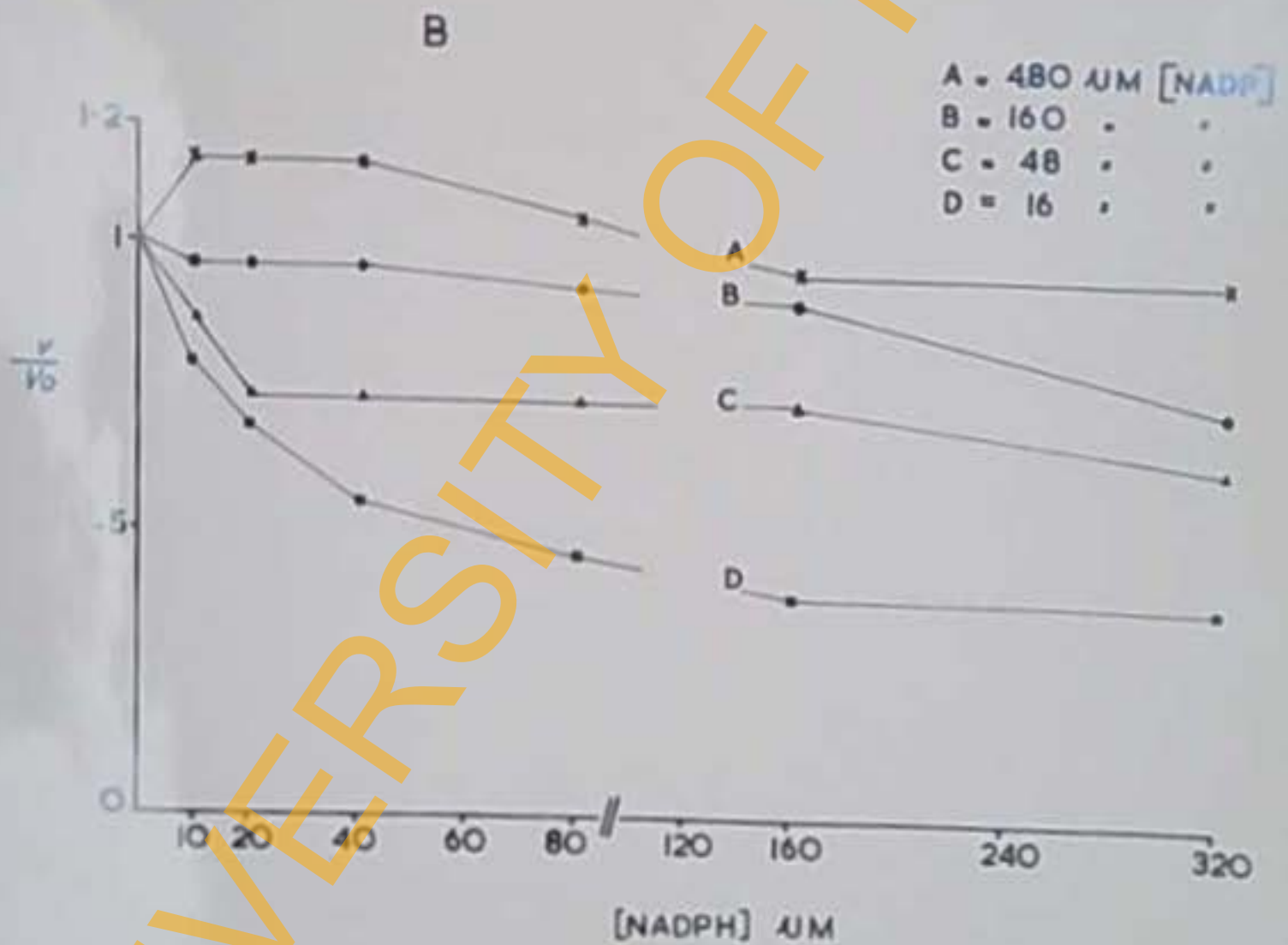Ita-Bale variant due to lack of sufficient enzyme.

FIG. 17(a). Interaction of glucose-6-phosphate dehydrogenase with NADPH. Experimental conditions as for Fig. 15(a). Concentration of NADPH as indicated on abscissa and concentration of NADP as indicated on the data. Plot of the reaction velocity (v) normalised to the velocity of the uninhibited reaction (Vo). See Table XIV

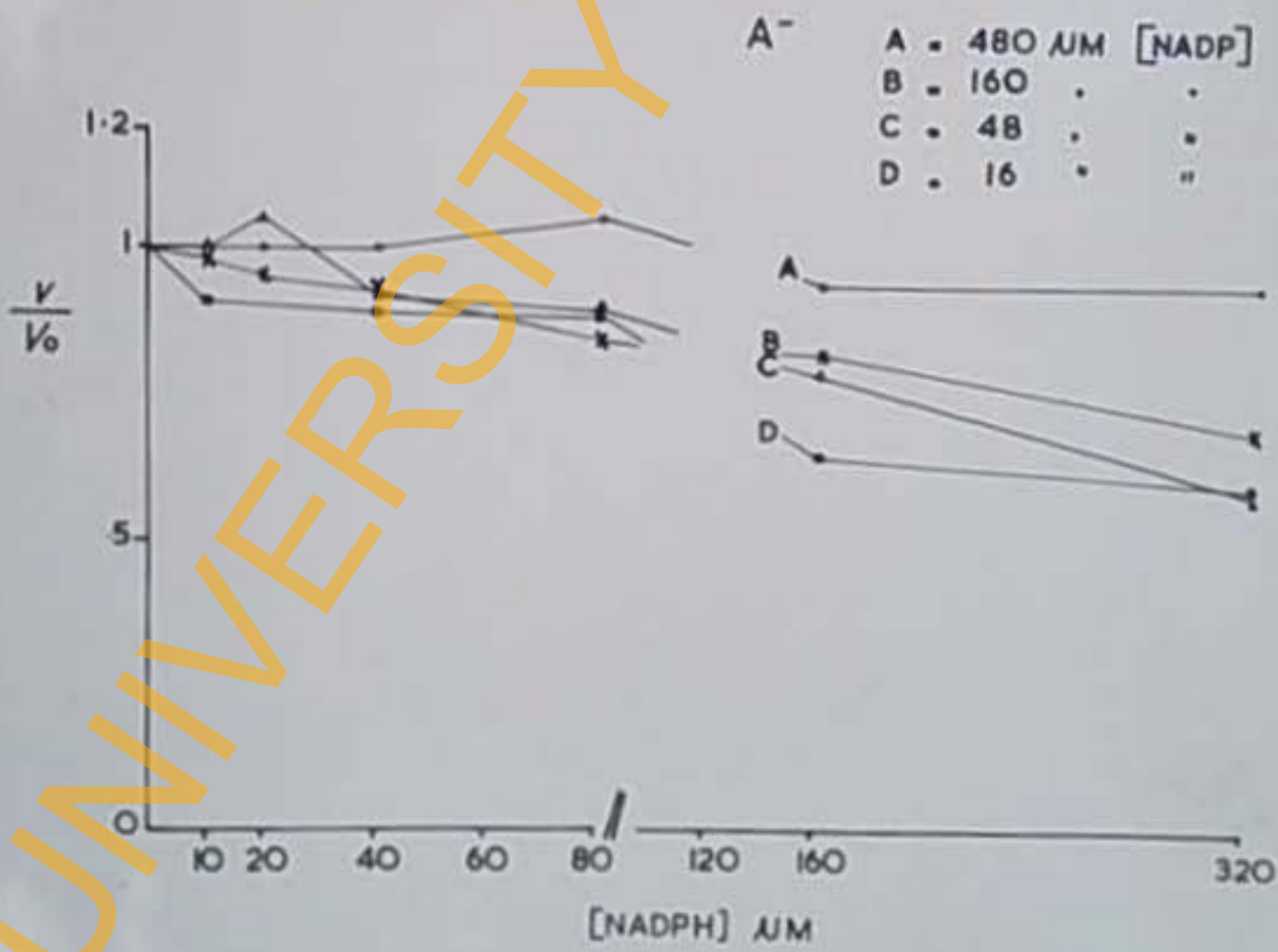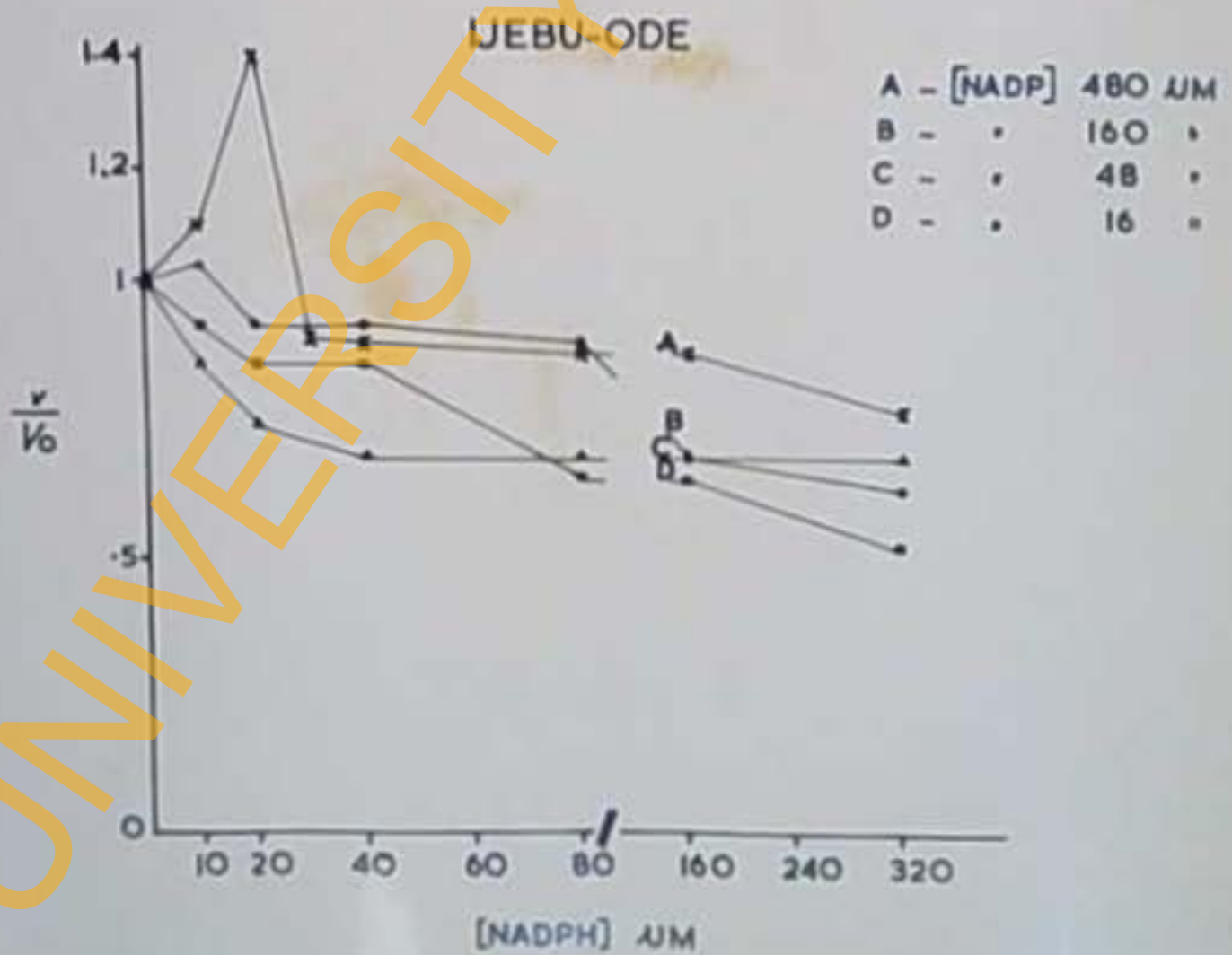Fig. 17(b). Same legend as in Fig. 17(a).

