HUMAN RED CELL NADP-DEPENDENT XYLITOL DEHYDROGENASE: KINETIC AND GENETIC STUDIES.

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A Thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, for the Degree of Doctor of Philosophy.

Johannesburg 1984

DECLARATION

I declare that this thesis is my own, unaided work and that it has not been submitted for a degree at any other University.

Anthony Bruce Lane

ANTHONY BRUCE LANE

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ABSTRACT

A deficiency of the enzyme NADP dependent xylitol dehydrogenase (L-xylulose reductase) has previously been found to be the cause of chronic essential pentosuria. Essential pentosuria is a recessively inherited condition which is marked by the continual excretion of relatively large amounts of the enzymes substrate, L-xylulose. The major objective of the study described was to find a simple method for the identification of individuals who are heterozygous for the "pentosuria" and normal alleles. The pentosuria allele could then be used as a gene marker in linkage studies aimed at mapping the L-xylulose reductase locus. A L-xylulose reductase assay suitable for the identification of carriers of essential pentosuria was developed and tested on members of a South African Lebanese family in which the inheritance of pentosuria had previously been suggested to be dominant. It was found that family members could, on the basis of their L-xylulose reductase activities, be classified as either normal, heterozygous or homozygous for the pentosuria allele. Measurements of serum L-xylulose concentrations revealed that pentosuria is, contrary to the previous report, recessively inherited in this family.

A sample of the local Ashkenazi Jewish population was screened for pentosuria carriers. Six out of the 237 individuals screened were found (on the basis of their L-xylulose reductase activities and from the results of a loading test), to carry the pentosuria allele. The frequency of the pentosuria allele in this population was estimated from the apparent heterozygote frequency to be 0.0127. Linkage analyses were carried out on the families of the identified heterozygotes and on members of the Lebanese family mentioned above.

No evidence of tight linkage was found between the pentosuria allele's locus and those coding for various red cell antigens, red cell enzymes and serum proteins.

Kinetic, chromatographic and electrophoretic studies revealed that the red cells of normal individuals contain two distinct L-xylulose reductases, a minor and a major isozyme. Pentosurics lack the major isozyme but appear to have approximately normal amounts of the minor isozyme. The minor isozyme is electrophoretically distinct from the major isozyme, has markedly higher Michaelis constants for the substrates L-xylulose and xylitol and shows a lower pH optimum when catalysing the oxidation of xylitol. Electrophoresis also revealed that liver tissue contains two L-xylulose reductases which occur in similar proportions to those of red cells but which migrate at slightly different rates.

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ABBREVIATIONS AND EXPLANATORY NOTE ON NOMENCLATURE

ACD	acid citrate dextrose anticoagulant solution
ATP	adenosine triphosphate
°C	degrees centigrade
cm	centimeter
CMC	carboxymethyl cellulose
E	extinction (optimal density)
EDTA	ethylenediaminetetra-acetic acid
g	gram
g	acceleration due to gravity
G6PD	glucose-6-phosphate dehydrogenase
GSH	reduced glutathione
GSSH	oxidised glutathione
Hb	haemoglobin
hr	hour
K_m	Michaelis constant
ℓ	litre
mg	milligram
mins	minutes
n	nano
NAD	nicotinamide-adenine dinucleotide (NAD^+)
NADH	reduced nicotinamide-adenine dinucleotide ($NADH+H^+$)
NADP	nicotinamide-adenine dinucleotide phosphate ($NADP^+$)
NADPH	reduced nicotinamide-adenine dinucleotide phosphate ($NADPH+H^+$)
SD	standard deviation
Tris	tris (hydroxymethyl) aminomethane
U	unit

UDP	uridine diphosphate
UTP	uridine triphosphate
v	volume
$\underline{v}$	reaction velocity
V_{max}	maximum reaction velocity
w	weight

Note on the nomenclature used for the enzymes and the reactions catalysed by them

Since the reaction catalysed by NADP-dependent xylitol dehydrogenase (L-xylulose reductase), L-xylulose + $NADP+H^+ \rightleftarrows$ xylitol + $NADP^+$, is reversible, the enzyme, when catalysing what is called the forward reaction: L-xylulose + $NADPH+H^+ \rightarrow$ xylitol + $NADP^+$, has been referred to as L-xylulose reductase. When catalysing the reverse reaction: xylitol + $NADP^+ \rightarrow$ L-xylulose + $NADPH+H^+$, the enzyme has been referred to as xylitol dehydrogenase or NADP-linked xylitol dehydrogenase to prevent confusion with NAD-linked xylitol dehydrogenase which is not the subject of this study.

1. INTRODUCTION

1.1. General introduction

Chronic essential pentosuria in man is an inherited condition which is brought about by a deficiency of L-xylulose reductase (EC.1.1.1.10) (Wang and van Eys, 1970). In normal individuals, L-xylulose reductase catalyses the conversion of L-xylulose to xylitol at rates which prevent the accumulation of L-xylulose (Freedberg *et al.*, 1959). Individuals with a deficiency of L-xylulose reductase are unable to carry out this conversion rapidly enough and L-xylulose consequently reaches abnormally high concentrations in their urine and plasma (Bozian and Touster, 1959; Freedberg *et al.*, 1959). Although pentosurics apparently enjoy perfect health (Janeway, 1906; Lasker, 1955), their relatively large and constant urinary L-xylulose output aroused interest, and research carried out as a result, led ultimately to the uncovering of the glucuronic acid pathway. In addition, these studies shed light on the biosynthesis of L-ascorbic acid. Garrod (1908a) made a profound statement while speaking about metabolic processes during his first Croonian lecture when he said:

> "Such knowledge as we have of these steps is derived from casual glimpses afforded when as the outcome of one of Nature's experiments, some particular line is interfered with and intermediate products are excreted incompletely burnt."

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In this introduction an attempt will be made to illustrate how one such 'glimpse' led to the elucidation of the glucuronic acid pathway and, further, to summarise what is known about essential pentosuria and mammalian L-xylulose reductase.

The first reported case of essential pentosuria was that of Salkowski and Jastrowitz (1892). Many more cases were described in the following decade and when Garrod delivered his famous Croonian lectures in 1908 he could refer to 30 reported cases (Garrod, 1908b). Garrod dealt with essential pentosuria in his fourth lecture and correctly categorised it with albinism, alcaptonuria and cystinuria as an 'inborn error of metabolism'. At this time the excreted sugar had not been identified, although Salkowski and Jastrowitz (1892) had found that it was not fermented by yeast and that it yielded an osazone with a melting point of 159°C, a characteristic which suggested that it might be a pentose. Neuberg (1900) suggested that the excreted sugar was arabinose, but Zerner and Waltuch (1913) showed that this was not so, and Levine and La Forge (1914) and Zerner and Waltuch (1914) independently identified it as L-xylulose. L-xylulose is like glucose, a reducing sugar, and it is this property which led to the discovery of most pentosurics, as tests aimed at identifying a diabetic through the detection of reducing substances in urine will, of course, yield a positive result in an individual with pentosuria (Larson *et al.*, 1941; Wright, 1961).

Another way in which pentosurics came to light was as a result of the abuse of drugs like morphine. The attention of Salkowski and Jastrowitz (1892) was drawn to the first case because he was a morphine addict whose urine, on being tested for reducing substances, gave a positive result. Soon after this, the urine of four other morphine users was also found to yield positive results (Reale, 1894; Caporali, 1896), and Caporali (1896) claimed that pentosuria could be induced by injecting morphine into healthy dogs. After the morphine injections were stopped, the urine of these four morphine

addicts as well as the urine of the dogs returned to normal. Salkowski and Jastrowitz reported that their patient continued to excrete the pentose after the morphine injections had ended.

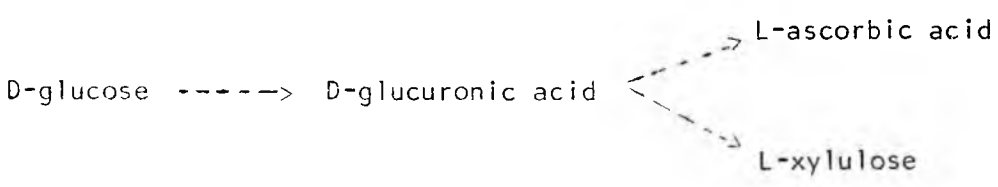
The effects of another drug on pentosuria led to a further advance in this field. Margolis (1929) noted, as have others since (Enklewitz and Lasker, 1935; Larson *et al.*, 1941; Touster *et al.*, 1955), that the amount of L-xylulose excreted by pentosurics tends to remain relatively constant. One of his pentosuric patients, who happened to suffer from migraine, was noticed to excrete more L-xylulose on days following migraine attacks. The drug amidopyrine had been taken by the subject for the migraine headaches and Margolis demonstrated that administration of the drug on 'non-headache days' dramatically increased the patient's L-xylulose excretion. Since then a variety of drugs with diverse structures have been found to increase L-xylulose excretion by pentosurics (Enklewitz and Lasker, 1933, 1935).

A possible explanation for this phenomenon was provided by Enklewitz and Lasker (1933), who were aware that many drugs form complexes with D-glucuronic acid before being excreted. They suggested that D-glucuronic acid production could be stimulated as a result of its excretion with these drugs and further, that D-glucuronic acid was a precursor of L-xylulose. Enklewitz and Lasker (1935) gained hard evidence in support of their hypothesis by showing that L-xylulose output in a pentosuric subject could be greatly increased by the administration of D-glucuronic acid. It is now firmly established that D-glucuronic acid is a precursor of L-xylulose, but the way, or ways, in which various drugs increase its rate of formation are still not fully understood. Barbital,

for instance, stimulates D-glucuronic acid formation but is not excreted as a glucuronic acid conjugate (Burns *et al.*, 1957). Some but not all of the drugs which stimulate D-glucuronic acid production increase levels of liver uridine disphosphate glucose dehydrogenase (Hollman and Touster, 1962), an enzyme involved in D-glucuronic acid synthesis.

When D-glucuronic acid was found to be a precursor of L-ascorbic acid, as well as of L-xylulose (Isherwood *et al.*, 1953), the main thrust of research became aimed at finding out how L-ascorbic acid is formed. As intermediates in L-ascorbic acid biosynthesis were identified, it became obvious that L-ascorbic acid and L-xylulose are indeed formed along the same biochemical pathway (in animals and plants capable of synthesizing L-ascorbic acid that is).

The steps in L-ascorbic acid and L-xylulose biosynthesis were worked out as follows: Longenecker and his collaborators (Musalin *et al.*, 1939; Longenecker *et al.*, 1940) found that a number of substances which were known to be detoxified through D-glucuronic acid conjugation, increased the urinary excretion of L-ascorbic acid in rats. Mosbach and King (1950) and Eisenberg and Gurin (1952) established by means of radioactive tracer studies that the carbon skeleton of D-glucuronolactone can be derived *in vivo* from D-glucose without rearrangement of the carbon atoms. The following scheme summarises what was known at this stage:



Isherwood *et al.* (1953) attempted to work out the mechanism of L-ascorbic acid biosynthesis from glucose and galactose by administering a variety of possible intermediates to cress seedlings and rats, and then noting which of these stimulated L-ascorbic production. They found that D-glucuronolactone, D-galacturonolactone, L-gulonolactone and L-galactonolactone would do this and accordingly postulated the following pathway (and a homologous pathway starting with D-galactose):
D-glucose → D-glucuronic acid → L-gulonic acid → L-ascorbic acid.
Lactones rather than the corresponding acids which they spontaneously give rise to on hydrolysis, have been used in this type of work because they are more readily taken up by cells (Pacham and Butler, 1954; Touster *et al.*, 1955). The acids and not their lactones, however, appear to be the true intermediates (Touster, 1959), with the exception of L-gulunolactone rather than L-gulonic acid which is a precursor of L-ascorbic acid (Burns and Evans, 1956).

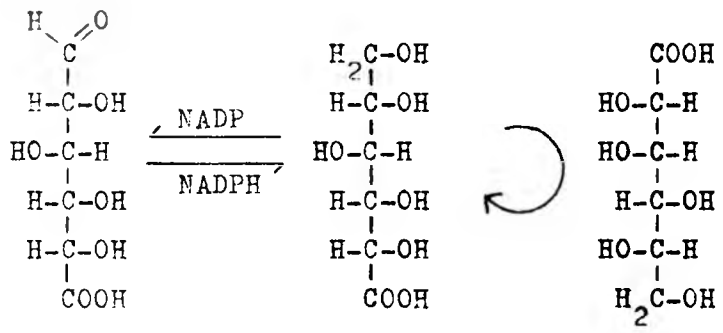
Horowitz and King (1953) were able to recover labelled D-glucuronic and L-ascorbic acids from the urine of chlorotone stimulated rats after labelled D-glucose had been injected into them. In addition, they found that injection of labelled D-glucuronolactone in place of labelled D-glucose resulted in four to six times more labelled ascorbic acid being excreted which was additional evidence that D-glucuronic acid is an intermediate in L-ascorbic acid formation and, further, that it is a more immediate precursor than D-glucose.

Burns and Evans (1956) confirmed the findings of Isherwood *et al.* (1953) that L-glucuronolactone can be converted to L-ascorbic acid in both unstimulated and chlorotone stimulated rats, and obtained

evidence that L-gulonic acid is an intermediate in the process. Finally, Hassan and Lehninger (1956) detected an enzyme in liver extracts which could convert D-glucuronic acid to L-gulonic acid in the presence of NADPH. The following scheme depicts what was known by 1956:

$$\text{D-glucose} \rightarrow \text{D-glucuronic acid} \underset{\text{NADP}}{\overset{\text{NADPH}}{\rightleftharpoons}} \text{L-gulonic acid} \rightarrow \text{L-ascorbic acid.}$$

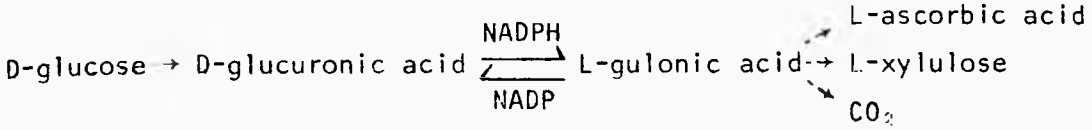
The formation of L-gulonic acid from D-glucuronic acid involves a so-called inversion which is brought about as follows: When the aldehyde group at C1 of D-glucuronic acid is reduced, the carboxyl group at C6 becomes the most highly oxidised end carbon, and is therefore numbered C1 by convention (Figure 1.1). The arrangement of H atoms and OH groups at the 'new' C5 of gulonic acid corresponds to the arrangement at C2 of L-glyceraldehyde and so what was formerly a D-sugar derivative now becomes an L-series sugar derivative. Horowitz *et al.* (1952) presented evidence for this inversion when they found that D-glucose labelled at the C1 position was converted to L-ascorbate labelled at the C6 position in rats. Touster *et al.* (1955) suggested that such an inversion also takes place in the formation of L-xylulose and that this is followed by the loss of the carbonyl bearing carbon. Touster *et al.* (1957) provided experimental evidence for this when they found that 6-^{13}C-D-glucuronolactone fed to a pentosuric subject increased the production of unlabelled L-xylulose, whereas when 1-^{13}C-D-glucuronolactone was fed it was converted to 5-^{13}C-L-xylulose. Burns and Kanfer (1957) showed that rat kidney preparations could convert L-gulonic acid (or its lactone) to L-xylulose and that the CO_2 produced in the process contained the original carboxyl carbon (C6) of L-gulonic acid. The scheme given



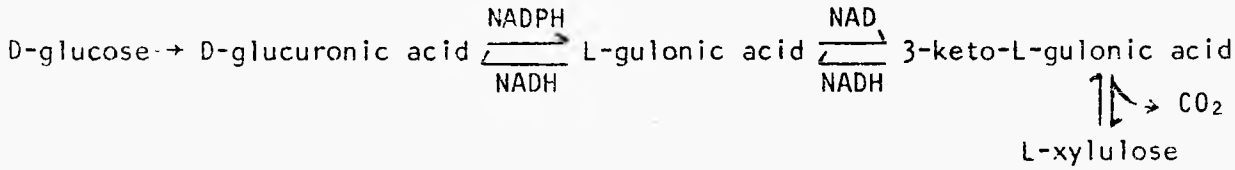
D-glucuronic acid L-gulonic acid

Figure 1.1. The transformation of D-glucuronic acid to L-gulonic acid through the reduction of the (C1) aldehyde group of D-glucuronic acid.

below outlines what was known at this stage, i.e. in 1957:



In summary, it was known by 1957 that the formation of both L-ascorbic acid and L-xylulose had the following in common: They shared the precursors D-glucose, D-glucuronic acid and L-gulonic acid, and in the process of their formation, an inversion of the original carbon skeleton of D-glucuronic acid occurred. From L-gulonic acid, the pathway leading to L-ascorbic acid (L-gulonic acid → 2-keto-L-gulonolactone → L-ascorbic acid) (Kanfer *et al.*, 1959) branches away from that leading to the formation of L-xylulose. The next step in the formation of L-xylulose from D-gulonic acid is the oxidation of L-gulonic acid to 3-keto-L-gulonic acid by a β-L-hydroxy acid dehydrogenase which was discovered and partially purified by Ashwell *et al.* (1960). Winkelman and Ashwell (1961) then demonstrated that L-xylulose could be made from 3-keto-L-gulonic acid (with the simultaneous release of CO_2) by an enzyme from guinea pig liver. The following scheme summarises what was known at this stage:



A mechanism for the formation of L-xylulose from D-glucuronic acid had been found, but this did not explain what happened to L-xylulose in normal individuals. Hiatt (1958a), using radioactive tracers and a 'ribose-trapping' technique, showed that L-xylulose can be an intermediate in the production of ribose and that this pathway is blocked in pentosurics. An alternative explanation for essential pentosuria had

previously been put forward by Knox (1958), who suggested that pentosurics may be unable to reabsorb L-xylulose from the fluid in their kidney tubules. This latter explanation was shown to be incorrect by Bozian and Touster (1959) and Freedberg *et al.* (1959), who found that plasma as well as urinary L-xylulose levels were raised in pentosuric subjects.

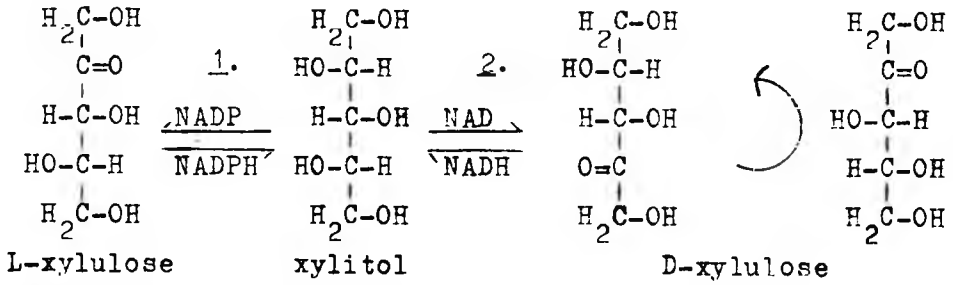
Guinea pig liver slices and homogenates are capable of removing L-xylulose from solution and guinea pig liver mitochondria are able to convert L-xylulose to xylitol (Touster *et al.*, 1954, 1955, 1956). Hollmann and Touster (1956) found that NADPH serves as the hydrogen donor for this L-xylulose reductase (NADP-linked xylitol dehydrogenase) catalysed reaction and that guinea pig liver mitochrondria also contain an NAD dependent xylitol dehydrogenase which has properties in common with the cytosol enzyme described by Blackley (1951) and McCorkindale and Edson (1954). The NADP- and NAD-linked enzymes constitute a system for the transformation of L-xylulose to D-xylulose (Hollman and Touster, 1956), as diagrammed in Figure 1.2.

The last step in the glucuronic acid pathway results in the formation of D-xylulose-5-phosphate which is also a pentose phosphate shunt intermediate. Hickman and Ashwell (1958) purified a kinase capable of catalysing the reaction:

D-xylulose + ATP → D-xylulose-5-phosphate + ADP,

and Dayton *et al.* (1958), Hiatt (1958b, 1958a) and Eisenburg *et al.* (1959) used radioactive tracers to obtain evidence that the two pathways merge *in vivo*.

D-glucuronic acid may be produced by either of two routes. The first is through the oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid (Strominger *et al.*, 1954),



1. NADP-linked xylitol dehydrogenase.

2. NAD-linked xylitol dehydrogenase.

Figure 1.2. The system for the interconversion of D- and L-xylulose discovered by Hollmann and Touster (1956).

followed by the release of D-glucuronic acid-1-phosphate by a pyrophosphatase (Kornberg, 1955). D-glucuronic acid-1-phosphate is finally converted to D-glucuronic acid by the removal of phosphate (Ginsberg *et al.*, 1958). The other source of D-glucuronic acid is from *myo*-inositol. Charalampous and Lyras (1957) have shown that rat kidney extracts are able to convert *myo*-inositol to D-glucuronic acid and Charalampous (1960) subsequently purified the enzyme which catalyses this oxidative cleavage. Hankes *et al.* (1969) found that pentosurics, as a result of the block in the glucuronic acid pathway are able to catabolise *myo*-inositol at less than one-tenth the normal rate. Figure 1.3 outlines the whole glucuronic acid pathway.

Although the glucuronic acid pathway had been elucidated by 1960 and the site of the block resulting in pentosuria known (Hiatt, 1958a; Eisenberg *et al.*, 1959; Touster, 1959), no one actually demonstrated a deficiency of NADP-linked xylitol dehydrogenase in individuals with pentosuria until 1970. In that year, Wang and van Eys (1970) were able to show that red blood cells from pentosurics have abnormally low NADP-linked xylitol dehydrogenase and by implication, low L-xylulose reductase activities. The reason for this delay is that the enzyme occurs mainly in liver and kidney and the removal of tissue from an otherwise healthy pentosuric would not have been justified.

1.2. Incidence and inheritance of pentosuria

Pentosuria occurs mainly in Ashkenazi Jews (Garrod, 1908b; Margolis, 1929; Lasker, 1952; Mizrahi and Ser, 1963). Touster (1959), for example, noted that of the 200 or so cases described prior to 1959,

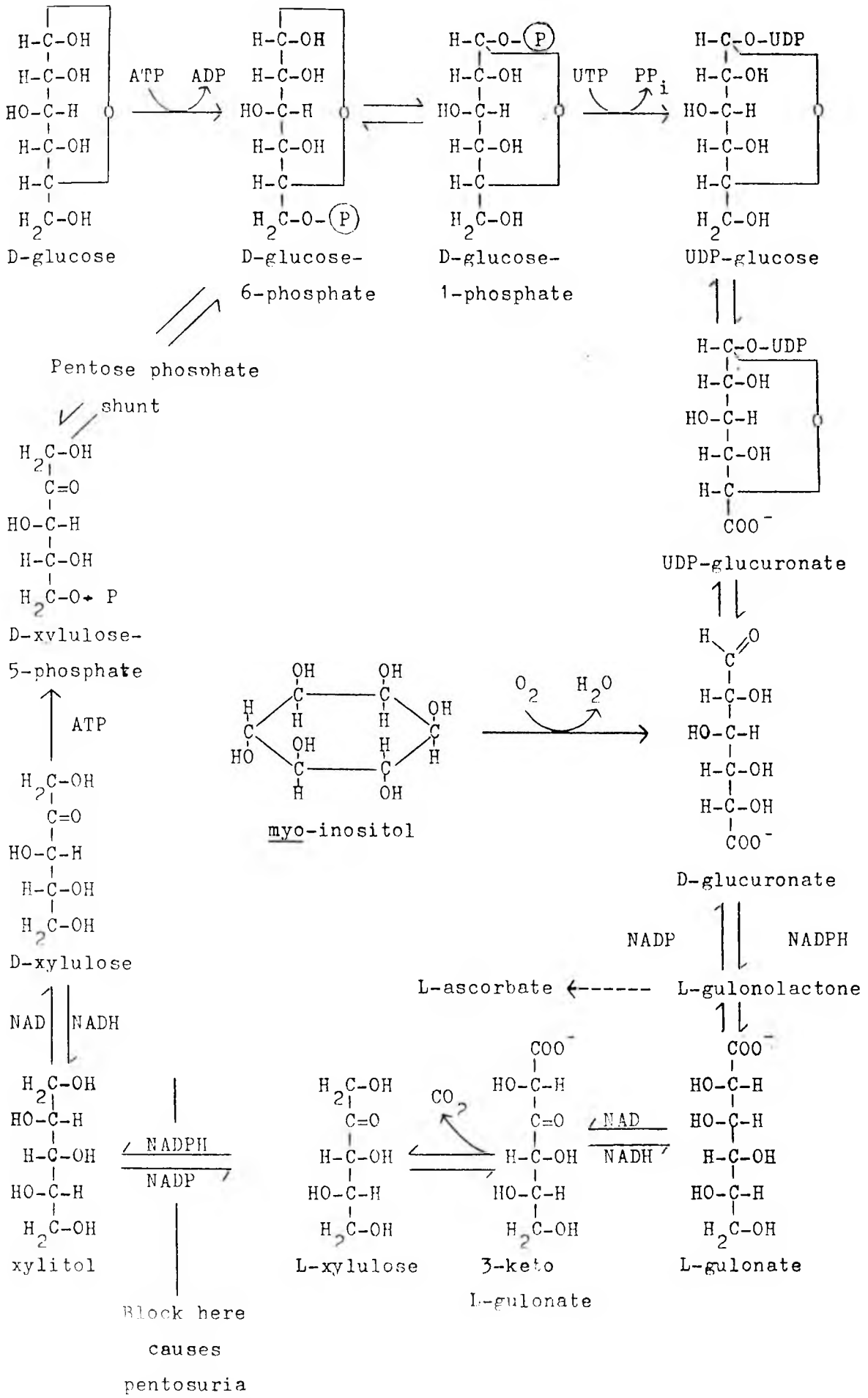


Figure 1.3. The glucuronic acid pathway.

only two were non-Jewish. The last mentioned pentosurics (sisters) were South Africans of Lebanese descent (Barnes and Bloomberg, 1953). Khachadurian (1962) has since reported twelve additional cases (members of three families) from Lebanon. Since pentosuria has only been recorded in Ashkenazi-Jews and Lebanese, the likelihood exists that the pentosuria alleles in these two groups are the same. Two considerations bear on this idea:

1. Doubts have been raised about whether the forefathers of the Ashkenazim originally came from Palestine (Koestler, 1976).
2. Pentosuria has not been recorded in any other group of Jews apart from the Ashkenazim (Mizrahi and Ser, 1963).

The Ashkenazim, as opposed to the Sephardic Jews who live or lived until recent times along the shores of the Mediterranean sea, are the Yiddish-speaking Jews from Eastern Europe. It is generally believed that the Ashkenazim are descended from Jews who originated in Palestine, resided in Germany and France during medieval times and then moved eastwards into Lithuania, Latvia, Poland and Russia. Unfortunately, the historical evidence for this is meagre and Koestler (1976) has advanced the idea that members of a Turkish tribe, the Kazars, who once lived in the area between the Black and Caspian seas and whose rulers embraced Judaism in about 740 AD moved westwards and northwards and founded the Ashkenazim. Mourant *et al.* (1978) after comparing blood group frequencies in various Sephardic and Ashkenazi populations state the following:

> "When however we compare Ashkenazim with Sephardim we find that there are indeed systematic differences between them. But these are so small that we can hardly avoid the conclusion that the two populations have a common origin."

As there is no doubt that the ancestors of the Sephardim came from

Palestine, Mourant *et al's* findings strengthen the evidence that the ancestors of the Ashkenazim came from there as well.

With regard to the second point, that pentosuria does not occur in any other group of Jews, one may note that neither does Type 1 Gaucher's nor Niemann-Pick disease Type A. Tay-Sachs disease is another inherited condition which occurs mainly in Ashkenazi Jews, although Goodman (1979) states that there have been a few cases in Sephardic Jews. Although natural selection has been suggested as being the reason for the increased frequency of the Tay-Sachs allele in Ashkenazi Jews (Myrianthopoules and Aronson, 1966), it has been pointed out by Wagener *et al.* (1978) that the populations who surrounded the Ashkenazim in Eastern Europe and who lived under similar conditions, showed no such increase. If natural selection on its own was not responsible for the high Tay-Sachs allele frequency in the Ashkenazim, then genetic drift, alone (Wagener *et al.*, 1978) or in conjunction with natural selection (Chakravarti and Chakraborty, 1978), or founder effect (Chase and McKusick, 1972), would appear to be the most likely alternatives. The same process or processes could have also brought about the increase in the frequency of the pentosuria allele in the Ashkenazim.

The familial occurrence of essential pentosuria has been underscored by a number of authors, for example, Garrod (1908b), Margolis (1929) and Lasker *et al.*, (1936). Margolis stated that about one-third (twenty-four) of all pentosurices (seventy-eight) reported by 1929 belonged to only nine families. Attention has also been drawn to the fact that pentosuric offspring result more frequently from consanguineous matings than from matings of unrelated

individuals (Garrod, 1908b; Lasker *et al.*, 1936; Khachadurian, 1962). The fact that the parents of pentosurics are usually not affected themselves (Lasker *et al.*, 1936), added to the above, is evidence that the allele for pentosuria is recessive in effect. Schultz (1938), however, found the condition in two generations of the same family and on the strength of this, suggested that pentosuria could be inherited as an autosomal dominant with incomplete penetrance. Evidence against Schultz's suggestion was provided by Lasker *et al.* (1936) who noted that none of the ten normal sibs of pentosurics known to them had given rise to any pentosuric offspring. The family described by Schultz is probably an instance of so-called pseudominanance: an affected individual fortuitously married an individual heterozygous for the same allele and the couple were thus able to produce homozygous offspring. This is a phenomenon likely to appear for recessive traits when the particular allele has a high frequency in the population.

On the other hand, results published by Politzer and Fleischmann (1962) during an investigation of the South African Lebanese family in which essential pentosuria occurred, could best be interpreted in terms of a dominant mode with incomplete penetrance. It is possible that the 'Lebanese pentosuria allele' differs from the 'Ashkenazi-Jewish allele' and causes a more severe deficiency of L-xylulose reductase and hence pentosuria in heterozygotes. However, the criterion used for classifying individuals in the study of Politzer and Fleischmann, viz. whether urinary L-xylulose could be consistently detected (no quantitative data are given), makes the validity of the classification doubtful since Touster *et al.* (1954, 1955) have found that heterozygotes tend to have urinary levels of L-xylulose which are slightly raised relative to the traces present

in the urines of normal individuals. Furthermore, inheritance of pentosuria in the other Lebanese families was recessive (Khachadurian, 1962).

A more accurate way of distinguishing between pentosurics, carriers of a single pentosuria allele and normal individuals is that used by Freedberg *et al.* (1959) and Kumahara *et al.* (1961). Their method was to obtain urine and serum from fasting pentosurics and their close relatives as well as from presumably normal individuals before giving these subjects a quantity of the L-xylulose precursor, D-glucuronolactone. The L-xylulose concentrations in serum and urine samples obtained at intervals over the following three hour period were then measured. The D-glucuronolactone brought about large increases in the serum and urine L-xylulose concentrations of pentosurics compared with those observed in the controls, while heterozygotes showed intermediate increases. The drawbacks of the method are firstly, it is difficult to persuade individuals to subject themselves to a test of this sort since swallowing the D-glucuronolactone is unpleasant not to mention the repeated blood and urine samples required and the considerable diarrhoea which may result (personal experience). Secondly, measuring L-xylulose in the serum and urine samples is time consuming, and the potential for variation, independent of that due to variation in the amount of L-xylulose reductase present in each subject, must be great when one considers the many physiological and metabolic steps required for the uptake and transport of D-glucuronolactone from the gut and its transformation into the L-xylulose which finally appears in the plasma and urine.

1.3. Mammalian xylitol dehydrogenases

Hollmann and Touster (1956) detected two enzymes capable of catalysing the conversion of xylitol to xylulose in guinea pig liver mitochondria. One of the enzymes required NADP to act as coenzyme and appeared to have an absolute specificity for xylitol (L-xylulose and NADPH were the products), while the other enzyme required NAD as coenzyme and could act on ribitol as well as xylitol (the products being either D-ribulose or D-xylulose and NADH). Hollmann (1960) described a method for separating and partially purifying these two mitochondrial enzymes and reported that the NADP-dependent enzyme had a Km for xylitol of $2{,}54 \times 10^{-2}$ M and that *p*-mercuribenzoate was a strong inhibitor. Further, he found that the equilibrium constant of the catalysed reaction:

$$\text{xylitol} + \text{NADP}^+ \rightleftharpoons \text{L-xylulose} + \text{NADPH} + \text{H}^+$$

was $2{,}97 \times 10^{-11}$ which meant that equilibrium at physiological pH values would strongly favour the formation of xylitol. This, together with the enzyme's specificity for L-xylulose suggested that it could be used to measure L-xylulose concentrations. The second (NAD-linked) xylitol dehydrogenase resembles the cytosolic sorbitol dehydrogenase described by Blackley (1951) and McCorkindale and Edson (1954), but differs in that it is mitochondrial and does not appear to act on D-fructose and L-sorbose.

Ironically, most of the NADP-dependent xylitol dehydrogenase (L-xylulose reductase) of guinea pig liver is non-mitochondrial and was overlooked by Touster's group until 1969 (Arsenis and Touster, 1969). In the meantime, Hickman and Ashwell (1959) obtained a crude preparation of NADP-linked xylitol dehydrogenase from acetone powders

of guinea pig liver which almost certainly included the cytoplasmic enzyme. Arsenis and Touster (1969) later discovered the cytosolic enzyme when they observed that acetone powders prepared from whole liver homogenates contained far more activity than could be attributed to the mitochondria alone. The cytosol enzyme identified by Arsenis and Touster appeared to behave differently from that obtained by Hickman and Ashwell, in that their enzyme required the presence of $MgCl_2$ for full activity, while the enzyme in the preparation of Hickman and Ashwell did not. Another difference was that the enzyme of Arsenis and Touster could be almost completely inhibited by iodoacetate, while the enzyme of Hickman and Ashwell could not be inhibited at all.

L-xylulose reductase occurs mainly in liver and kidney tissue (Hollmann and Touster, 1956; Kumahara *et al.*, 1961). Kumahara *et al.* (1961) found activity in normal human liver (and also in rat and guinea pig liver and kidney tissue), but failed to detect activity 'in leukocytes, erythrocytes, saliva, semen or adipose tissue of normal human subjects'. Bässler and Reimond (1965) discovered, however, that red blood cells are able to metabolise xylitol, which suggested that they might possess some of the enzymes necessary for the formation of its precursors. Wang and van Eys (1970) subsequently detected both NAD and NADP-linked xylitol dehydrogenases in human red blood cells and found that red cells from pentosurics show the expected deficiency of the NADP-linked enzyme. Wang and van Eys (1970) reported a mean activity level of 28,8 ± 9,7 nmoles/minute/g haemoglobin for normals and a mean level of 5,2 units for pentosurics, which is approximately eighteen per cent of the normal mean. Only one presumed heterozygote

was investigated and his level of activity (19,0 units) was approximately sixty-six per cent of the normal mean. Judging from these data, the prospects for accurate heterozygote identification at population level did not appear promising. If for instance two standard deviations are subtracted from the normal mean, a value of 9,4 units is arrived at which is approximately one-third of the normal mean and much lower than the level found in the heterozygote.

Wang and van Eys (1970) also estimated the variant enzyme's Michaelis constants for NADP and xylitol and came to the following conclusions:

> "The Michaelis constant for xylitol on the NADP-linked dehydrogenase in pentosuric blood appeared normal. Therefore the decreased NADP affinity of the enzyme is apparently the molecular abnormality that causes the enzymatic disorder in pentosuria."

1.4. Aims of the present study

1. To gather basic biochemical information about the red cell L-xylulose reductases (NADP-linked xylitol dehydrogenases) of normal and pentosuric individuals.
2. To devise a technique which would discriminate between normals, pentosuria carriers and pentosurics on the basis of their red cell L-xylulose reductase activities.
3. To determine the frequency of pentosuria carriers in the local Ashkenazi-Jewish population.
4. To search for linkage between the L-xylulose reductase locus and an array of other gene loci.
5. To reinvestigate the South African Lebanese family studied by Politzer and Fleischmann (1962) in order to establish whether the apparent dominant mode of inheritance found by them is also manifest at the enzyme level.