Fig. 17(c).   Same legend as Fig. 17(a).
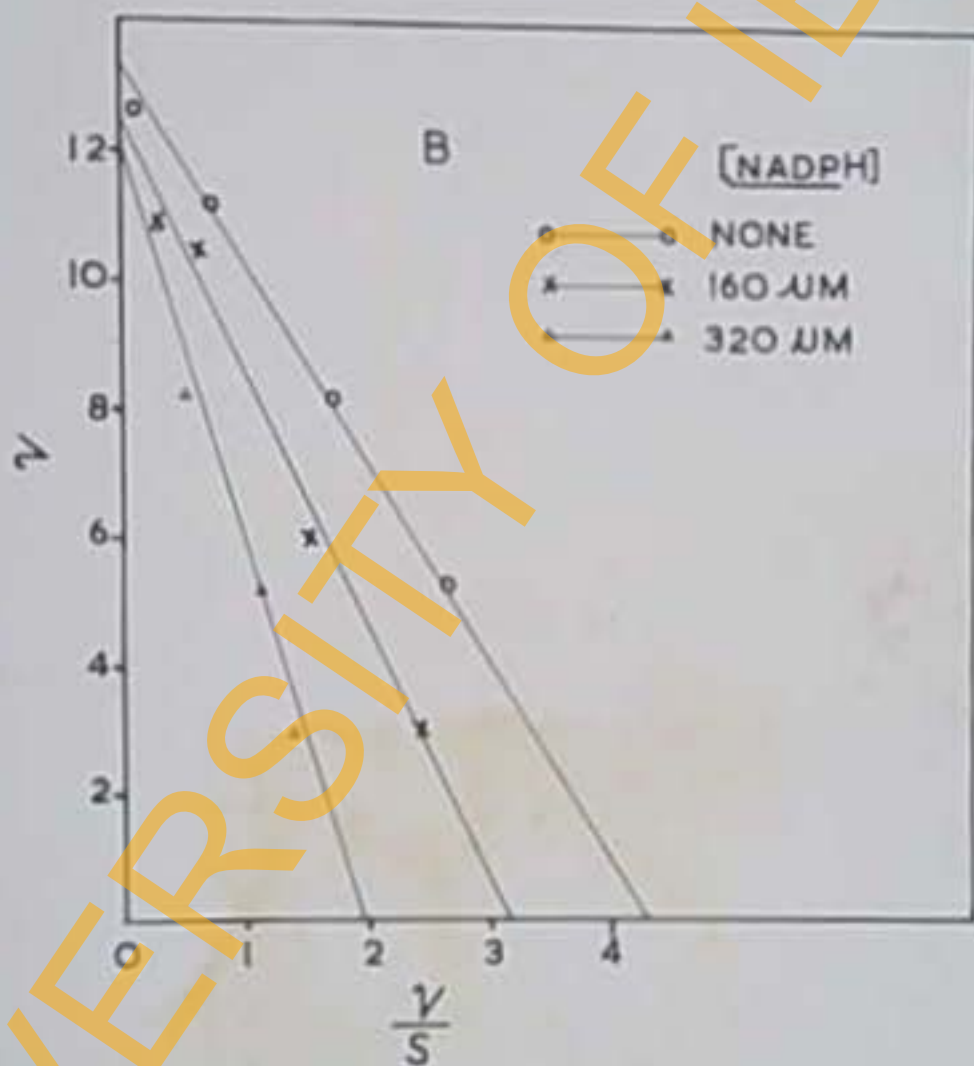
Fig. 18(a). Part of the data of Figs. 17(a), (b), (c) plotted
according to Dixon and Webb (1964) where v is the
velocity and s is the concentration of NADP.
The K_i values obtained are in Table XV.
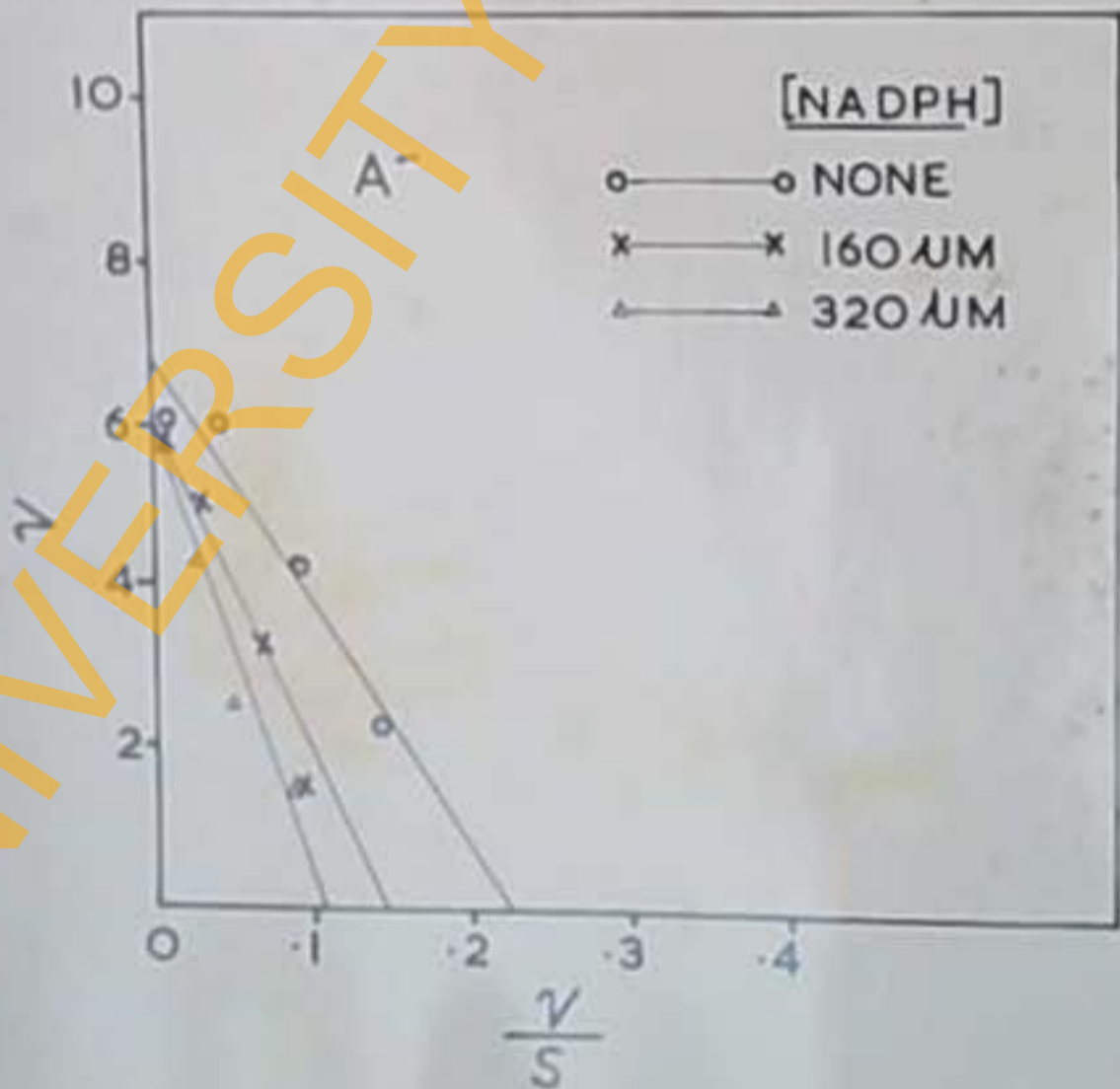
Fig. 18(b). Same legend as Fig. 18(a)
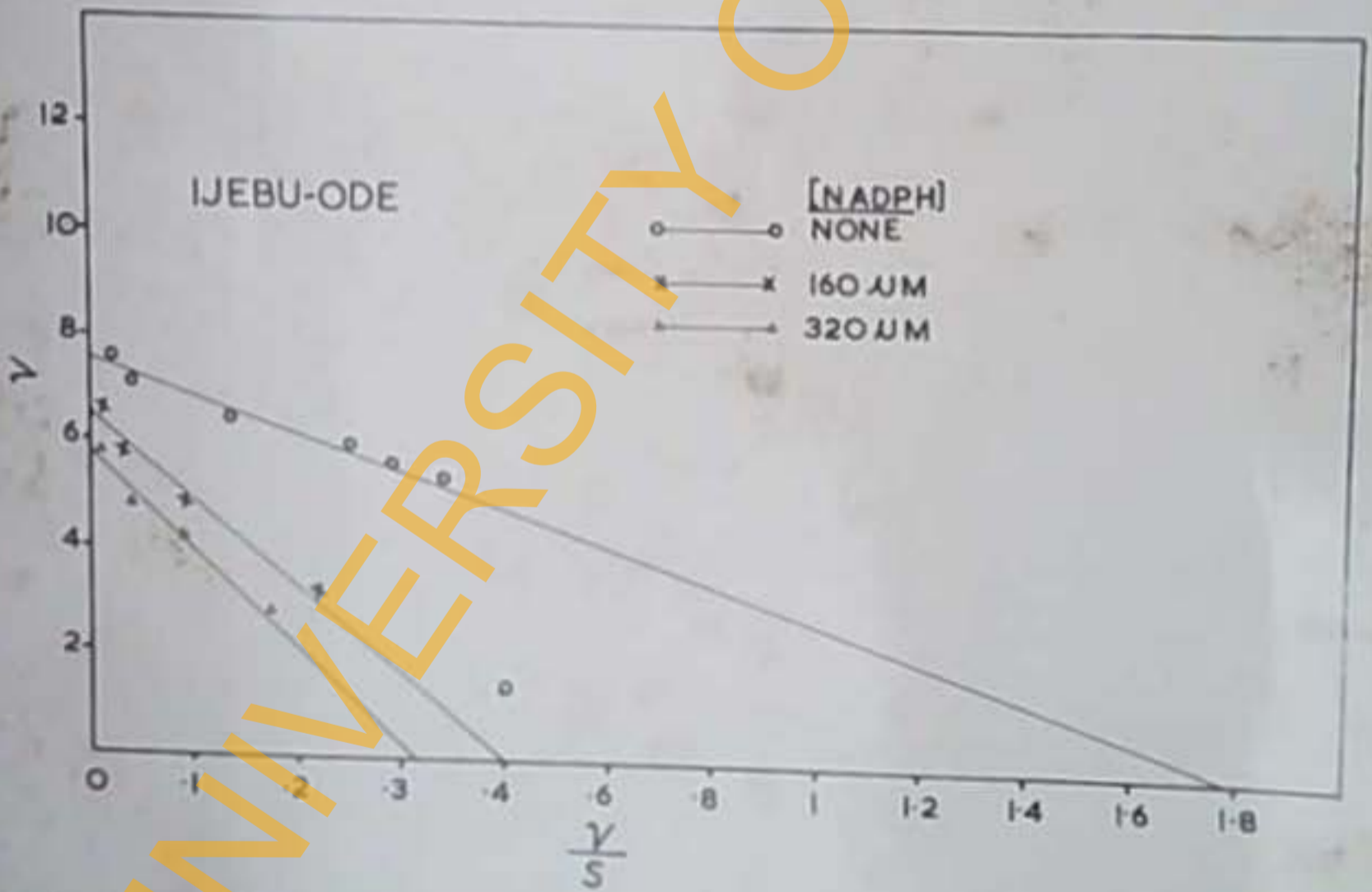
Fig. 18(c).   Same legend as Fig. 18(a).
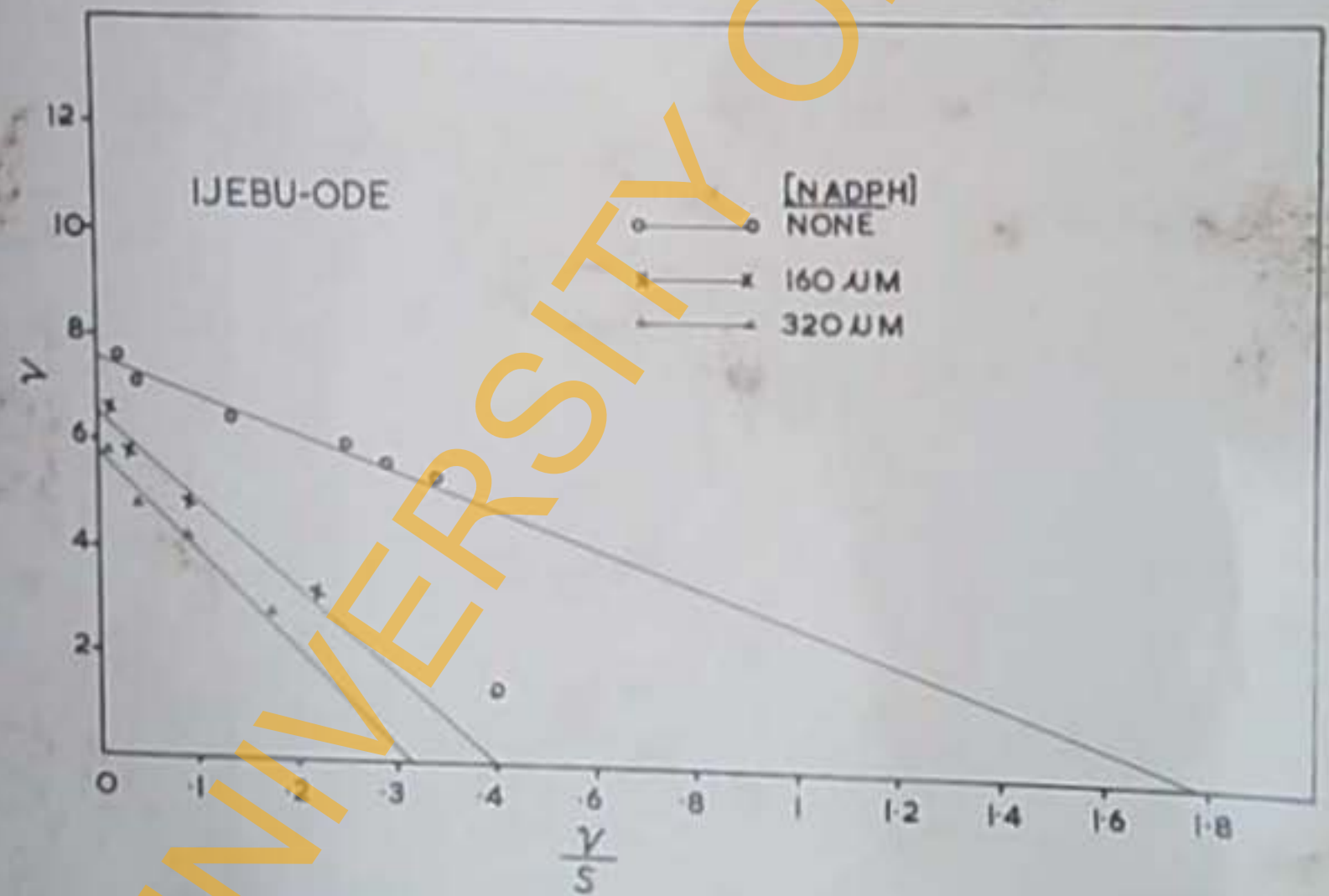
Fig. 18(c). Same legend as Fig. 18(a).

TABLE XV

INHIBITION CONSTANTS FOR INTERACTION BETWEEN G6PD VARIANTS AND NADPH

	K_i µM at 160 µM	320 µM NADPH
A	445	405
B	553	260
Ijebu-Ode	98.3	58

The concentration of the substrate, NADP, varied between 16 and 480 µM while the concentrations of the inhibitor, NADPH, employed are 160 and 320 µM, hence two values of K_i.

disclose that a competitive component exists, but is partly obscured by other features which will be discussed below. From the linear curves it is possible to calculate values of K_i. However, these are in turn dependent on the concentration of NADP. Therefore, more than one value of K_i is obtained for each variant and some are tabulated in Table XV.

D I S C U S S I O N

(a) <u>Interaction between G6PD and NADP</u>: The data presented in Figure 15 clearly indicate that the affinity of red cell glucose-6-phosphate dehydrogenase for NADP is a function of the concentration of NADP. This result will be in consonance with any assumption that the enzyme bears more than one binding sites for NADP, and that the binding of the first molecule of NADP increases the affinity of one or more other sites for this substrate. The double-bended substrate saturation function kinetics of B and Ita-Bale, like that of A (Luzzatto, 1967), are reminiscent of the oxygen dissociation curve of oxyhaemoglobin (Haldane & Priestly, 1935) and of the affinity of aspartate transcarbamylase for aspartate (Gerhardt & Pardee, 1963). The sigmoid curves obtained in this work suggest that G6PD is made of sub-units and these sub-units undergo flexible interaction on binding NADP. The enzyme molecule as a whole undergoes a conformational change and causes an increase in the affinity for the substrate. This possible mechanism is consistent with the powerful and convincing model of Monod, Wyman & Changeux (1965) for allosteric and co-operative effects in regulatory enzymes based on the assumptions of a

"symmetry principle". It is however, impossible on the basis of kinetic data alone, to distinguish between such a model and one in which co-operation between two substrate molecules depends, for instance, on their interaction at overlapping binding sites, without a conformational change in the enzyme.

The shape of the NADP saturation curves for A⁻ and Ijebu-Ode are sharply different from those of the other variants and appear at first sight to follow classical Michaelis-Menten kinetics. However, the data in Table XIII indicate that there is still in them a transition from low to high affinity for NADP, when the concentration of the latter is increased. The kinetics obtained indicates that this transition occurs already at very low NADP concentration, with the result that sigmoidal curves cannot be obtained. The possibility that some of the differences might be due to artifacts arising during purification cannot however be completely excluded. It is conceivable that the enzymes might have been modified with respect to certain binding sites for NADP and that as a result the enzyme displays no apparent sub-unit interaction in the form of sigmoidal kinetics. This possibility is considered in view of the fact that certain enzymes, regulatory in function, can be modified by changes in environmental conditions, which entail an increased affinity towards the substrate (Gerhart & Pardee, 1963).

It is noteworthy that Kirkman & Hendrickson (1962) and Tsutsui & Marks (1962) have independently reported that the sedimentation of erythrocyte glucose-6-phosphate dehydrogenase in sucrose density gradients is affected by the NADP concentration. The forms of the enzyme having lower and higher sedimentation rates may be involved in a monomer-dimer equilibrium or may represent interconvertible conformational forms without difference in their state of aggregation. In either case, one could suggest that the change in sedimentation behaviour induced by NADP also corresponds to the transition from lower to high affinity for NADP, observed in these studies.

(b) Interaction between G6PD and NADPH: The action of NADPH (Figure 17 (a), (b) and (c)) on the activity of various enzyme types may be interpreted as resulting from the combination of two effects.

(1) There is enhancement of the affinity for NADP at low concentrations of NADPH (Luzzatto, 1967). This results in an actual activation at low concentrations of NADPH, observed for all variants, although at different concentrations of NADP.

(2) There is inhibition, at higher NADPH concentration, partially competitive in nature (see Figure 18), which is more marked the lower the concentration of NADP, and which is characteristic for each variant, A⁻ being the most resistant among those tested.

The emerged complex kinetics of the NADPH effect may be rationalised on the basis of some postulations. First an attempt will be made to describe the effect of NADPH in general, and then the distinct

features exhibited by the different enzyme variants will be discussed.

It is suggested that NADPH can interact with two different kinds of sites in the G6PD molecule. One kind of site is the same which binds the substrate, NADP. At these sites NADPH can replace NADP to all intents and purposes, except of course as a substrate. When the NADP concentration is in the range where G6PD has low affinity for it, NADPH will therefore help the transition to the state of high affinity, and can in such circumstances show an activating effect, as was described for enzyme type A (Luzzatto, 1967). When the NADP concentration is in the range where G6PD already has high affinity for this substrate, NADPH will compete for the NADP-binding site, and will thus show an inhibitory action with competitive features. When all NADP binding sites are saturated, NADPH can bind at different site. Under these conditions it can change the conformation of the enzyme in a favourable sense and activation will again be observed. This has been found for variants B and Ijebu-Ode at concentrations of NADP above 160 uM (Table XIV and Figure 17). Similar examples have been described in the case of other enzymes. Thus, the activating effect of adenosine triphosphate (ATP) on aspartate transcarbamylase (Gerhart & Pardee, 1963) and of valine on threonine deaminase (Changeux, 1963) are similar examples. Furthermore, even the possibility of the same substance acting as an activator and as an inhibitor does have a precedent in the

case of maleate on aspartate transcarbamylase (Gerhart & Pardee, 1963). The different kinds of sites at which NADPH can bind would ordinarily be called allosteric (Monod et al. 1963).