CHAPTER 2

2. SUBJECTS, MATERIALS AND METHODS

2.1. Subjects studied

2.1.1. Pentosuric subjects and relatives

The author was fortunate to have the co-operation of two known pentosuric subjects. The first of these, RB, is an Ashkenazi Jewish man who was born in Breslau (now Wroclaw), Poland, in 1908, to apparently unrelated parents. At six years of age his urine was investigated because of (as far as he can remember) an attack of influenza, and found to contain abnormal amounts of a reducing substance which was subsequently found to be pentose. At thirty-eight years of age, RB underwent a medical examination in order to obtain life insurance cover. Once again his urine was found to contain an abnormal amount of some reducing substance (Benedict's test) from which a phenylosazone could be prepared, indicating that it was a sugar (Bloomberg *et al.*, 1946). The sugar was not fermented by yeast which showed that it was neither glucose nor fructose but it gave rise to a 'heavy bluish-green precipitate' when subjected to Bial's Orcein test, suggesting that it was a pentose. Further investigations by Barnes and Bloomberg (1953) made the diagnosis even more certain. These workers cocrystalised phenylhydrazone prepared from the patient's urinary sugar with a phenylhydrazone prepared from D-xylulose and observed the different (from either single osazone) form of the crystals. The melting point of the cocrystalised phenylosazones was found to be ± 40°C higher than either of the unmixed phenylosazones, a finding which suggested that the urinary sugar was L-xylulose. Finally, the subject's urinary sugar was

found to migrate at the same rate as pure xylulose when chromatographed on paper. Unfortunately this subject has no children and none of his immediate relatives were available for study.

The second pentosuric subject studied, HK, (formerly HS), was the elder of two Lebanese sisters encountered by Barnes and Bloomberg (1953). HK's two grandmothers were sisters who, together with their husbands, emigrated from Lebanon to South Africa in 1902 (Politzer and Fleischmann, 1962). HK's younger sister was originally examined because of her susceptibility to epileptiform attacks. Abnormal amounts of a reducing substance at first thought to be glucose were detected in her urine, but this was later shown to be L-xylulose. Urine from HK, who in contrast to her sister appeared to be quite healthy, was then tested and found also to contain large amounts of L-xylulose. Politzer and Fleischmann (1962) reinvestigated the Lebanese sisters and tested urine specimens from a further 126 members of their family. HK was also one of the pentosurics used by Hankes *et al.* (1969) to demonstrate that *myo*-inositol is almost completely catabolised via the glucuronic acid pathway.

HK and a number of her relatives were again investigated as part of the present study.

2.1.2. Ashkenazi-Jewish subjects

The South African Ashkenazi-Jewish population is mainly descended from immigrants from Lithuania, Latvia, Poland and Russia, who settled in South Africa between 1880 and 1937 (Saron, 1965). The subjects of the present study were medical students at the local

university and some of the students' close relatives. The students (237), after being lectured on inherited metabolic disorders, availed themselves of an opportunity to be tested for Tay-Sachs allele heterozygosity and for the presence of other gene markers including the essential pentosuria allele.

2.1.3. Non-Jewish subjects

Of the seventy-four non-Jewish subjects studied, fifty-eight were a random sample of Afrikaans speaking South Africans who are descended from mainly Dutch, French and German immigrants who came to South Africa in the second half of the 17th Century. The remaining sixteen subjects comprised nine Caucasians of English extraction, three Indians (Caucasians), two individuals of mixed Negro and Caucasian descent and two South African Negroes.

2.2. Materials

Deionised, distilled water was used throughout and all purchased chemicals were reagent grade.

2.2.1. The L-xylulose used in this study

The production of L-xylulose was undertaken because this substrate was not commercially available. The method described by Touster (1962) for its preparation was followed with one modification: the chromatography solvent used was ethyl acetate-pyridine-water (6:3:2 v/v) instead of 1-butanol-pyridine-water (10:3:3 v/v).

Procedure: Fifteen g of L-xylose (Sigma) was refluxed for 4,5 hours in 150 mℓ dry pyridine (Merck product No. 7463). The

pyridine was then removed by evaporation under reduced pressure in a rotary evaporator (temperature ± 70°C) and the resulting syrup mixed with 50 mℓ water. The water, together with a small amount of pyridine still present, was removed by evaporation on a rotary evaporator as before, but on this occasion and during all subsequent evaporation steps, the temperature was kept at 44°C. The remaining dark brown syrup was dissolved in 45 mℓ of warm water and then mixed with 2,5 g of activated charcoal. The mixture was stirred until the charcoal had been wet, after which the charcoal was separated from the L-xylulose containing solution by Büchner filtration. The charcoal was washed twice, each time with a volume of water equal to the original filtrate and all the filtrates finally combined. The volume of the combined filtrates was reduced by means of a rotary evaporator and the preparation then dried further under a stream of nitrogen. The resulting viscous syrup was dissolved in 25 mℓ of 96% ethanol and placed in a refrigerator to facilitate the crystallisation of L-xylose. After several hours, 3 mℓ of diethyl ether were added to aid further crystallisation of L-xylose. Similar additions of ether, totalling 25 mℓ, were made during the next 48 hours. The mixture was then placed in a -20°C freezer for 4 days before the L-xylose crystals were separated from the L-xylulose solution by Büchner filtration. The L-xylose crystals were washed several times with cold absolute ethanol and the washings combined with the original filtrate. The ethanol was evaporated in a stream of nitrogen and the remaining oily-syrup dissolved in 15 mℓ of 96% ethanol. Approximately 0,625 mℓ quantities of resulting sugar solution were applied as bands, 7,5 cm from the top edges of 18,5 x 22 inch sheets of Whatman 3 MM filter paper.

Descending chromatography with ethyl acetate-pyridine-water (6:3:2 v/v) as solvent was then carried out for approximately 4,5 hours. L-xylulose was located on the chromatograms with the aid of guide strips, each approximately 0,5 cm wide, which had been cut longitudinally from the chromatograms and stained. Staining of the strips was done by spraying them with napthoresorcinol reagent and then heating at 105°C for 10 minutes. Napthoresorcinol reagent was made by dissolving 0,1 g napthoresorcinol in 50 mℓ ethanol and mixing this with 50 mℓ 0,25 N HCl and 5 mℓ of orthophosphoric acid (specific gravity 1,85). L-xylulose containing portions of the chromatograms were cut into pieces of approximately 0,5 x 4 cm, saturated with water and then pulped by vigorous stirring with a glass rod. The L-xylulose solution was removed by Büchner filtration and the pulp washed with a volume of water equal to the filtrate. After vigorous stirring, the eluate was removed by Büchner filtration and the procedure repeated once more. The filtrates were combined and dried, firstly in a rotary evaporator and then under a stream of nitrogen.

2.3. Basic methodology

2.3.1. Red blood cell preservation and recovery

Whole blood containing ACD as anticoagulant was centrifuged at 1 000 x g for 5 minutes and the plasma removed by aspiration. The volume of packed red cells was estimated and, using a vortex mixer to facilitate rapid mixing, an equal volume of preserving fluid was added dropwise to the cells. The preserving fluid/red cell mixture was then stored at -20°C until required.

The preserving fluid contained the following substances dissolved

in a final volume of one litre:

Tri-potassium citrate (monohydrate)	19, 4 g,
Sodium dihydrogen orthophosphate (dihydrate)	3,10 g,
Disodium hydrogen orthophosphate (dihydrate)	3,50 g,
Glycerol	400 mℓ.

When required, the red cell/glycerol mixtures were thawed and then dialysed against 0,9% (w/v) NaCl for at least 3 hours at 0 - 4°C. After this, the cells were washed by the method described in 2.3.2.

2.3.2. Haemolysate preparation

Whole blood containing citrate-phosphate-dextrose (CPD) or acid citrate-dextrose (ACD) anticoagulant was mixed with approximately 2 volumes of saline (0,9% NaCl) and the cells then pelleted by centrifugation at 1 000 x g for 4 minutes. The supernatant and the buffy coat were aspirated and the cells then washed 3 times by suspending them in approximately 9 volumes of saline, pelleting them by centrifugation and aspirating the supernatant. The packed cells were then chilled in an ice-water bath for at least 5 minutes before being disrupted in a MSE Soniprep 150 sonicator fitted with a microprobe.

2.3.3. Haemoglobin estimation

The method described by Beutler (1975) was followed with the modification that a C4 Coulter counter standard was used in place of a cyanmethaemoglobin standard. A ferricyanide-cyanide reagent (Drabkin's solution) was prepared by dissolving 200 mg $K_3Fe(CN)_6$, 50 mg KCN and 1 g of $NaHCO_3$ in one litre of water. Aliquots of haemolysate or haemolysate containing assay mixture (usually 10 to

50 microlitres) were diluted with Drabkin's solution to a final volume of 3 mℓ. After mixing and standing at room temperature for 15 minutes, the extinction at 540 nm of each sample was measured against Drabkin's solution in a double beam spectrophotometer. The extinction readings were then converted to milligrams of haemoglobin by means of a standard curve. Measurements for the standard curve were obtained by diluting (in triplicate) a commercially available C4 Coulter counter standard and treating aliquots of this in the way described for haemolysates.

2.3.4. Measurement of NADP and NADPH

In instances where the NADP concentrations had to be known with precision, the method described in the Boehringer Mannheim publication entitled 'Biochemica information I (1973)' was used with the modification that a Tris buffer was substituted for the triethanolamine buffer specified.

Two batches of the following mixture were made up in 3 mℓ cuvettes:

2,69 mℓ of 0,1 M Tris-HCl buffer pH 7,6,

0,1 mℓ of 0,1 M $MgCl_2$,

0,1 mℓ of 14 mM D,L-isocitric acid,

0,1 mℓ of the NADP solution (test solution ± 1,3 µmoles/mℓ).

The solutions were used to zero a double beam spectrophotometer set at 340 nm and a trace made on the recorder chart. Ten microlitres (± 0,1 U) of isocitrate dehydrogenase (Boehringer) were added to the experimental cuvette and the change in extinction due to the formation of NADPH, followed on the recorder. When the reaction had gone to completion, a further 10 µℓ of isocitrate dehydrogenase solution were added to the

experimental solution and the additional change in extinction if any, recorded. The determination was repeated and the mean change in extinction due to the formation of NADPH, substituted in the following equation:

$$\text{Concentration of NADP in the test solution } (\mu\text{moles/m}\ell) = \frac{3 \times \Delta E_{340\ nm}}{0,622}$$

NADPH concentrations were measured according to the method described in Beutler (1975): One-hundred µℓ of 1M Tris-HCl buffer, pH 8,0, containing EDTA (5 mM) were added to 850 µℓ of water in a one mℓ cuvette. The extinction at 340 nm was measured against a water blank and then 50 µℓ of the NADPH solution (approximately 2 mg/mℓ) were added to the buffered solution and the extinction again measured. The difference between the first and second extinction values was then divided by 3,11 to yield the NADPH concentration of the original solution in µmoles/ℓ. The procedure was repeated and the mean of the two estimates taken.

2.3.5. L-xylulose quantitation

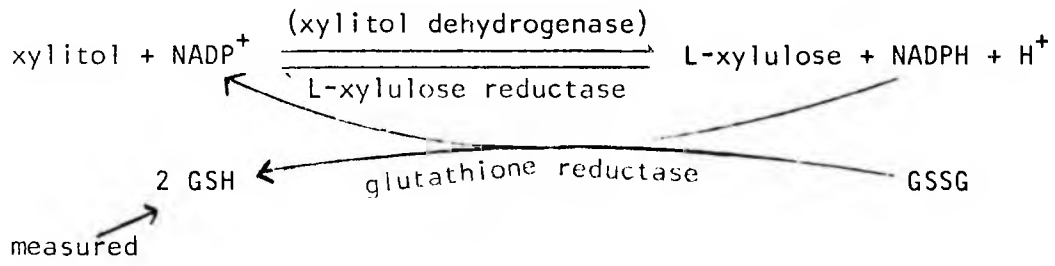
The method described by Hickman and Ashwell (1959) for quantitating L-xylulose was followed without modification except that pigeon liver L-xylulose reductase (Sigma) was used instead of guinea pig liver L-xylulose reductase. The assay contained the following substances in a final volume of 1 mℓ:

Tris-HCl buffer pH 7,0	40	µmoles (Tris),
Cystein	1	µmole,
NADPH	100	µmoles,
$MgCl_2$	5	µmoles,
L-xylulose	± 0,05	µmoles.

The blank solution contained water in place of L-xylulose. Blank and sample solutions were equilibrated to 30°C and a recorder trace of the extinction reading at 340 nm then made. Three microlitres of L-xylulose reductase suspension (approximately 0,133 U) were added to both the blank and sample solutions and the resulting change in extinction followed on the recorder. When the reaction and gone to completion, (when approximately all the L-xylulose present had been reduced to xylitol), the difference between the initial and final extinction values was used to calculate how much NADPH had been oxidised and hence how many µmoles of L-xylulose were originally present.

2.3.6. The glutathione method for assaying xylitol dehydrogenase activity

The glutathione method (Wang and van Eys, 1970) involved coupling the xylitol dehydrogenase catalysed reaction to the reduction of oxidised glutathione (catalysed by glutathione reductase) as diagrammed:



The reduced glutathione (GSH) produced was then measured by a modification of the method of Beutler *et al.* (1963).

2.3.6.1. Reagents

1. Precipitation solution: 1,67 g glacial metaphosphoric acid, 0,2 g Na_2 EDTA and 30 g NaCl dissolved in water and made up to 100 mℓ.
2. Phosphate solution: 0,6 M Na_2HPO_4.

3. DTNB reagent: 40 mg 5,5'dithiobis-(2-nitrobenzoic acid) per 100 mℓ of 1% (w/v) sodium citrate.
4. Standard reduced glutathione solution: 0,1 mM GSH in precipitation solution.
5. Tris buffer: 86,53 g Tris dissolved in approximately 800 mℓ of water and then adjusted to pH 7,0 with concentrated HCl before being made up to one litre.
6. Magnesium chloride solution: 7,26 g $MgCl_2.6H_2O$/ℓ.
7. Nicotinamide solution: 8,72 mg/10 mℓ water.
8. Oxidised glutathione solution: 43,7 mg/10 mℓ water.
9. NADP solution: 7,87 mg NADP (disodium salt)/10 mℓ water.
10. Xylitol solution: 1,597 g/10 mℓ water.

2.3.6.2. Procedure

A separate incubation solution for each haemolysate assayed, was constituted by mixing:

Tris buffer	0,35 mℓ (250 µmoles/assay),
Magnesium chloride solution	0,35 mℓ (12,5 µmoles/assay),
Nicotinamide solution	0,35 mℓ (2,5 µmoles/assay),
Oxidised glutathione solution	0,35 mℓ (2,5 µmoles/assay),
NADP solution	0,35 mℓ (0,35 µmoles/assay),
Xylitol solution	1,00 mℓ (1,05 mmoles/assay),
Haemolysate (neat)	0,25 mℓ,
Water	0,50 mℓ.

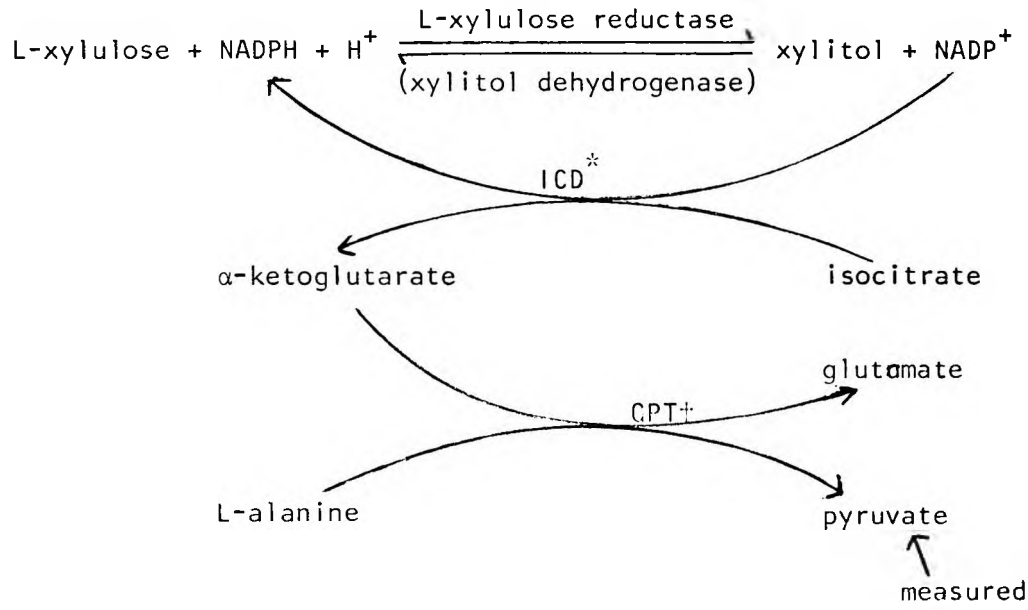
Blanks contained water in place of xylitol. One mℓ aliquots of incubation solution (and corresponding blank solution) were incubated for various times before stopping the enzyme catalysed reactions by

the addition of 1,5 mℓ of precipitation solution to each. The mixtures were stood for 3 minutes and then centrifuged for 5 minutes at 2 000 x g. One mℓ of the cleared supernatant was taken from each tube and mixed with 2 mℓ phosphate solution and 0,5 mℓ DTNB colour reagent. Extinction values at 412 nm were then read on a double beam spectrophotometer (one cm light path) against water. One mℓ aliquots of GSH standard solution, freshly made up, were treated in the same way as the assay solutions and read against a blank solution containing water in place of GSH. The amount of haemoglobin present in each assay was measured by taking 50 µℓ aliquots of the complete reaction mixture and treating these as described under haemoglobin estimation.

Wang and van Eys (1970) do not provide many details, but the method was modified as follows: The stopped reaction mixtures were cleared of precipitated protein by centrifugation instead of filtration. The amount of GSH produced per assay was increased by using a neat haemolysate in place of a once diluted haemolysate. The intensity of colour due to the GSH formed was increased by halving the amount of precipitation solution added and by mixing the deproteinated supernatant with half the prescribed volume of double the prescribed strength phosphate solution.

2.3.7. The pyruvate method for assaying L-xylulose reductase

The pyruvate method involved coupling the L-xylulose reductase catalysed reaction to two other enzyme-catalysed reactions:



*ICD = isocitrate dehydrogenase.

†GPT = glutamate-pyruvate transaminase.

Pyruvate formed as a result of the L-xylulose reductase catalysed reaction was measured by the method described by Chen *et al.*, (1972).

2.3.7.1. Reagents

1. Colour reagent: 0,1 g 2,4-dinitrophenylhydrazine/100mℓ of 20% concentrated HCl.

2. The following components were mixed together and made up to a final volume of 5 mℓ with water:

46,8 mg	L-alanine	(reaction mixture concentration = 50 mM),
26,7 mg	D,L-isocitric acid	(" " " = 1,5 mM),
2,0 mℓ	of 46,85 mM $MgCl_2$ solution	(" " " = 2,7 mM),
1,88 mℓ	of 1 M Tris-HCl buffer at pH 7,0	(" " " = 0,1 mM),

6,0 U isocitrate dehydrogenase (Boehringer) (reaction mixture concentration = 0,64 U/mℓ),

12,0 U glutamate-pyruvate transaminase (Boehringer) (" " " = 1,28 U/mℓ),

3. NADPH (tetrasodium salt), 2 mg/mℓ) (" " " - 0,24 mM).

4. L-xylulose, 74 mM (" " " = 7,4 mM).

5. Alcoholic KOH: 2,5 g KOH dissolved in 2,5 mℓ water and then made up to 100 mℓ with absolute ethanol.

6. Pyruvate standard: 0,4 mM sodium pyruvate in water.

2.3.7.2. Procedure

A 1,2 mℓ aliquot of solution 2 was mixed with 0,6 mℓ of neat haemolysate. Four 400 µℓ aliquots of this mixture were pipetted into separate tubes and 50 µℓ of NADPH solution added to each. One of the four tubes was placed in a 30°C waterbath for 5 minutes and then 50 µℓ of L-xylulose solution added to start the reaction. Reactions in the three remaining tubes were started at 20 minute intervals in the same way. Eighty minutes after the reaction in the first tube had begun, all four tubes were removed from the waterbath and their enzyme catalysed reactions stopped by the addition of 0,5 mℓ of colour reagent. The resulting mixtures were allowed to stand at room temperature for 5 minutes before one mℓ of toluene was added to each and the mixtures vortexed for approximately 20 seconds. The tubes were sealed with Parafilm 'M' and centrifuged at 1 500 x g for 5 minutes. After centrifugation, 0,5 mℓ of the upper phase from each tube was mixed with one mℓ of alcoholic KOH and the extinction at 490 nm read 15 minutes later. A factor for the conversion of

extinction readings to moles of pyruvate formed was obtained with every batch of haemolysates assayed by treating 0,5 mℓ aliquots of standard pyruvate solution as well as 0,5 mℓ aliquots of water in the same way as the solutions taken from the waterbath. Ten µℓ aliquots of the solution 2-haemolysate mixture were added to 3 mℓ volumes of Drabkin's solution for haemaglobin estimation.

2.4. Kinetic, chromatographic and electrophoretic studies

2.4.1. Preparation of enzyme for kinetic studies

Kinetic studies were carried out on enzyme preparations which had been freed of haemoglobin by ion-exchange chromatography.

2.4.1.1. Ion-exchanger preparation

Before use, the ion-exchanger (Whatman CM 52) was precycled according to the manufacturer's instructions. Equilibration was achieved by suspending the ion-exchanger in approximately 15 volumes of equilibration buffer and then adjusting the pH to that required by the addition of either the acidic or basic component of the buffer. When the required pH had been reached, the ion-exchanger was washed (on a Büchner funnel) with approximately 10 times its volume of equilibration buffer. In cases where the exchanger was required for reuse, it was poured into a Büchner funnel and 10 to 20 volumes of 2 M NaCl allowed to percolate through it. This was followed by 20 volumes of deionised water and finally by sufficient equilibration buffer to bring the pH of the effluent to that of the equilibration buffer.

Following equilibration, the ion-exchanger was mixed with enough equilibration buffer to make pouring easy. The slurry was then poured into a measuring cylinder and allowed to settle at (4°C) for at least 12 hours. The volume of the settled ion-exchanger as well as the total volume (supernatant + ion-exchanger) was then noted so that the volume of resuspended slurry necessary for any required bed volume could be calculated by simple proportion.

2.4.1.2. Separation of L-xylulose reductase from haemoglobin

Washed red cells were disrupted by sonication and the resulting haemolysate diluted with an equal volume of cold 10 mM sodium phosphate buffer at pH 6,5. The diluted haemolysate was then mixed with 5 times its volume of settled CMC which had been equilibrated with 10 mM sodium phosphate at pH 6,5 and freed of excess moisture by Büchner filtration. The haemolysate-CMC mixture, which had the consistency of fairly stiff porridge, was stirred occasionally over a period of 10 minutes before the enzyme containing solution was separated from the CMC-bound haemoglobin by Büchner filtration. The volume of the eluate was measured and the CMC then washed with an equal volume of cold equilibration buffer. The eluates were pooled and NADP added to a final concentration of 20 µM. The solution was then centrifuged for 20 minutes at 38 000 x g (4°C) and the supernatant mixed with sufficient ammonium sulphate to provide a final concentration of 2,72 M. After the ammonium sulphate had dissolved, the preparation was stood at 0°C for 10 minutes and then centrifuged at 38 000 x g for 10 minutes. The supernatant was discarded and the precipitated material, which included L-xylulose reductase,

stored at 0 - 4°C until required. Enzyme solutions made by dissolving the precipitated material obtained in this way have been referred to as crude (haemoglobin free) preparations.

2.4.1.3. Preparation of the normal major isozyme

The normal major isozyme was prepared by either column chromatography (see section 2.4.3.1) or by one of the two following methods:

Method 1

Ammonium sulphate precipitated material obtained in the way described above (section 2.4.1.2) was dissolved in equilibration buffer (10 mM sodium phosphate at pH 5,7), containing 20 µmoles NADP/ℓ at the rate of one mℓ of buffer for every 10 mℓ of original neat haemolysate used. The solution was dailysed against equilibration buffer for 2 hours at 0 - 4°C and then centrifuged for 10 minutes at 38 000 x g (4°C). The clear supernatant was diluted with an equal volume of equilibration buffer and then mixed with a batch of CM-cellulose equilibrated to pH 5,7 at the rate of 0,075 mℓ of packed ion-exchanger for every mℓ of neat haemolysate originally used. The ion-exchanger-enzyme slurry was kept at 0°C for 10 minutes with occasional stirring after which the major isozyme which had adsorbed onto the ion-exchanger was pelleted by centrifugation (one minute at 1 000 x g). The supernatant which contained the minor isozyme and some contaminating major isozyme was frozen for later use. The CM-cellulose with the adsorbed major isozyme was washed with three, 50 mℓ volumes of equilibration buffer by suspending the exchanger in the buffer and then pelleting it by centrifugation and discarding the supernatant. The major isozyme was

then eluted from the ion-exchanger by mixing it with one mℓ of 600 mM NaCl in 0,2 M sodium phosphate at pH 7,0 for every mℓ of dialysed enzyme preparation originally applied. After standing at 0°C for 5 minutes with occasional stirring, the ion-exchanger was pelleted by centrifugation and the supernatant which contained most of the activity present, retained. Two further elutions were performed in the same way. Before use in kinetic studies, the eluted enzyme was dialysed against 10 mM sodium phosphate buffer at pH 7,0 for 2 to 3 hours at 0 - 4°C.

Method 2

Ammonium sulphate precipitated material obtained in the way described in section 2.4.1.2 was mixed with 20 mM sodium phosphate buffer at pH 5,7 at the rate of 2 mℓ of buffer for every 10 mℓ of original neat haemolysate used. Sucrose, to a final concentration of 10% w/v was added to the supernatant before it was applied to a Sephadex G-25 (fine) column which had been equilibrated with 20 mM sodium phosphate at pH 5,7. The volume of enzyme solution applied to the column was limited to less than 20% of the column's bed volume so that complete desalting would be ensured. The column was developed with equilibration buffer under gravity and the protein solution which eluted after the void volume, collected by hand using the small traces of haemoglobin which were present as a marker. The desalted enzyme solution was mixed with a batch of CMC equilibrated with 10 mM sodium phosphate at pH 5,7, and the isozymes further separated as described for Method 1. The eluted major isozyme was finally desalted on the same Sephadex column which had been re-equilibrated with 20 mM sodium phosphate at pH 7,0.

2.4.1.4. Preparation of the normal minor isozyme

The minor isozyme containing supernatants which had been reserved during preparation of the major isozyme were thawed, pooled and centrifuged for 10 minutes at 35 000 x g at 4°C. The supernatant (27,6 mℓ) was freed of traces of contaminating major isozyme by passing it through a 20 mℓ (bed volume) column of CMC which had been equilibrated with 20 mM sodium phosphate at pH 5,7. The minor isozyme was then precipitated by the addition of ammonium sulphate to a final concentration of 2,72 M and pelleted by centrifugation at 35 000 x g for 10 minutes at 4°C. The pellet was dissolved in 5 mℓ of 20 mM sodium phosphate buffer at pH 7,0 and then desalted on a Sephadex G-26 (fine) column as described in section 2.4.1.3.

Before use in kinetic studies, the preparation was electrophoresed (see section 3.3.3.2) in order to ascertain that no major isozyme was present.

2.4.2. Kinetic studies

Kinetic studies were carried out on both crude (haemoglobin free) enzyme preparations as well as on preparations of the normal major and minor isozymes.

2.4.2.1. Kinetic studies on crude preparations

Michaelis constant and pH-activity studies were performed on crude (haemoglobin free) enzyme preparations.

2.4.2.1.1. Michaelis constants

a) K_m for NADP:

The ammonium sulphate precipitated material obtained after the removal of haemoglobin (see section 2.4.1.2) was dissolved in a small volume (2 - 10 mℓ) of a solution containing 2,7 mM EDTA (pH 7,0) and 7 mM β-mercaptoethanol and then dialysed at 0 - 4°C for 2 to 4 hours. After dialysis, the enzyme solution was centrifuged at 38 000 x g for 10 minutes at 4°C. A stock NADP solution (containing approximately 1,5 mg of NADP disodium salt/mℓ) was prepared and assayed as described in section 2.3.4. A series of dilutions which would provide reaction mixture concentrations of between 4,7 and 28,64 µM were made from this stock. Reaction mixtures were constituted as follows:

0,3 mℓ	Tris-HCl buffer 1 M at pH 7,0 (final concentration = 0,1 M)
0,3 mℓ	27 mM $MgCl_2$ (final concentration = 2,7 mM)
0,3 mℓ	NADP solution
1,6 mℓ	3,286 M xylitol (final concentration = 1,75 M)
0,5 mℓ	enzyme preparation
3,0 mℓ	

Changes in fluorescence due to the production of NADPH were followed on a Baird-Atomic Fluorimet fluorometer fitted with filters IX0 and 0Y13. Preliminary tests showed that blanks were unnecessary as no change in fluorescence took place in the absence of xylitol. Reciprocals of reaction velocity were plotted against the corresponding reciprocals of substrate concentration and a regression line (obtained by the method of least squares using a Hewlett-Packard calculator with Hewlett-Packard programmes, Stat 1-05A and Stat 1-22A) fitted. The apparent K_m was then obtained from the reciprocal of the x-axis intercept. The

determination was repeated with fresh enzyme preparations from non-Jewish subjects on two further occasions and also on enzyme prepared from the Ashkenazi and Lebanese pentosurics.

b) K_m for xylitol at pH 7,0:

Enzyme was prepared from normal and pentosuric individuals in the way described for the NADP K_m determinations. The same basic assay was also used except that in some determinations, reactions were followed by monitoring changes in extinction at 340 nm in a spectrophotometer. The NADP concentration employed was 1,5 mM and the highest and lowest xylitol concentrations used with enzyme from normals were 0,197 and 0,0328 M respectively. Three determinations were made with enzyme from normals and two with enzyme from the pentosuric subjects. The xylitol concentrations used with enzyme from the pentosurics ranged between 0,29 and 1,75 M.

A direct comparison of the rates at which enzyme (in crude preparations) from a pentosuric and a normal catalysed the reverse reaction at different xylitol concentrations was made as follows: Crude (haemoglobin free) enzyme preparations were obtained from a normal and a pentosuric individual and assayed at a xylitol concentration of 1,753 M. Quantities of the two enzyme preparations which contained approximately equal activities (at a xylitol concentration of 1,753 M) were then allowed to catalyse and reverse reaction at two lower xylitol concentrations (0,877 and 0,584 molar). The results were finally represented in the form of a Lineweaver-Burk plot.

c) K_m for xylitol at pH 9,5:

The ammonium sulphate precipitated material obtained (see section 2.4.1.2) from approximately 20 mℓ of neat haemolysate was dissolved

in 2 mℓ of a buffer which was made by adjusting a solution containing 0,2 M Tris, 0,2 M glycine and 0,2 M sodium dihydrogen orthophosphate to pH 9,5 with 1ON NaOH. Twenty µmoles of NADP were added to the enzyme solution before dialysing it for 3 hours against one litre of the above buffer. Reaction mixtures were constituted as follows:

0,3 mℓ dialysis buffer (pH 9,5)

0,2 mℓ dialysed enzyme solution

0,1 mℓ NADP solution (final concentration 0,254 mM)

0,4 mℓ xylitol solution at various concentrations

1,0 mℓ

The range of xylitol concentrations used with the enzyme from pentosurics was 0,266 to 1,6 moles/ℓ and with enzyme from normal individuals, 0,152 to 0,32 moles/ℓ.

d) K_m for L-xylulose:

The ammonium sulphate precipitated material obtained after removal of haemoglobin (see section 2.4.1.2) was dissolved in a solution containing EDTA (2,7 mM, pH 7,0), β-mercaptoethanol (7,0 mM) and NADPH (10 µM) and then dialysed for 3 hours against one litre of the same solution before being centrifuged (at 38 000 x g for 10 minutes at 4°C) to remove material which precipitated during dialysis.

A solution of L-xylulose was prepared, and a series of dilutions made from this. Reaction mixtures (pH 7,0) contained 0,1 M Tris, 2,7 mM $MgCl_2$, 0,25 mM NADPH, L-xylulose at various concentrations and enzyme preparation. The L-xylulose concentrations employed, ranged between 0,111 and 4,2 mM when using enzyme from normals and 0,133 and 0,204 M when using enzyme from pentosurics. Reactions were followed by monitoring changes in extinction at 340 nm in a double

beam spectrophotometer. Blanks were identical to the experimental solutions except that they contained water in place of L-xylulose.

e) K_m for NADPH:

The K_m for NADPH of enzyme from normal individuals was estimated using the same basic reaction mixture as specified for the xylitol K_m estimations except that NADP was omitted and L-xylulose at concentrations of between 7,4 mM and 16,6 mM was used in place of xylitol. A stock NADPH solution was prepared and its concentration determined spectrophotometrically. A series of dilutions of the stock were made to provide reaction mixture NADPH concentrations of between 6 and 128 µM. Reaction rates were measured in a double beam spectrophotometer by following changes in extinction at 340 nm. Blank solutions consisted of the complete reaction mixture minus L-xylulose.

2.4.2.1.2. pH-activity studies

a) Reverse reaction (xylitol dehydrogenase activity), enzyme from a normal individual:

Enzyme from a normal individual was separated from haemoglobin and concentrated by ammonium sulphate precipitation as described in section 2.4.1.2. The precipitate was dissolved in 7 mℓ of 2,7 mM EDTA (pH 7,0) which contained NADP at a concentration of 60 µM. The preparation was centrifuged for 20 minutes at 35 000 x g (4°C) and the clear supernatant retained.

A solution containing 0,2 moles of Tris, 0,2 moles of glycine and 0,2 moles of sodium dihydrogen orthophosphate per litre was made up and volumes of this adjusted with either 10N NaOH or concentrated HCl to pH values ranging from 6,0 to 12,0.

Rates of the xylitol dehydrogenase catalysed reactions were then measured (at three different xylitol concentrations) at various pH values. Reaction solutions contained Tris, glycine and sodium orthophosphate each at a concentration of 0,1 M; 0,25 mM NADP and xylitol at a final concentration of either 0,8, 0,072 or 0,038 M. A separate blank (containing all reagents except xylitol) was prepared for each assay. Reaction rates were measured in a double beam spectrophotometer by monitoring changes in extinction at 340 nm.

After the reactions had proceeded for a sufficient interval, the assay mixtures were reserved and their pH values measured. Reciprocals of the rates measured with each of the three xylitol concentrations at each pH were plotted against reciprocals of the corresponding xylitol concentrations and theoretical V_{max} and K_m values estimated from regression equations calculated by the method of least squares.

b) Reverse reaction (xylitol dehydrogenase activity), enzyme from a pentosuric individual:

The method followed was the same as that used with enzyme from the normal individual except that reaction rates were measured at one xylitol concentration only (0,8 M) because the amount of enzyme available was limited.

c) Forward reaction (L-xylulose reductase activity), enzyme from a normal individual:

The method followed was the same as that used when exploring the reverse reaction pH-activity relationship, except that L-xylulose and NADPH were used in place of xylitol and NADP. The final NADPH

concentration was 0,24 mM and the three L-xylulose concentrations employed at each pH were 7,400, 0,672 and 0,352 mM.

The effect of pH on the L-xylulose reductase activities of the normal minor isozyme and of enzyme from the pentosurics was not investigated because these enzymes have high Michaelis constants for L-xylulose and the determinations would consequently have required large amounts of L-xylulose.