The mechanism envisaged for the complex role of NADPH may be described more fully as follows. The slope of the substrate saturation functions in the form of Hill's plots (Figure 16) in which the value of n is approximately 2 for each variant, (Table XIII), points to the existence of at least two sub-units in G6PD (Yoshida (1966) suggested the existence of six sub-units)). The data in this work indicate that the sub-units in G6PD interact strongly. NADPH, which is structurally almost identical to NADP, can bind at the substrate-binding site but weakens the sub-unit interactions. The groups in the active site become favourably arranged for substrate binding, and the affinity increases. This is an example of the "co-operative heterotropic effect" postulated by Monod, Wyman & Changeux (1965). The same mechanism of weakening sub-unit interactions, operates on the binding of the substrate. This may be indicated as "co-operative homotropic effect". At high NADPH concentration and low NADP concentration, the binding of NADPH strengthen sub-unit interactions. When the interactions become stronger, the active site on each sub-unit becomes more slightly distorted and as a result substrate affinity is reduced. It is thus clear that interplay between NADP and NADPH, leading to enzyme activation or inhibition according to their absolute and relative concentrations may constitute a refined mechanism

for the regulation of activity of glucose-6-phosphate dehydrogenase and therefore of the pentose-phosphate pathway in the red cell.

This regulatory mechanism would be based on the interaction of NADP and NADPH at the first kind of site namely - the substrate binding site. Whether the binding of NADPH to the other kind of site that has been postulated, (at which NADPH has an activating effect even at high NADP concentration) has any physiological significance is doubtful. This is because such an effect has been observed only at NADP concentration above 100μM, namely of an order of magnitude that probably never obtains _in vivo_, at least in the erythrocyte.

Whereas it is clear that the interpretation of the kinetics of interaction of G6PD and NADPH that have been described above are largely speculative, there is no doubt that whatever the precise molecular mechanism of the interaction of the enzyme with NADP and NADPH, such interactions are subject to change as a result of genetic mutation. Such changes are quite remarkable. Thus, if we refer again to the normal B type, we observe that inhibition by NADPH is quite pronounced at the concentration of NADP of 16 μM, it is less at 48 μM, it is almost abolished at 160 μM and _activation_ is observed at 480 μM NADP. The behaviour of Ijebu-Ode is qualitatively similar although, from the quantitative point of view, the inhibition is less marked at 16 μM

and the activation is less marked at 480 μM. The results previously obtained with variant A (Luzzatto, 1967) were quite similar, except that activation was not observed even at very high NADP concentration, and the inhibition at very low NADP concentration was more marked. The variant that clearly sets itself aside is A⁻. This variant seems to be insensitive to NADPH compared to the others, both in the sense of inhibition and in the sense of activation. Thus, significant activation is never observed and significant inhibition occurs only either at very high NADPH concentration or at very low NADP concentration and even under these conditions it is less pronounced than with any other variant. The possible physiological significance of the last result in terms of the metabolism of G6PD-deficient red cells will be discussed later (Chaper VII).

CHAPTER VI

EFFECT OF TEMPERATURE AND OF SULPHYDRYL GROUP REAGENTS ON GENETIC VARIANTS OF G6PD

Amongst the techniques that are used for the characterisation of enzymes, those based on the effect of physical or chemical denaturating agents have always ranked high. In the present work, the study of thermal inactivation has been extended to include not only the conventional technique of the time course of inactivation at 37° but rather to observe the inactivation produced by short incubations at gradually increasing temperatures (thermal inactivation profile). Among the inhibitors, those affecting thiol groups were selected because it was already known that they affect at least some of the G6PD variants (Kirkman 1962; Chung & Langdom 1963b), and because of the indirect evidence suggesting that thiol groups may be involved in the active centre of this enzyme (see chapter IV).

(a) Effect of temperature of the activity of G6PD Variants:

It has been previously observed (Luzzatto & Allan, 1965) that red cell G6PD is quite stable to temperatures of 55° and over: at higher temperatures it undergoes a sharp inactivation. As a result, if the enzyme is pre-incubated for a short time (.7 minutes in this work) at the given temperature and then re-tested at room temperature a 'melting curve' is obtained. Figure 19 (a) & (b) shows the data for enzymes A and A⁻. Figure 19 (c) & (d) shows
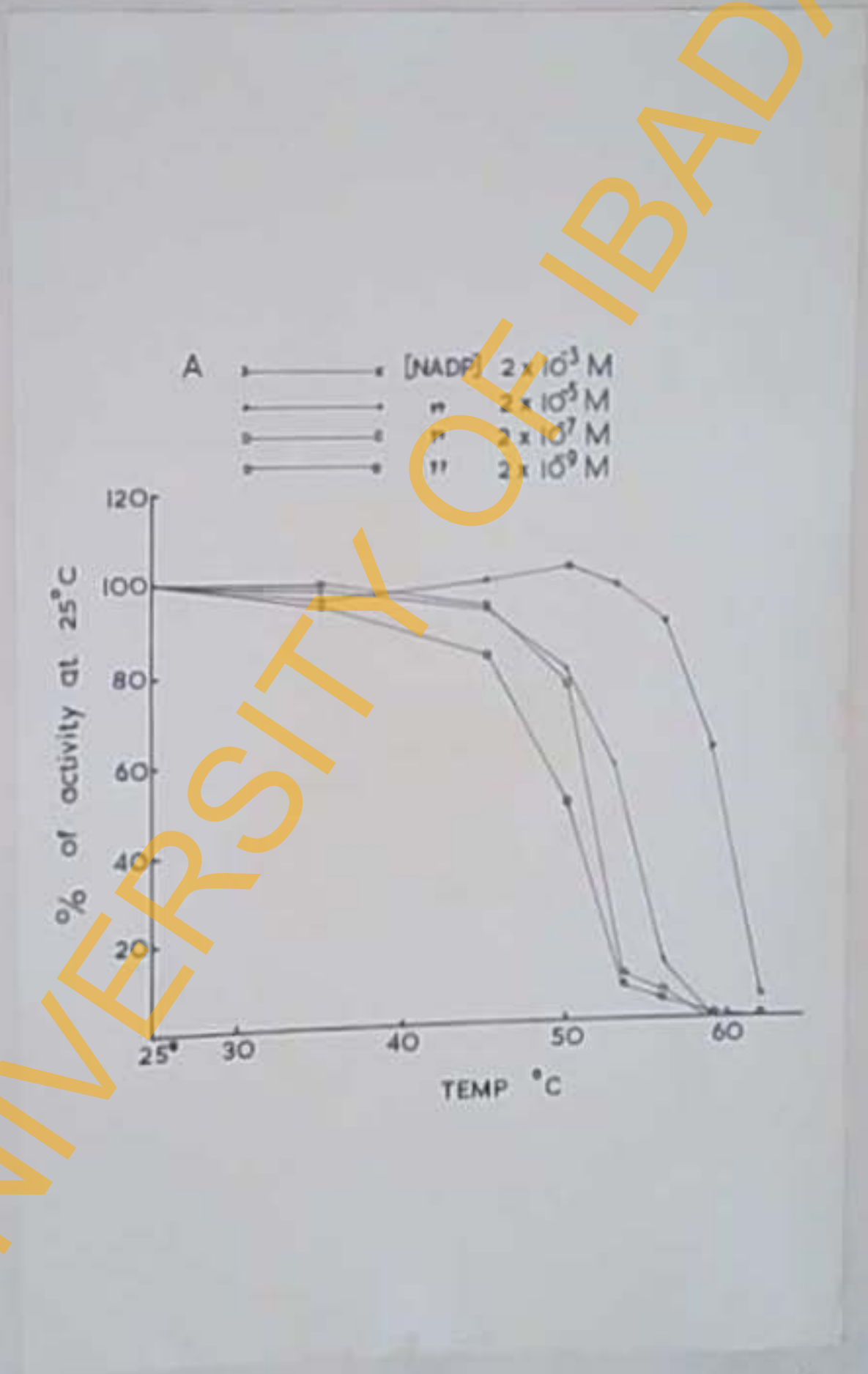
Fig. 19(a). Thermal inactivation profile of G6PD variants at various NADP concentrations as indicated. See expt. techniques (chapter II) for details. The data are for A
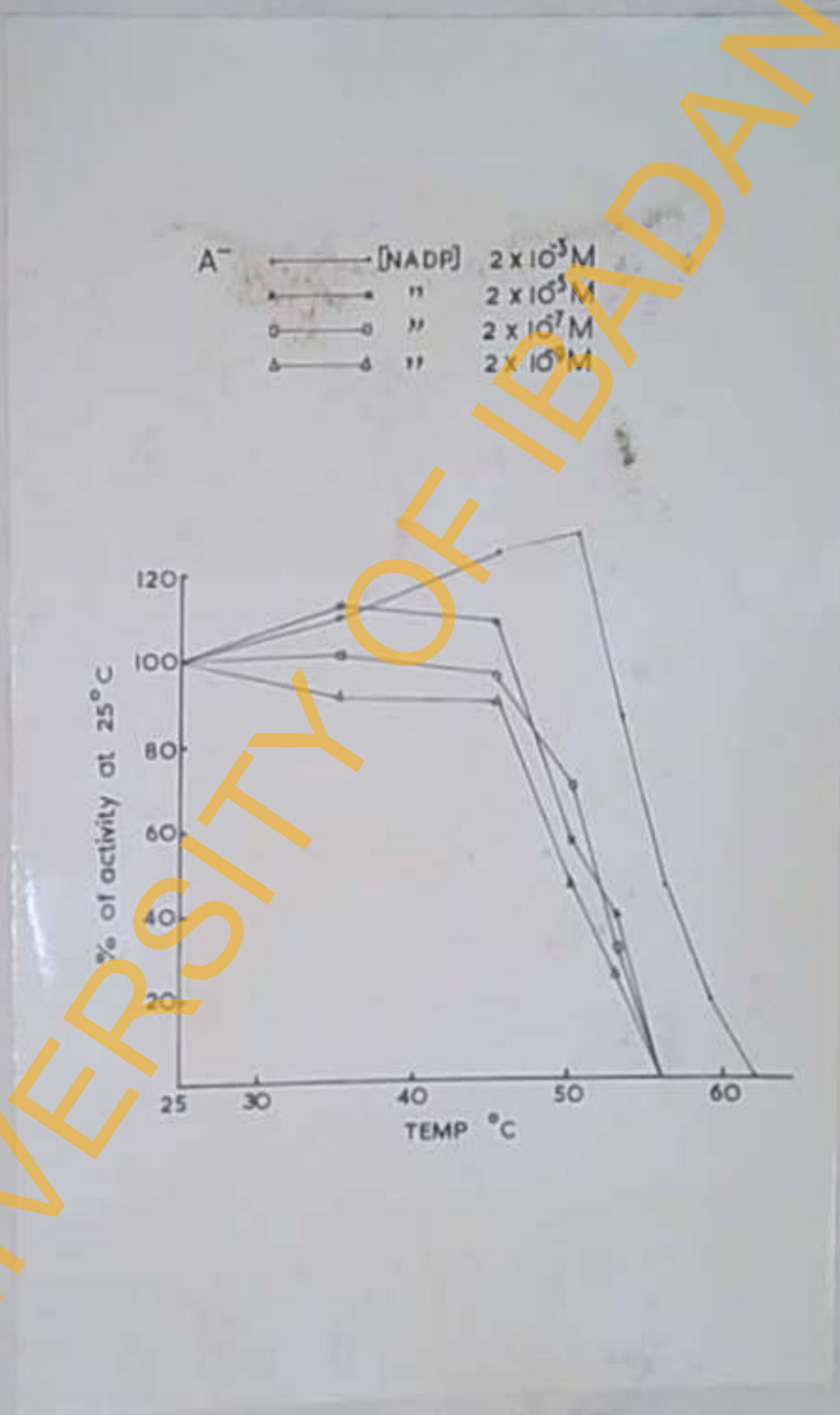
Fig. 19(b). Enzyme type A⁻. See Fig. 19(a) for legend.
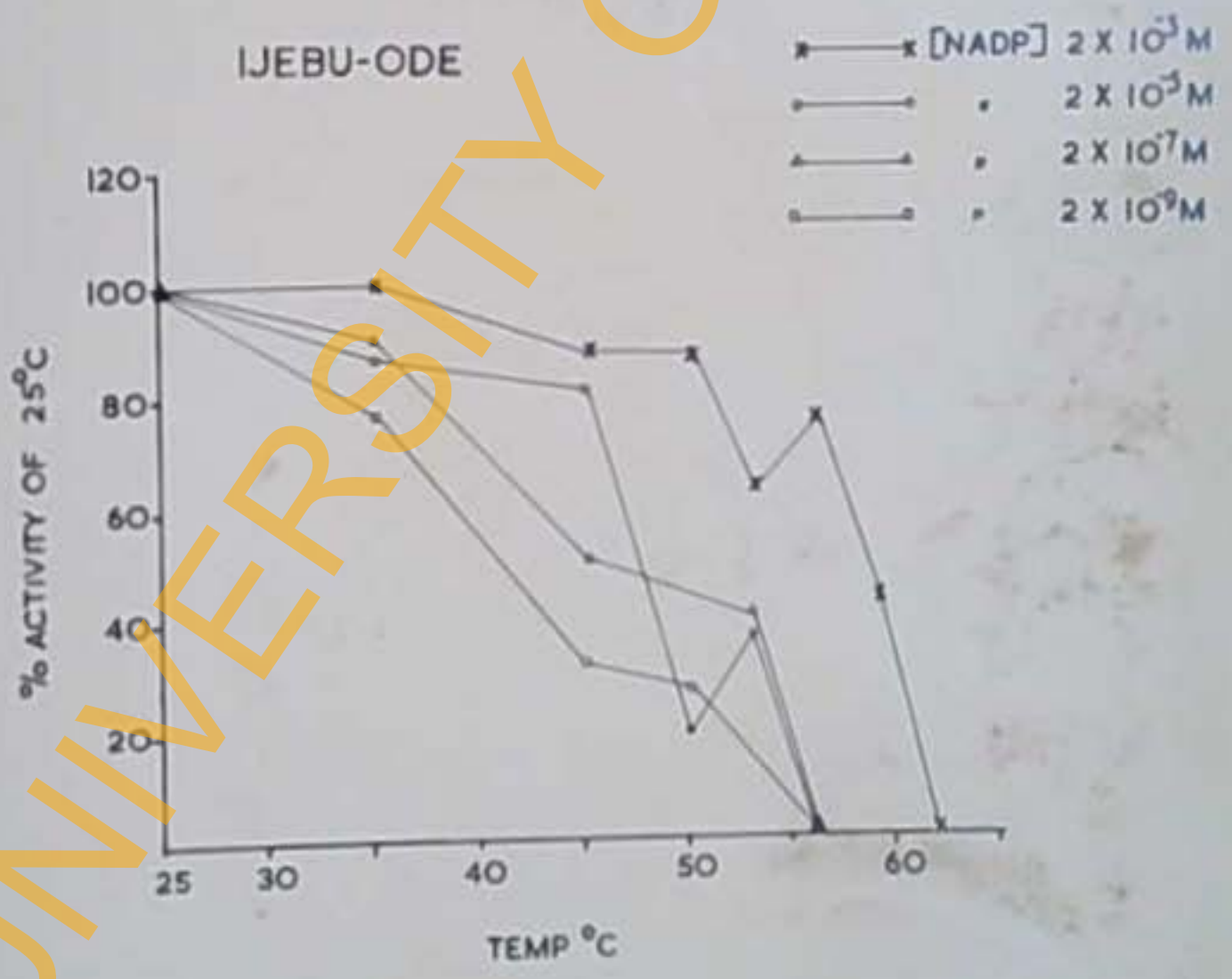
Fig. 19(c). Enzyme type Ijebu-Ode. See Fig. 19(a) for legend.
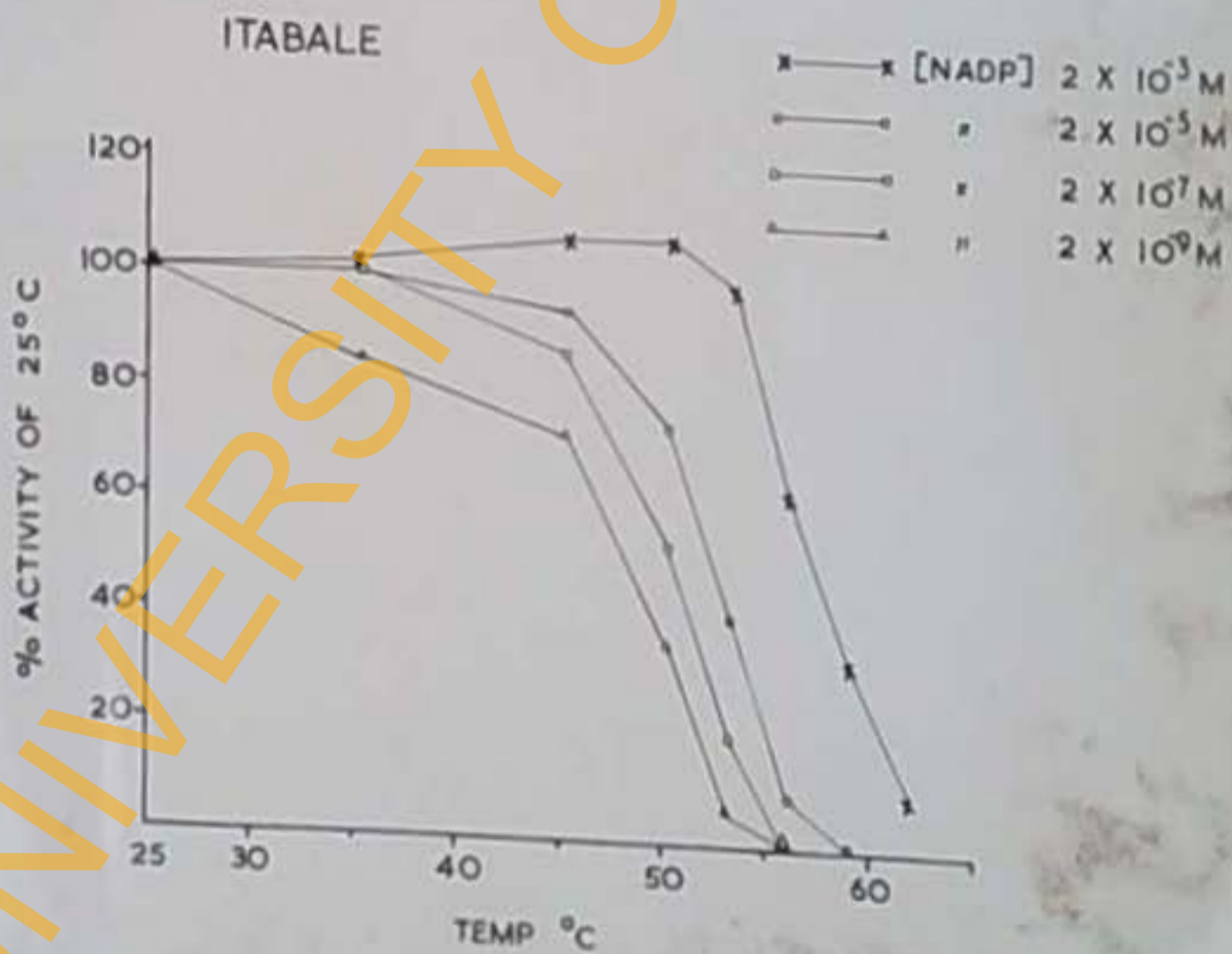
ITABALE

Fig. 19(d). Enzyme type Ita-Bale. See Fig. 19(a) for legend.

TABLE XVI

TRANSITION TEMPERATURE (^{o}C) OF THERMAL INACTIVATION OF G6PD VARIANTS AS A FUNCTION OF NADP CONCENTRATION

G6PD Variants	[NADP]			
	2×10^{-3}M	2×10^{-5}M	2×10^{-7}M	2×10^{-9}M
A	59	53.5	51.5	50.0
A^{-}	56	51.5	51	50.0
B	58.5	51.5	44	43
Ijebu-Ode	58	47.5	45	41
Ita-Bale	57	52	50	48