2.4.2.2. Kinetic studies on the major isozyme of normal individuals

2.4.2.2.1. Michaelis constants

a) K_m for xylitol:

A preparation of the normal major isozyme (13 mℓ) was mixed with 16 µmoles of NADP and then dialysed for 2 hours against two litres of 10 mM sodium phosphate buffer at pH 7,0. The dialysed preparation was divided into 200 µℓ aliquots and frozen. The aliquots of enzyme solution were thawed (two at a time) immediately before use and mixed with Tris-glycine buffer (which had been adjusted to pH 9,0 with NaOH) and the other components of the reaction mixture, minus xylitol. After temperature equilibration, xylitol at the same temperature was added to the experimental solution (an equal volume of water was added to the blank) and the enzyme catalysed formation of NADPH followed at 340 nm on a double beam spectrophotometer. Reaction mixtures contained 0,05 M Tris, 0,05 M glycine, 0,25 mM NADP, xylitol at concentrations ranging between 0,078 and 1,0 M and enzyme solution. The determination was repeated once with a fresh enzyme preparation.

b) K_m for NADP:

The enzyme used for this determination was prepared by the second of the methods described in section 2.4.1.3. After the enzyme solution had been desalted on a Sephadex G-25 (fine) column it was divided into 300 µℓ aliquots and frozen. A solution of NADP was prepared and its concentration measured as described in section 2.3.4. The frozen aliquots of enzyme solution were thawed immediately before use and mixed with Tris-glycine buffer (which had been adjusted to pH 9,0 with NaOH) and the remaining components of the reaction solution barring xylitol. The mixtures were allowed to equilibrate to 30°C before reactions were started by the addition of xylitol to the experimental solutions (water in place of xylitol was added to the blanks). Changes in extinction at 340 nm were then monitored in a double beam spectrophotometer. The reaction mixtures contained 0,05 M Tris, 0,05 M glycine, 1,4 M xylitol, NADP at concentrations ranging between 6,19 and 37,13 µM and enzyme solution. The K_m was estimated from a Lineweaver-Burk plot as before.

c) K_m for L-xylulose:

The preparation of enzyme for this determination was the same as that used for the xylitol K_m (at pH 9,0) except that the buffer against which the enzyme was finally dialysed contained 10 µM NADPH in place of NADP and the pH of the reaction mixture buffer (0,2 M Tris, 0,2 M sodium orthophosphate adjusted with NaOH) was 7,0. The reaction mixture contained 0,05 M Tris, 0,05 M sodium orthophosphate, 0,24 mM NADPH, enzyme solution and L-xylulose at concentrations ranging from 0,38 to 24,00 mM. Blank solutions contained water in place of L-xylulose. Reactions were followed by monitoring changes in

extinction at 340 nm and the K_m was finally estimated from a Lineweaver-Burk plot.

d) K_m for NADPH:

The enzyme preparation used for this K_m estimate was passed through a Sephadex G-25 (fine) column (enzyme solution volume equalled approximately 20% of column bed volume) in order to replace the NaCl containing buffer which had been used to elute the enzyme from the final batch of ion-exchanger, with a 20 mM sodium phosphate buffer at pH 7,0. This was also done to remove possible traces of NADP which may have remained in the enzyme solution from earlier steps in its preparation.

A stock NADPH solution was prepared and its concentration measured as described in section 2.3.4. The buffer used in the reaction mixture was the same as that used when estimating the enzyme's K_m for L-xylulose. Reaction mixtures contained 0,05 M Tris, 0,05 M sodium orthophosphate, 16,1 mM L-xylulose, enzyme solution and NADPH at concentrations ranging between 9 and 100 µM. The rates at which NADPH disappeared from reaction mixtures were followed in a double beam spectrophotometer. The blank solution contained all components of the reaction mixture except NADPH. A second blank containing all the components of the reaction mixture minus L-xylulose was also prepared as a check.

2.4.2.2.2. pH-activity studies

a) Reverse reaction (xylitol dehydrogenase activity):

Enzyme for this investigation was prepared and dialysed by the first method described in section 2.4.1.3. In other respects, the method followed is the same as that used with the crude preparation

of normal enzyme already described except that some additional points were obtained for pH values close to the enzyme's apparent pH optimum on the day following the main determination and the buffer strength was reduced to half that used before.

b) Forward reaction (L-xylulose reductase activity):

Enzyme for this investigation was prepared and dialysed as described in section 2.4.1.3. The method followed was the same as that described for the pH-activity investigation done on the normal crude enzyme preparation except that the buffer concentration was reduced by half.

2.4.2.3. Kinetic studies on the minor isozyme of normal individuals

2.4.2.3.1. Michaelis constants

a) K_m for xylitol:

The method followed was identical to that used for measuring the K_m for xylitol of enzyme in crude preparations from pentosurics.

b) K_m for L-xylulose:

The method followed for this determination was the same as that used for estimating the K_m for L-xylulose of enzyme in crude preparations from the pentosuric subjects. The L-xylulose concentrations employed ranged from 12,5 to 87,8 mM.

2.4.2.3.2. pH-activity

The method followed was the same as that used when investigating the pH-activity relationship of enzyme from pentosurics except that the

buffer concentration was halved and the xylitol concentration increased from 0,8 M to 1,4 M which would be closer to a saturating substrate concentration.

2.4.3. Chromatography and electrophoresis

Red cell L-xylulose-reductase isozymes from both normal and pentosuric individuals were chromatogrammed and electrophoresed. In addition, an electrophoretic comparison was made of liver and red cell isozymes.

2.4.3.1. Chromatography

The xylitol dehydrogenase present in 43 mℓ of freshly prepared haemolysate from an individual with normal enzyme activity was separated from haemoglobin by the method described in section 2.4.1.2. The ammonium sulphate precipitate obtained was dissolved in 5 mℓ of 10 mM sodium phosphate buffer at pH 5,7 (equilibration buffer), mixed with 40 µmoles of NADP and then dialysed for 90 minutes against two litres of equilibration buffer. Following dialysis, the preparation was centrifuged for 20 minutes at 35 000 x g (4°C) and 7 mℓ of the resulting supernatant applied to the top of a 160 mℓ (bed volume) CMC column which had been equilibrated with the above buffer. As soon as the enzyme solution had entered the column, a linear gradient of NaCl in equilibration buffer (0 - 600 mM NaCl over a volume of 400 mℓ) was applied. The column (cross-sectional area = 5.2 cm^2) was developed at a flow rate of one mℓ per minute and 5 mℓ fractions were collected. The relative amount of protein in each fraction was gauged by measuring its extinction at 280 nm. In addition, the extinctions at 410 nm of the last 7 to 20 fractions were also measured in order to detect the trace amounts of haemoglobin which

began to elute at this stage. The relative xylitol dehydrogenase activity of each fraction was estimated by adding 300 µℓ of the fraction to 300 µℓ of a reaction mixture whose preparation is described below. The samples were incubated at 30°C for 30 minutes and then 1,4 mℓ of 1,0 M Tris-HCl at pH 7,0 were added to each and fluorescence due to NADPH which had formed, measured on a Perkin-Elmer 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 347 nm and 460 nm respectively. A standard was prepared by adding a known quantity of NADPH (5,28 nmoles in 5 µℓ) to 300 µℓ of one of the void volume fractions and treating this in the same way as the 300 µℓ aliquots taken from the other fractions.

Reaction mixture preparation: 7,55 g xylitol, 10 mg NADP (disodium salt), 0,6 g Tris and 0,375 g glycine were dissolved in approximately 15 mℓ of water and the pH adjusted to 10,5 with 10 N NaOH before making the volume up to 20 mℓ.

The separation method described above was followed on a number of occasions using enzyme prepared from normal individuals, both pentosurics (HK and RB) and a daughter of HK.

2.4.3.2. Electrophoresis

Fresh haemolysates were prepared from whole blood or from dialysed and washed preserved red cells (see section 2.3.2). The liver specimens used were obtained at autopsy and had been stored frozen at -70°C for varying periods ranging from 3 to 21 months. Two of the liver specimens were from individuals who died of different apparently inherited (undiagnosed) metabolic disorders and the third specimen was from a healthy individual who died a violent death.

Homogenates were prepared by adding approximately 2 volumes of water to small pieces (± 3 mm^3) of freshly thawed liver and disrupting these by hand in a ground glass homogeniser (0°C). The homogenates were then centrifuged at 9 000 x g for 3 minutes and the xylitol dehydrogenase activities of supernatants adjusted to approximately 0,38 µmoles/hour/100 µℓ.

Electrophoresis was carried out in starch gels using a modification of the buffer system employed by Fildes and Parr (1963). The gel buffer consisted of 0,01 M sodium phosphate buffer at pH 7,0, and the electrode buffer of 0,2 M sodium phosphate at pH 7,0 instead of the 0,1 M sodium phosphate buffer (pH 7,0) used by Fildes and Parr. This modification was made because the original buffer sometimes failed to adequately control the pH of the gel. NADP (20 µM) was included in the gel buffer and in the electrode buffer of the cathode tank.

Starch gels were made by swirling mixtures of starch and gel buffer (25 g hydrolysed starch, Merck, and 250 mℓ gel buffer) in one litre Erlinmeyer flasks over a Bunsen flame until the mixtures became viscous. Five µmoles of NADP were then added to each molten gel before it was 'degassed' by attaching the flask to a vacuum pump for about 10 seconds. The degassed gels were poured into perspex trays (20 cm long, 13 cm wide and 1 cm deep) and left to set for 30 to 40 minutes. The gels were then covered with thin polythene sheets and placed in a refrigerator until required (gels were used within 24 hours of preparation). Whatman 17 MM filter-paper rectangles (approximate size: 0,8 x 0,4 cm) were soaked with neat haemolysate or enzyme solution and inserted 4 cm from the cathode end of each gel. Gels were then placed between cathode and anode buffer compartments and connected to these by lint wicks which had been soaked

in electrode buffer. Two glass rods (approximately 0,5 cm in diameter) were placed on either side of the sample bearing inserts to hold the polythene sheet (which was temporarily removed during sample application) clear of the gel at the origin. Electrophoresis was carried out at a constant voltage of 6,4 v/cm for 16 hours in a refrigerator at approximately 4°C.

Following electrophoresis, the gels were cut horizontally into two slices by passing a length of taut narrow gauge stainless steel wire through each and the slices then separated. A sheet of Whatman 3 MM filter paper of the same size and shape as the cut surface of the gel was soaked in staining solution and then laid over the surface. The gels were incubated at 37°C and then viewed under long wavelength ultraviolet light.

Staining solutions

Three different staining solutions were used:

1. A stain which was best for detecting the major isozyme of normals by its xylitol dehydrogenase activity.
2. A stain suitable for detecting both major and minor isozymes of normals as well as the residual enzyme of pentosurics by their xylitol dehydrogenase activities.
3. A stain for detecting L-xylulose reductase activity.

Stain 1

The substances listed below were dissolved in approximately 7 mℓ of water, and the pH adjusted to 10,5 with 10 N NaOH before making the volume up to 10 mℓ.

0,15 g glycine (final concentration 0,2 M),
1,52 g xylitol (final concentration 1,0 M),
2,0 mg NADP (disodium salt) (final concentration 0.25 mM).

Stain 2

The substances listed below were dissolved in approximately 7 mℓ of water and the pH adjusted to 9,5 with 10 N NaOH before making the volume up to 10 mℓ.

0,15 g glycine (final concentration 0,2 M),
0,24 g Tris (final concentration 0,2 M),
4,56 g xylitol (final concentration 3,0 M),
2,0 mg NADP (disodium salt) (final concentration 0,25 mM),
10,0 mg $MgCl_2.6H_2O$ (final concentration 4,93 mM).

Stain 3

Prepared by mixing -

0,2 mℓ of 1,0 M Tris-HCl buffer, pH 7,0 (final concentration 0,2 M Tris),
0,2 mℓ of 27 mM $MgCl_2$ (final concentration 2,7 mM),
0,6 mg NADPH (tetrasodium salt) (final concentration ± 0,36 mM),
0,266 mℓ of 1,5 M L-xylulose (final concentration 0,2 M) and
1,334 mℓ water.

2.4.4. The effect of various substances on enzyme activity

2.4.4.1 Nicotinamide on xylitol dehydrogenase activity

This effect was demonstrated as follows: A fresh haemolysate was prepared from a normal individual and aliquots of this assayed by the glutathione method in the presence of either 0, 0,1, 1,0 or 10 mM nicotinamide. The amount of haemoglobin in each assay was measured in order to correct for errors made in pipetting the haemolysate.

2.4.4.2. Magnesium chloride and other salts on xylitol dehydrogenase and L-xylulose reductase activity

The following experiment was performed with the aim of establishing whether it was necessary to add $MgCl_2$ to systems for assaying red cell

xylitol dehydrogenase activities. Enzyme which had been separated from haemoglobin and concentrated by ammonium sulphate precipitation as described in section 2.4.1 was dissolved in 2 mℓ of a solution containing 60 µM NADP, 7 mM ß-mercaptoethanol and 2,7 mM EDTA (pH 7,0) and then dialysed (0 - 4°C) against one litre of the same solution for 3 hours. The xylitol dehydrogenase activity of the preparations was then assayed in the presence and absence of 2,7 mM $MgCl_2$ using a reaction mixture which contained 0,1 M Tris (pH 7,0), 2,7 mM $MgCl_2$ (or water), 0,25 mM NADP, 1,75 M xylitol and enzyme solution. Reactions were followed by monitoring changes in extinction at 340 nm in a double beam spectrophotometer. The blank contained water in place of xylitol.

The next experiment was carried out on a preparation of the normal major isozyme (see section 2.4.1.3). Before use, the enzyme solution was desalted on a Sephadex G-25 (fine) column (the volume of enzyme solution applied was 23,7% of the column volume). The desalted enzyme's L-xylulose reductase activity was then assayed in the presence and absence of 5 mM $MgCl_2$. The reaction mixture contained 0,05 M Tris and 0,05 M sodium orthophosphate (adjusted to pH 7,0 with 10 N NaOH), 5 mM $MgCl_2$ (or water), 0,24 mM NADPH, 16,1 mM L-xylulose and enzyme solution.

The third experiment was done with the aim of finding a possible metal activator of the major isozyme of normal individuals. A preparation of the normal major isozyme was concentrated by ammonium sulphate precipitation. The precipitated protein obtained after centrifugation (38 000 x g for 10 minutes) was resuspended in approximately 2 mℓ of supernatant and 40 µmoles of NADPH added. The suspension was then divided into two equal portions; one of these (0,55 mℓ) was dialysed

against one litre of 2,7 mM EDTA (pH 7,0) while the other was dialysed against one litre of 10 mM sodium phosphate buffer (pH 7,0) for 2 hours. One-hundred µℓ aliquots of both the EDTA and phosphate dialysed enzyme solutions were separately mixed with 100 µℓ volumes of various salt solutions ($MgCl_2$, $MnCl_2$, $CuSO_4$, $FeSO_4$ and $Zncl_2$) each at a concentration of 2 mM. The enzyme-salt solution mixtures were stood at approximately 4°C for one hour and their xylitol dehydrogenase activities then assayed. The reaction mixture concentrations of the various salts was 0,2 mM.

2.5. Enzyme storage and stabilisation

2.5.1. Storage in red cells and haemolysates

Three methods of storing the xylitol dehydrogenase of red cells were investigated.

Method 1

A preparation of washed, packed red cells was divided into aliquots and these stored at -20°C for various times.

Method 2

Fresh, packed red cells were mixed with preserving solution as described in section 2.3.1 and separate aliquots of the mixture stored frozen at -20°C.

Method 3

Whole blood was mixed with the anticoagulant ACD at the rate of 6,66 mℓ of blood/mℓ of ACD and stored at 4°C.

When required, the preserved cells (Method 2) were thawed and then dialysed overnight against saline at 4°C. After dialysis, the cells were subjected to the washing procedure described under haemolysate preparation (see section 2.3.2) and then lysed by 3 cycles of freezing

and thawing. Red cells stored as part of whole blood (Method 3) were washed and lysed in the same way as the dialysed preserved cells. Haemolysates which resulted from the storage conditions of Method 1 were used directly after thawing. Xylitol dehydrogenase activities were determined by the glutathione method.

2.5.2. Storage in concentrated ammonium sulphate solutions

For practical purposes, an idea of the rate at which crude (haemoglobin free) preparations of L-xylulose reductase from normal individuals lost activity when stored precipitated in 2,72 M ammonium sulphate solutions was obtained as follows: The method used to obtain crude (haemoglobin free) preparations of enzyme (see section 2.4.1.2) was followed until just before the ammonium sulphate precipitation step. β-mercaptoethanol and NADP were added to the enzyme solution (final concentrations: 7 mM and 20 μM respectively) and L-xylulose reductase then precipitated by the addition of ammonium sulphate as described in section 2.4.1. After standing at 0°C for 15 minutes, the precipitated material was pelleted by centrifugation at 38 000 x g for 10 minutes at 4°C and all but 5 to 10 mℓ of the supernatant discarded. The precipitated material was resuspended and the mixture stored at 4°C. Aliquots of the suspension were withdrawn and assayed after various times of storage. The experiment was repeated with the omission of β-mercaptoethanol.

2.5.3. Enzyme stabilisation

Crude (haemoglobin-free) preparations from normal individuals were found to lose activity fairly rapidly unless stored frozen or as ammonium sulphate precipitates. The following experiments were

therefore carried out in order to determine whether enzyme inactivation could be prevented by the presence of various substances.

One mℓ aliquots of enzyme solution, prepared by the method described in section 2.4.1.2, were mixed with 0,5 mℓ volumes of solutions which contained different combinations of the following substances: NADP, β-mercaptoethanol, EDTA and xylitol. The final concentrations of the above substances (when present) were: 10 µM NADP, 2,7 mM EDTA, 7 mM β-mercaptoethanol and 0.1 M xylitol. Table 2.1 shows the different combinations of these substances which were mixed with separate aliquots of enzyme solution. The mixture comprising combination 11 plus enzyme solution was frozen at -20°C while the remaining 10 enzyme-stabilising solution mixtures were incubated at 30°C for 15 hours. All eleven preparations were then assayed.

In the next experiment a crude haemoglobin-free preparation of enzyme from a normal individual was mixed with NADP, β-mercaptoethanol and EDTA (pH 7,0) so that final concentrations of these substances (10 µM NADP, 7 mM β-mercaptoethanol and 2,7 mM EDTA) were equal to those recommended for G6PD stabilisation (Betke *et al.*, 1967). Aliquots of the enzyme solution were then heated for 25 minutes at 48°C in the presence of different amounts of NADP (9 to 129,5 µM). The aliquots were then cooled and their activities compared to similar aliquots which had remained at 0°C during the heating period.

In the following experiment, activities present in aliquots of enzyme solution were again measured after these had been heated in the presence of a more continuous range of NADP concentrations. Enzyme was separated from haemoglobin and precipitated by ammonium sulphate as described in section 2.4.1.2. The precipitate was pelleted by

TABLE 2.1. Combinations of potential stabilising substances added to aliquots of enzyme solution. The appearance of crosses in the columns opposite the chemicals listed indicates that 100 µℓ of a solution of that substance was present in the mixture added to the enzyme solution before incubation. The minus signs indicate that 100 µℓ of water was added in place of the chemical to the left of it. Combination 1 for example contained all 4 chemicals while combination 11 contained none of them.

	Combinations										
Stabilising solution	1	2	3	4	5	6	7	8	9	10	11
NADP	+	-	+	+	+	-	+	-	-	-	-
Xylitol	+	+	-	+	+	-	-	+	-	-	-
β-mercaptoethanol	+	+	+	-	+	-	-	-	+	-	-
EDTA (pH 7,0)	+	+	+	+	-	-	-	-	-	+	-

centrifugation and then dissolved in 3 mℓ of cold sodium EDTA at pH 7,0. The resulting solution was then dialysed against one litre of the same EDTA solution (0 - 4°C) for 3 hours. One hundred µℓ aliquots of dialysed enzyme were added to 100 µℓ aliquots of 0,1 M Tris-HCl buffer at pH 7,0 and then mixed with different amounts of a NADP solution and water to produce a series of 300 µℓ buffered enzyme solutions with NADP concentrations ranging between 0 and 110 µM. Duplicate solutions, containing NADP at the highest concentration were stored on ice while the rest were heated for 25 minutes at 48°C. After heating, the enzyme solutions were cooled on ice, their NADP concentrations equalised and then both heated and unheated solutions assayed.

Once the red cells of normal individuals had been found to contain two L-xylulose reductases, the thermal lability of the major isozyme in the presence of different concentrations of NADP was investigated. Before use, the enzyme solution (5,5 mℓ) was passed through a 40 mℓ (bed volume) Sephadex G-25 (fine) column which had been equilibrated with 20 mM sodium phosphate at pH 7,0 in order to remove NaCl left over from the preparation procedure. Three hundred µℓ aliquots of enzyme solution were heated at 40°C for 0, 10 or 20 minutes and then cooled and assayed in order to obtain an idea of the enzyme's stability. The heating procedure was repeated at 42°C both in the presence and absence of 254 µM NADP and the aliquots assayed after cooling. Finally, aliquots of buffered enzyme solution containing different concentrations of NADP (0 - 100 µM) were heated at 42°C for 40 minutes. After heating, the aliquots were cooled, their NADP concentrations were equilised (100 µM) and their activities measured.

2.6. Population screening and family studies

The L-xylulose reductase assay used in the population screening and family studies was the pyruvate method described in section 2.3.7.

2.6.1. Establishment of a normal range

Before attempting to use the pryuvate assay method for the identification of heterozygotes, the range of red cell activities in a sample of 74 non-Jewish subjects was determined. Blood for this purpose was collected in vacutainers containing ACD and haemolysates were prepared and assayed within 2 days of collection.

2.6.2. Pentosuric family study

When the pyruvate assay method had been found to differentiate clearly between pentosurics and normals, blood was obtained from the family members of one of the pentosurics (HK) and assayed in order to ascertain whether the pentosuria allele (as judged by the levels of L-xylulose reductase activity) could be detected in heterozygotes. A portion of the blood taken from each individual was also used for gene marker studies.

2.6.2.1. Measurement of serum and plasma L-xylulose concentrations

Serum and plasma L-xylulose concentrations in samples from family members were measured by the method described by Kumahara *et al.* (1961). Samples were deproteinised by adding a one-tenth volume of 72% perchloric acid to them and then centrifuging at 38 000 x g for 5 minutes at 0 - 4°C. Aliquots of supernatant were then adjusted to approximately pH 7,2 by the addition of 0,2 N KOH and the clear

supernatants remaining after precipitation of potassium perchlorate assayed. One mℓ assay mixtures were constituted as follows:

0,10 mℓ 0,4 M Tris-maleate buffer at pH 7,5,

0,10 mℓ 10 µM cystein solution,

0,10 mℓ 50 mM magnesium chloride solution,

0,40 mℓ deproteinised serum or plasma,

0,19 mℓ water,

0,01 mℓ L-xylulose reductase suspension in 3 M ammonium sulphate (equivalent to 0,42 U).

The total change in extinction at 340 nm due to the reduction of L-xylulose was used to calculate the amount of NADP formed and hence the amount of L-xylulose originally present in each sample.

2.6.3. Screening the Ashkenazi-Jewish sample

Haemolysates, prepared by sonicating dialysed, washed, preserved red cells from 237 Ashkenazi-Jewish medical student volunteers were assayed by the pyruvate method.

2.6.3.1. Follow-up studies

Repeat determinations of haemolysate L-xylulose reductase activity were carried out on fresh specimens obtained from the 7 individuals whose activities had been found to be more than 2 SD below the sample mean. Samples obtained from available close relatives of these individuals were also assayed.

Six of the 7 'low activity' individuals were then subjected to the D-glucuronolactone loading test described by Kumahara *et al.* (1961). Subjects were asked to fast overnight and on the following morning a sample of blood (10 mℓ) was drawn from each. They were then given 25 g of D-glucuronolactone by mouth and one hour later a second blood

sample was drawn. The samples were allowed to clot at 0 - 4°C for approximately 4 hours before the serum was removed. The L-xylulose concentrations of the serum samples were then measured in the way described in section 2.6.2.1.

2.6.4. Linkage analysis

Linkage analysis was carried out on data collected on members of the Lebanese and Ashkenazi-Jewish families in which pentosuria alleles were segregating.

2.6.4.1. Gene marker studies

Gene marker studies were carried out (by the author's colleagues) on donated blood from members of the families in which the pentosuria allele had been found to be segregating. The phenotypes of all available subjects were established for the following systems:

Red cell enzyme systems	Red cell antigen groups	Serum protein systems
Acid phosphatase (ACP_1)	ABO	Properdin Factor B (Bf)
Adenosine deaminase (ADA)	MNSs	Group spectific component (Gc)
Adenylate kinase (AK_1)	Rh	Haptoglobin (Hp)
Carbonic anhydrases I and II (Ca_1 and Ca_2)	Duffy	Transferrin (Tf)
Esterase D (EsD)	Kell	
Glutamate pyruvate transaminase (GPT_1)	P	
Glucose-6-phosphate dehydrogenase (G6PD)		
Glyoxalase I (GLO_1)		
Peptidases A and B (Pep A and Pep B)		
Phosphoglucomutases (1 and 2) (PGM_1 and PGM_2)		
6-Phosphogluconate dehydrogenase (PGD)		

Blood grouping was carried out according to methods in Race and Sanger (1968). Methods used for the red cell enzyme systems appear in Harris and Hopkinson (1976). Typing of the serum protein systems was carried out by the following methods: Properdin factor B (Bf) and group specific component (Gc) types were identified after electrophoresis by the method of Teisberg (1970) and immunofixation by the method of Alper and Johnson (1969). Additional information about the Gc subtypes was obtained through isoelectric focusing by the method of Kühnl *et al.* (1978). Haptoglobin and transferrin phenotypes were determined by the methods described by Giblett (1969).

2.6.4.2. Lod score determination

Morton's sequential test (Morton, 1955) was used for evaluating the likelihood of linkage between the major L-xylulose reductase gene locus and the loci of those polymorphic genes (listed in section 2.6.4.1) which showed useful variation. For two generation pedigrees where mating had been of the double back-cross type, the logs of the odds (lod scores) were obtained from the Table which appears in Race and Sanger (1968). The lods for more complicated pedigrees were obtained by computer using the LIPED 3 programme devised by Ott (1977) for the Elston-Stewart algorithm (Elston and Stewart, 1971).

CHAPTER 3

3. RESULTS

3.1. L-xylulose production

A comparison of the separation of L-xylulose obtained with the two chromatography solvents is shown in Figure 3.1. It can be seen that the ethyl acetate-pyridine-water solvent brought about a better separation of L-xylulose from the other sugars present, in a given time, than the 1-butanol-pyridine-water solvent. An indication of the purity of the L-xylulose produced can be obtained from Figure 3.2 which shows the presence of only one substance as revealed by the napthoresorcinol stain.

3.2. Demonstration of low enzyme activities in pentosurics by the glutathione and pyruvate methods

Figures 3.3 and 3.4 show the results obtained when haemolysate xylitol dehydrogenase and L-xylulose reductase activities of a normal and a pentosuric individual were compared. From Figure 3.3, which shows the results obtained by the 'glutathione method', it can be seen that a significant non-linear blank reaction occurred in both assays. A straight line, indicating that the xylitol dehydrogenase catalysed reaction was linear, could be obtained by subtracting the blank from the experimental values. Assays done by the pyruvate method (Figure 3.4) showed a much smaller blank reaction than that observed with the glutathione method. The results presented in Figure 3.3. confirm the finding of Wang and van Eys (1970) that pentosurics have a deficiency of red cell NADP-linked xylitol dehydrogenase activity. The results obtained by the pyruvate method (Figure 3.4) demonstrate that pentosurics have the corresponding, expected deficiency of

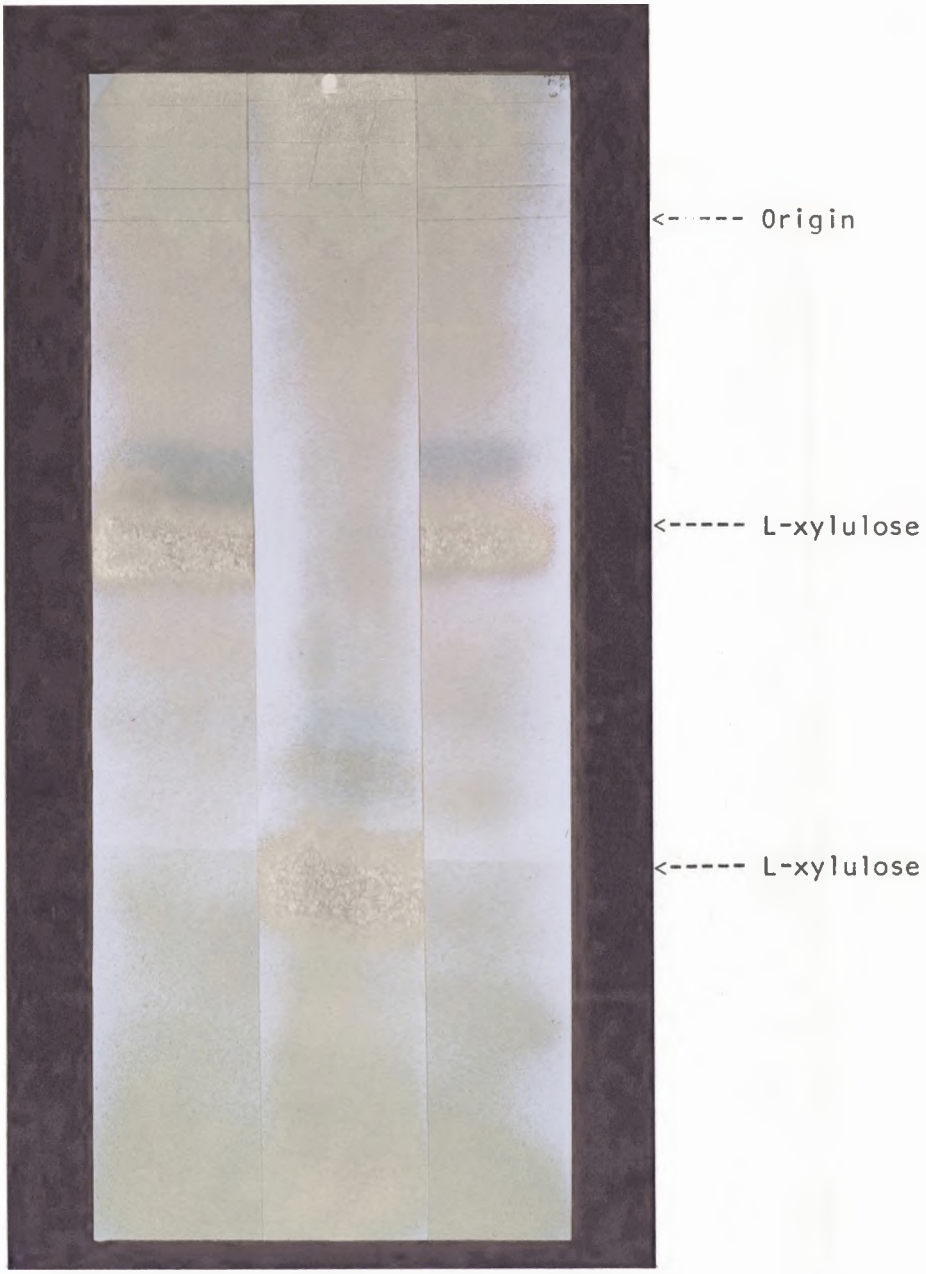


Figure 3.1. Sections of two chromatograms which were developed with different solvents. The central strip (labelled 11) was part of a chromatogram which had been developed with the ethyl acetate-pyridine-water solvent and the strips on either side of it were parts of a chromatogram which was developed with 1-butanol-pyridine-water. The dark-grey bands on the chromatograms are due to the presence of L-xylulose, while the faint brown and blue bands are due to the presence of other pentoses which formed during the refluxing step.

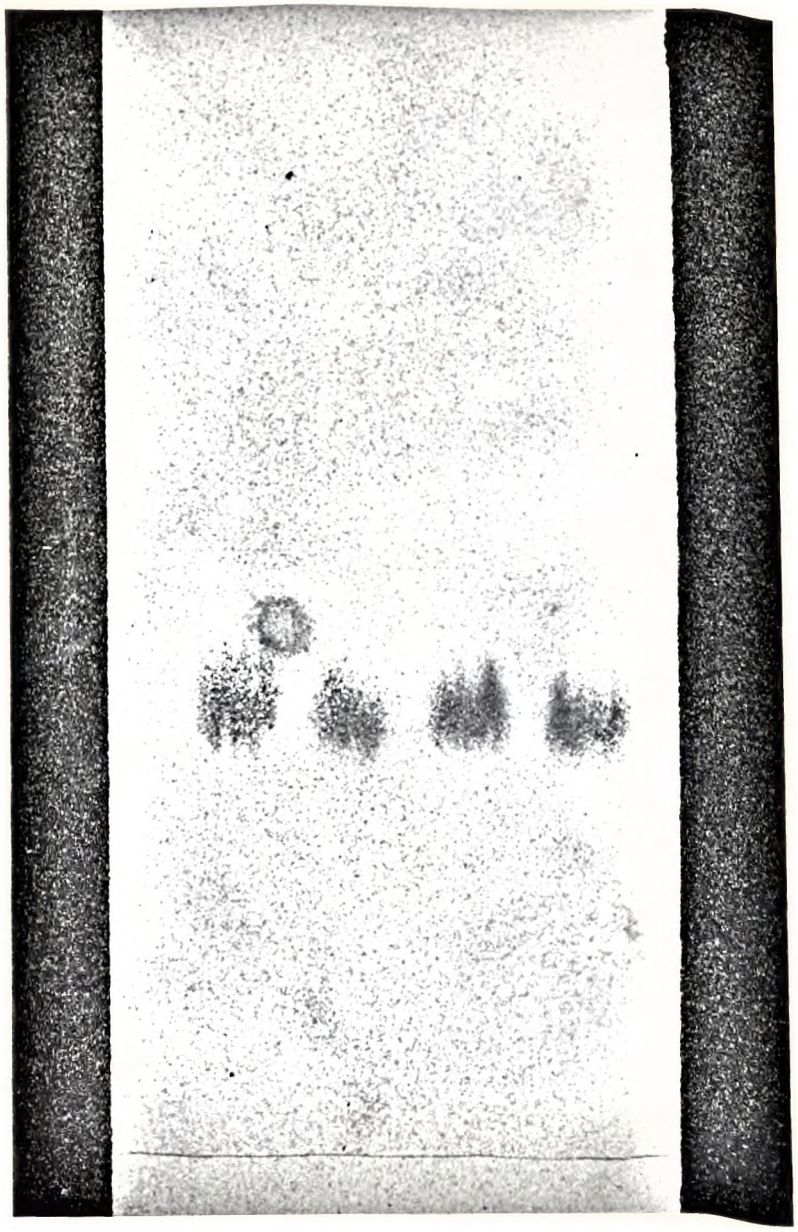


Figure 3.2. A chromatogram (developed with ethyl acetate-pyridine-water) which was run to check the purity of the L-xylulose produced. Four applications of the same L-xylulose solution were made. No other bands apart from those due to L-xylulose were visible on the chromatogram.

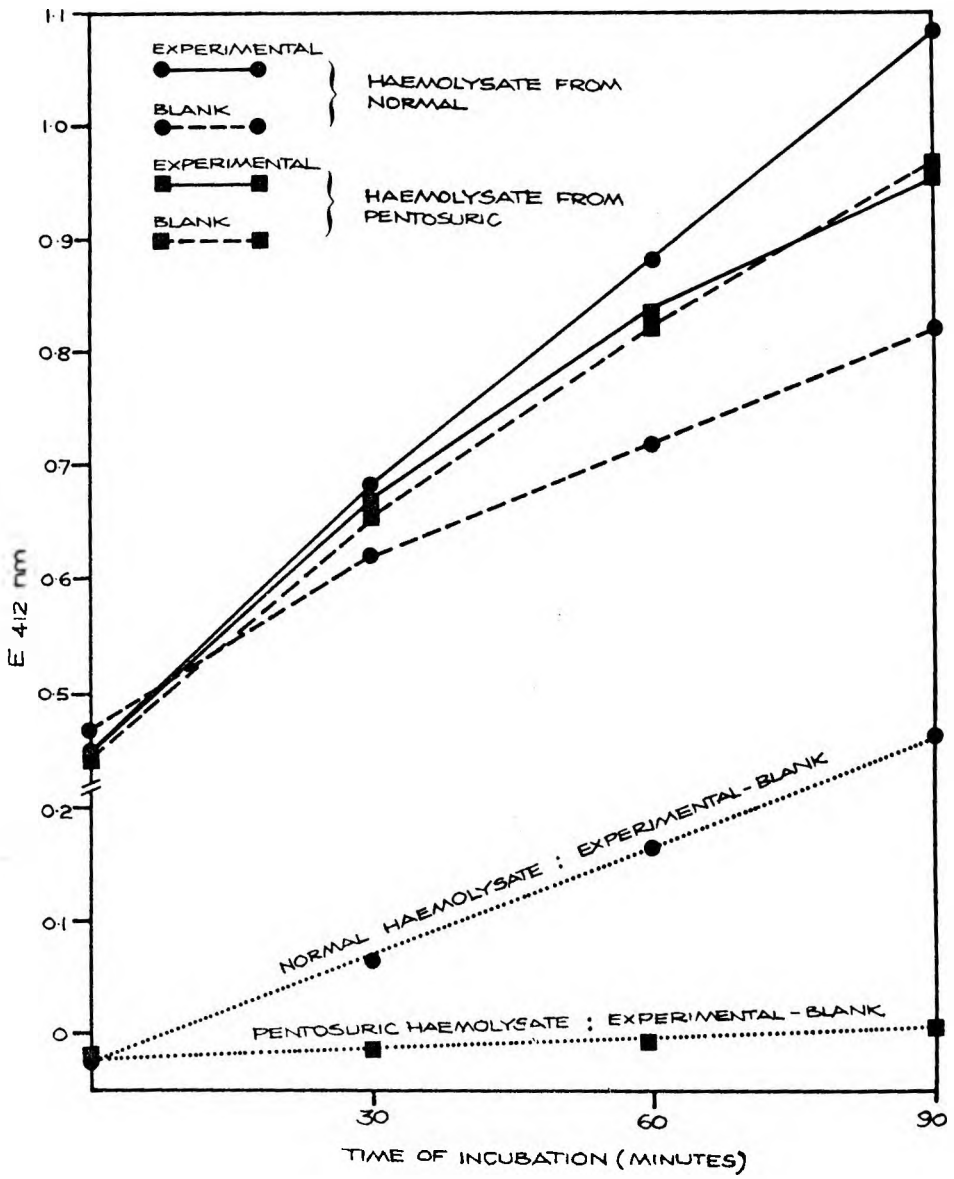


Figure 3.3. A comparison of normal and pentosuric haemolysate xylitol dehydrogenase activities as measured by the glutathione method. The lower curve for each assay was obtained by subtracting the blank from the experimental readings. The activities of the normal and pentosuric haemolysates were 1.819 and 0.048 μ moles/hr/g Hb respectively.