similar data for Ijebu-Ode and Ita-Bale. It is seen that Ita-Bale conforms remarkably to the pattern observed for enzyme type A. The shape of the curves obtained is quite similar and also the relationship between the family of curves as a function of NADP concentration is quite similar. The transition temperature (defined as the temperature at which 50% of the enzyme is inactivated) is found to be 57°C for Ita-Bale as compared to 59°C for A (in excess of NADP, 1 mM). By contrast, the profiles observed for Ijebu-Ode are quite abnormal in shape. It is clear that in this variant there is a peculiar and remarkable change in the way the enzyme behaves with respect to temperature especially in relation to the stabilizing effect of NADP. Thus, even in excess of NADP (1 mM), it is clear that the melting curve is much less sharp for Ijebu-Ode than for A or Ita-Bale. Furthermore, there are irregularities in the profile, such as a peculiar reactivation around 56°C. It is when the concentration of NADP is decreased that the thermal instability of Ijebu-Ode becomes far more apparent. At 10^{-5} M NADP the enzyme is already markedly unstable anywhere above 45°C, although it again undergoes a peculiar reactivation at about 47°C. A similar phenomenon is again observed at the 10^{-7} M NADP. At this concentration of NADP, and at lower concentration (10^{-9} M) the instability of the enzyme is extreme and considerable inactivation is observed even at room temperature. Thus, at low concentrations of NADP the transition temperature for this enzyme is exceptionally low, around 36°C.
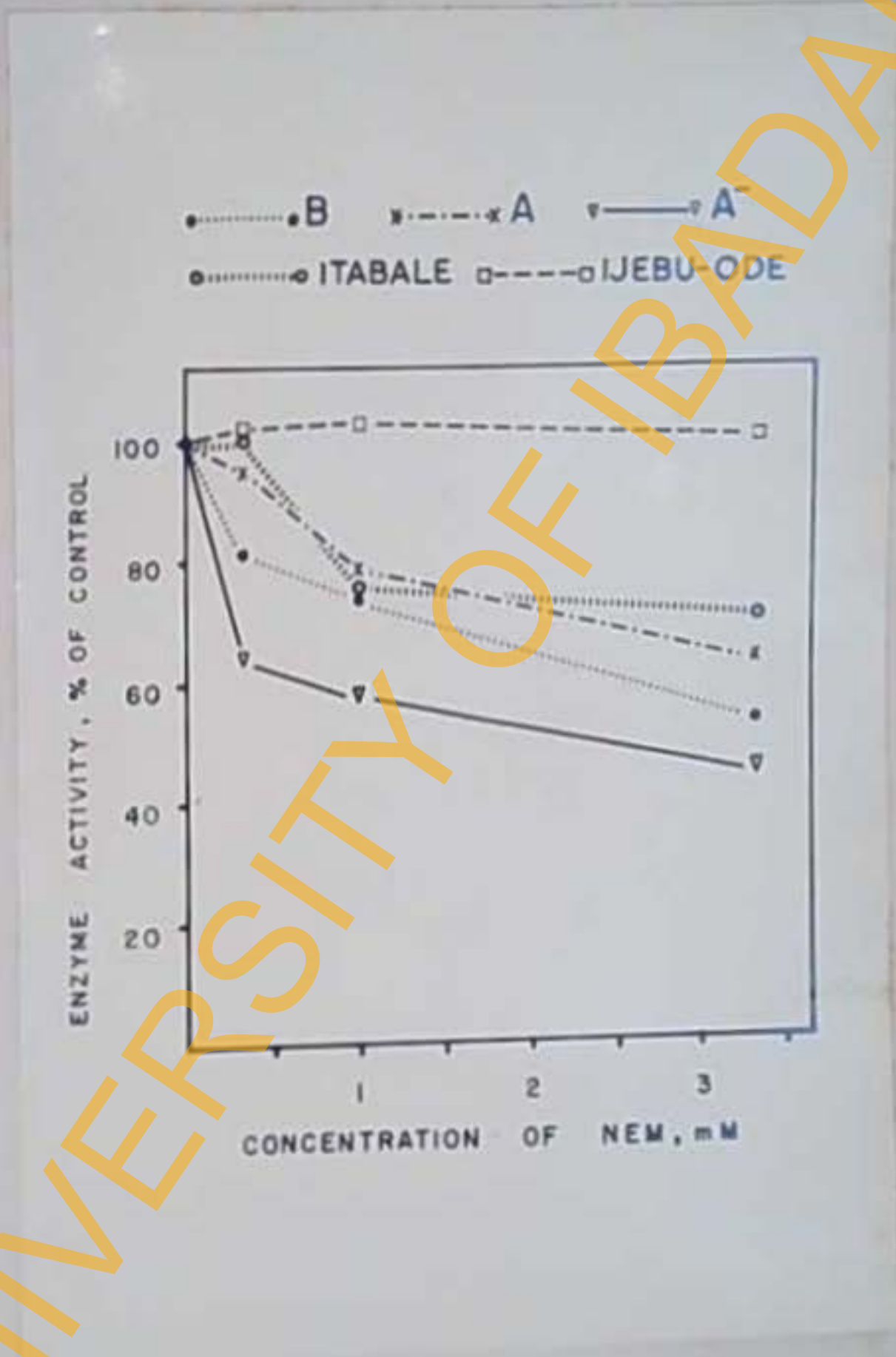
Fig. 20. Inhibition of G6PD variants by N-ethylmaleimide (NEM). Experimental conditions are the same as described under experimental techniques except that the buffer was 20 mM Tris.HCl pH 8.6 containing MgSo$_4$ 10 mM.

×————————× A; ∇————————∇ A⁻; o·····o B;

□– – –□ Ijebu-Ode; o– – —o Ita-Bale.
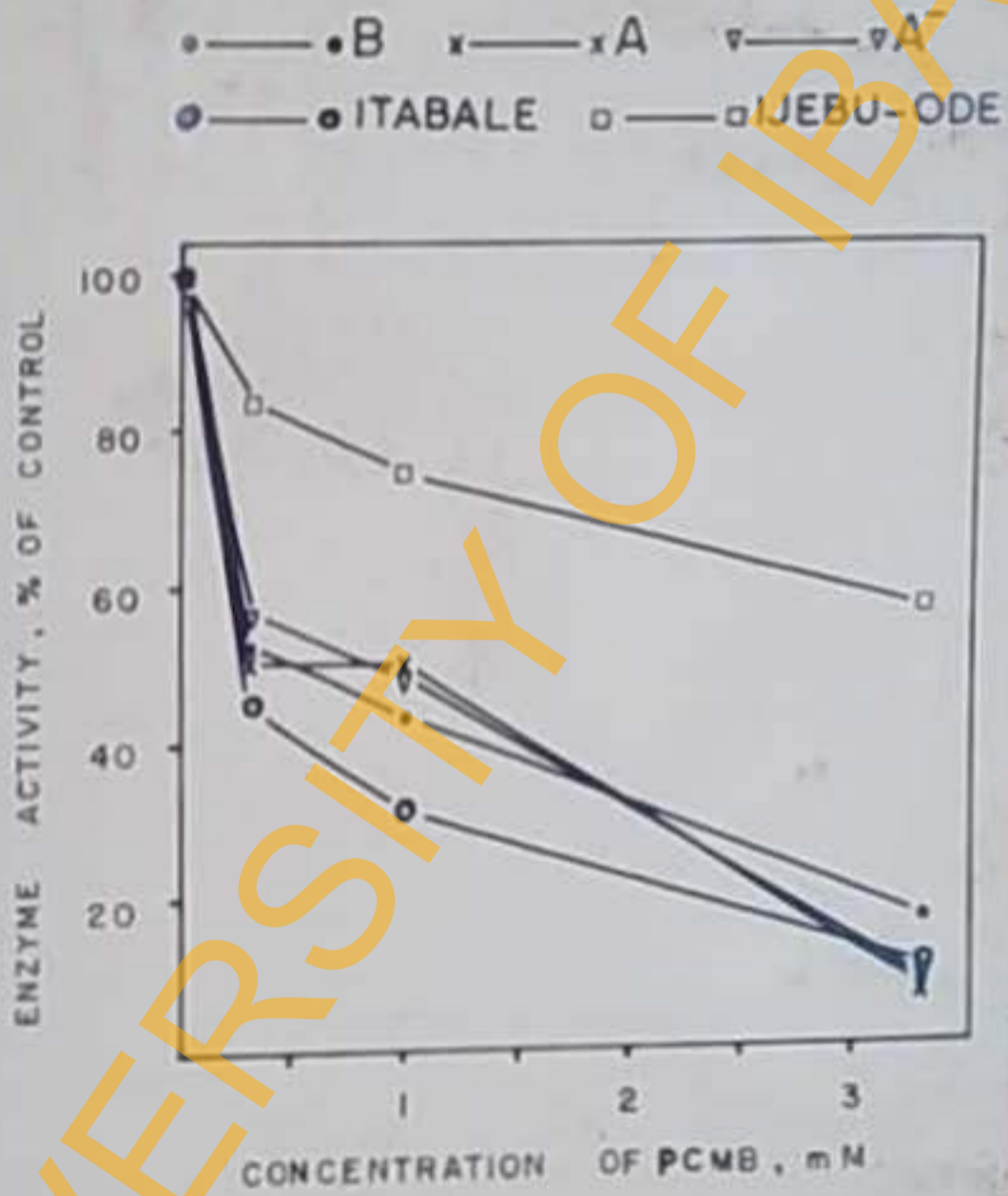
Fig. 21. See Fig. 20 for legend. Inhibition of G6PD variants by p-chloromercuribenzoate (PCMB).

(b) <u>Effect of Sulphydryl group reagents</u>: Iodoacetic acid,
bromoacetic acid and iodoacetamide, _(from preliminary results) all have a mild, time-
dependent inhibitory action on the enzymes studied. For example,
after three hours pre-incubation with iodoacetamide, the
inhibition is 15-30% at 10 mM and 5-15% at 2 mM. With these
three reagents no significant differences were observed among the
five variants in either the time course or the extent of inhibition

On the other hand, NEM is a more powerful inhibitor (see
Fig. 20). Here the action is instataneous. The inhibition ranges
between 30 and 60% for the enzyme types A, B, A^- and Ita-Bale.
However, Ijebu-Ode stands out as being a completely refactory
to this inhibitor.