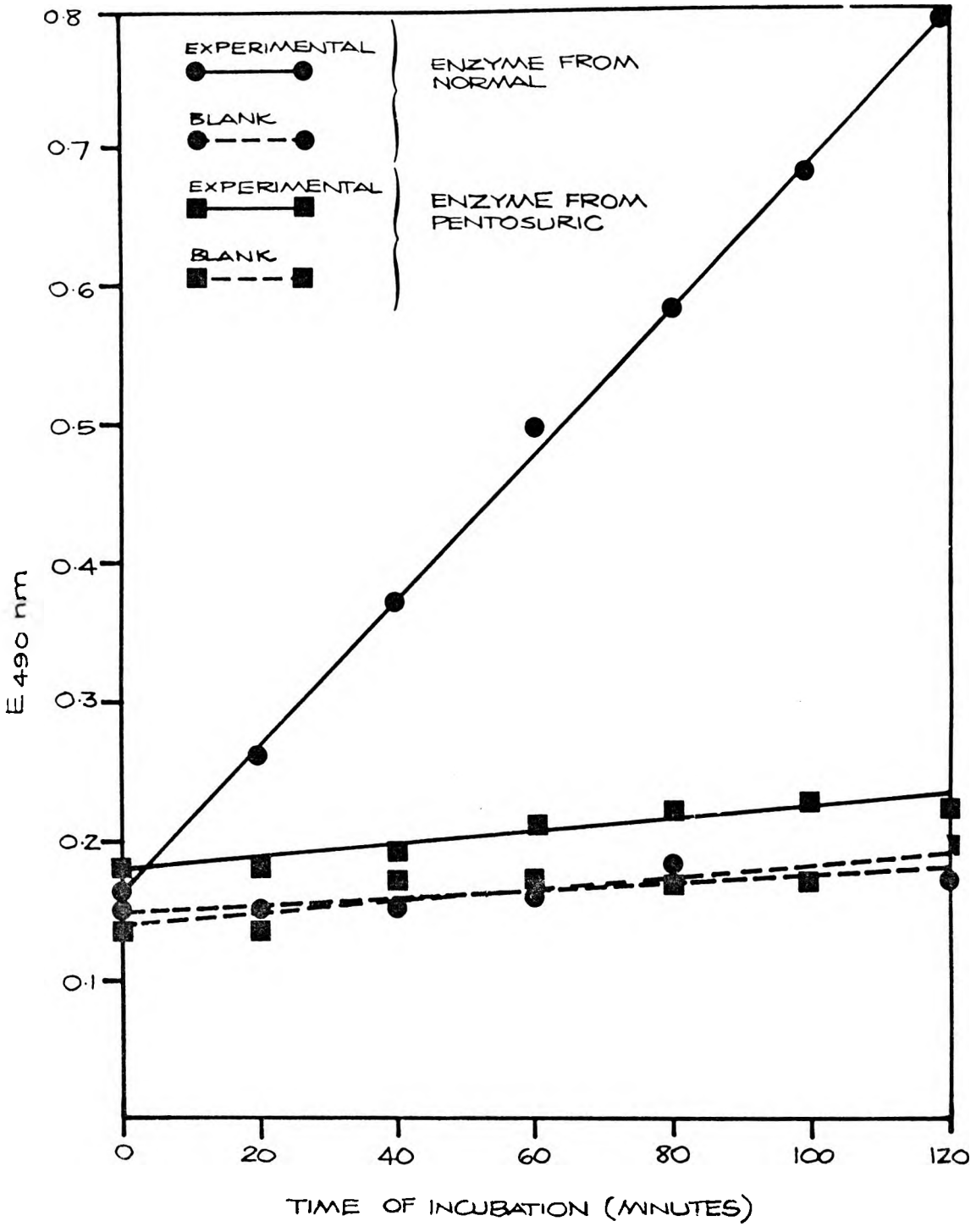


Figure 3.4. A comparison of normal and pentosuric L-xylulose reductase activities as measured by the pyruvate method. The activities of the normal and pentosuric haemolysates were 6.827 and 0.115 μ moles/hr/g Hb respectively.

L-xylulose reductase activity which is the actual cause of essential pentosuria.

3.3. Kinetic, chromatographic and electrophoretic studies

3.3.1. Enzyme preparation

The total enzyme recovered from haemolysates (from normals) after haemoglobin separation ranged between forty-five and sixty-one per cent of the original activity. Results of a typical separation appear in Table 3.1. Yields of crude haemoglobin-free enzyme activity/mℓ of haemolysate from the pentosuric subjects were between 0,0131 and 0,0257 µmoles/hour. Preparations of the normal major isozyme made from crude haemoglobin-free extracts contained between twenty and fifty per cent of the activity originally present in the crude preparations.

3.3.2. Michaelis constants and the effects of pH on enzyme activity

3.3.2.1. Studies on crude enzyme preparations

Figures 3.5 to 3.12 are Lineweaver-Burk plots of results obtained with crude, haemoglobin-free preparations of enzyme from the pentosuric subjects and from normal individuals. From Table 3.2 in which the K_m values obtained are listed, it can be seen that the K_m for NADP of the 'pentosuric enzyme' is very similar to that of the normal enzyme. These two enzymes, however, have strikingly different Michaelis constants for xylitol (see Figure 3.8). The results shown in Figure 3.9 allow a direct comparison to be made between the rates at which enzyme from a pentosuric and a normal individual catalysed the reverse reaction at different xylitol concentrations. The Michaelis constants of the normal and 'pentosuric' enzymes for L-xylulose

TABLE 3.1. The separation of xylitol dehydrogenase from haemoglobin using the CMC batch method.

	Volume (mℓ)	Total haemoglobin (g)	Activity (µmoles/hour/mℓ)	Total activity (µmoles/hour/total volume)
Haemolysate	30,0	4,96	0,559	16,77
Suspension of material precipitated in ammonium sulphate solution	7,2	0,01	1,382	9,95

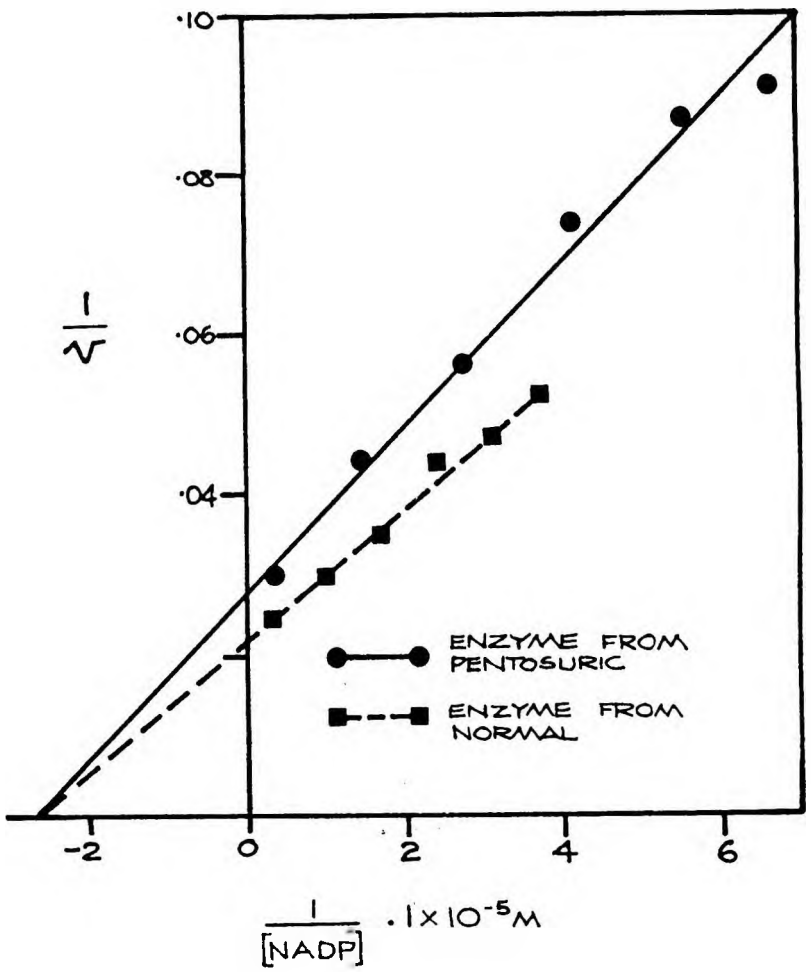


Figure 3.5. Lineweaver-Burk plots used to estimate the "normal" and "pentosuric" enzymes K_m values for NADP (at pH 7.0). The apparent K_m for NADP of enzyme from the normal individual was 3.88 μM and that of enzyme from the pentosuric (H.K.), 3.78 μM. A similar determination carried out on enzyme from pentosuric R.B. yielded an apparent K_m of 6.67 μM.

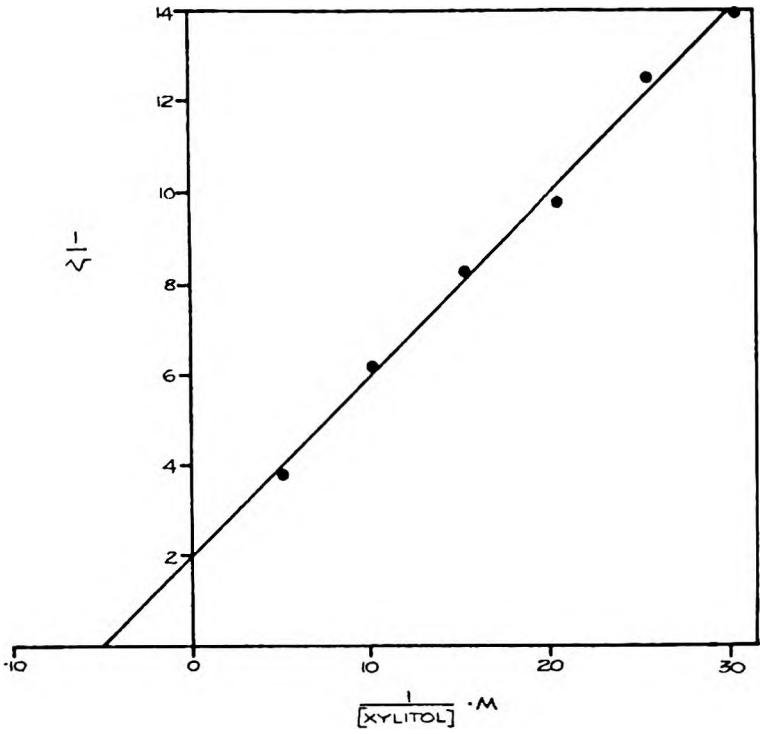


Figure 3.6. Lineweaver-Burk plot of data used to estimate the K_m for xylitol of enzyme prepared from a normal individual (reaction pH 7.0). The reciprocal of the x-axis intercept yields an apparent K_m of 0.206 M. Two additional determinations of this K_m yielded values of 0.199 M and 0.139 M.

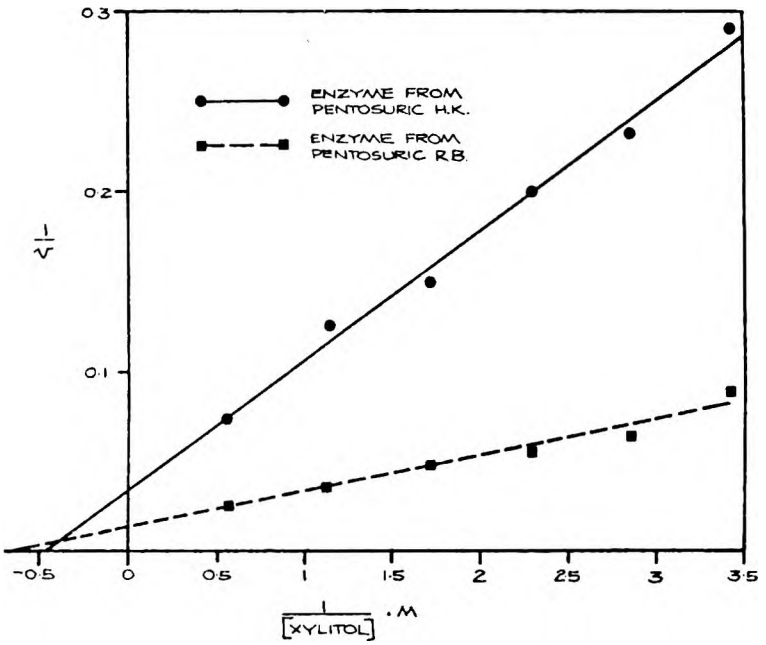


Figure 3.7. Lineweaver-Burk plots of data (obtained at a reaction pH of 7.0) used to estimate the K_m for xylitol of enzyme prepared from the two pentosuric subjects (H.K. and R.B.). The apparent K_m for xylitol of enzyme from H.K. was 2.383 M and that of enzyme from R.B., 1.537 M.

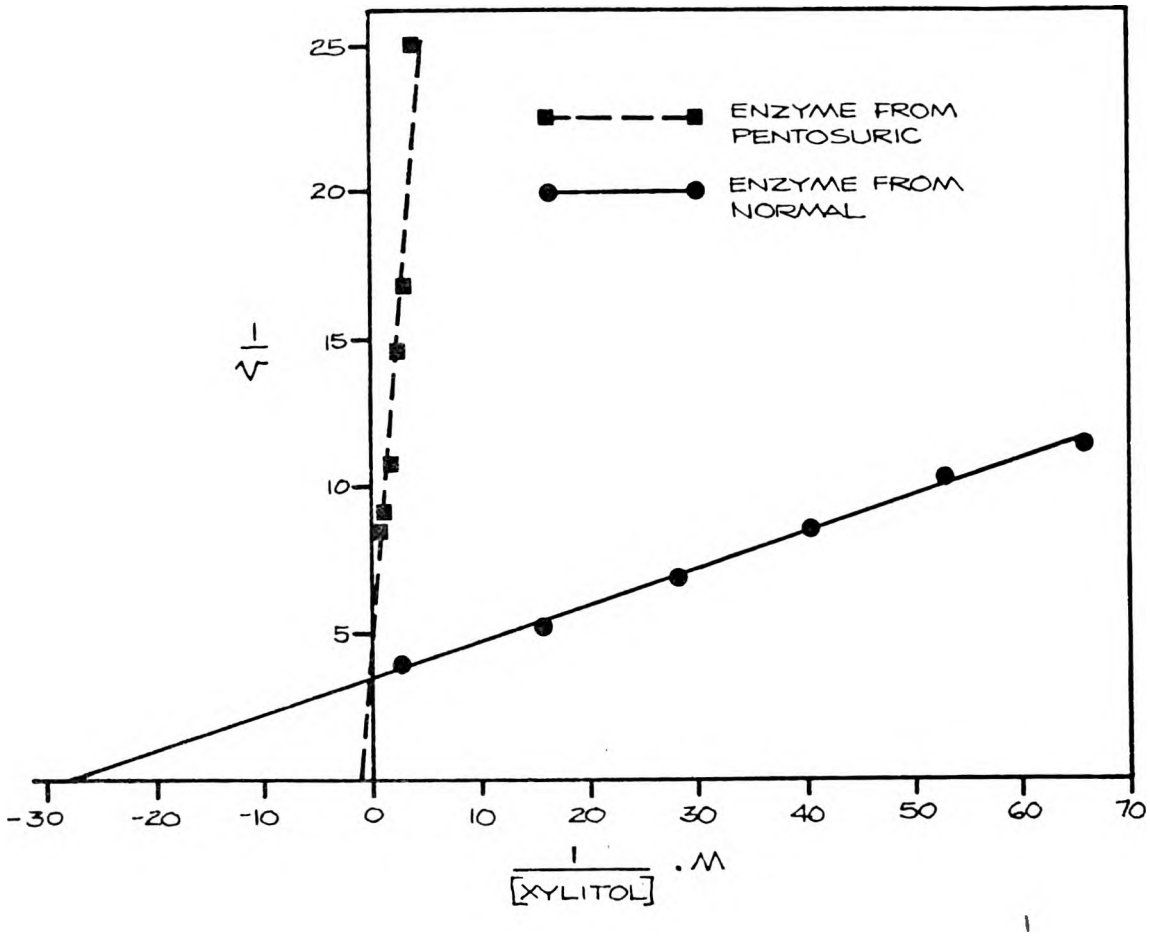


Figure 3.8. Lineweaver-Burk plots of data used to estimate the normal and "pentosuric enzyme's" Michaelis constants for xylitol at pH 9.5. The 'normal' enzyme's apparent K_m for xylitol was 36.2 mM while that of the 'pentosuric enzyme', was 654 mM.

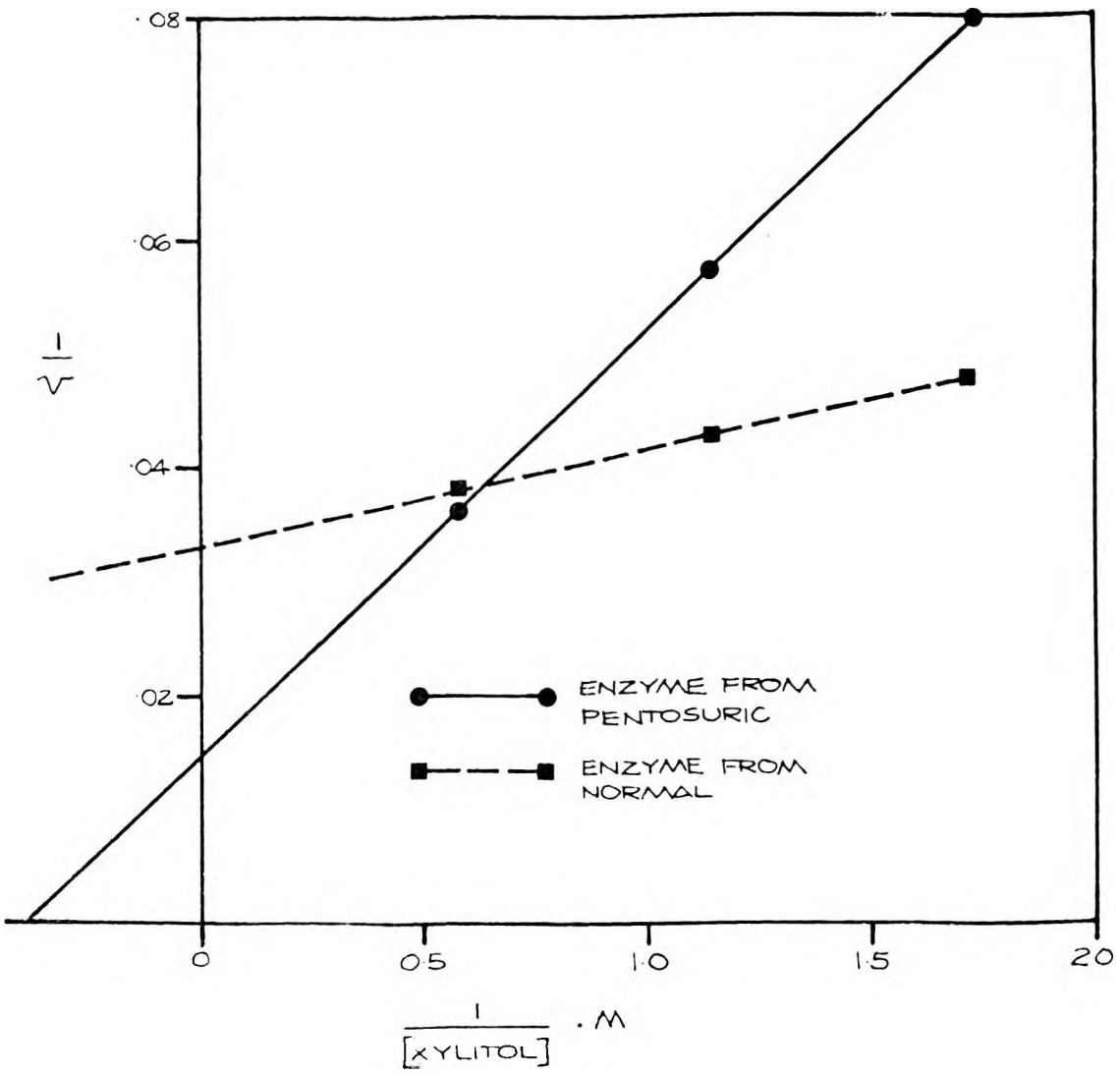


Figure 3.9. The results of a direct comparison of rates at which enzyme from a pentosuric and a normal individual catalysed the reverse reaction (pH 7.0) at various xylitol concentrations. The "pentosuric" and "normal" enzyme preparations were adjusted (at the highest xylitol concentration used) to approximately the same activity. The rates at which the adjusted pentosuric and normal enzyme preparations catalysed the reverse reaction were then measured at two lower xylitol concentrations.

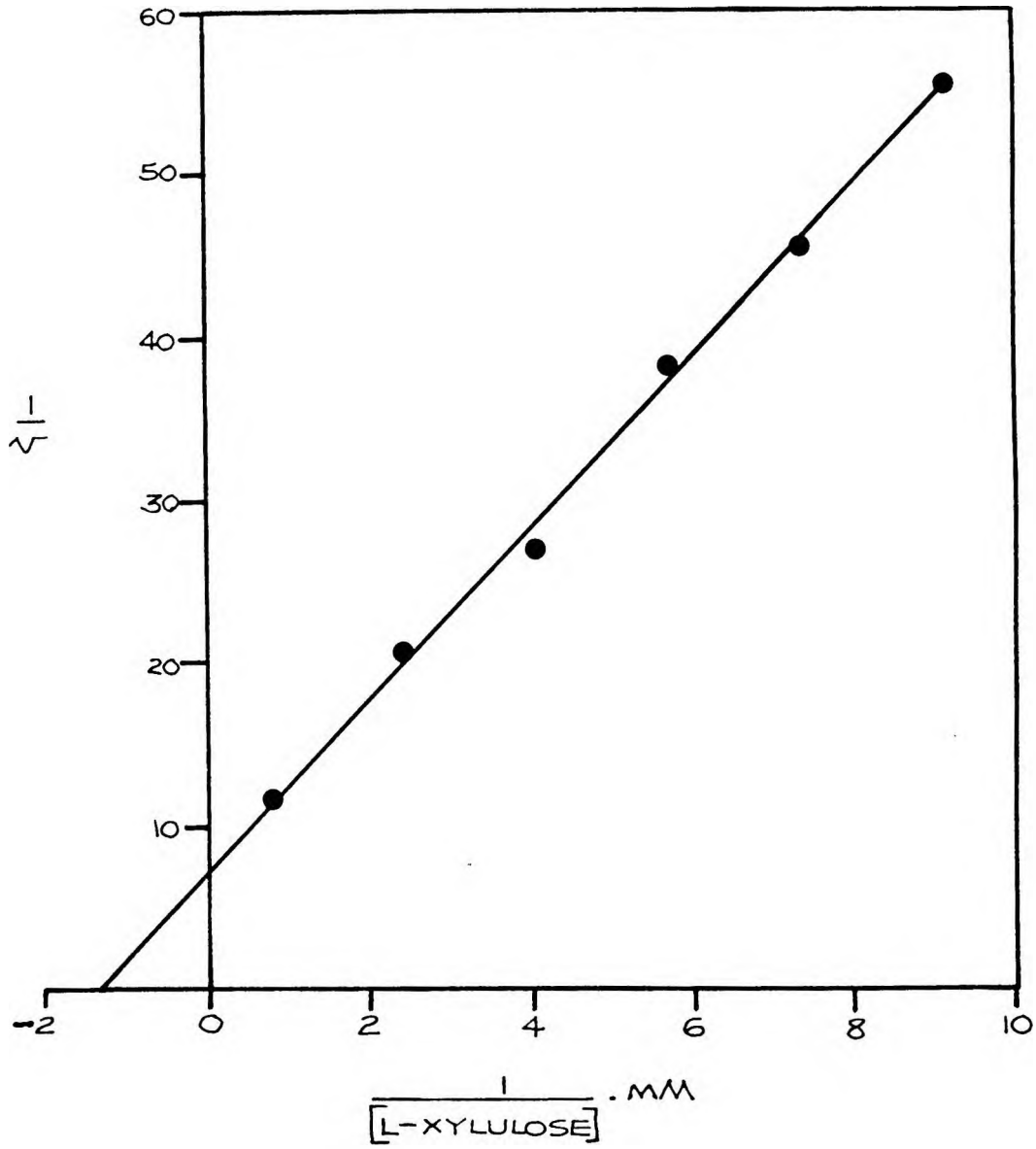


Figure 3.10. Lineweaver-Burk plot of data used to estimate the K_m for L-xylulose of enzyme prepared from a normal individual (reaction pH 7.0). The reciprocal of the x-axis intercept yields and apparent K_m of 0.773 mM. Two additional determinations of this K_m gave values of 0.527 and 1.026 mM.

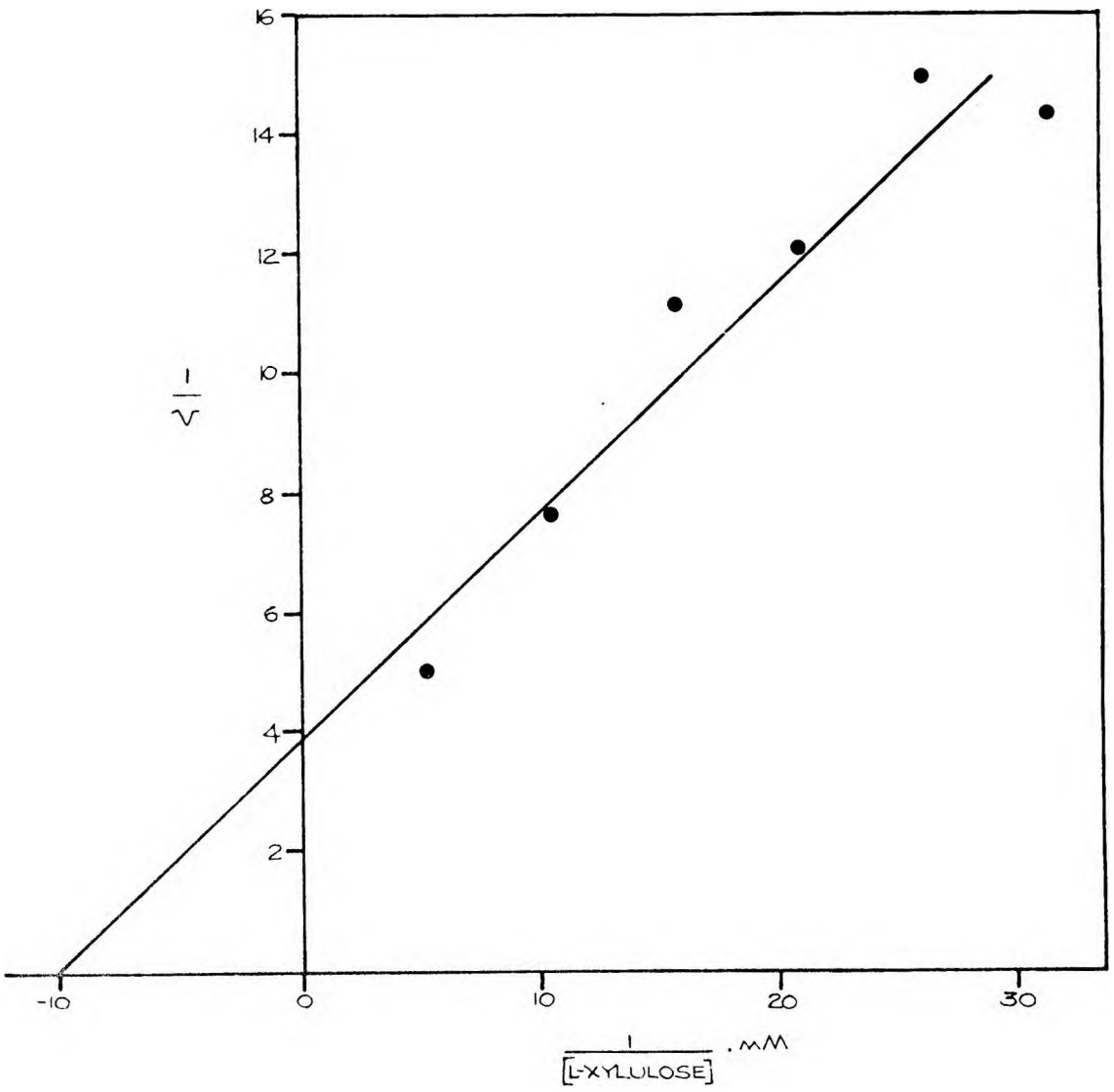


Figure 3.11. Lineweaver-Burk plot of data used to estimate the K_m for L-xylulose of enzyme prepared from pentosuric H.K. (reaction pH 7.0). The reciprocal of the x-axis intercept yields an apparent K_m of 0.0971 M. A similar determination with enzyme from pentosuric R.B. yielded an apparent K_m of 0.0454 M.

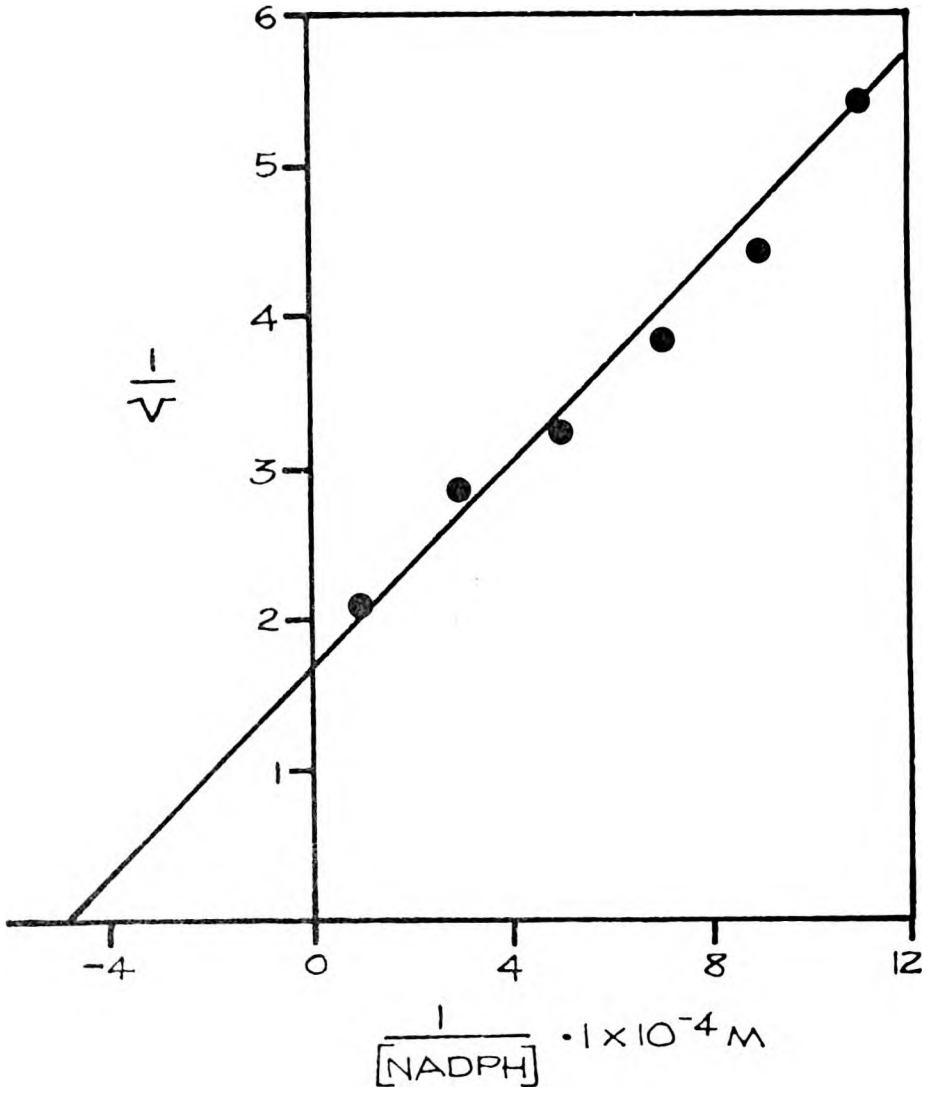


Figure 3.12. Lineweaver-Burk plot of data used to estimate the K_m for NADPH of enzyme from a normal individual (reaction pH 7.0). The reciprocal of the x-axis intercept yields an apparent K_m of 20.52 µM. Additional determinations of this K_m yielded values of 27.97 µM, 7.81 µM, 18.01 µM, 32.91 µM and 49.75 µM.

TABLE 3.2. The averaged apparent Michaelis constants for enzyme in crude preparations from normal and pentosuric individuals.

	K_m	
Substrate (pH)	Normal	Pentosuric
NADP (7,0)	6,66 µM	4,42 µM
Xylitol (7,0)	0,181 M	1,910 M
Xylitol (9,5)	0,036 M	0,654 M
L-xylulose (7,0)	0,775 mM	97,057 mM
NADPH (7,0)	26,16 µM	

differ even more strikingly than their corresponding Michaelis constants for xylitol (see Figures 3.10 and 3.11 and Table 3.2). The normal enzyme's K_m for NADPH (forward reaction) was measured for completeness (see Figure 3.12) but not that of the 'pentosuric enzyme' because this would have required saturating concentrations of the second substrate, L-xylulose, which was in short supply.

pH activity studies: Ideally, the effect of pH on reaction rates should be measured at saturating substrate concentrations because the rate at which an enzyme catalyses its reaction may be influenced by two pH related factors, namely, the enzyme's affinity for its substrates at a particular pH and its ability to actually catalyse the reaction at that pH (Dixon and Webb, 1964). The use of saturating substrate concentrations eliminates the affinity factor but, it is not always feasible (for reasons of solubility, lack of substrate, substrate inhibition, etc.), to use saturating substrate concentrations. An alternative approach, and the one used here for the normal enzyme, is to determine V_{max} values at different pHs. The results of the pH-activity studies done on the normal enzyme are shown in Figures 3.13 and 3.14. The rough Michaelis constant estimates obtained while estimating the V_{max} values are also shown in the Figures. The xylitol dehydrogenase and L-xylulose reductase activities of enzyme from normals had very different pH optima (10,5 - 11,5 and 5,5 - 6,5 respectively) and the apparent K_m values for xylitol and L-xylulose showed marked variations with pH. The pH-activity determination carried out on the 'pentosuric enzyme' was only done for the reverse reaction and then using only a single xylitol concentration because the amount of enzyme available was limited. The apparent pH optimum of the 'pentosuric enzyme' (see Figure 3.15) when catalysing the

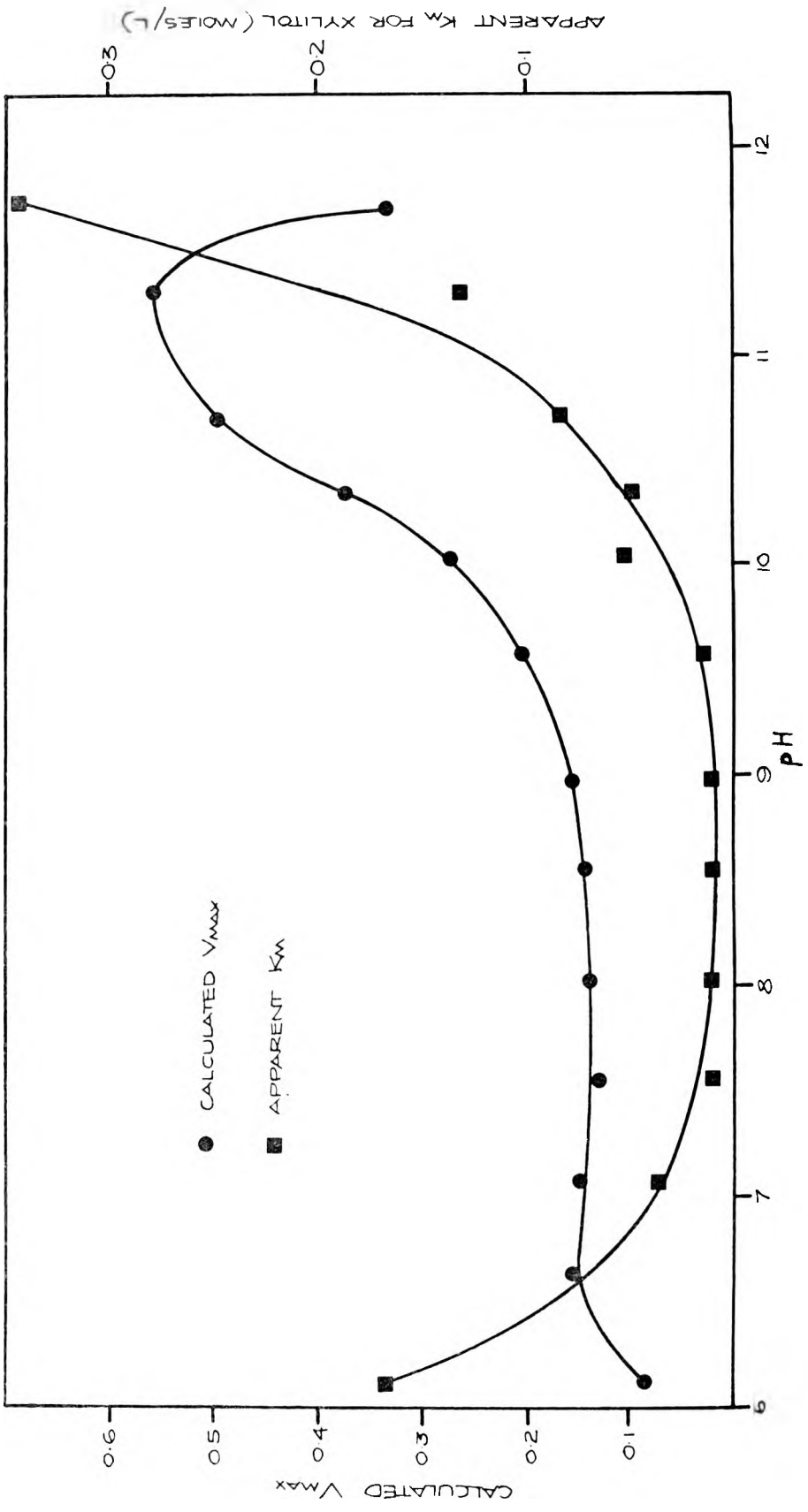


Figure 3.13. The effect of pH on the xylitol dehydrogenase activity of a crude (haemoglobin-free) preparation of enzyme from a normal individual.

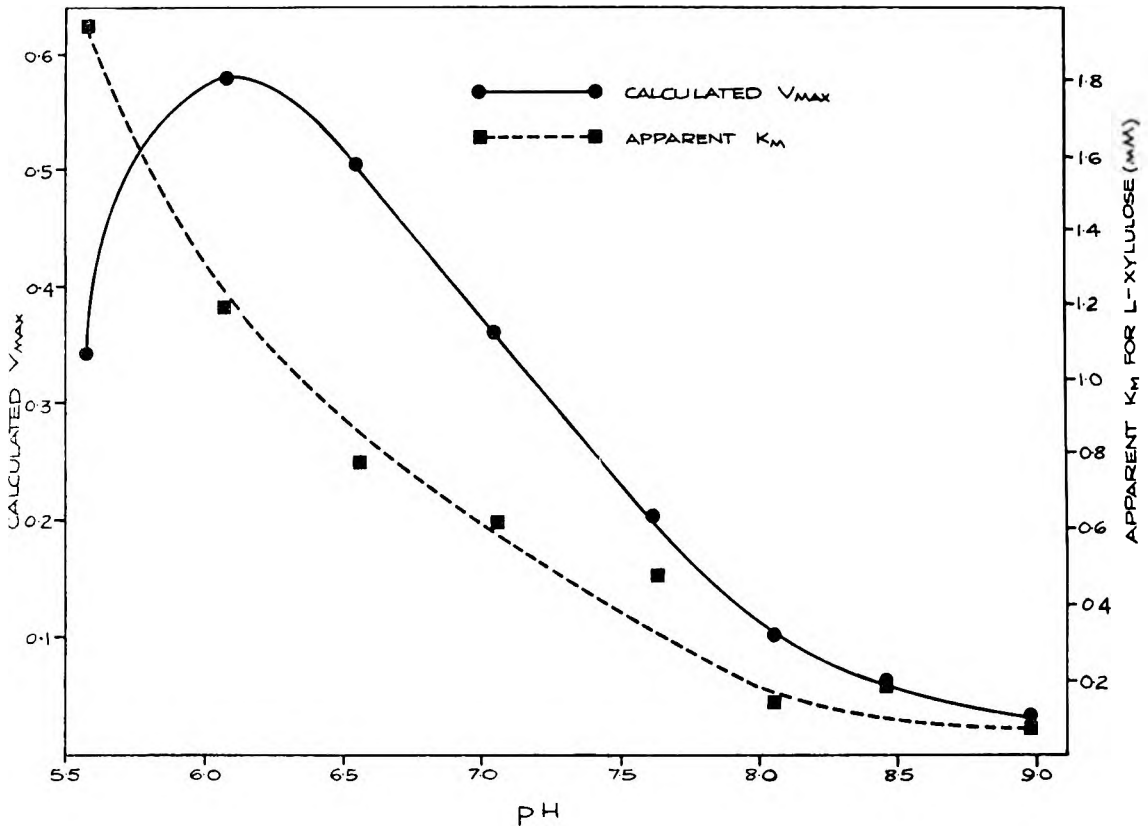


Figure 3.14. The effect of pH on the L-xylulose reductase activity of a crude (haemoglobin-free) enzyme preparation from a normal individual.

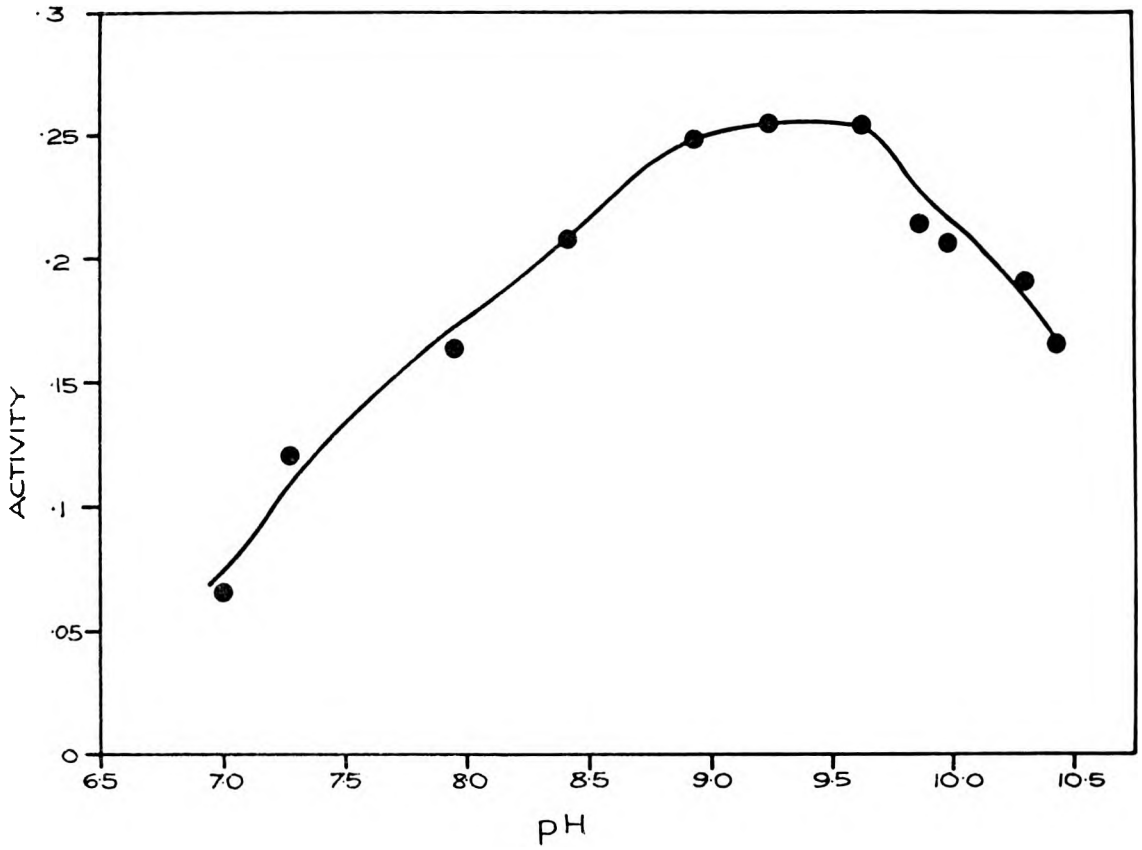


Figure 3.15. The effect of pH on the xylitol dehydrogenase activity of a crude (haemoglobin-free) enzyme preparation from a pentosuric subject.

reverse reaction, was between pH 9,0 and 10,0, which is lower than that of normal enzyme (see Figure 3.13) which was approximately 11.

3.3.2.2. Major and minor isozymes from normals

When haemolysates and crude haemoglobin-free preparations from normal individuals had been found to contain small amounts of a second L-xylulose reductase (called the minor isozyme - see section 3.3.3.2), additional kinetic studies were carried out on this as well as on the major isozyme. The Lineweaver-Burk plots obtained are shown in Figures 3.16 to 3.21 and the Michaelis constants which were estimated from these are listed in Table 3.3. Table 3.3 also shows results obtained under similar conditions with crude, haemoglobin-free preparations from normals and pentosurics. From the Table it can be seen that estimates of the normal major isozyme Michaelis constants for L-xylulose and NADP are similar to those obtained with crude haemoglobin-free preparations from normals It will also be noted that the normal minor isozyme Michaelis constants for xylitol and L-xylulose are almost identical to those estimated for the 'pentosuric enzyme'. Estimates of the normal major isozyme Michaelis constants for xylitol and NADP (made for the sake of characterising the enzyme) were carried out at pH 9,0 because in this region small variations in pH due to experimental error would have minimal effects on the K_m values (see Figure 3.22a).

The effect of pH on the xylitol dehydrogenase and L-xylulose reductase activities of the major and minor isozymes are shown in Figures 3.22 to 3.24. The shapes of the pH-activity curves obtained with the normal major isozyme are similar to those obtained with the crude haemoglobin-free preparations from normals. In addition, pH was found to have a similar influence on the major isozyme Michaelis

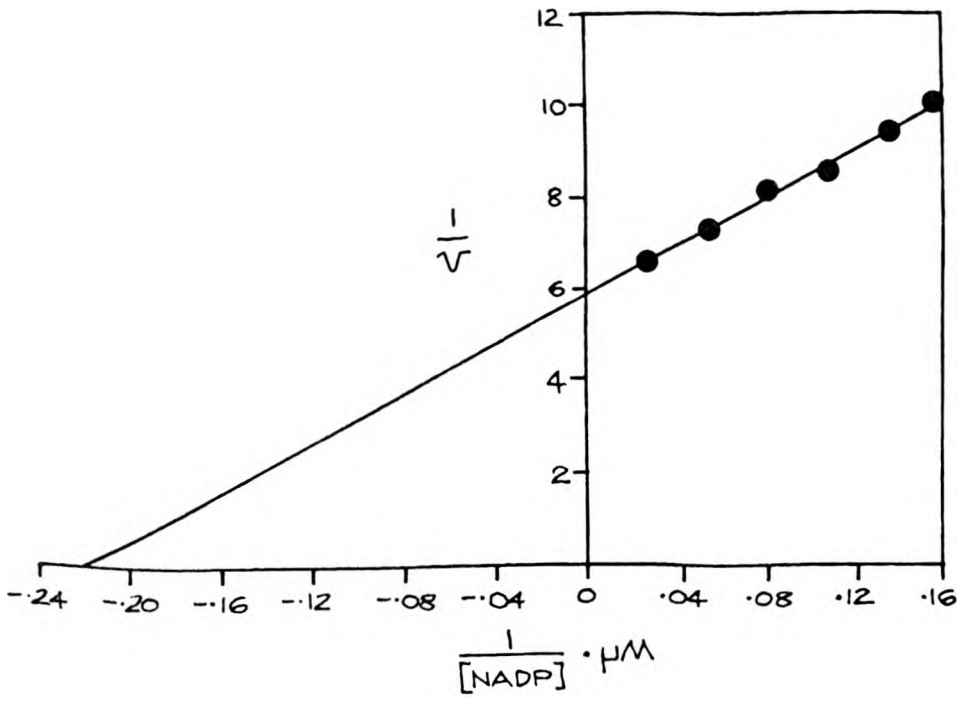


Figure 3.16. Lineweaver-Burk plot of data used for the estimation of the normal major isozymes Michaelis constant (at pH 9.0) for NADP. The receiprocal of the x-axis intercept yields an apparent K_m of 4.476 μM.

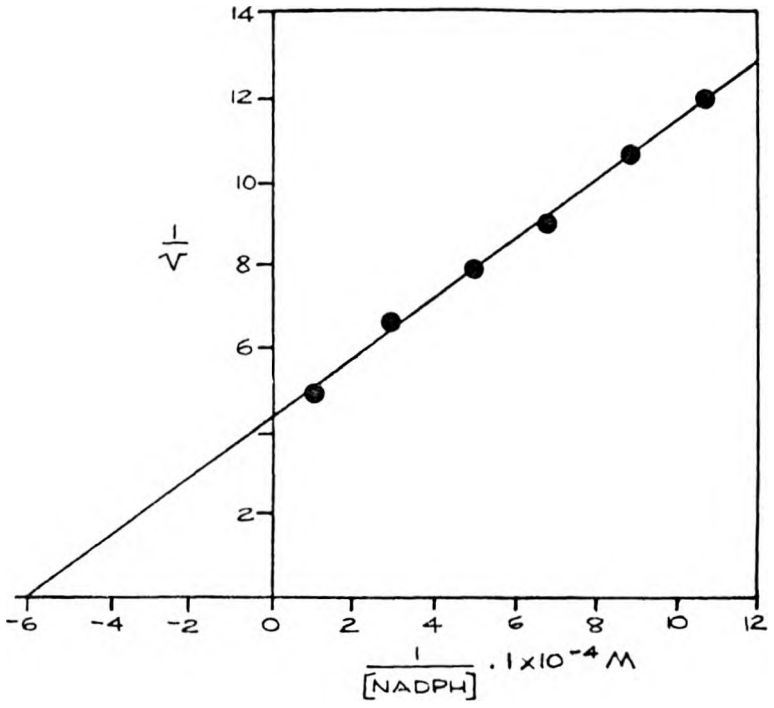


Figure 3.17. Lineweaver-Burk plot of data used for the estimation of the normal major isozymes Michaelis constant (at pH 7.0) for NADPH. The receiprocal of the x-axis intercept yields and apparent K_m of 16.33 μM.

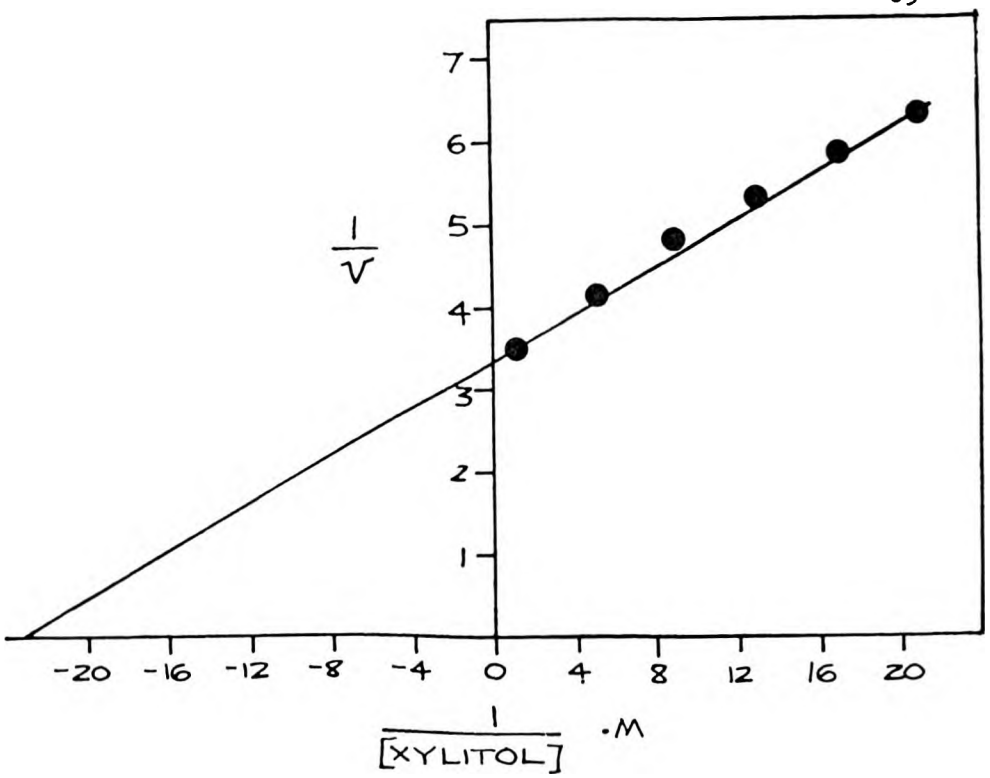


Figure 3.18. Lineweaver-Burk plot of data used for the estimation of the normal major isozymes Michaelis constant (at pH 9.0) for xylitol. The reciprocal of the x-axis intercept yields an apparent K_m of 0.060 M.

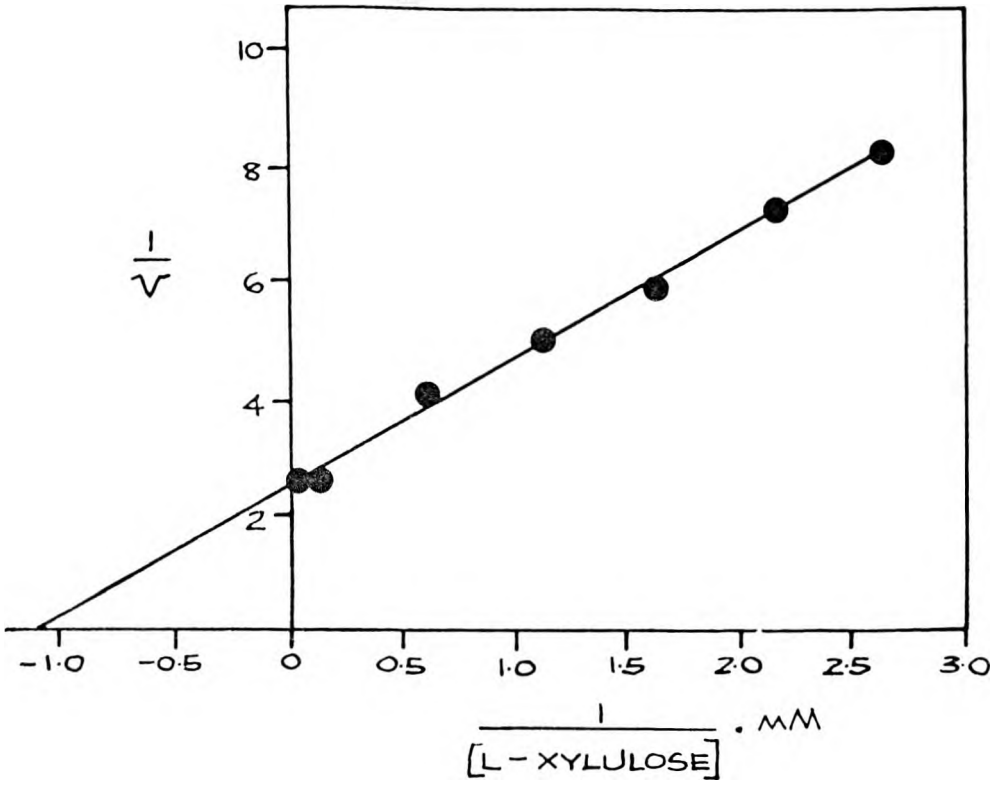


Figure 3.19. Lineweaver-Burk plot of data used for the estimation of the normal major isozymes Michaelis constant (at pH 7.0) for L-xylulose. The reciprocal of the x-axis intercept yields an apparent K_m for L-xylulose of 0.924 mM.

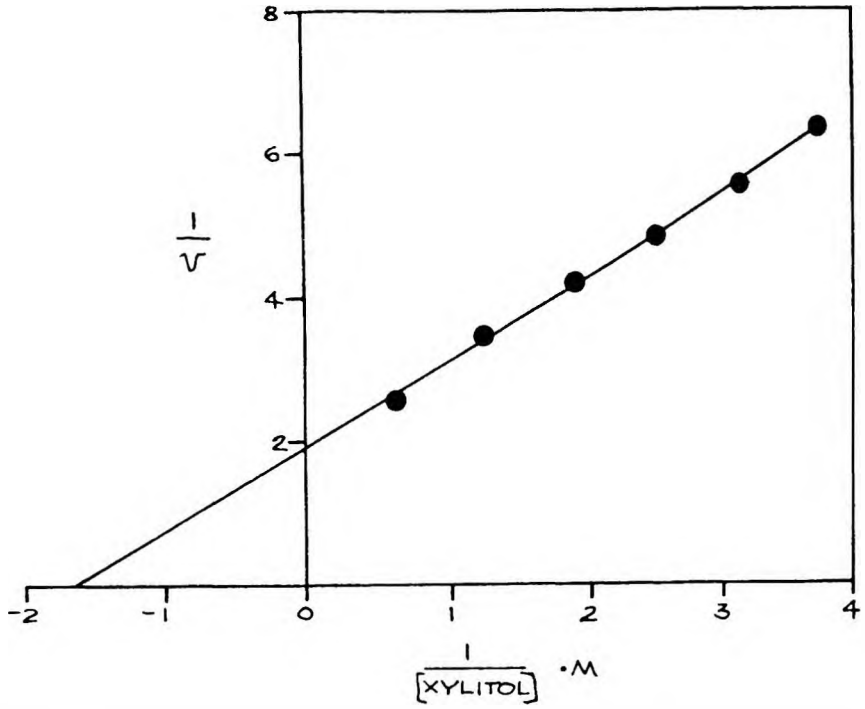


Figure 3.20. Lineweaver-Burk plot of data used for the estimation of the normal minor isozymes Michaelis constant (at pH 9.5) for xylitol. The reciprocal of the x-axis intercept yields an apparent K_m for xylitol of 0.617 M.

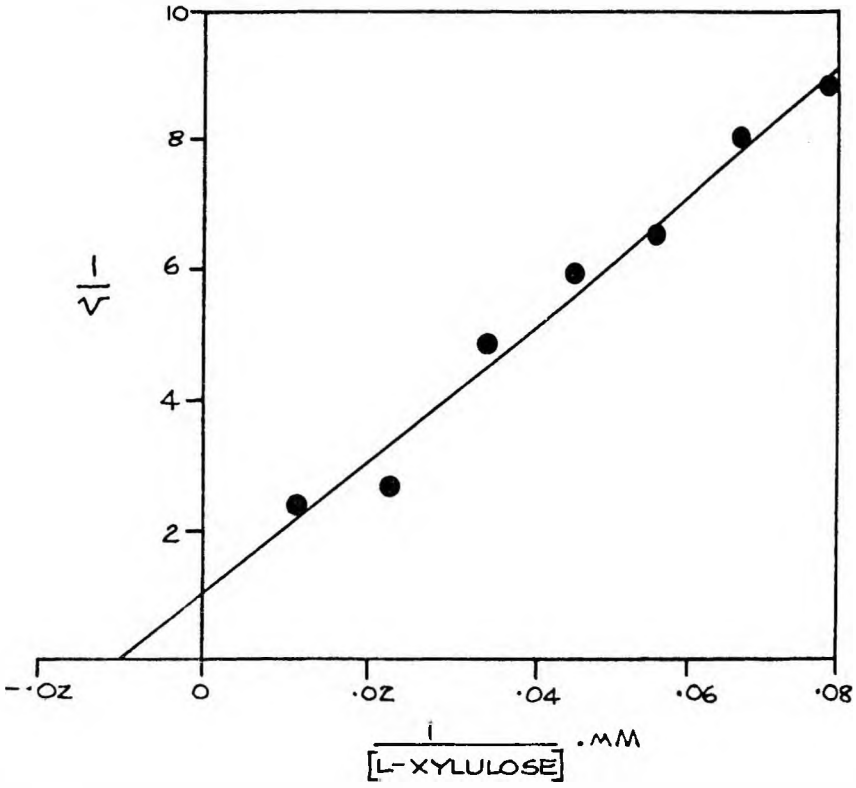


Figure 3.21. Lineweaver-Burk plot of data obtained on the normal minor isozyme at a reaction pH of 7.0. The reciprocal of the x-axis intercept yields an apparent K_m for L-xylulose of 0.0994 M.

TABLE 3.3. Apparent Michaelis constants determined for the normal major and minor isozymes. The values given on the right hand side of the table are the corresponding estimates obtained for enzyme in crude preparations from normals and pentosurics.

Substrate (pH)	Normal major isozyme	Normal minor isozyme	Crude preparations	
			Normals	Pentosurics
NADP (9,0)	4,476 µM			
Xylitol (9,0)	0,051 M			
Xylitol (9,5)		0,617 M		0,775 mM
L-xylulose (7,0)	0,924 mM	99,4 mM	0,775 mM	97,1 mM
NADPH (7,0)	16,33 µM		26,16	

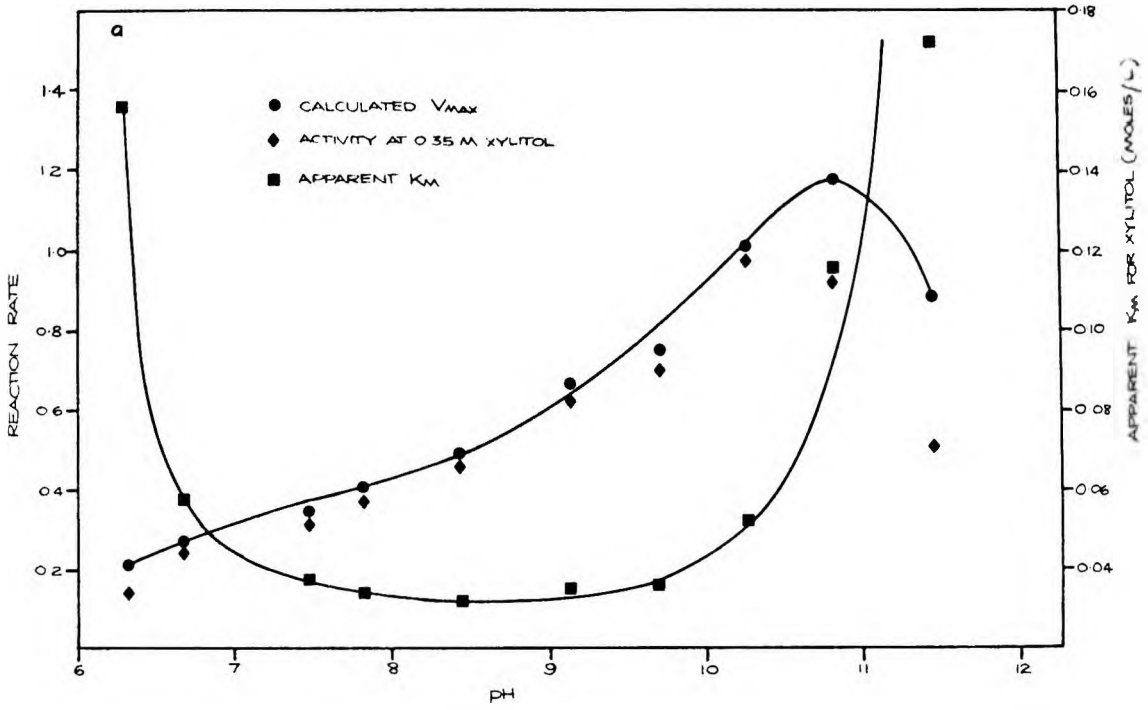


Figure 3.22. The effect of pH on the xylitol dehydrogenase activity of the normal major isozyme (a) results of the first determination; (b) additional data points obtained at pH values close to the apparent pH optimum.

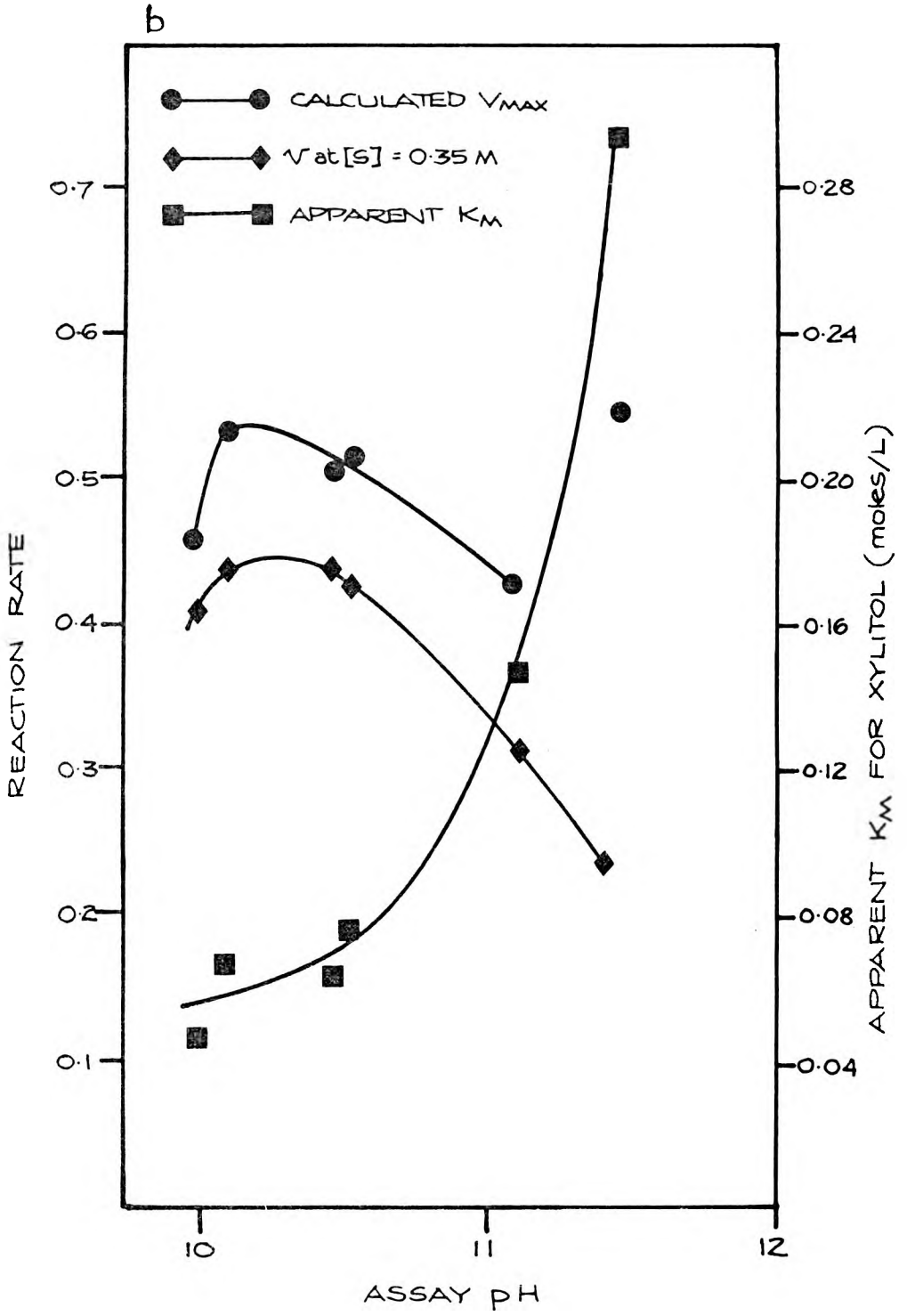

b
CALCULATED Vmax
V at [S] = 0·35 M
APPARENT KM
REACTION RATE
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·28
0·24
0·20
0·16
0·12
0·08
0·04
APPARENT KM FOR XYLITOL (moles/L)
10
11
12
ASSAY pH

Figure 3.22b

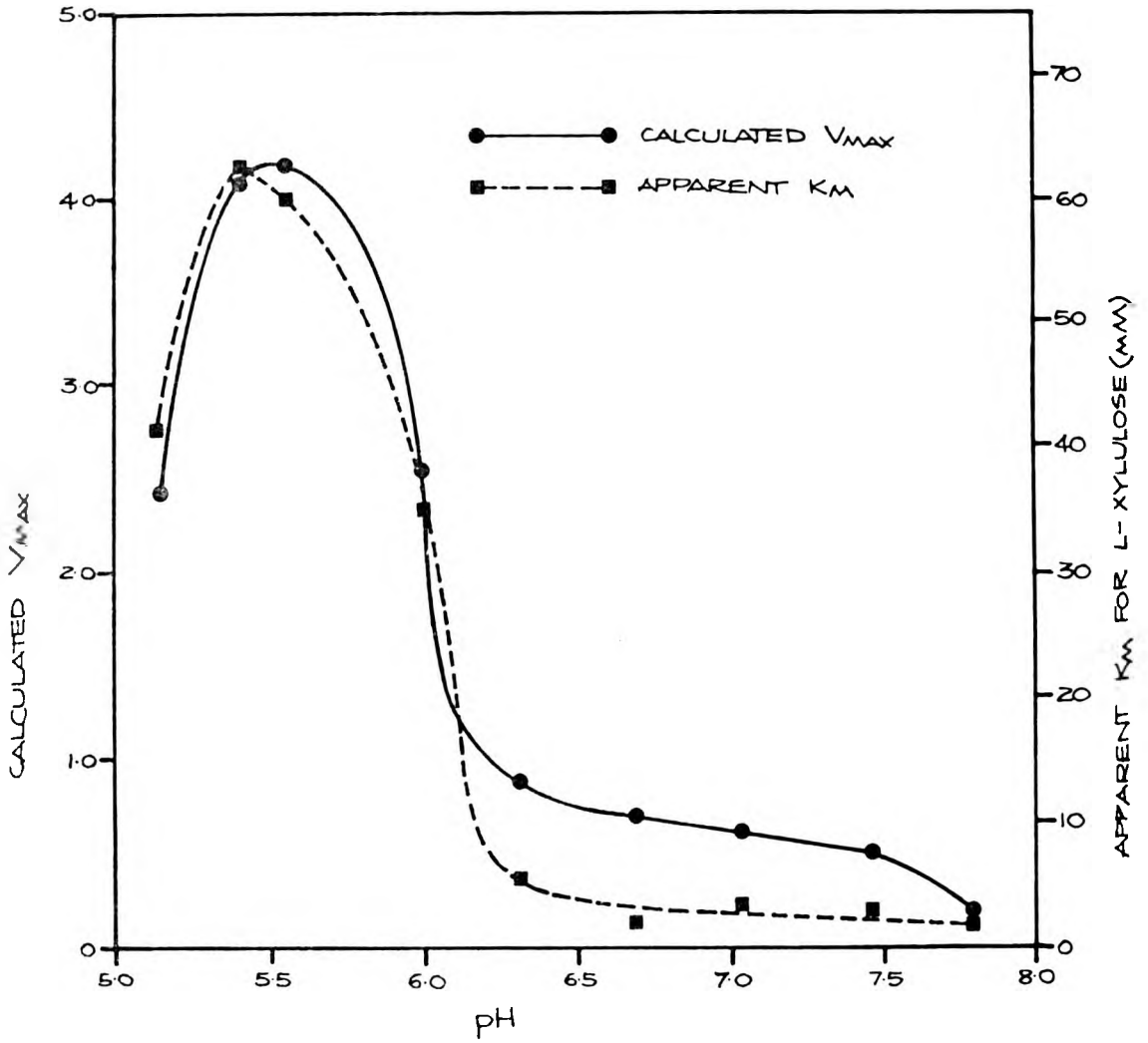


Figure 3.23. The effect of pH on the L-xylulose reductase activity of the major isozyme of normal individuals.