PCMB, a mercaptide-forming reagent, had already been found
to be a potent inhibitor of red cell glucose 6-phosphate
dehydrogenase (Khung and Langdon 1963 a). The inhibition is manifest within seconds after
the addition of the reagent to the reaction mixture. The effect
of relatively high concentration of PCMB on enzyme activity again
sharply differentiates between Ijebu-Ode, the most resistant
variant, and the other four that have been examined (Fig. 21).
At much lower concentrations a finer analysis is possible: this
was carried out for the A and A^- variants (Fig. 22). It is
found that A^- is significantly more susceptible to PCMB
inhibition than A. These results were
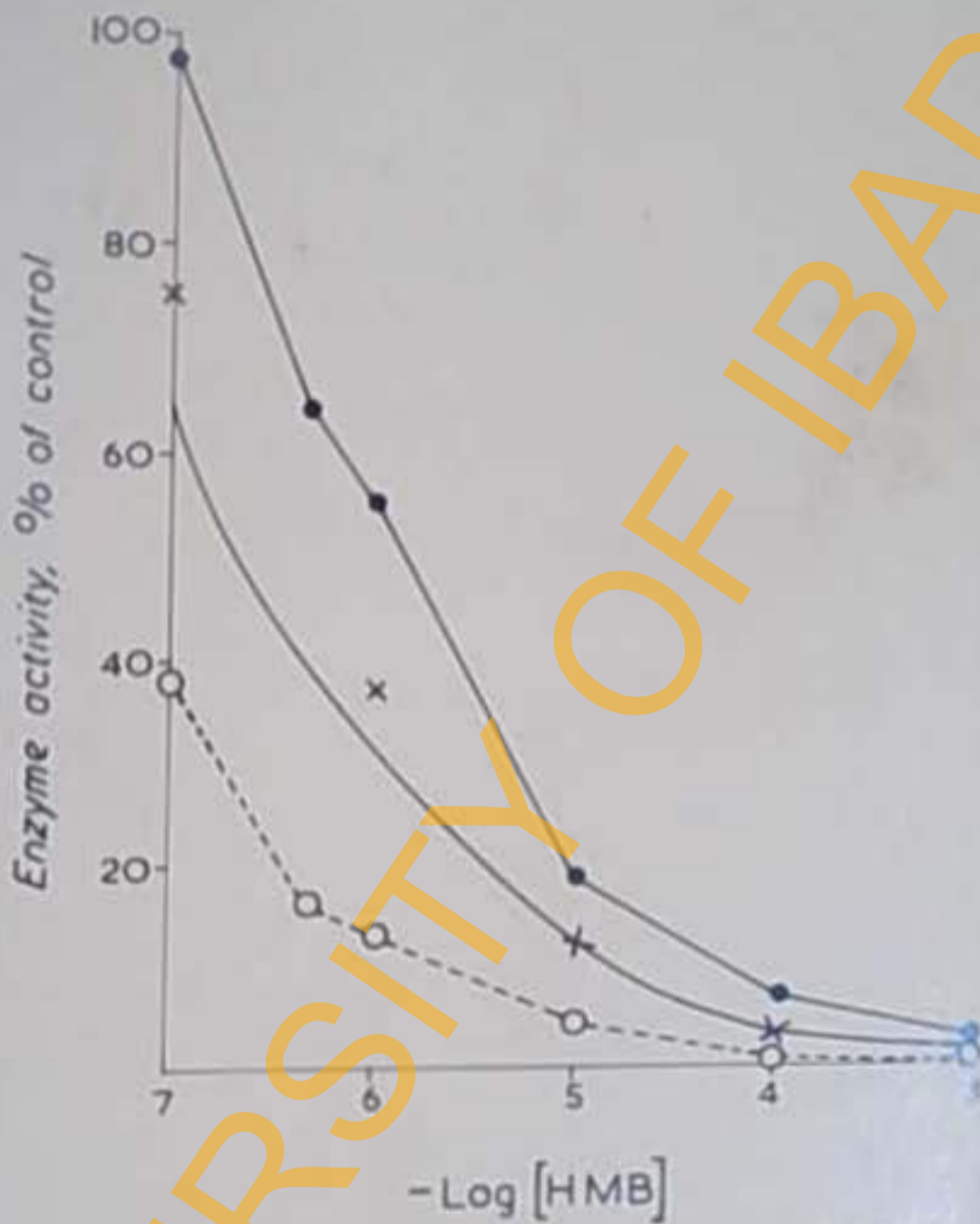
Fig. 22. Inhibition of G6PD variants A and A⁻ by low concentration of PCMB. Here the enzyme preparations were first incubated for 15mm at 22°C with various concentration of of PCMB in 5 mM phosphate buffer, pH 6.9, 50 mM KCl, 0.1 mH EDTA, and 1μM NADP. Enzyme activity was then measured in the usual assay system. PCMB concentrations shown on abscissa are the concentration in the assay mixture. Concentrations during pre-incubation were 2.3 fold higher at each point ——• A; o-- - —o A⁻ ; were ×——————× mixture of A and A⁻, experimental points. The middle full line indicates the calculated inhibition curve for the mixture if there is no interaction between the two preparations.

observed by adding the inhibitor, PCMB directly to the reaction mixture. However, it was possible to obtain a greater sensitivity in analysing the effect of the inhibitor by pre-incubating the enzyme at room temperature with PCMB and then assaying the remaining activity. Under such conditions, it was found that even much lower concentration of PCMB, of the order of 10^{-7}M would cause considerable inhibition, and that this effect was quite different for variants A and A⁻. Unfortunately, lack of sufficient amount of enzyme made it impossible to repeat the experiment for Ijebu-Ode and Ita-Bale.

D I S C U S S I O N

The results obtained with the five variants in the thermal inactivation profiles (Figs. 19, a - d) as a function of NADP⁺ concentration are discriminatory among the enzyme types (see table XVI). It is seen that, in excess of NADP, all four variants A, A⁻ Ijebu-Ode and Ita-Bale undergo activation at some point between room temperature and the temperature of inactivation: A 45° - 51°; A⁻ 30 - 51°; Ijebu-Ode 30 - 35°; and Ita-Bale 35 - 55°C. Abrupt inactivation takes place at high temperature - A (53°C), A⁻ 51°C and Ita-Bale 51°C. Ijebu-Ode had a rather unique inactivation profile. The shift in the region of "superactivity" may reflect differences in structural configuration among the variants. The thermal inactivation profile of A⁻ is slightly different from the data reported by Luzzatto and Allan (1965), perhaps because the purity of their

own enzyme was higher than the one used in this work. Moreover, their own A^- enzyme obtained from a single blood donor was tested whereas pooled A^- was used in this experiment. The significance of the latter point is not known yet. It is however striking that the transition temperatures for A and A^- in excess of NADP are still similar as in the previously reported data.

The inactivation profiles observed, especially those in excess of NADP, are reminiscent of the co-operative breakdown of a highly stabilised structure, such as a helix-random coil transition (Scheraga, 1963). On decreasing the concentration of NADP, there is a concomitant decrease first in the transition temperature, and then in the sharpness of the transition. This is consistent with the concept that NADP is specifically involved in stabilising the active conformation of the enzyme and is in agreement with previous findings by Marks et al. (1961), Kirkman & Hendrickson (1962), and Chung & Langdon (1963b).

It may be of some interest to speculate on the nature of the inactivation process. According to Sheraga (1963), if the inactivation does indeed correspond to the destruction of a helical region of the molecule, the length of this region will be of approximately 27 amino acid residues for a transition temperature of 59° (as found for A and A^-). Deviations from this value might be due either to the presence of residues which have a de-stabilizing action on the helical region, or might be due to a change in the length of the very same helical region, possibly

due to the presence of the proline residue.

The action of sulphydryl group reagents suggests that either sulphydryl groups are important as such for the activity of red cell G6PD, or that the attachment of these reagents to the enzyme molecule interferes with activity, for instance by steric hindrance of substrate binding. The latter hypothesis had been held true by Chung & Langdon (1963b), who demonstrated that NADP protects G6PD from inhibition by PCMB, indicating that sulphydryl groups might be involved in NADP binding. Whereas enzyme inhibition per se does not enable one to choose between the two hypotheses mentioned, the fact that Ijebu-Ode is much less inhibited than the other variants (Figs. 20, 21) also favours the seconds hypothesis. If indeed, an -SH residue, essential for activity were lacking by mutation in Ijebu-Ode, the enzymatic activity should be reduced; but this has been found not to be true. On the other hand, if in this variant, a cysteine residue has been lacking which is not essential for activity, but which in other variants serves as an attachment site for PCMB and NEM, then Ijebu-Ode would be expected to resist the effect of these reagents. The mild inhibition still exerted by PCMB (but not by NEM) on Ijebu-Ode may be explained by the ability of PCMB to react with groups essential for activity, other than -SH groups. The NEM may not be reactive towards these residues.

The rather subtle difference in the inhibitory action of PCMB on A and A⁻ again (chapter VI) indicates a structural difference between these two variants.

CHAPTER VII

GENERAL CONCLUSION

Comparison of genetic variants of human erythrocyte G6PD:

Though the five genetic variants of red cell glucose-6-phosphate dehydrogenase differ in their electrophoretic mobilities (Fig. 3) and in their reactivity under various conditions, they essentially perform similar biochemical functions. These variants constitute a glaring example of the effect of what are probably one point mutations in the gene specifying the structure of an individual protein.

The five G6PD types are similar in their affinities for G6P but have different affinities for 2dG6P. From the plots of $\log V_{max}$, $\log K_m$ and $\log \frac{V_{max}}{K_m}$ against pH, the presence of ionisable imidazolium and thiol residues in or near the active centres has been predicted for most of the variants, while the absence of thiol residue is postulated for Ijebu-Ode.

The work carried out on the pH-dependence of the two major kinetic parameters, V_{max} and K_m, may serve as a useful illustration of the potential and at the same time of the limitation of the kinetic approach to the structure of the active centre of an enzyme. Following the work of Dixon (1953), this analysis has been carried a long way towards inferring which ionisable groups are involved in the enzyme reaction.

In the case reported here, one had the additional advantage of being able to compare the behaviour of five different variants (see chapter IV). It was thus possible to make an "educated guess" as to which amino acid residues in the protein are involve. However, the limitations become clear when it is realized that groups which are quite remote from the active centre, especially if ionised, can affect crucially the behaviour of the protein towards ligands. In consideration of this, it will be essential to obtain direct information of the primary structure so that this could then be correlated with the results from the kinetic work. It is possible that if such data are collected for a number of enzymes, as they have already been collected to some extent in the case of abnormal haemoglobins (Wintrobe, 1967), the information obtained could be extrapolated to other cases even before the structural analysis is complete.

Structural differences among G6PD variants: Like for haemoglobin variants, it has been proved that erythrocyte G6PD variants A and B differ by a single amino acid substitution (Yoshida, 1967). It is reasonable to think that other variants will differ by alterations in the amino acid sequence of their primary structures. Such alterations, whether additions, deletions, or substitutions, will bring about differences in the tertiary structure of the enzyme proteins. The differences in the structure of their peptide chains resulting in a certain total net charge characteristic for each variant are reflected

in the differences in electrophoretic migrations. However, A and A⁻ G6PD types have identical migrations. It is possible that these two enzyme variants differ by alterations in the amino acid sequence of their primary structures and yet have identical net charge. Two proteins may also have identical amino acid sequence but differ in the folding of their peptide chains and thus in the tertiary and quartenary structures. This possibility is known to occur in sperm whales and pork insulin, two proteins which have identical amino acid sequence, yet differ in their reactions towards antisera (Berson & Yalow, 1961, 1963). On the basis of this evidence, the authors continued, it would seem that, in addition to amino acid sequence, the precise configuration of folding of the protein molecule is also determined by the genetic apparatus. However, it is desirable to obtain the amino acid sequence of G6PD type A⁻ and possibly those of other variants too as a basis for understanding the complex structures of the proteins.

Inactivation and reactivation of G6PD: Several workers have reported difficulties in the recovery of erythrocyte A⁻ enzyme type during purification by ion exchange chromatography. The instability of A⁻ may involve several mechanisms. Perhaps its instability is due to dissociation into inactive subunits, in which case the use of higher concentrations of NADP during the purification procedure might be sufficient to stabilize the enzyme. In contrast, rat mammary gland G6PD does not need NADP for its stability during purification (Levy, Raineri & Nevaldine, 1966). The findings of Bonsignore et al., 1968 (a) & (b) on metabolite

induced inactivation paralleled by splitting of red cell G6PD into lower molecular weight inactive subunits and subsequent reactivation of inactivated enzyme by NADP, confirms the stabilising role of this co-enzyme. Moreover, the metabolic cycle of G6PD as postulated by these authors may help to explain the fast rate of degradation of enzyme type A^- in vivo in the sense that such metabolite inactivated A^- variant may not be easily reactivated. An experimental analysis of the interactions of several genetic variants of red cell G6PD with these metabolites, with a view to finding out whether the inactivation and reactivation processes are characteristic for each variant, is desirable.