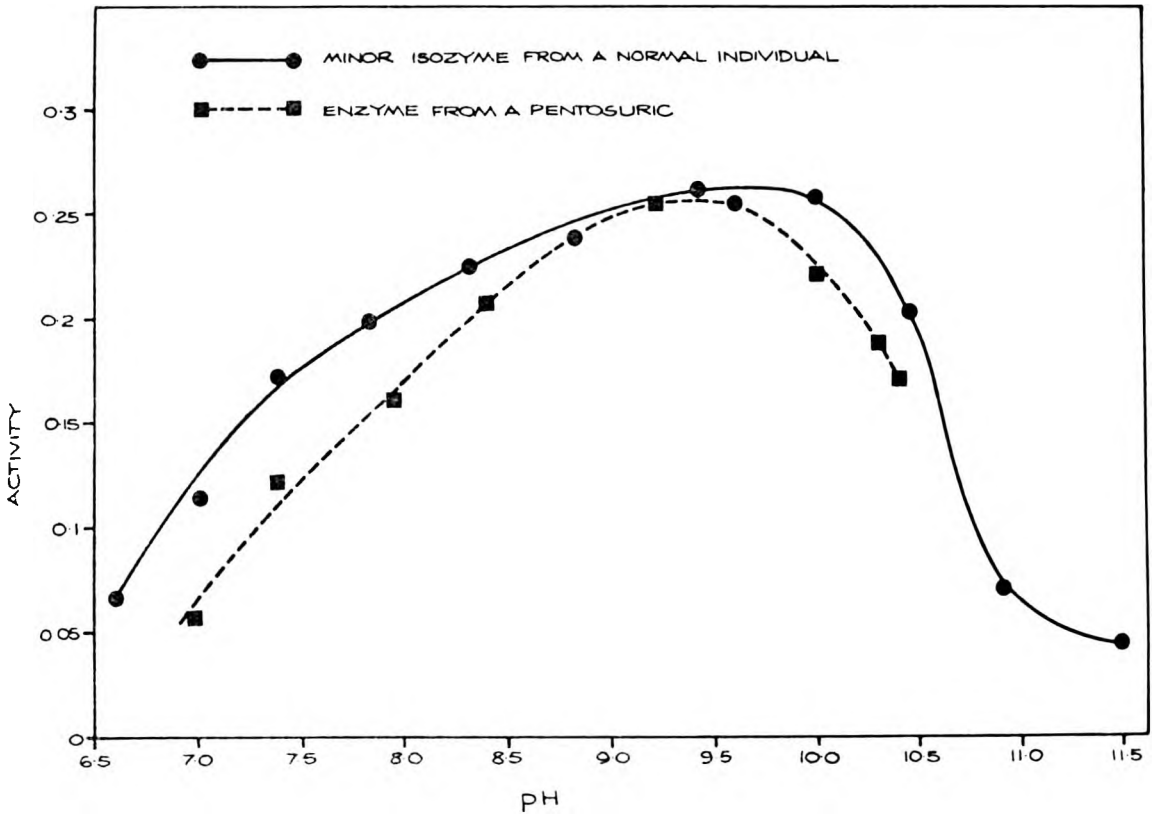


Figure 3.24. The effect of pH on the xylitol dehydrogenase activity of the minor isozyme from a normal individual. The curve obtained with a preparation of enzyme from a pentosuric (already presented in Figure 3.15) is also shown in the Figure.

constants for xylitol and L-xylulose to that found for enzyme in crude haemoglobin-free preparations from normal individuals. It is interesting that the major isozyme Michaelis constants for xylitol and L-xylulose are high at pH values close to its pH optima; one would expect enzymes to have high affinities for their substrates at their pH optima. The shape of the pH-activity curve obtained with the minor isozyme from normals (Figure 3.24) can be seen to be very similar to that obtained with a crude haemoglobin-free preparation of enzyme from a pentosuric and this, together with the similarity of their Michaelis constants for xylitol and L-xylulose and their apparently identical electrophoretic mobilities (see Figures 3.26 and 3.28) suggests that the 'pentosuric enzyme' and the minor isozyme from normals are the same. Table 3.4 lists the apparent pH optima of the major and minor isozymes as well as estimates obtained for enzyme in crude haemoglobin-free preparations from normals and pentosurics.

3.3.3. Chromatography and electrophoresis

3.3.3.1. Chromatography

Chromatography of crude preparations of L-xylulose reductase from normal individuals (Figure 3.25) revealed two well separated peaks of activity. The first peak to elute (just after the void volume) was called the minor isozyme while the second peak (which eluted before the traces of haemoglobin present) was called the major isozyme. In contrast, enzyme preparations from both pentosurics lacked the second (major) peak while an enzyme preparation from a carrier of the pentosuria allele (individual III-2 in Figure 3.36) contained both minor and major isozymes, but the major isozyme peak was reduced in comparison to the pattern obtained for normal individuals.

TABLE 3.4. The pH optima obtained from the pH-activity curves shown in Figures 3.13 to 3.15 and 3.22 to 3.24.

Enzyme source	Reaction catalysed	Apparent pH optimum
Crude preparation from normal	Reverse	10,5 - 11,5
Major isozyme from normal	Reverse	10,0 - 10,5
Crude preparation from normal	Forward	5,5 - 6,5
Major isozyme from normal	Forward	± 5,5
Minor isozyme from normal	Reverse	± 9,5
Crude preparation from pentosuric	Reverse	9,0 - 10,0

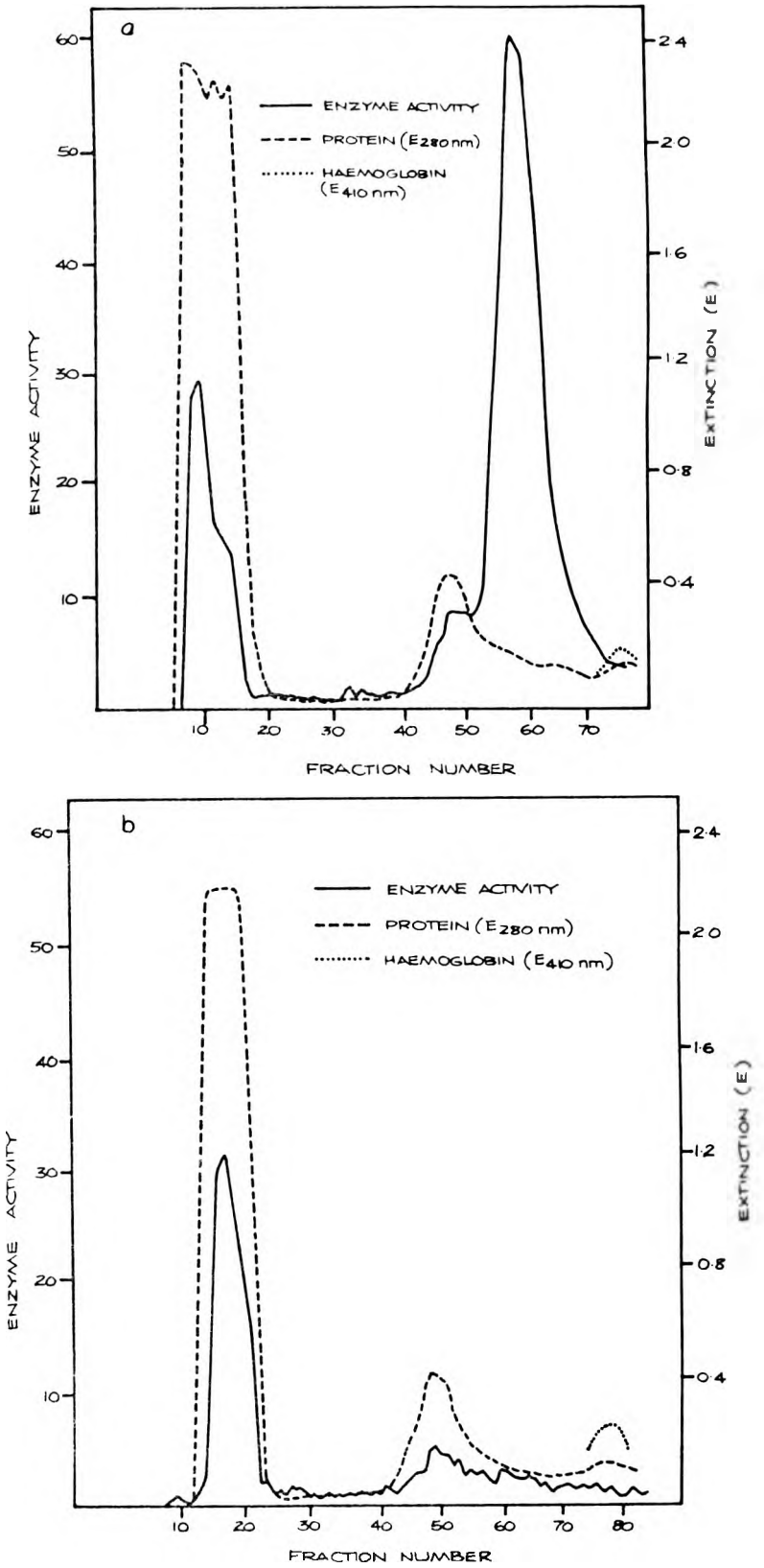


Figure 3.25. Chromatography of xylitol dehydrogenase from (a) a normal individual, (b) a pentosuric and (c) a carrier of the pentosuria allele. Extinction values at 280 nm represent the elution of protein, while those measured at 410 nm represent the elution of trace amounts of haemoglobin.

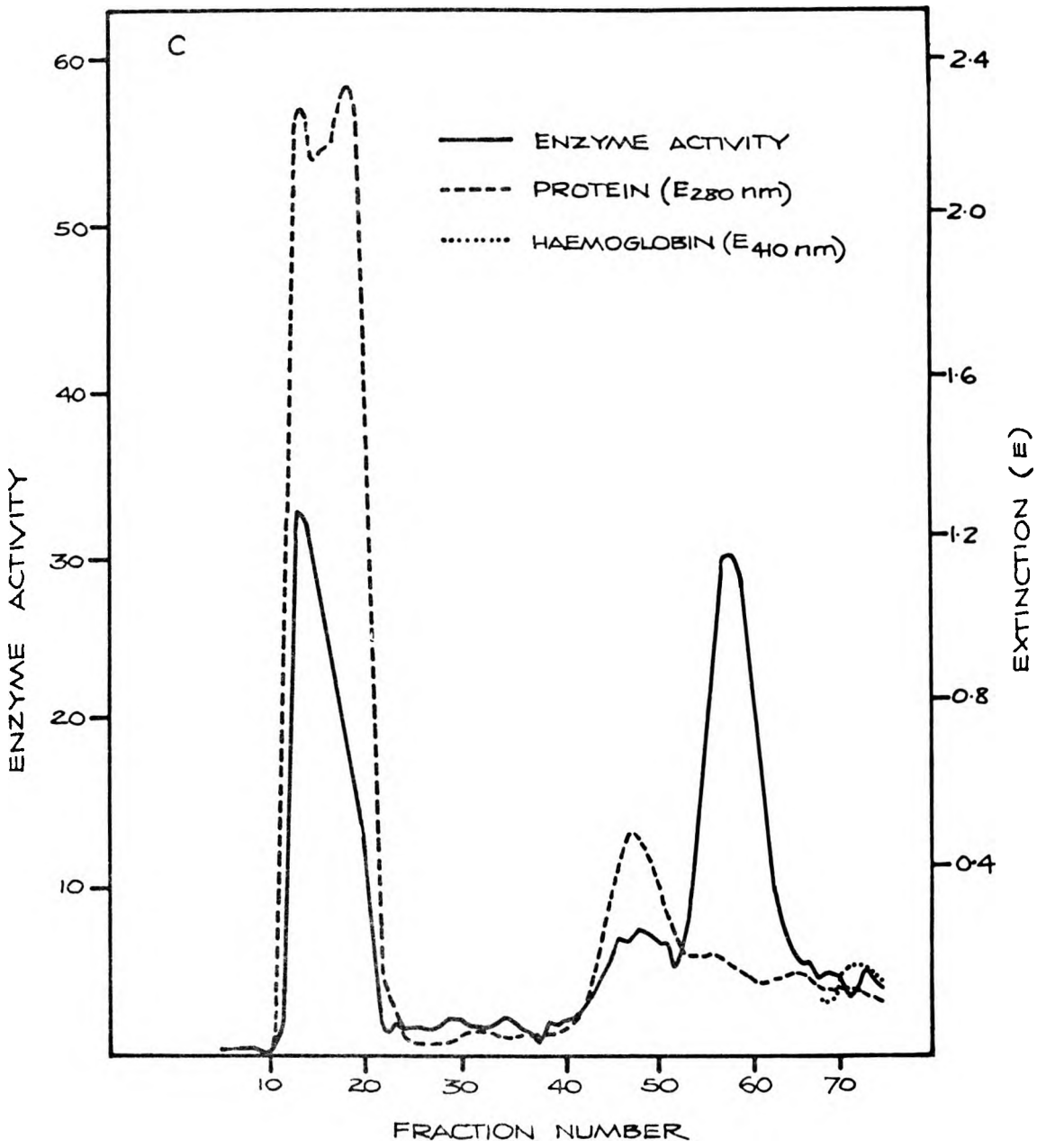

C
ENZYME ACTIVITY
PROTEIN (E280 nm)
HAEMOGLOBIN (E410 nm)
ENZYME ACTIVITY
EXTINCTION (E)
FRACTION NUMBER
60
50
40
30
20
10
2·4
2·0
1·6
1·2
0·8
0·4
10
20
30
40
50
60
70

Figure 3.25c

3.3.3.2. Electrophoresis

Results obtained by electrophoresis of human liver and crude haemolysate L-xylulose reductases are presented in Figures 3.26 to 3.28. A preparation of the minor isozyme from the red cells of a normal individual was also electrophoresed in the gel shown in Figure 3.28. The absence of the major isozyme in a haemolysate from a pentosuric (RB) can be seen in Figure 3.26. Haemolysates from the two carriers, the daughter of pentosuric HK and one of the Ashkenazi-Jewish carriers, MR (individual III-11 in Figure 3.40) which were electrophoresed on the same gel (Figure 3.26), showed both isozymes, but the staining of the major isozyme appeared to be less intense than the major isozyme in samples from normal individuals. A comparison of the mobilities of the major red cell and liver isozymes can be made from Figures 3.27a and 3.27b. The liver major isozyme migrated more rapidly than the red cell major isozyme. The gel shown in 3.27b, was stained under conditions which revealed the minor isozymes more clearly. It can be seen from Figure 3.27b that the minor isozyme of liver also migrates slightly faster than its red cell counterpart. The fact that both minor isozymes (red cell and liver) were best revealed by a stain which had a high xylitol concentration (compare Figures 3.27a and b) and further, that they occur in similar proportions in red cells and liver, suggests that they are homologous. The significance of the faint bands close to the origin in Figure 3.27b is not understood.

Different slices of the same gel, one stained in a way which revealed xylitol dehydrogenase activity and one stained by a solution which revealed L-xylulose reductase activity, are shown in Figure 3.28a and b. This was done in order to show that the same enzymes which

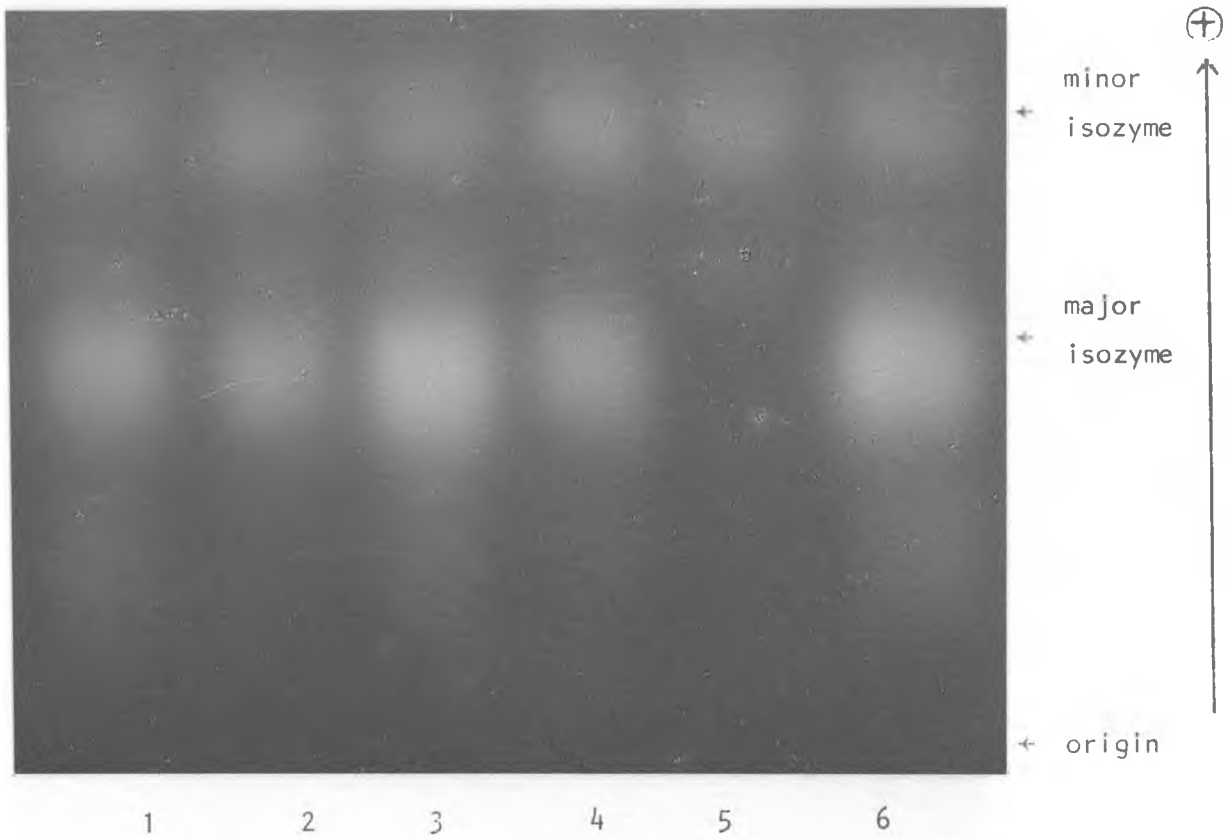


Figure 3.26. An electrophoretic comparison of red cell NADP-linked xylitol dehydrogenases from two normal individuals (lanes 1, 3 and 6), two carriers of the pentosuria allele (lanes 2 and 4) and pentosuric R.B. (lane 5).

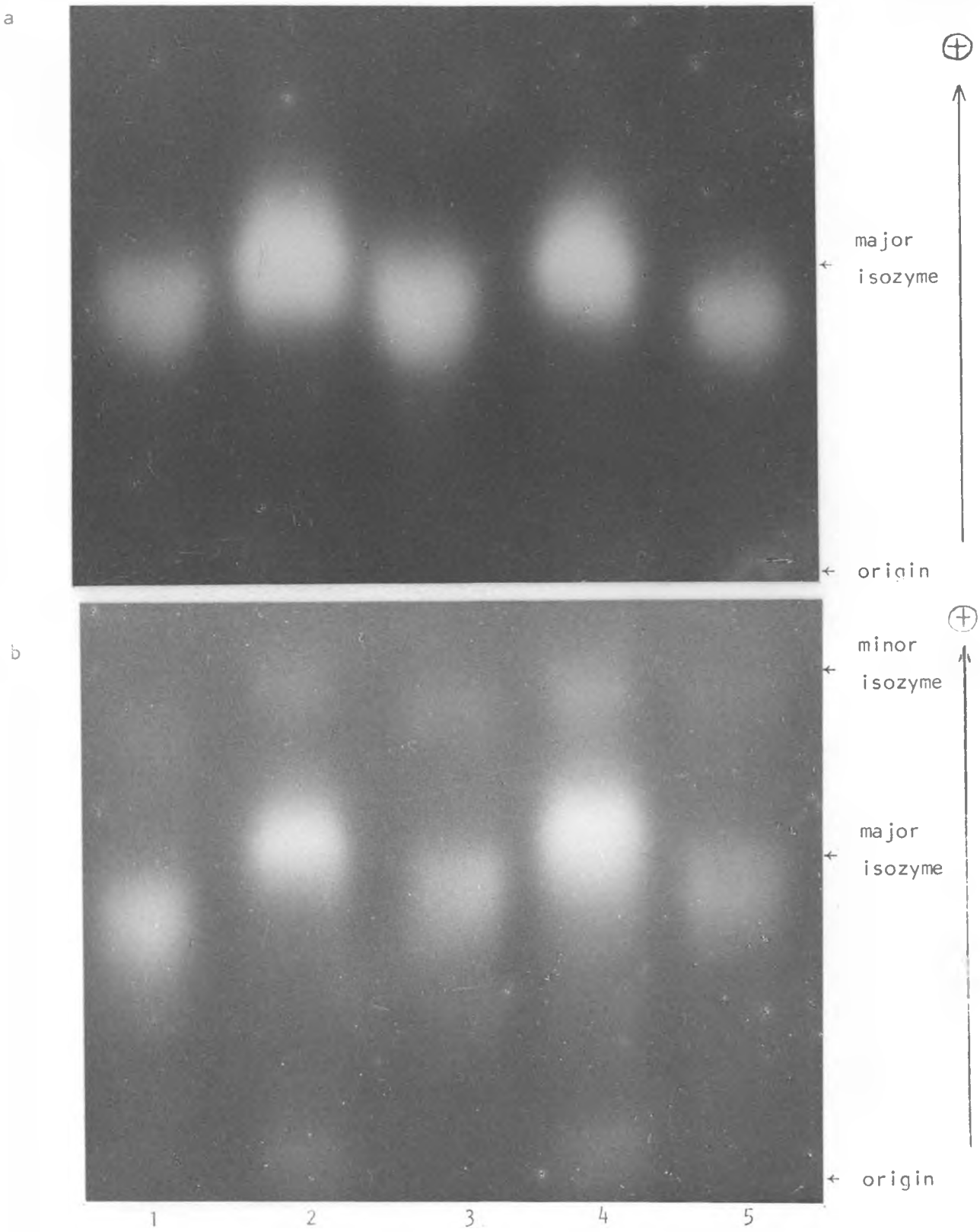


Figure 3.27. An electrophoretic comparison of red cell and liver NADP-linked xylitol dehydrogenases (L-xylulose reductases). Lanes 1, 3 and 5 contained red cell isozymes and lanes 2 and 4, liver isozymes.

(a) Gel stained under conditions favourable for major isozyme activity.
(b) Top slice of the same gel stained under conditions more favourable to the minor (rapidly migrating) isozymes.

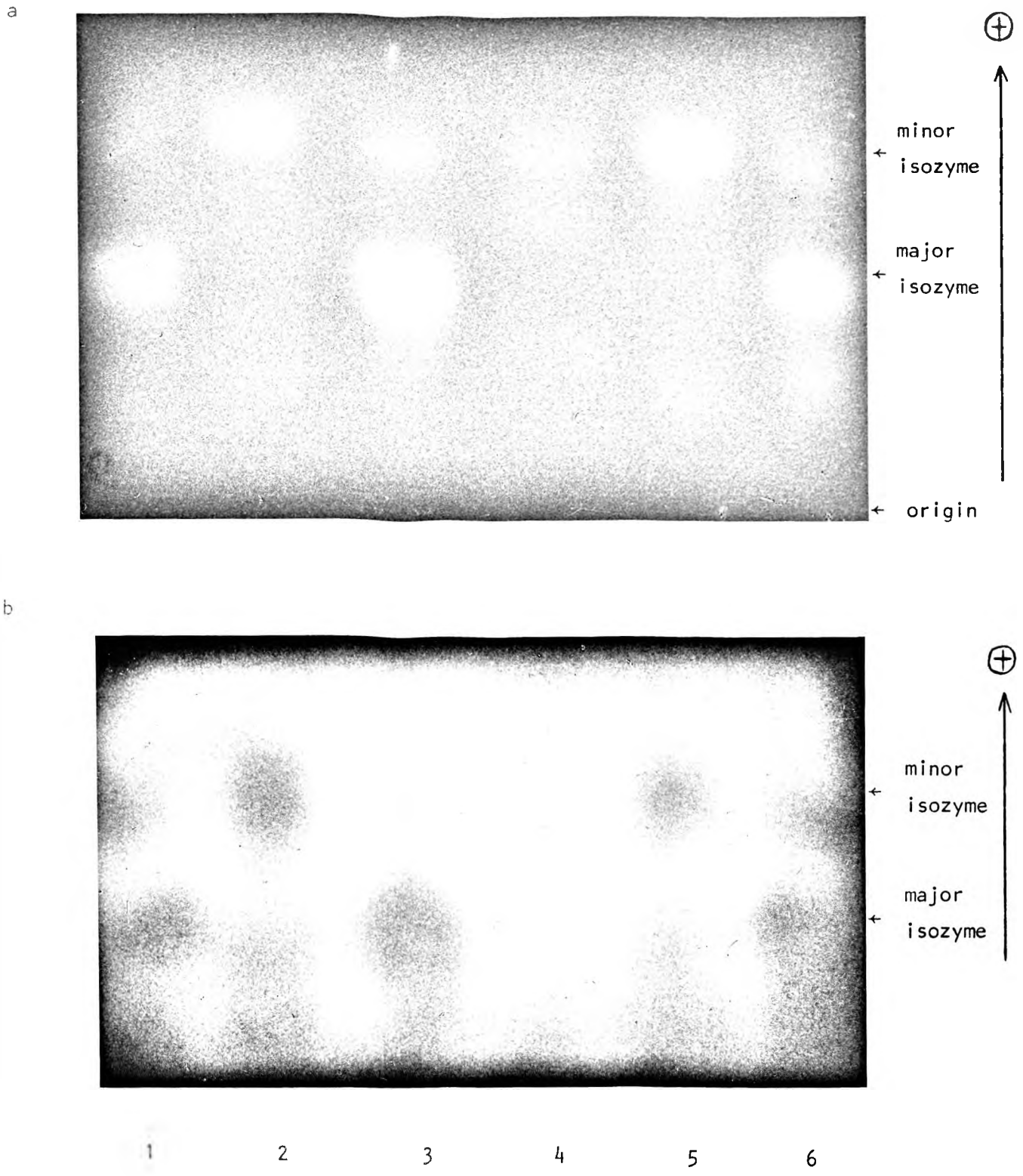


Figure 3.28. An electrophoretic demonstration of the correspondence between NADP-linked xylitol dehydrogenase activity and L-xylulose reductase activity. Lanes 1, 3 and 6 contained enzyme from a normal individual; lanes 2 and 5, a preparation of the minor isozyme from a normal individual and lane 4 enzyme from a pentosuric (H.K.).

(a) Bottom slice of the gel stained by xylitol dehydrogenase activity.
(b) Top slice of the gel stained by L-xylulose reductase activity.

catalyse the NADP-linked dehydrogenation of xylitol, also catalyse the reduction of L-xylulose. Two bands are visible in the lanes containing enzyme from pentosurics in Figures 3.26 and 3.28a. The top (anodal) band is the minor isozyme while the lower band may be an altered form of the major isozyme. The extra bands visible in Figures 3.28a and b may be deterioration products of the major and minor isozymes, but this is not certain. One of these bands, the shadow seen in lane 2 of Figure 3.28b at a position just below the level of the major isozyme band, was caused by the presence of a yellowish substance which apparently lacked L-xylulose reductase activity but which was accentuated when the gel was illuminated from below while being photographed.

3.3.4. The effects of various substances on enzyme activity

Initial assays of NADP-linked xylitol dehydrogenase activity were done (according to the recommendation of Wang and van Eys, 1970), in the presence of nicotinamide (0,714 mM in the 'glutathione' assay and 2,4 mM in the 'spectrophotometric' assay). Preliminary results (not presented) suggested that crude haemoglobin-free preparations from normal individuals had a higher enzyme activity when nicotinamide was left out of the assay system. The results presented in Table 3.5 show that nicotinamide concentrations of 1 and 10 mM markedly inhibited haemolysate NADP-linked xylitol dehydrogenase activity.

In spite of the fact that Hickman and Ashwell (1959) found 'no evidence for a metal cofactor' for guinea pig liver L-xylulose reductase, magnesium chloride has been included in L-xylulose reductase assay systems by a number of researchers (Freedberg *et al.*, 1959; Arsenis and Touster, 1969; Wang and van Eys, 1970). During the

TABLE 3.5. The effect of different nicotinamide concentrations on red cell NADP-linked xylitol dehydrogenase activity.

Nicotinamide concentration mM	Inhibition (%)
0	0
0,1	0
1,0	8,6
10,0	25,7

present study, no noticeable difference in activity was detected when dialysed crude haemoglobin-free preparations of enzyme from a normal individual were assayed in the presence and absence of 2,7 mM magnesium chloride. However, a small (1,4 per cent) increase in activity was observed when a 'desalted' preparation of the normal major isozyme was assayed in the presence of 5 mM magnesium chloride (see Table 3.6). Whether this apparent increase is significant is not known.

The effects of a number of other salts on the activity of the normal major isozyme were also investigated (see Figure 3.29). Only manganese chloride appeared to increase enzyme activity but this was found to be an artifact as the 'increase' would take place even in the absence of enzyme. It can also be seen from the Figure that the presence of EDTA appeared to protect the enzyme from inactivation by copper.

3.4. Enzyme storage and stabilisation

3.4.1. Storage in red cells, haemolysates and in concentrated ammonium sulphate solutions

The efficacy of different methods of storing crude L-xylulose reductase is shown in Figure 3.30. It can be seen that the enzyme lost activity rapidly when stored as part of a frozen haemolysate whereas full enzyme activity was maintained for seventeen days in ACD blood stored at 4°C. After this enzyme activity decreased, falling to eighty-four per cent after thirty-two days. Long term storage was best in frozen preserved red cells which lost less than eight per cent of their enzyme activity after 114 days storage. The storage of ammonium sulphate precipitated enzyme in solutions containing 2,95 M ammonium sulphate and 20 µM NADP is shown in Figure 3.31. It can be seen from the Figure that

TABLE 3.6. Activity of a 'desalted' preparation of the normal major isozyme in the presence and absence of 5 mM magnesium chloride

	Activity (µmols/hour/100 µℓ of enzyme solution)	
With added $MgCl_2$ (final concentration = 5 mM)	0,0134	0,0139
Without added $MgCl_2$	0,0135	0,0141

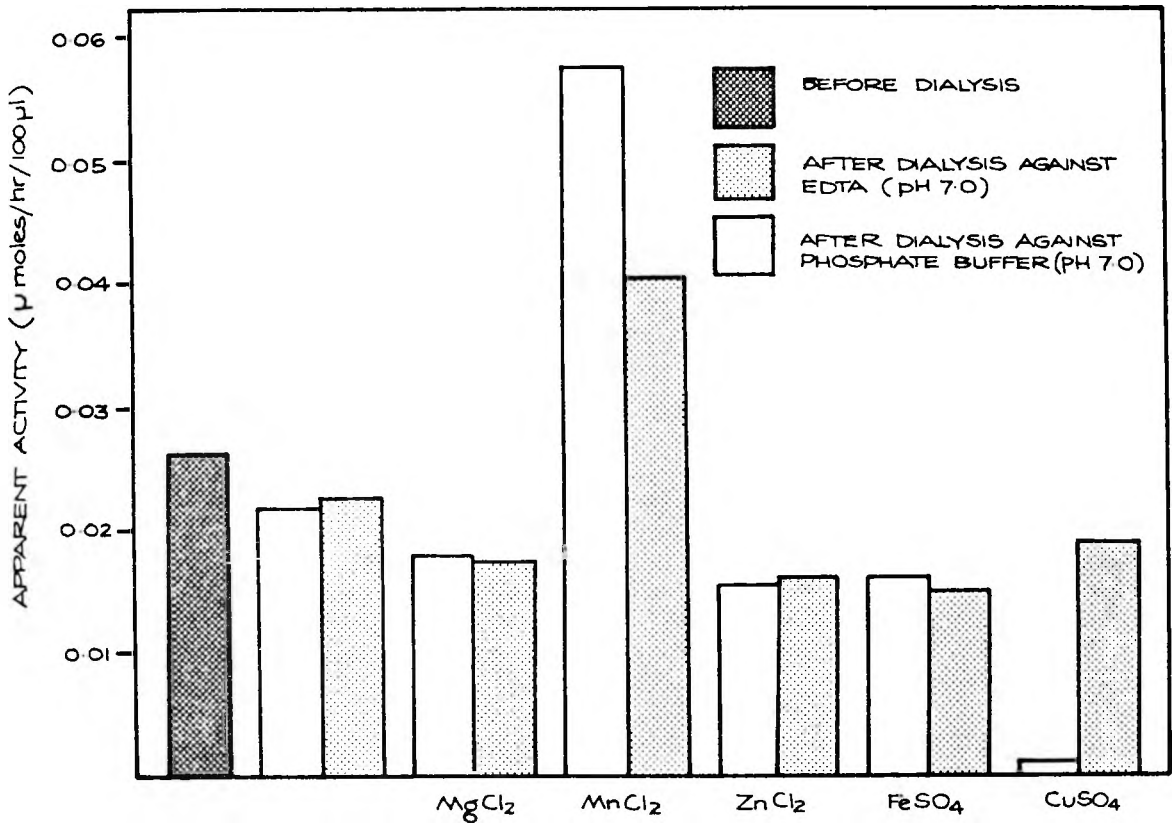


Figure 3.29. The effects of various salts on the xylitol dehydrogenase activity of the normal major isozyme. Enzyme was either dialysed against 2.7 mM EDTA (pH 7.0) or 10 mM sodium phosphate buffer (pH 7.0) and then stored for 1 hour at 0-4°C in the presence of various salts (1 mM final concentration) before being assayed.

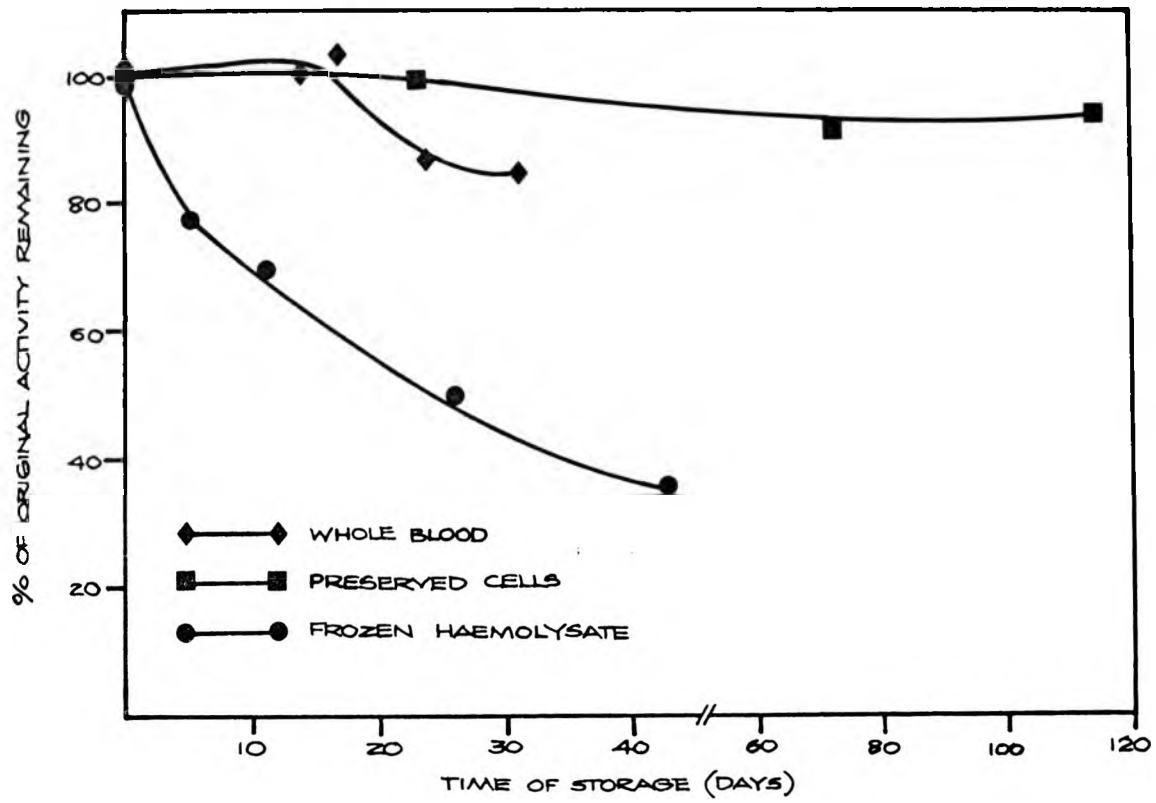


Figure 3.30. Enzyme activity remaining in preserved red cells, red cells of whole blood and haemolysates after various times of storage.

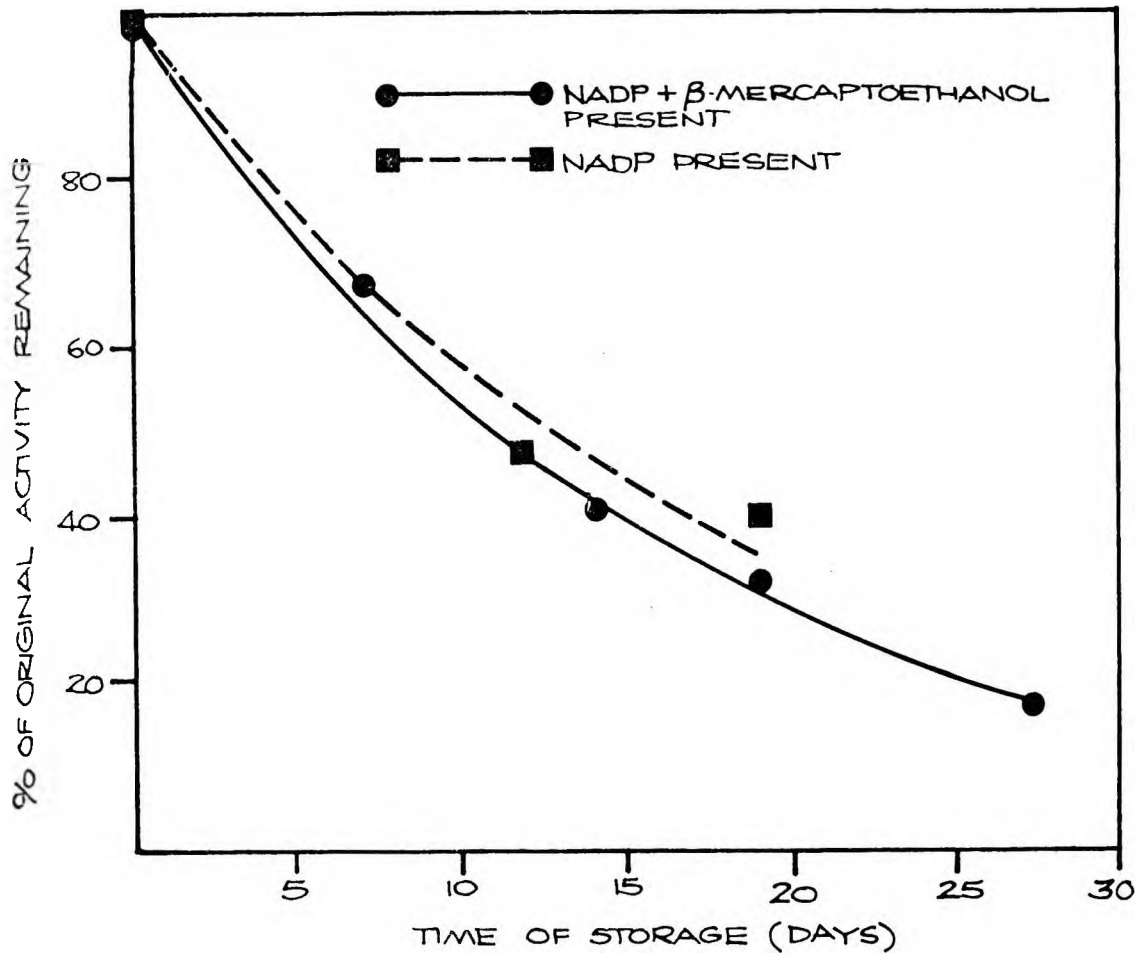


Figure 3.31. The decrease in activity with time of crude haemoglobin free preparations of normal red cell L-xylulose reductase when stored precipitated in 2.9 M ammonium sulphate (at ± 4°C in the presence of 20 µM NADP and 7 mM β-mercapto-ethanol).

the presence of 7 mM β-mercaptoethanol had no preservative effect on enzyme activity.

3.4.2. Enzyme stabilisation

The protective effect of NADP on L-xylulose reductase in preparations from normal individuals is demonstrated by the results shown in Figures 3.32 to 3.34 and Table 3.7. From Figure 3.32, it can be seen that the aliquots of enzyme solution which contained NADP during storage (at 30°C for fifteen hours) had more activity after storage than those which contained no NADP. Table 3.7 shows that relatively high NADP concentrations were able to safeguard enzyme in crude haemoglobin-free preparations from being inactivated by heat (48°C for twenty-five minutes) while Figure 3.33 illustrates that high NADP concentrations had the same effect on the normal major isozyme. Figure 3.34 shows how the normal major isozyme and enzyme in crude haemoglobin-free preparations from normals lost activity when heated in the presence of various lower concentrations of NADP. Heating resulted in an initial drop in activity in all samples (even at the highest NADP concentrations used). Apart from this, no further drop occurred in aliquots containing between 100 and 40 μM NADP but aliquots which contained less than 40 μM NADP showed a further drop in activity.

3.5 Population screening and family studies

3.5.1. Determination of the normal range of L-xylulose reductase activity

The range of L-xylulose reductase activities in haemolysates from the sample of non-Jewish individuals is presented in Figure 3.35. The

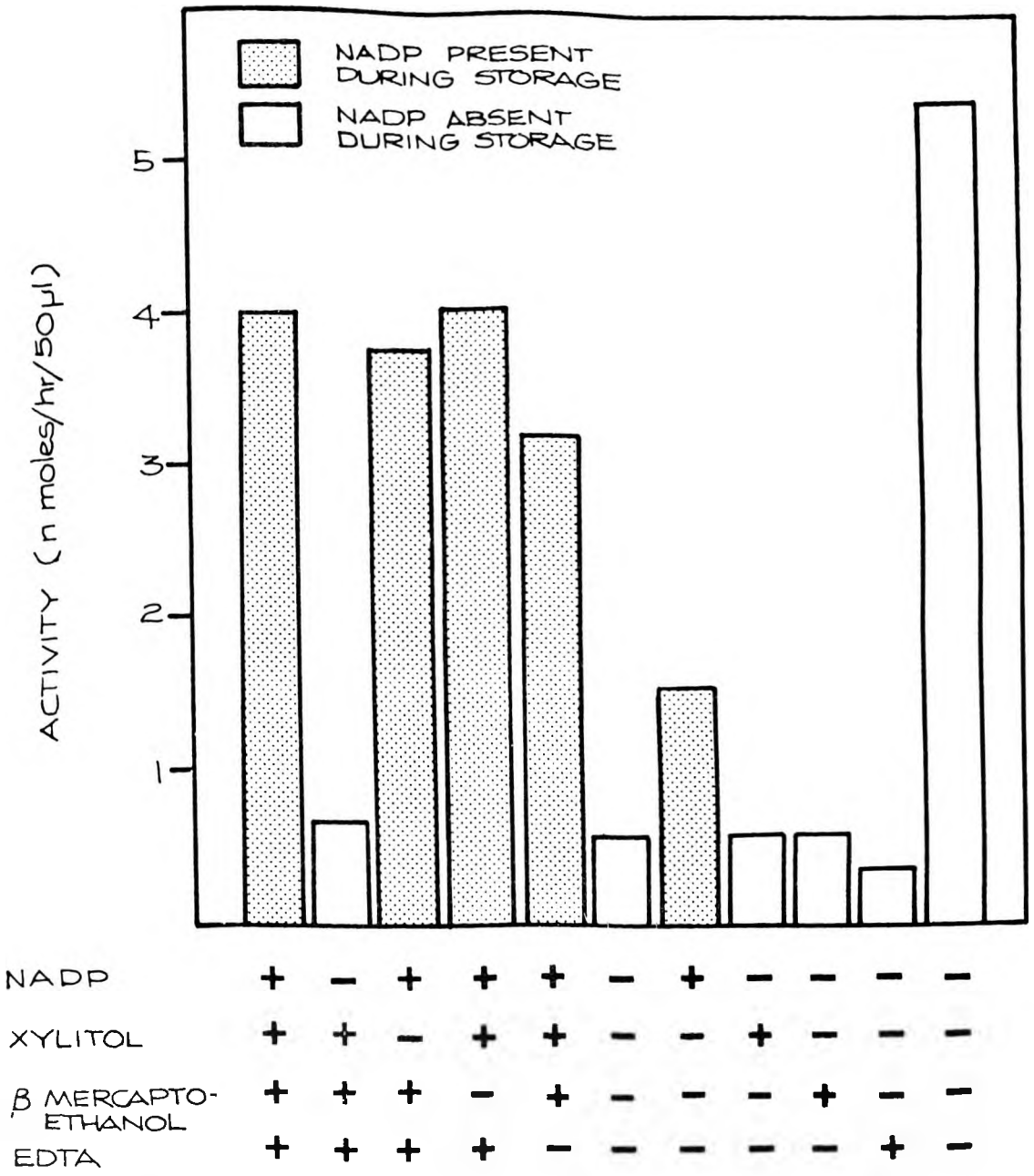


Figure 3.32. Results of the experiment designed to test different combinations of NADP, xylitol, β-mercaptoethanol and EDTA for their ability to protect L-xylulose reductase against inactivation during storage. The combinations of substances present during storage are indicated by the plus and minus signs beneath the histogram. Preparation 11 had no additives and was stored frozen at -20°C while the remaining preparations were stored at 30°C for fifteen hours. The initial activity of the enzyme preparation was 9.41 nmoles/hr/50 µl.

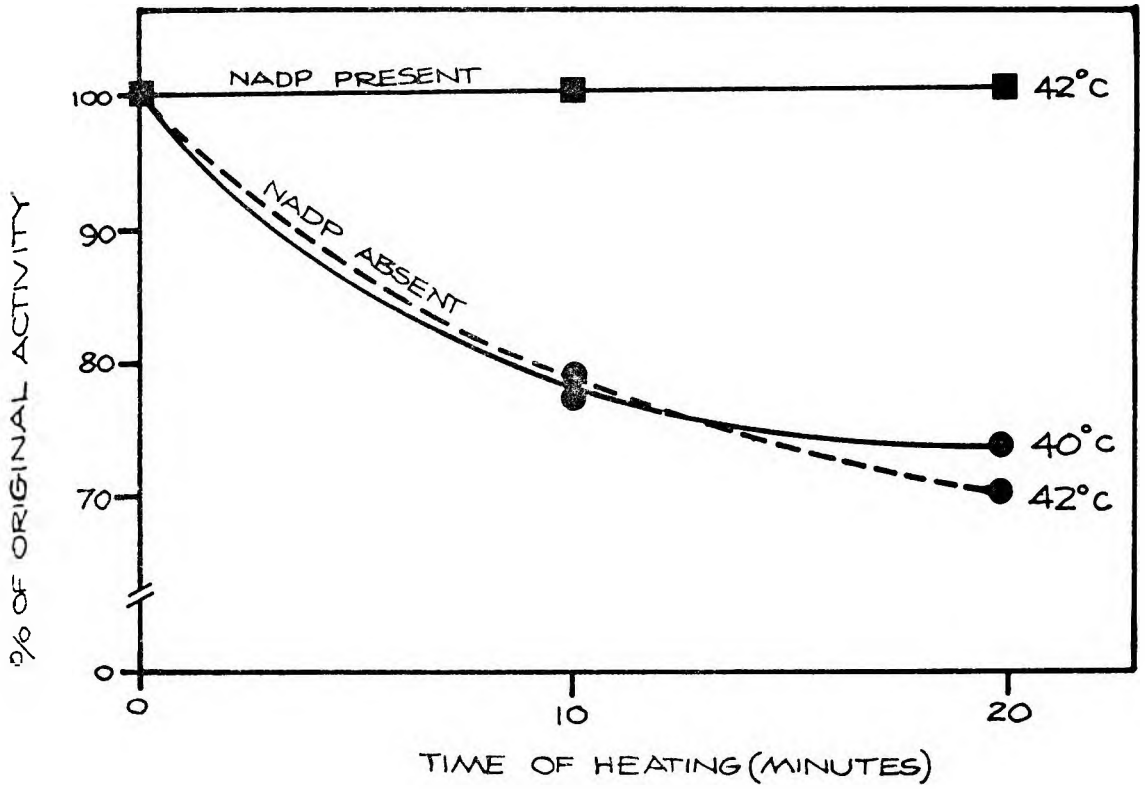


Figure 3.33. Effect of a relatively high NADP concentration (254 μM) on the stability of the normal major isozyme at various temperatures.

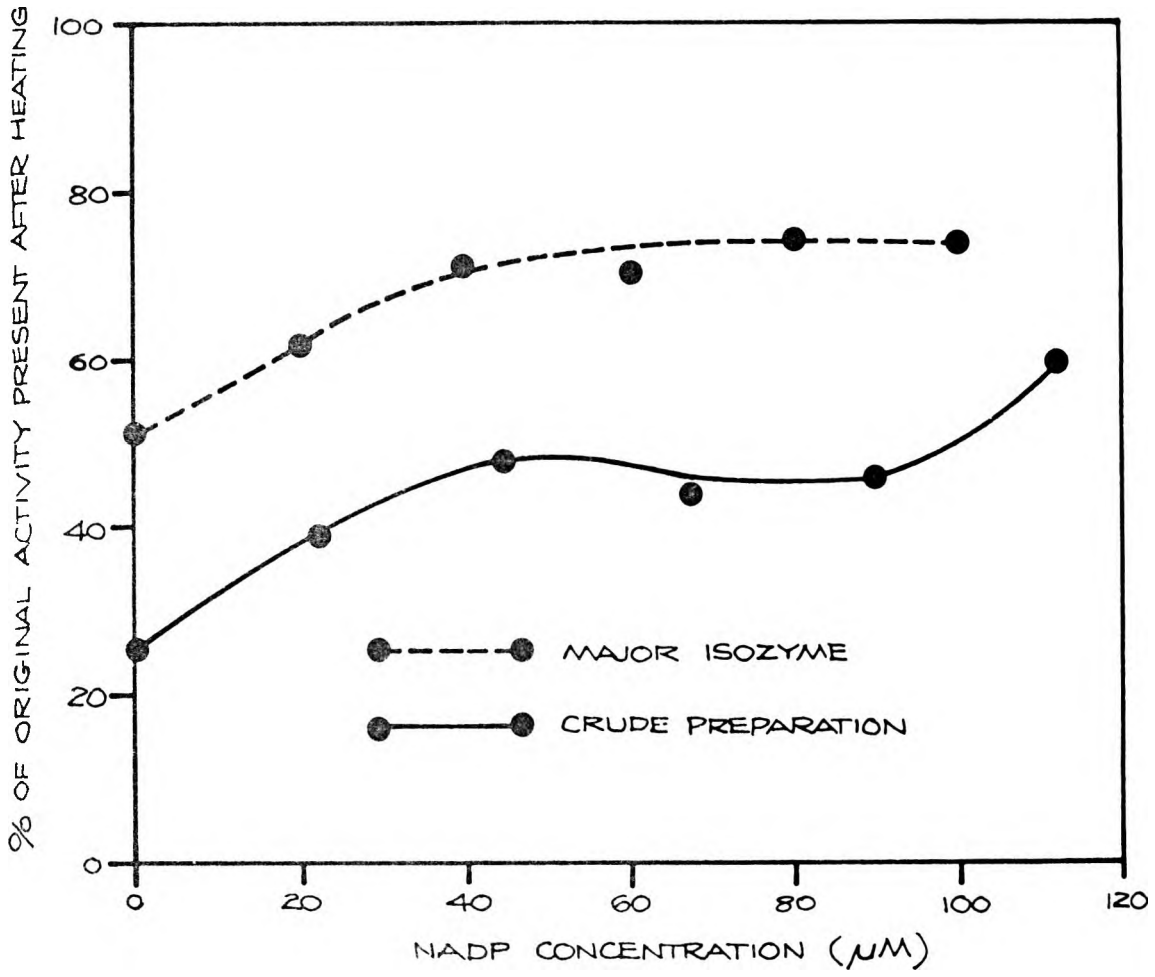


Figure 3.34. The effect of various concentrations of NADP on the stability of the normal major isozyme and on enzyme in a crude haemoglobin free preparation from a normal individual.

TABLE 3.7. Protection of L-xylulose reductase in a crude haemoglobin-free preparation from a normal individual against thermal inactivation (48°C for twenty-five minutes) by different concentrations of NADP.

NADP concentration µM	Activity before heating (nmols/hour/50 µℓ)	Activity after heating (nmols/hour/50 µℓ)	Apparent change in activity (%)
9,00	1,16	0,41	-64
69,25	1,32	1,27	- 4
129,50	1,31	1,41	+ 8

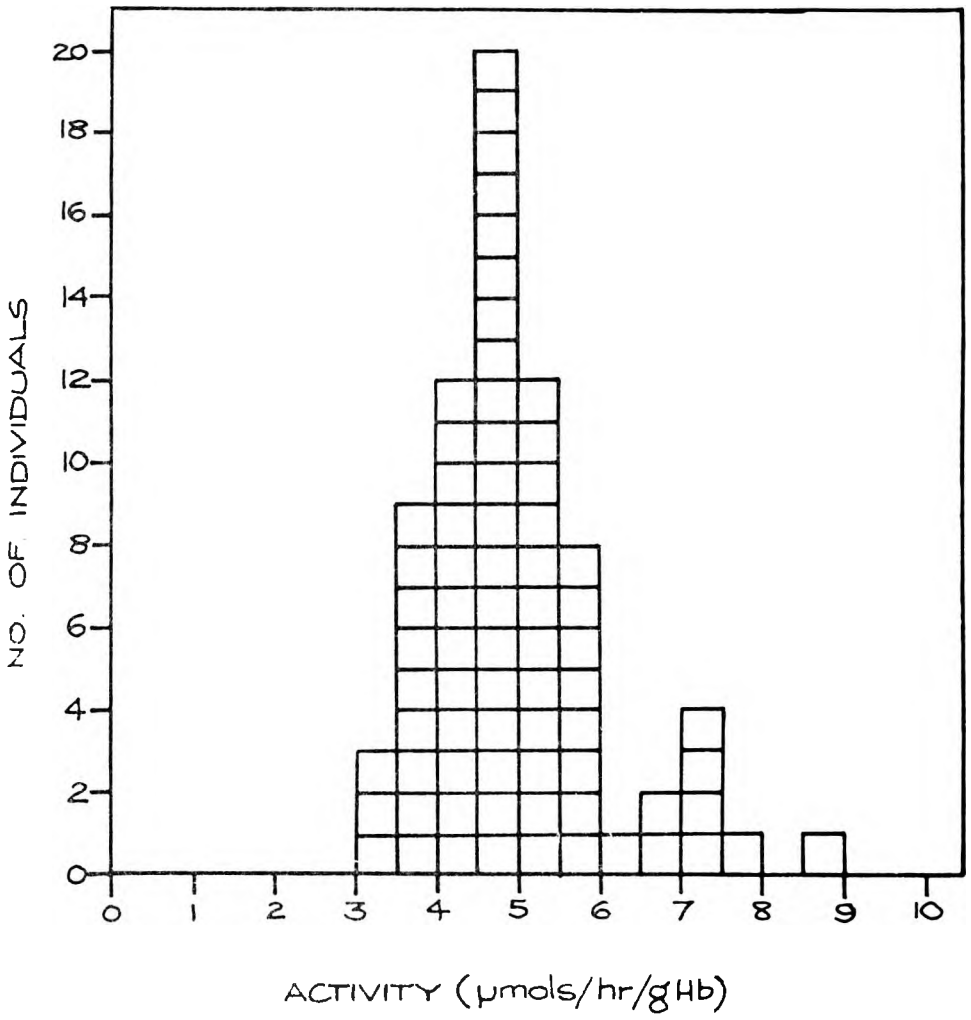


Figure 3.35. Distribution of haemolysate L-xylulose reductase activities in a sample of seventy four non-Jewish individuals as measured by the pyruvate method.

mean activity (± 1 SD) was 4,97 ± 1,10 µmoles/hour/g Hb. There is a suggestion of a second mode at approximately 7 µmoles/hour/g Hb which could be due to genetic variability or sampling and or experimental error.

3.5.2. Lebanese family study

After a normal range had been established, the pyruvate method was applied to samples from members of a family in which the pentosuria allele was known to be segregating (see Figure 3.36). Individual family members could on the basis of their red cell L-xylulose reductase activities be assigned to one of three categories:

1. Those who had normal levels of activity (within two standard deviations of the normal mean) and who were considered to be homozygous for the normal allele.
2. The proband who showed approximately zero activity under the conditions of the assay and who is considered to be homozygous for the pentosuria allele.
3. A group who had intermediate levels of activity (more than two standard deviations below the normal mean) and who are believed to be heterozygous for the normal and pentosuria alleles.

The suggestion made by Politzer and Fleischmann (1962) that pentosuria is dominantly inherited in this family was investigated by measuring serum L-xylulose levels in some of its members (see Figure 3.37). It was found that family members could be divided into only two classes on the basis of their serum L-xylulose levels:

1. The proband who showed the characteristic high L-xylulose level of a pentosuric, and
2. the remaining family members who had barely measurable serum (or plasma) L-xylulose levels.

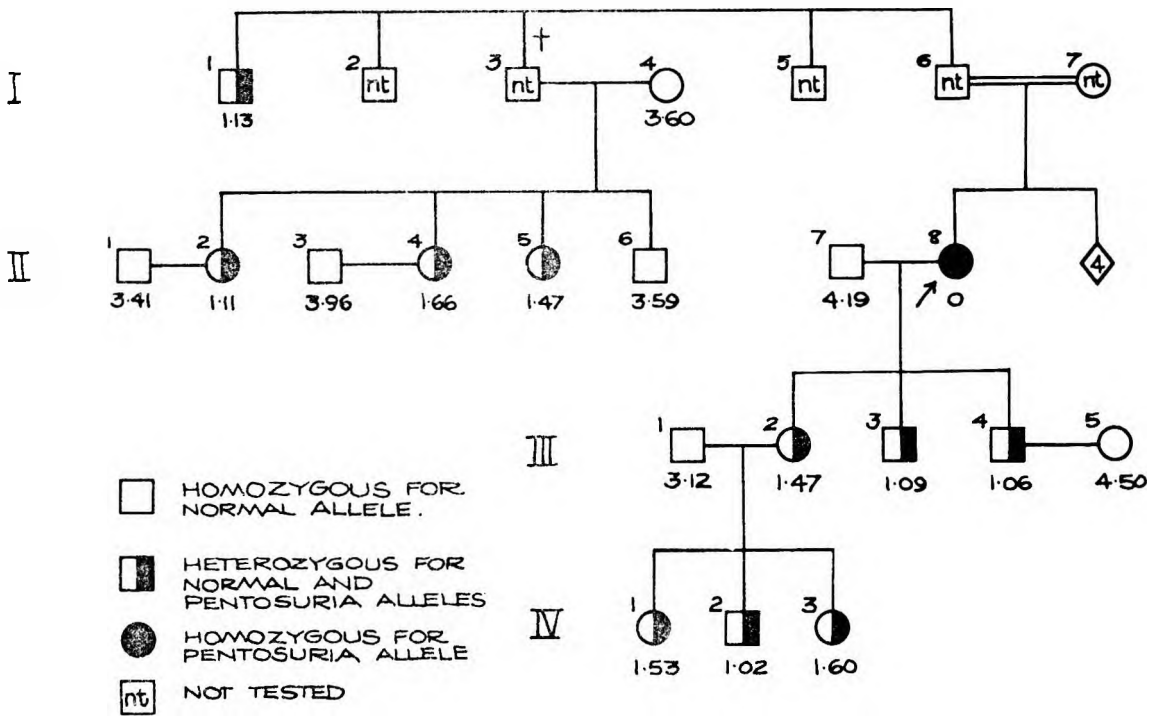


Figure 3.36. Haemolysate L-xylulose reductase activities (μ moles/hr/g Hb) in samples from the Lebanese pentosuric (H.K.) and some of her relatives (mean activity ± 1 SD for normals was 4.97 ± 1.09 μmoles/hr/g Hb). Note that individuals I-6 and I-7 are related to each other (first cousins).

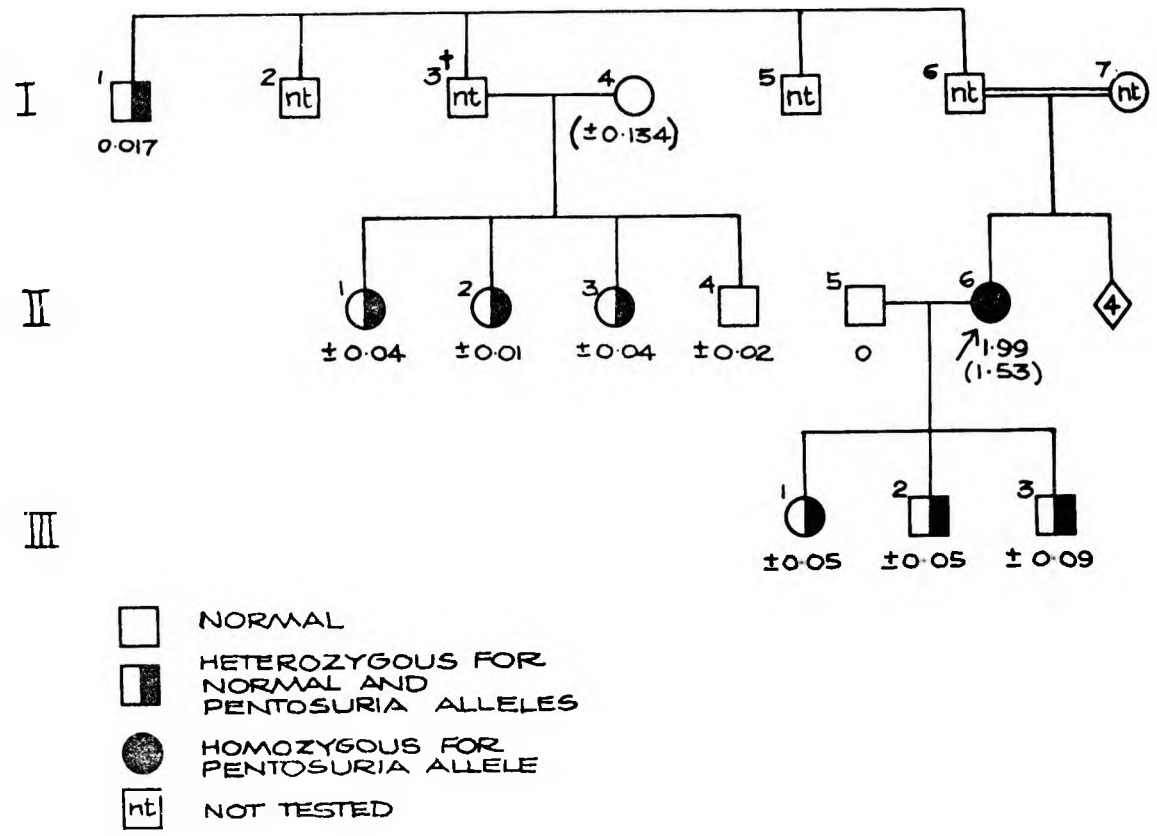


Figure 3.37. Part of the Lebanese family pedigree showing serum L-xylulose concentrations (mgs/100 ml) in samples from various family members. The values found in plasma are shown in brackets. Genotypes were assigned on the basis of red cell L-xylulose reductase activities.

The Ashkenazi pentosuric's plasma L-xylulose level was measured for the sake of comparison and found to be 1,54 mg/100 mℓ which was almost identical to that found in the plasma of the Lebanese pentosuric (1,53 mg/100 mℓ). Although several members of the Lebanese family had L-xylulose reductase activities which were more than two standard deviations below the normal mean, only one of these, the proband (who showed approximately zero activity) had the characteristic high serum L-xylulose level of a pentosuric. It seems, therefore, that pentosuria is inherited as a recessive in this family too.

3.5.3. Ashkenazi-Jewish sample

Results obtained when a sample of the local Ashkenazi-Jewish population was screened for pentosuria carriers appear in Figure 3.38. The mean red cell L-xylulose reductase level (± 1 SD) was 4,97 ± 1,08 µmoles/hour/g Hb which is almost identical to that found in the non-Jewish sample (4,97 ± 1,10 µmoles/hour/g Hb). The distribution of activities in the Ashkenazi sample, however, differs from that of the non-Jewish sample in that there appears to be a second mode of low activities which is largely due to the presence of the pentosuria allele in this population.

The results of the D-glucuronolactone loading test subsequently carried out on six of the seven individuals comprising the low activity group are presented in Table 3.8. It can be seen that all but one of them showed large increases in serum L-xylulose concentrations in response to the load of the L-xylulose precursor administered. The increases recorded are similar to those found by Kumahara *et al.* (1961) for heterozygotes.

If it is assumed that none of the individuals whose activities fell

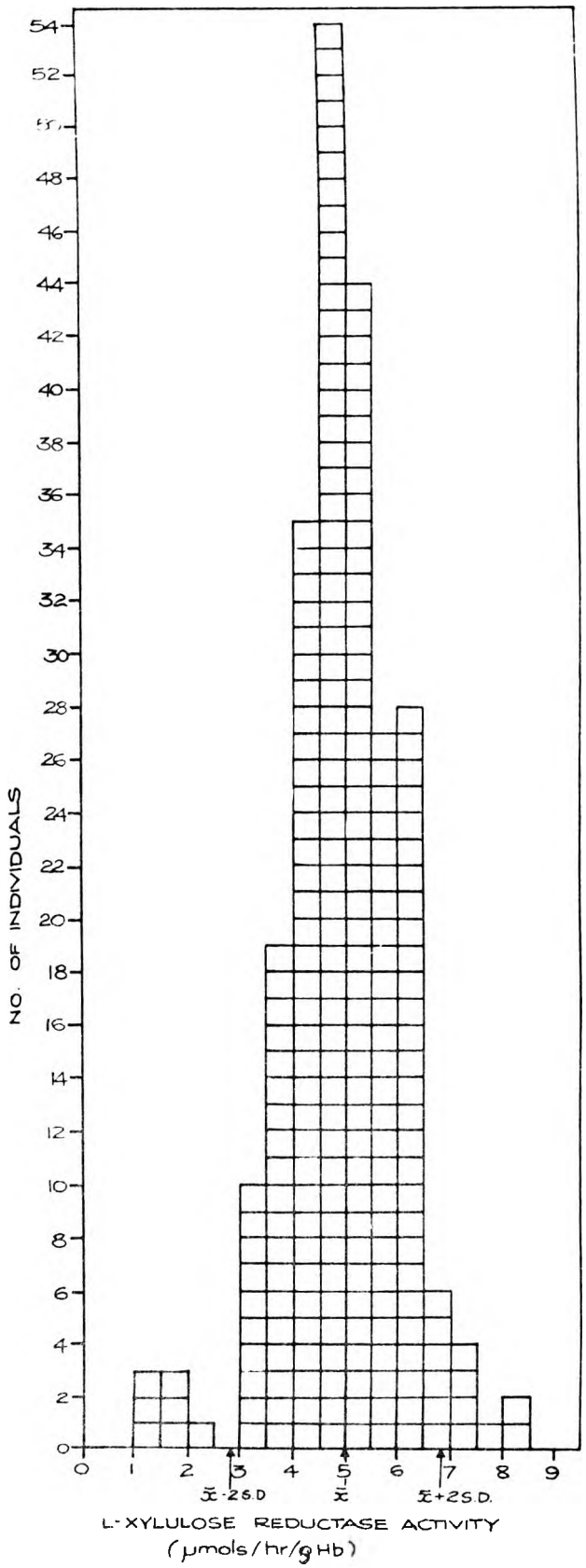


Figure 3.38. Distribution of haemolysate L-xylulose reductase activities in a sample of 237 Ashkenazi-Jewish medical students.

TABLE 3.8. Serum L-xylulose concentrations of six individuals comprising the low activity group and six non-Jewish controls before and after administration of D-glucuronolactone. The mean increase (± 1 SD) in the low activity individuals (excluding subject CZ) was 0,708 ± 0,291 and that in the control group 0,195 ± 0,062; $t = 4.25$, $p<0.01$.

Subjects	Serum L-xylulose (mg/100 mℓ)		
	Before glucuronolactone	One hour after glucuronolactone	Increase
Low activity individuals			
FA	0,053	1,204	1,151
PJ	0,166	0,577	0,411
DM	0,115	0,702	0,587
OO	0,113	0,949	0,836
MR	0,200	0,753	0,553
CZ	0,108	0,101	-
Non-Jewish controls			
SH	0,096	0,335	0,239
TJ	-	0,195	0,195
AL	0,022	0,306	0,284
DL	0,040	0,185	0,145
KR	0,088	0,281	0,193
MR	0,105	0,218	0,113

about the second (high activity) mode are heterozygous for the pentosuria allele, then the frequency of this allele as estimated from the apparent heterozygote frequency is 0,0127.

3.5.3.1. Investigation of relatives of the low activity individuals

The parents of four of the 'low activity' individuals identified in the Ashkenazi-Jewish sample were available for investigation and in each case one of them also had a L-xylulose reductase level which was more than two standard deviations below the Ashkenazi sample mean (see Figure 3.39). A fifth individual's parents were not available, but some of his relatives, representing three generations of this family showed L-xylulose reductase levels which were similar to his see Figure 3.40).

3.5.4. Linkage analysis

The segregation of the pentosuria allele and alleles of the polymorphic systems listed in Section 2.6.4 was followed in six families (five Ashkenazi and one Lebanese). No close linkage between the major L-xylulose gene locus and any of the other polymorphic loci investigated was apparent (see Table 3.9). The highest lod score obtained was 0,795 in favour of linkage with the 6-phosphogluconate dehydrogenase locus. Of the fifteen polymorphic systems for which lods could be calculated, seven showed some positive scores. The 'positive score systems' can be ranked in order of the numbers of offspring used in the calculation of the lods so that some idea of their possible significance can be obtained. The lods in favour of linkage with GPT_1 were calculated from matings which yielded twenty-two offspring. PGM_1 was next with twenty offspring, then *Gc* and *Bf* with twelve offspring each followed by *MNSs* with eleven and *PGD* with four offspring.

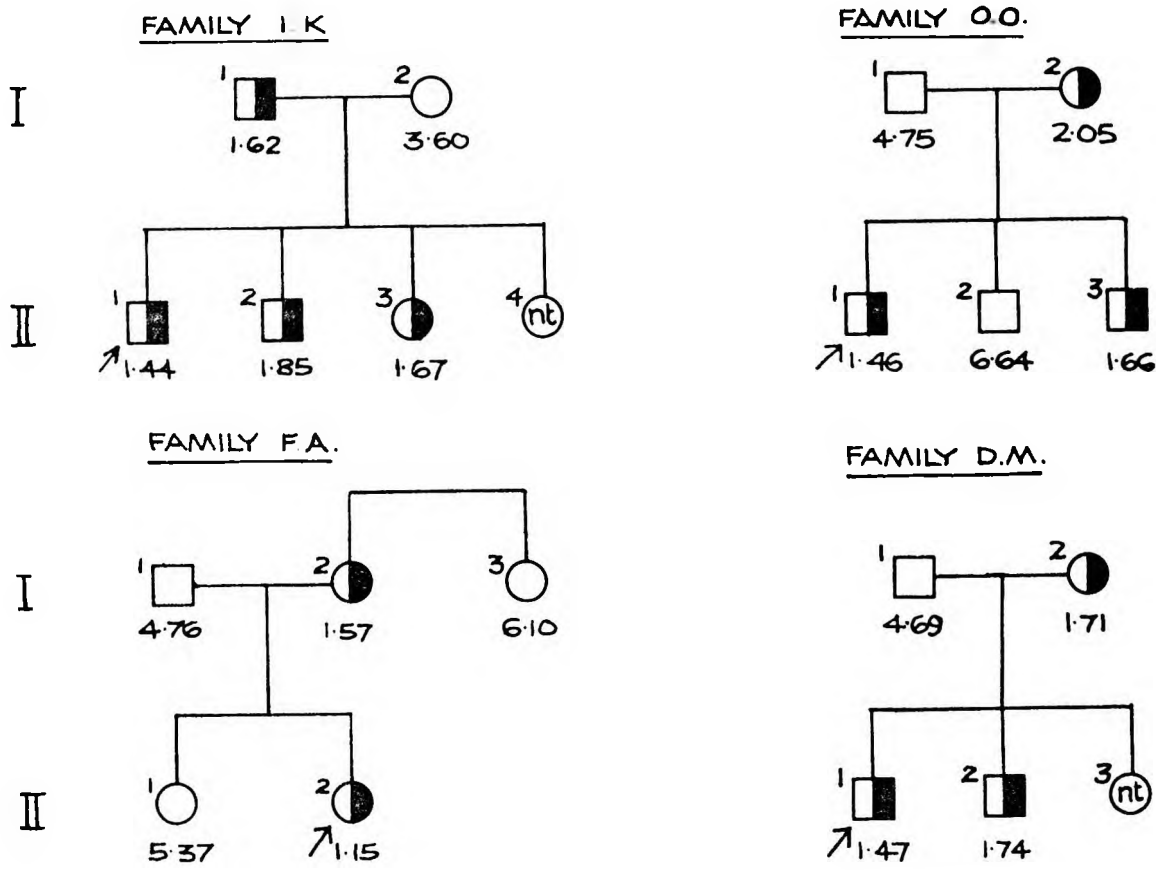


Figure 3.39. Pedigrees of four of the Ashkenazi-Jewish students who are believed to carry the pentosuria allele. Haemolysate L-xylulose reductase activities (μ moles/hr/g Hb) appear below the symbols. The activity level for normals was 4.07 ± 1.09 units; nt = not tested.