Effect of inhibitors: The observed variations associated with interactions of the enzyme variants with the inhibitors - NEM and PCMB, and the thermal inactivation profile studies all point towards differences in the structural configurations among the enzyme types. The postulation about the absence of thiol residue in or near the active centre of Ijebu-Ode is also well supported by the effect of inhibitors on the enzyme.

Regulatory properties: The sigmoidality of rate-concentration curves for NADP (Figs. 15 (a) & (b) has qualified G6PD to be placed on the list of regulatory proteins. The interpretation of the mechanism of such a kinetics, readily invokes the concept of subunit interactions (see Gerhart & Pardee 1952, 1963; Changuex, 1963; Monod et al., 1965). It is difficult to provide an

alternative model at the moment. The sigmoidal curves have for the first time provided evidence for the difference in the kinetics of A and A⁻ G6PD types. It had been shown by Yoshida et al (1967), that A and A⁻ enzyme types are similar in catalytic and immunological properties. Even though the curve for A⁻ appears at first sight consistent with the classical hyperbolic Michaelis-Menten model, the calculated dissociation constants point to the likelihood of the existence of a transition from low to high affinity at very low concentration of NADP. It is difficult to extrapolate the results of in vitro kinetic studies to the conditions in the living systems because of the possibility of existence of modifying factors in vivo. Moreover, the fact that kinetic data fit a certain hypothesis does not constitute proof.

(See Table XII)

The crucial evidence that the reported sigmoidal saturation curve reflects primarily the specific interaction between G6PD and NADP, lies probably in the demonstration that such interaction is subject to change as a result of genetic mutation. Thus, while the saturation curves for enzymes A and B are quite similar, those for A⁻ and Ijebu-Ode are quite different and distinct from each other. The physiological implications of this finding may become fully apparent only if suitable investigation can be carried out on intact cells. However, certain likely speculations are already possible.

Reference is particularly being made to the significance of these curves to the metabolism of G6PD deficient red cells.

Two main differences are apparent between the A and B variants on one side and the A⁻ on the other side.

(1) A⁻ has a much more nearly hyperbolic saturation curve for NADP.

(2) A⁻ is far less sensitive to inhibition by NADPH.

These differences may become quite crucial when one tries to envisage how they will reflect on the activity of the pentose phosphate parthway within red cells containing either the A or the B variant as opposed to the A⁻ variant. This, one can try to do first with regard to "normal" conditions (under which the accurate pentose phosphate pathway operates only at a very low rate), and then with regard to conditions under which it is markedly stimulated.

(a) Under normal conditions, if the NADP concentration in the red cells is within the range where the A and B variants are in the state of low affinity for NADP, it is apparent from the saturation curves (see Fig. 15, a & b) that their G6PD is operating at a rate which is a very small fraction of V_{max}. By contrast at the same NADP concentration, the A⁻ variant will be already much nearer to saturation, and operating almost at the rate of V_{max}. In addition, since a measurable concentration of NADPH is always present in red cells, this will have a certain inhibitory action on the A and B variants but not on the A⁻ variant. Both these facts will contribute to a relatively greater activity of A⁻ compared to A and B. In order at least to attempt a quantitative evaluation of the difference in activity, one makes use of the most recent available figures on the concentrations of NADP and

NADPH in the red cells, as reported by Gross, Schroeder & Gabrio (1966). Assuming an intracellular concentration of approximately 32 μM for NADP and approximately 24 μM for NADPH, and making reference to Figures 15 and 17, it is possible to calculate that, under such conditions, the B variant will function at approximately 42% of its maximum rate, but A⁻ will function at approximately 83% of its maximum rate. This almost two-fold difference in the percentage activity of the two variants will already compensate to considerable extent for the lower level of enzyme in the "deficient" cells. When it is considered that the determination of pyridine nucleotides is subject to considerable error, and that it is impossible to know whether the saturation curves obtained truly reflect the condition (pH, ions, etc.) prevailing within the red cells, it is conceivable that this compensation might be even more effective. Basically, it is suggested that the peculiar differences in kinetic behaviour of the A⁻ variant compared with A and B might explain a well known paradoxical observation, namely that the metabolism of G6PD-deficient cells, and particularly the rate of the pentose phosphate pathway in them, is not significantly abnormal. It is suggested that under physiological conditions the activity of the A⁻ enzyme in the cell is either normal or very nearly normal.

(b)   A very different situation will prevail once an oxidative stress is imposed upon the red cells. In terms of regulations of G6PD activity such as stress will probably produce a change

in the ratio of NADP to NADPH, namely, an increase in NADP and a decrease of NADPH. Again this will entail two differences between the normal and the deficient red cells. In red cells containing G6PD, A or B, the increase in NADP concentration causes the transition of the enzyme from low affinity to high affinity, and hence the velocity of the enzyme reaction is remarkably increased. Furthermore, the decrease in NADPH concentration will entail considerable release from inhibition. Both of these changes will result in increased G6PD activity and much faster operation of the pentose phosphate pathway. By contrast, in G6PD-deficient cells, the increase in NADP concentration will increase the activity of the enzyme only very slightly, since the enzyme is already working at near-maximum velocity. The decrease in NADPH concentration will not enhance G6PD activity since no inhibition was taking place. Thus, A⁻ containing red cells have almost no way of increasing the rate of operation of the pentose phosphate pathway when this is demanded.

The detailed kinetic analysis of the interaction of G6PD variants with NADP and NADPH thus provides a plausible explanation for their performance _in vivo_. Clearly, only experiment in which the intracellular rate of operation of G6PD can be evaluated will demonstrate whether such an interpretation is entirely valid.

The complex interaction of G6PD variants with NADPH points to the likelihood that this metabolite serves as a regulator of the first reaction of the pentose phosphate pathway. The negative

feedback mechanism implied is not too surprising as such a
mechanism is now known to operate extensively in micro-organisms. (Gerhart and Pardee
The mechanism of action of NADPH probably involves conformational
alterations through subunit interactions. The inhibition may
implicate other mechanism besides the involvement of the subunits,
such as partial inhibition, whereby the rate constants for the
breakdown of the enzyme-substrate (ES) and enzyme-substrate-
inhibitor (ESI) complexes are different. The work of Glock & McLean
(1955) has suggested that the ratio of NADP/NADPH is of
significance in the control of the pentose phosphate pathway.
The rate of oxidation of NADPH serves as a regulator of the rate
of reaction of G6PD. The existence of activating, active and
regulatory sites as postulated in this report, in parallel to
what has been observed in micro-organisms still requires
conclusive experimental proof. The existence of regulation of
pentose phosphate pathway may not be an exclusive prerogative of
G6PD alone. It is possible that other enzymes and metabolites
associated with this pathway may participate in this regulatory
exercise as a cumulative phenomenon. The work of Bossignore et al.
(1968a, 1968b) on metabolite induced inactivation of erythrocyte
glucose-6-phosphate dehydrogenase by glucose-6-phosphate, gluconate
6-phosphate, NADPH and an unidentified inactivating enzyme lends
support to the suggestion of cumulative or concerted regulatory
control of pentose phosphate pathway.

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mechanism is now known to operate extensively in micro-organisms. *Gerhart and Pardee. 196*
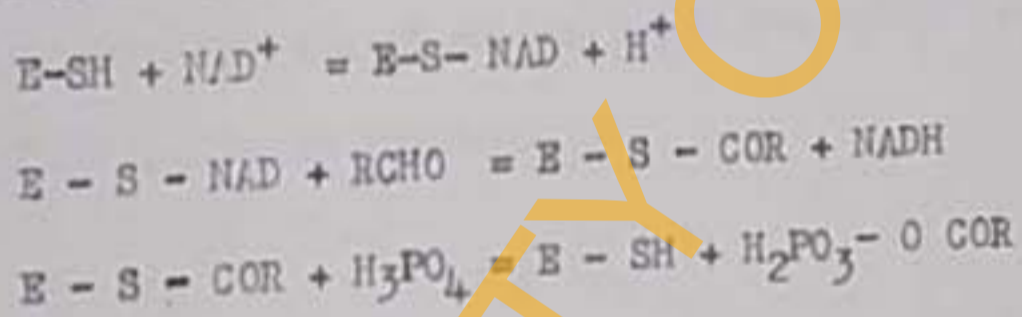The mechanism of action of NADPH probably involves conformational
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The rate of oxidation of NADPH serves as a regulator of the rate
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6-phosphate, NADPH and an unidentified inactivating enzyme lends
support to the suggestion of cumulative or concerted regulatory
control of pentose phosphate pathway.

MECHANISM OF ENZYME ACTION

The present ideas about the mechanism of action of dehydrogenase enzymes are well illustrated by the case of glyceraldehyde-3-phosphate dehydrogenase (Dixon and Webb, 1964). The enzyme has been crystallised both from yeast and from muscle. For both, the affinity for nicotinamide adenine dinucleotide (NAD) is much higher than for the other substrate. The protection offered by NAD against inhibition of the enzyme by some thiol reagents (Shifrin and Kaplan, 1960) is an indication that NAD is bound to the enzyme protein through thiol groups. The part of NAD at which the thiol group combines is not known. Based primarily on such data, the generally accepted scheme of action is as follows:-

$$E-SH + NAD^+ = E-S-NAD + H^+$$

$$E - S - NAD + RCHO = E - S - COR + NADH$$

$$E - S - COR + H_3PO_4 = E - SH + H_2PO_3 - O COR$$

In the case of glucose-6-phosphate dehydrogenase the same scheme is likely to hold. It is known that the affinity of G6PD for NADP is higher than for G6P. The protection offered by NADP against inhibition of G6PD by PCMB (see p. 21) suggests also that this co-enzyme is bound to thiol groups in the enzyme protein. The presence of thiol group(s) in the active centre of G6PD is supported by the data on inhibition by PCMB and NEM presented in Figs. 20 and 21.

In addition to the thiol groups, Soldin and Balinsky (1966) proposed the presence of an imidazolium residue in the active centre. The data presented here (see Fig. 13; p. 80 and 81) are also in agreement with this suggestion.

However, an imidazolium residue may not be critical. If it were, A and Ita-Bale which may "lack" histidine (inferred from the absence of imidazolium residue, see Fig. 13) would be deficient, and they are not. Alternatively, it is possible that the imidazolium residues in these variants are critical but are now in a different environment with the result that their pKs have been modified.

Similarly, the absence of thiol residues postulated for Ijebu-Ode (p. 81 and 119) may also imply either that the general mechanism above is wrong or that the thiol residues are present in this variant in a different environment, which prevents the binding of PCMB and NEM and which also modifies the pK of the thiol residue to abolish the wave observed at pH 7.7 in the other variants.

Finally, even though the results reported and discussed in this thesis point out some of the differences and similarities in the _in vitro_ kinetics and properties of the five genetic variants of erythrocyte glucose-6-phosphate dehydrogenase in Nigeria, the data are by no means conclusive as to what actually happens _in vivo_. The fact that the Ijebu-Ode and Ita-Bale variants are neither associated with enzyme deficiency nor do entail any clinical abnormality, clearly confirms that genetic variation of G6PD is not synonymous with deficiency.

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