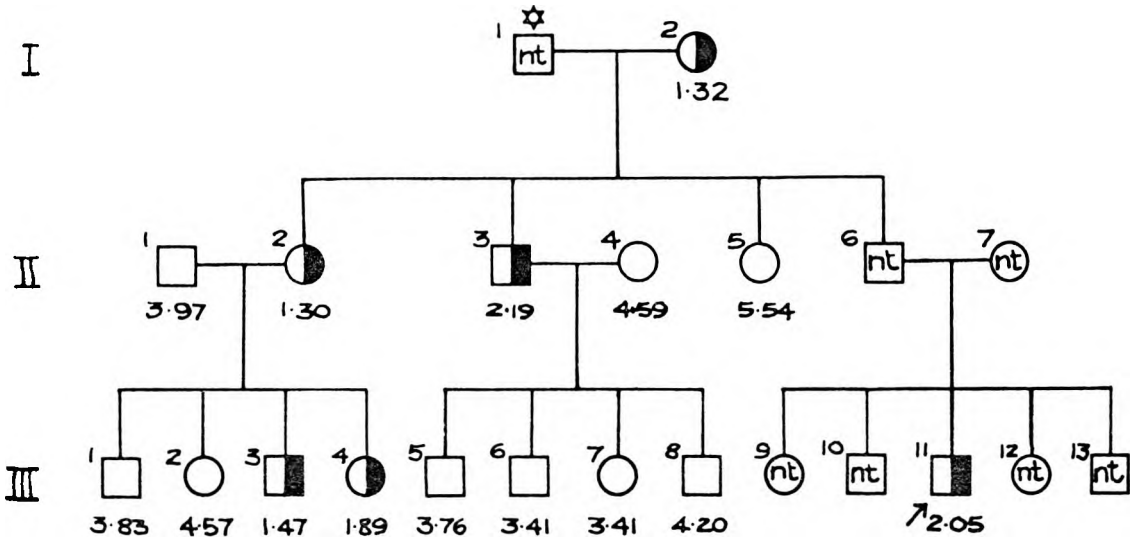


Figure 3.40. Pedigree of Ashkenazi-Jewish student M.R. Haemolysate L-xylulose reductase activities (μ moles/hr/g Hb) appear below the symbols representing those individuals studied. The mean activity level found for normals was 4.97 ± 1.09 units; nt = not tested.

TABLE 3.9. Lod scores determined for pedigrees in which the pentosuria allele is segregating.

Locus	Number of families	Number of children	Assumed recombination fraction				
			0,05	0,1	0,2	0,3	0,4
ABO	1	2	0,154	0,131	0,085	0,041	0,011
Duffy	1	2	-0,825	-0,528	-0,243	-0,099	-0,024
Kell	2	5	-1,361	-0,833	-0,364	-0,144	-0,034
MNSs	4	11	-0,433	-0,052	0,139	0,111	0,038
Rh	3	17	-3,770	-2,215	-0,898	-0,324	-0,060
ACP_1	1	4	-1,461	-0,899	-0,392	-0,152	-0,035
AK_1	1	3	-0,536	-0,305	-0,121	-0,045	-0,010
ESD	1	2	0,154	0,131	0,085	0,041	0,011
GLO_1	2	6	-1,069	-0,580	-0,202	-0,064	-0,012
GPT_1	5	22	-2,185	-0,983	-0,078	0,164	0,138
PGD	1	4	0,795	0,708	0,513	0,297	0,094
PGM_1	4	20	-1,174	-0,307	0,211	0,219	0,078
Hp	2	14	-0,825	-0,528	-0,243	-0,099	-0,024
Gc	1	12	-0,060	0,159	0,284	0,263	0,164
Bf	1	12	0,007	0,006	0,004	0,003	0,001

The *ABO* and *ESD* are last with two offspring each. Two of the loci for which positive scores were obtained occur on the short arm of chromosome 1. They are *PGD* which is situated between the p terminus and p34, and PGM_1 which is situated at p22.1 (Human Gene Map, 1983).

CHAPTER 4

4. DISCUSSION

4.1. Enzyme studies

Since the demonstration by Wang and van Eys (1970) that a deficiency of red cell NADP-dependent xylitol dehydrogenase activity occurred in pentosurics and that a decreased level existed in a heterozygote, the identification of heterozygotes by their low red cell xylitol dehydrogenase activities has appeared to be feasible. The main difficulty with this approach is that red cell enzyme activities, even of normal individuals, are very low. As an indication of this, Kumahara *et al.* (1961) failed completely to detect NADP-linked xylitol dehydrogenase activity in red cells when surveying various human 'tissues and secretions' for its presence. Wang and van Eys (1970) reported a mean level for normals of only 29 nmoles/minute/g Hb (1,74 µmoles/hour/g Hb) which, for the sake of comparison, is approximately 1/400th the haemolysate activity of the familiar enzyme glucose-6-phosphate dehydrogenase (Beutler, 1975). The mean haemolysate L-xylulose reductase activity for normals as measured by the pyruvate method in the present study was found to be 4,97 µmoles/hour/g Hb.

Preliminary attempts to assay the NADP-linked xylitol dehydrogenase activity of crude haemolysates, by direct spectrophotmetric monitoring of NADPH produced through its action, were unsuccessful because of the low activities combined with the relatively high haemoglobin concentrations. The glutathione method (Wang and van Eys, 1970) and the pyruvate method could be successfully applied because these assay systems allow relatively large amounts of haemolysate to be used without interference by haemoglobin because the latter is precipitated before the reaction product is measured.

Results obtained by the glutathione method confirmed Wang and van Eys's finding that pentosurics have a deficiency of NADP-linked xylitol dehydrogenase activity, while measurements made by the newly developed pyruvate method demonstrated that the observed deficiency of xylitol dehydrogenase activity corresponds to a deficiency of L-xylulose reductase activity which is the actual cause of essential pentosuria.

4.1.1. Kinetic studies on crude preparations of enzyme

Kinetic studies carried out by Wang and van Eys (1970) led them to the conclusion that the residual enzyme of pentosurics was altered in a way which lowered its affinity for NADP and that this, or rather its implied impaired inability to bind NADPH (they did not follow the reaction in the forward, *in vivo* direction), resulted in the enzyme being unable to adequately catalyse the dehydrogenation of L-xylulose. They state:

> "The Michaelis constant for xylitol on the NADP-linked dehydrogenase in the pentosuric blood appeared normal.... Therefore the decreased NADP affinity of the enzyme is apparently the molecular abnormality that causes the enzymatic disorder in pentosuria."

Attempts to verify this were hampered by the haemoglobin in haemolysates and so a batch method for its removal was developed and kinetic studies carried out on the resulting crude haemoglobin-free preparations.

Michaelis constants were initially measured at pH 7,0, the pH at which Wang and van Eys (1970) obtained the best discrimination between normals and pentosurics and the pH at which they carried out their Michaelis constant studies. The first surprising result obtained was that enzymes from normals and pentosurics showed

similar K_m values for NADP, a finding which was at variance with that of of Wang and van Eys (1970). The second significant finding was that enzyme prepared from pentosurics had a much higher K_m for xylitol than the enzyme obtained from normal individuals and this led in turn to the finding that the enzyme from pentosurics also had a much higher K_m for L-xylulose. It is realised that estimates of the K_m for NADP of the enzyme from pentosuric subjects are likely to be inaccurate because the concentration of the second substrate, xylitol, could not be raised to levels which would have been saturating for the enzyme. Because of this, it was not strictly correct to regard the enzyme as if it were catalysing a single substrate reaction when arriving at an estimate of the K_m.

The influence of pH on the rates at which red cell L-xylulose reductase catalyses the forward and reverse reactions was then investigated using crude haemoglobin-free preparations from normal individuals. Reaction rates at three different xylitol (for the reverse reaction) and L-xylulose (for the forward reaction) concentrations were measured at each pH so that theoretical V_{max} values could be estimated from Lineweaver-Burk plots. This procedure was followed because the substrate affinities of many enzymes change with changing pH (Dixon and Webb, 1964) and it is therefore desirable to either work with saturating substrate concentrations if this is possible or, if it is not, to calculate theoretical V_{max} values. NADP and NADPH concentrations were kept constant in the pH-activity studies because the enzymes have high affinities for these substrates and it is likely that the concentrations of these substances were saturating over the range of pH values used. The range of pH values used in these investigations was limited in the acid region by the instability of NADPH. The relationship between pH and xylitol dehydrogenase activity of enzyme from a pentosuric was investigated at only one xylitol concentration because of a lack

of enzyme. The pH-activity curve obtained is, therefore, only a rough representation of the actual relationship in this case. The K_m for NADPH of the enzyme from pentosurics was not measured because this would have required saturating concentrations of L-xylulose, the second substrate, and this was in short supply.

Results of the first set of pH-activity studies indicated the need for further K_m studies at pH values different from 7,0. The K_m for xylitol of the enzyme from pentosurics was compared with that of enzyme from normal individuals at a reaction pH of 9,5 which appears to be close to the pH optimum of the 'pentosuric enzyme'. Further, pH-activity studies indicated that the Michaelis constants for xylitol and L-xylulose of enzyme from normal subjects changed radically with pH. Estimates of the Michaelis constants for these substrates were accordingly made at pH values where possible variations in pH would have had minimal effects (pH 9,0 for xylitol and NADP and pH 7,0 for L-xylulose and NADPH).

4.1.2. Chromatography and electrophoresis

After NADP had been found to stabilise the xylitol dehydrogenase of crude haemoglobin-free preparations from normal individuals, attempts were made to find a concentration of NADP which would provide adequate protection for the enzyme but which would not be too expensive to use. Experiments aimed at finding such a concentration showed that the relationship between the enzyme's thermolability and the NADP concentration was not a simple one. It was thought that this could be due to the presence of more than one enzyme in the crude preparations, each with a different degree of stability at different NADP concentrations. Crude haemoglobin-free preparations

from the red cells of normals were subsequently chromatographed in columns of CMC equilibrated to pH 5,7. Two well separated peaks of activity were found to elute from the columns; the first (called the minor isozyme) eluted immediately after the void volume and the other (called the major isozyme), further along the applied NaCl gradient. Crude enzyme preparations from pentosuric subjects contained only the isozyme corresponding to the first peak (the minor isozyme) while a preparation from the daughter of one of the pentosurics showed both peaks, but with the second peak (the major isozyme) reduced. These findings were confirmed by electrophoresis performed on haemolysates obtained from normals, pentosurics and heterozygotes. Ironically, the NADP-stability curve obtained with preparations of the major isozyme of normals still had the same shape as that found with crude preparations. Haemolysates electrophoresed and stained at high concentrations of xylitol (reverse reaction) or L-xylulose (forward reaction) showed that the minor isozyme of normals and the enzyme from pentosurics migrate at similar if not identical rates. A number of other faint bands could also be seen on zymograms which had been stained at high xylitol concentrations. The significance of these bands is not understood although it is possible that the faint band just cathodal to the 'fast band' of pentosurics (see Figures 3.26 and 3.28a) is an altered form of the major isozyme.

Electrophoresis of liver NADP-linked xylitol dehydrogenases from three individuals who were presumably non-pentosurics revealed in each case the presence of two isozymes which occur in similar proportions to those of red cells. Although minor differences in the migration rates of the liver and red cell isozymes were apparent, it seems likely that these respective isozymes are coded by the same

genes and then post-transcriptionally modified in different ways in liver and red cells or reticulocytes. There are precedents for this: adenosine deaminase for instance exists in different (inter-convertible) forms in various human tissues (Akedo *et al.*, 1972; Hirschhorn, 1975). β-Hexosaminidase B is present in one form in a variety of tissues but in several forms in plasma (Price and Dance, 1972).

4.1.3. The effect of various salts and of nicotinamide on enzyme activity

Among the minor points checked during this study, was whether the addition of magnesium chloride to assay systems was necessary. All previously published assays (Hollmann and Touster, 1956; Hickman and Ashwell, 1959; Freedberg *et al.*, 1959; Wang and van Eys, 1970) incorporated magnesium chloride but there are conflicting reports about whether its presence is necessary for the full activation of at least one L-xylulose reductase, that from guinea pig liver (Hickman and Ashwell, 1959; Arsenis and Touster, 1969). The inclusion of magnesium chloride in assay mixtures which have high pH values, results in the formation of a precipitate which interferes with the spectrophotometric monitoring of reactions and it was left out in pH optimum determinations for this reason. Although the methods used to remove magnesium chloride from enzyme solutions in experiments aimed at determining whether its presence is necessary (dialysis against EDTA and chromatography on Sephadex G-25 columns) may not have been completely successful, it seems likely that its addition to assay systems is not essential. In the experiment in which enzyme was either dialysed against EDTA or phosphate buffer before being incubated with various salt solutions, the preparation which had been dialysed against EDTA showed approximately

the same activity as that which had been dialysed against phosphate buffer. None of the salt solutions (including magnesium chloride) appeared to activate the enzyme even though manganese chloride was thought, initially, to produce such an effect. Subsequent experiments showed, however, that this was an artifact.

Wang and van Eys (1970) claimed that nicotinamide increases red cell NADP-linked xylitol dehydrogenase activity and, accordingly, included it in their 'glutathione' and 'spectrophotometric' assays at concentrations of 0,14 and 2,5 mM respectively. It was found during the present study that the omission of nicotinamide had little effect on activities as measured by the glutathione method but that the presence of this substance at concentrations of 1,0 and 10,0 mM caused a definite inhibition of product formation.

Addition of β-mercaptoethanol to assay systems had no effect and neither did its presence in suspensions of ammonium sulphate-precipitated enzyme noticeably prevent loss of activity during storage.

4.1.4. Kinetic studies on the major and minor isozymes of normal individuals

When it was found that at least two different L-xylulose reductases exist in red cells of normal individuals (the major and minor isozymes), additional kinetic studies were carried out on the separate enzymes. Results obtained with the major isozyme were similar to those obtained with crude (haemoglobin-free) preparations from normal individuals. This was not surprising because the minor isozyme (judging from the electrophoretic study) appears to make up only a small proportion of normal haemolysate activity, the balance

being due to the major isozyme.

The kinetic studies carried out on the minor isozyme of normal individuals yielded results which (as expected) were similar to those obtained with enzyme from pentosurics. The fact that the enzyme of pentosurics and the minor isozyme of normals are present at similar levels of activity in haemolysates, that they migrate at the same rate when electrophoresed and chromatographed and have similar substrate affinities and pH optima, strongly suggests that these are one and the same enzyme.

There are a number of possible explanations for the isozyme patterns observed in pentosurics and normals (see Figure 4.1). The single structural gene models involve a possible processing gene and are thought to be less likely explanations than the two gene models, since processing defects could be expected to cause changes in the amounts of the minor isozyme produced, something which was not apparent in samples from pentosurics. In addition, carriers of the pentosuria allele were found to have decreased amounts of the major isozyme, i.e. the pentosuria allele is codominant in effect at this level which is contrary to what would be expected in a case of a partial deficiency of a processing enzyme. Paigen (1979) makes the following observation with regard to this:

> "Fortunately, a simple test can almost always discriminate whether the mutation has occurred in the structural gene or at a locus concerned with post-translational processing. Mutations in structural loci are almost invariably expressed codominantly, whereas mutations in processing loci are generally dominant or recessive."

The simplest of the two structural gene models would seem to be the most likely since the more complex 'subunit model' should logically be extended to include a third isozyme (β_n) which

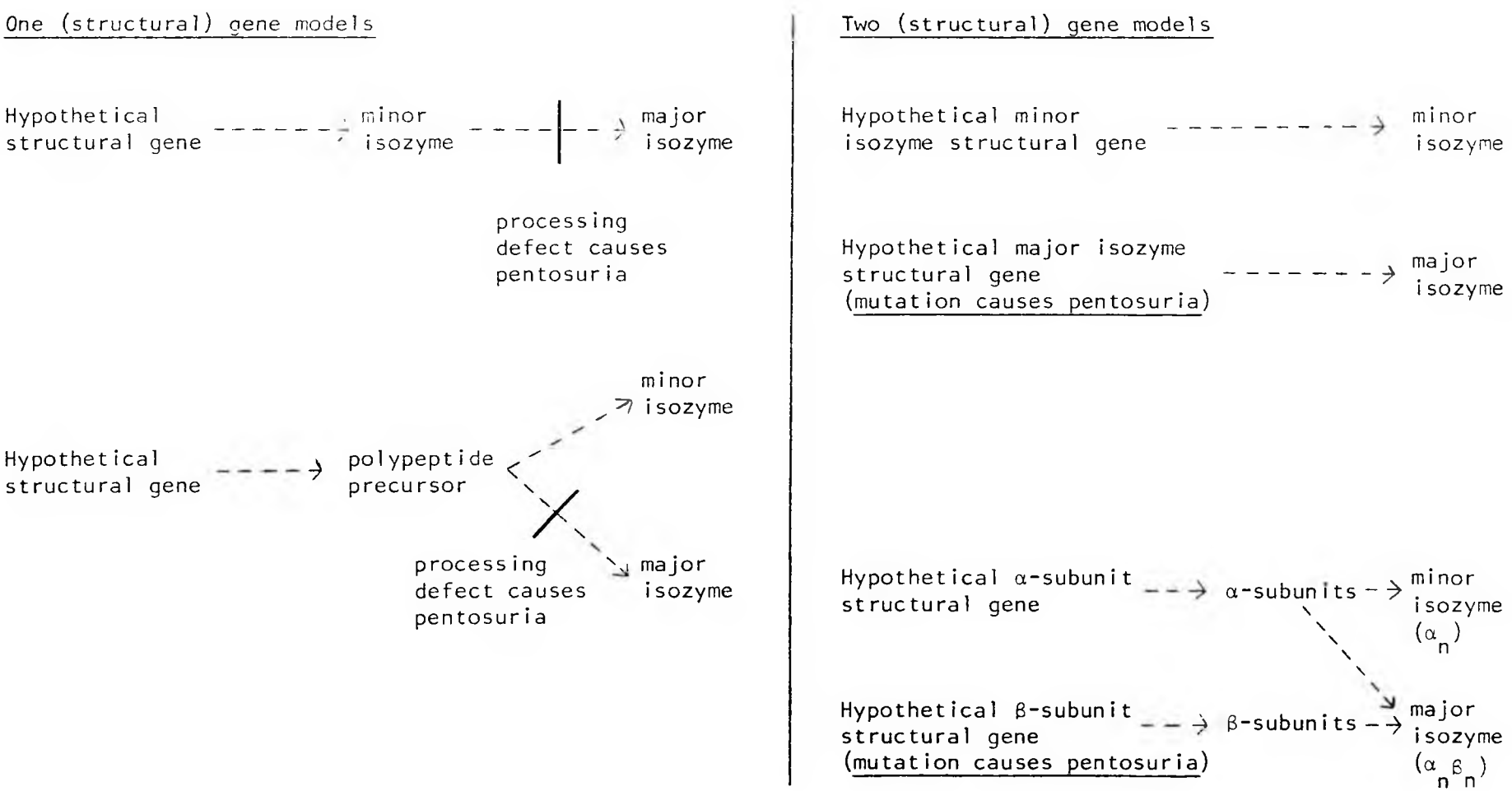


Figure 4.1. Possible explanations for the isozyme patterns observed in pentosurics and normals.

was not apparent in haemolysates. In addition, if pentosuria resulted from a lack of the hypothetical mixed oligomer $\alpha_n\beta_n$, due to a β gene mutation, then an increase in the hypothetical α-subunit oligomer (corresponding to the minor isozyme) may be expected, and no such increase was apparent. More elaborate extensions of the two structural gene models can be made, for instance by postulating the presence of a systematic regulatory gene (Paigen, 1979) for the major isozyme locus. In such a case, pentosuria could be the result of homozygosity for a mutated form of the regulatory rather than the structural gene. However, if the faint band just cathodal to what is believed to be the minor isozyme in electrophoresed samples from pentosurics can be shown to be an altered form of the major isozyme, then this would be evidence in favour of a structural rather than a regulatory gene mutation.

The presence of one out of two normally occurring L-xylulose reductases in pentosurics explains at least partly, the finding of Hankes *et al.* (1969) that pentosurics in spite of a block in the D-glucuronic acid pathway are still able to catabolise *myo*-inositol at slightly less than ten per cent of the normal rate. Hankes *et al.* state:

> "Pentosurics, with a block in the glucuronate-xylulose pathway, are able to catabolyse *myo*-inositol at less than 10% of the normal rate as judged by the conversion of inositol-^{14}C to glucose-^{14}C and $^{14}CO_2$. There is no way at present of knowing whether the pentosurics' ability to transform a little inositol to these products results from 'leakage' of L-xylulose past the metabolic block, or from the operation of an alternate metabolic route."

The presence of the minor isozyme in pentosurics clearly constitutes such a 'leak' but does not rule out the possibility of an alternate metabolic route.

Whether the minor isozyme is a hitherto undescribed enzyme or merely mitochondrial L-xylulose reductase is not known at present, although the second alternative seems unlikely because red cells invariably lack mitochondrial enzymes and, in addition, mitochondrial L-xylulose reductase is membrane bound. The minor isozyme's relatively high Michaelis constants for L-xylulose and xylitol may be an indication that these substances are not its usual substrates and that its main function is in some other pathway.

4.2. Population and family studies

Wang and van Eys's glutathione method for measuring haemolysate xylitol dehydrogenase activities has two main draw-backs: firstly, equilibrium strongly favours the reverse of the reaction catalysed and, secondly, some haemolysates bring about a significant non-xylitol dehydrogenase catalysed production of glutathione (the product measured) which has to be controlled for. The pyruvate method was the assay of choice for the population and family studies because it suffers from neither of these disadvantages; L-xylulose reductase activity is measured (the reaction proceeds in the favoured, *in vivo* direction), and no significant blank reaction takes place. Another reason for choosing the pyruvate method for the population and family studies was that there is a greater difference between the 'pentosuric' and 'normal' enzyme's Michaelis constants for L-xylulose than there is between their Michaelis constants for xylitol. By using L-xylulose as substrate, better discrimination could be achieved between normals and carriers of the pentosuria allele because a more effective substrate concentration could be chosen, i.e. one low enough to minimise the effect of the 'pentosuric enzyme' in samples from heterozygotes, while

still enabling the 'normal enzyme' to function at rates close to its V_{max}. Under such conditions, heterozygotes would be expected to show activities which are closer to the ideal level of fifty per cent of normal and pentosurics would be expected to have activities close to zero.

4.2.1. Investigations on the Lebanese family

After establishing a normal range by measuring activities in a sample of non-Jewish individuals, the pyruvate method was tried out on members of the Lebanese family in which a pentosuria allele was known to be segregating. It was found that individual family members could be placed into one of the following three categories on the basis of their L-xylulose reductase activities:

1. The proband (II-8 in Figure 3.36), a known pentosuric, showed approximately zero activity.
2. A group of individuals with normal levels of activity (above 3,5 units; the mean for normals being 4,97 ± 1,09 units).
3. A third group with intermediate levels (1,02 to 1,66 units), i.e. with levels which were well above zero but more than two standard deviations below the normal mean.

Since none of the non-Jewish subjects whose activities were measured when establishing the normal range had levels as low as those of the intermediate (third) group, it is assumed that these individuals are carriers of the pentosuria allele. The same family was studied by Politzer and Fleischmann (1962) who classified individuals as either normal or pentosuric according to whether L-xylulose could be consistently detected (by chromatography) in their urine. No quantitative data were presented and, since carriers sometimes excrete

more L-xylulose than the trace amounts present in the urine of normal individuals (Touster and Shaw, 1962), it is not surprising that Politzer and Fleischmann reached the following conclusion:

> "Acceptance of the recessive hypothesis would involve considerable assumptions, and we therefore favour the hypothesis that L-xylulosuria in this family is due to a dominant gene with poor penetrance."

Figure 4.2 allows a comparison to be made between Politzer and Fleischmann's interpretation of part of this family and that arrived at in the present study. It can be seen from Figure 4.2a that only the proband II-6 showed the high serum L-xylulose levels (measured in the present study) characteristic of pentosurics (Kumahara *et al.*, 1961, for comparison found levels of 1,2, 1,3 and 1,7 mg/100 mℓ in samples from three pentosurics), while individuals II-1, III-1 and III-2, who were classified by Politzer and Fleischmann as pentosurics, had bearly detectable levels. Figure 4.2b shows the author's interpretation, based on red cell L-xylulose reductase activities, of the same pedigree. The two classifications, Politzer and Fleischmann's and the author's, are applied to different phenotypic levels: Politzer and Fleischmann used as their criterion, the apparent presence or absence of L-xylulose in urine samples while the present author based his classification on measurements of enzyme activity. The high serum L-xylulose level of the proband correlates with her lack of L-xylulose reductase activity, but the low serum L-xylulose levels of the purported heterozygotes do not differ significantly from those of the normals, which suggests that pentosuria is recessively inherited in this family after all. Added evidence for this is that the only other reported cases of pentosuria in non-Jews occurred in three other Lebanese families (Khachadurian, 1962) and in each of these the condition was recessively inherited.

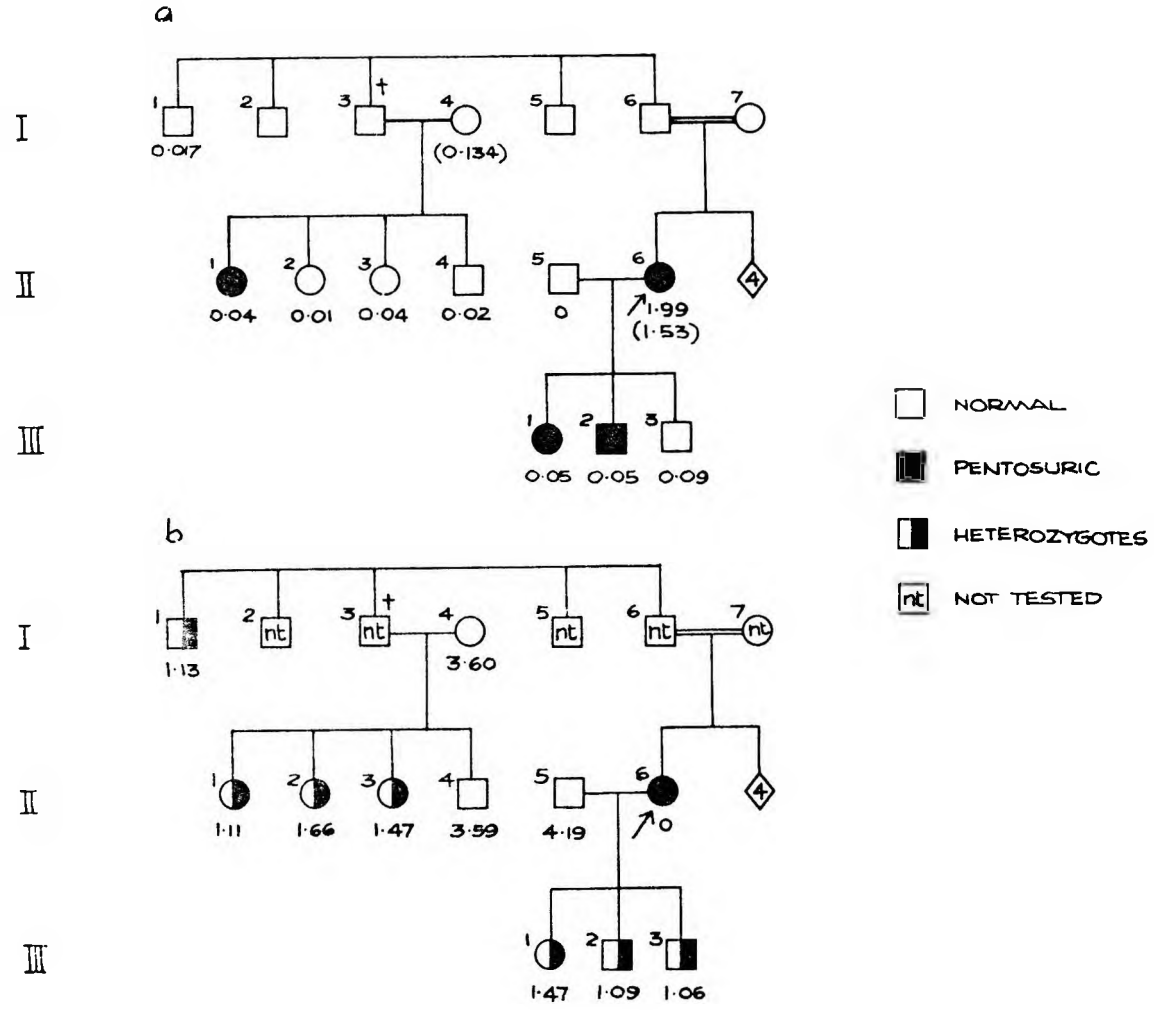


Figure 4.2. Pedigree of part of the Lebanese family studied.
(a) Shows Politzer and Fleischmann's interpretation of the family and
(b) shows the author's classification.
The figures below the symbols in (a) are the serum, and in brackets, plasma L-xylulose concentrations measured during the present study. The figures appearing below the symbols in (b) represent red cell L-xylulose reductase activities (the mean for normals was 4.97 ± 1.09 units).

Plasma L-xylulose concentrations in samples from the Lebanese and Ashkenazi pentosurics investigated in the present study were almost identical (1,53 and 1,54 mg/100 mℓ respectively), and electrophoresis of their red cell L-xylulose reductases produced the same zymogram pattern. In addition, both subjects showed approximately zero red cell L-xylulose reductase activity (as measured by the pyruvate method), and their residual L-xylulose reductase had similar Michaelis constants for xylitol, L-xylulose and NADP. These similarities do not necessarily mean that the Ashkenazi and Lebanese pentosurics have the same pentosuria causing alleles since any mutation which resulted in the absence of the major L-xylulose reductase isozyme would produce the same basic phenotype. The occurrence of pentosuria in two distinct groups (the Lebanese and Ashkenazim) may, as Khachadurian (1962) has pointed out, mean that their respective alleles arose on separate occasions. Tradition, however, and blood group data (Mourant *et al.*, 1976 and 1978), strongly suggest that the forefathers of the Ashkenazim came from the Middle-East. If this is so, then it is possible that one type of pentosuria allele was present in Middle-Eastern populations before the diaspora and that the founders of the Ashkenazim carried it with them to Europe. There are genetic similarities between the Ashkenazim and some of today's Lebanese populations which suggests that gene flow may have taken place between the populations to which their respective forefathers belonged. The Rh blood group system is most informative in this respect. Generally, people of the Middle-East (as well as the Ashkenazi and Sephardic Jews) show a relatively high frequency of the CDe haplotype and a relatively low cde frequency. In addition, cDe, a haplotype which was very likely introduced to the Middle-East as a result of interbreeding with African slaves (Patai and Wing, 1975), is elevated

in the Ashkenazim (and Sephardim) as well as in some of today's Lebanese populations. In accordance with the Rh evidence of African-Ashkenazi admixture, the Ashkenazim (as well as the Sephardim) show raised frequencies of other 'African alleles' such as the Duffy blood group *Fy* allele and the Kidd blood group Jk^a allele. Further evidence of similarities between the Ashkenazim and populations of the Middle-East was obtained by Wing (1974) who found that the Ashkenazi haptoglobin and red cell acid phosphatase frequencies resembled more closely those of Mediterranean non-Jewish populations than they did non-Jewish European populations.

If the forefathers of the Ashkenazim acquired the pentosuria allele in the Middle-East and took it with them to Europe, its frequency could have been increased there by the same process or processes which brought about the relatively high frequencies of alleles which cause conditions such as Tay-Sachs disease, Gaucher's disease dysautonomia, etc. Although the process whereby these genes reached their relatively high frequencies in the Ashkenazim cannot be identified with certainty so long after the establishment of the population, some inferences may be drawn all the same. Heterozygote advantage is often invoked as an explanation for the occurrence at polymorphic frequencies of alleles which are deleterious when homozygous, and although this almost certainly accounts for the high sickle cell, thalassaemia and variant G6PD gene frequencies in areas where malaria is endemic, it is difficult to find many other examples. Tentative evidence that heterozygote advantage could have been responsible for the high frequencies of some of the disease causing alleles in the Ashkenazim is the fact that three of the diseases (Tay-Sachs, Niemann Pick and Gaucher's disease) involve defects in sphingolipid catabolism which suggests that the same environmental factor

was countered in three similar but slightly different ways. Additional evidence favouring the idea that heterozygote advantage was responsible for the high Tay-Sachs allele frequency has been provided by Myrianthopoulos and Aronson (1966) who found that a sample of Tay-Sachs allele carriers gave rise to slightly more offspring (while living in eastern Europe) than a non-carrier control group did, although the difference was not statistically significant.

On the other hand, chance events can have an important effect on the composition of gene pools as is evidenced by the relatively high frequencies of deleterious dominant alleles in certain populations. One such example is the presence of the porphyria varigata gene at a high frequency in the South African Afrikaaner population (Dean, 1963). The fact that high frequencies of the Tay-Sachs, Gaucher and other disease causing alleles in the Ashkenazim go together with unusually low frequencies of alleles, such as that which causes phenylketonuria (Chase and McKusick, 1972), is tentative evidence that founder effect-genetic drift has operated on the gene pool of this group. The classic phenylketonuria allele is of particular interest since it occurs at appreciable frequencies in Sephardic and Oriental Jews as well as in European gentile populations, but is virtually unknown in the Ashkenazim (Cohen *et al.*, 1961). Additional evidence that founder effect-genetic drift played an important part in raising the frequencies of disease causing genes such as the Tay-Sachs and familial dysautonomia alleles in the Ashkenasim was obtained by tracing the ancestors of individuals who suffered from such diseases back to their place or origin in eastern Europe. Meals (1971) summarised the data which shows that the ancestors of Ashkenazi-Jews with differrent inherited diseases came from different

parts of eastern Europe. For instance, those with Tay-Sachs came mainly from the area surrounding Kaunas, Vilnius and Grodno in what is today the USSR, while those with dysautonomia came mainly from further south (from the area surrounding Krakow, Lvov and Budapest in southern Poland, the northwestern tip of Rumania and southwestern USSR).

4.2.2. Studies on the Ashkenazi-Jewish subjects

The distribution of red cell L-xylulose reductase activities in the Ashkenazi-Jewish sample appears to be bimodal. Parents and other close relatives of five out of the seven individuals in the low activity group were available for investigation, and in all five cases, one of the parents or close relatives (if the parents were not available) also showed similar low levels of activity. Six of the seven 'low activity' individuals were subjected to the glucuronolactone loading test (Kumahara *et al.*, 1961), and five of these showed the relatively large increases in serum L-xylulose concentrations characteristic of heterozygotes. The remaining individual did not show an increase and as her family were not available for investigation, she was assumed to be a non-carrier. One out of the seven low activity individuals was unwilling to undergo the loading test, but since one of his parents and two of his sibs had similar low L-xylulose reductase activity levels, he was considered to be a heterozygote. it is possible that some of the individuals in the high activity group were carriers of the pentosuria allele as well. However, a comparison of the allele's apparent frequency; q = 0,0127 (calculated from the apparent heterozygote frequency), with estimates arrived at by extrapolating from homozygote frequencies in other Ashkenazi populations; 0,0083 - 0,0125 (Lasker, 1952) and 0,0141 (Mizrahi and

Ser, 1963) suggests that very few, if any, heterozygotes were missed.

4.2.3. Linkage studies

Although L-xylulose reductase does not appear to be an important enzyme, its locus once mapped could serve as a 'landmark' and possibly facilitate further mapping of the human genome. Unfortunately, leukocytes (Kumahara *et al.*, 1961) and cultured skin fibroblasts (personal observation) do not exhibit appreciable L-xylulose reductase activity which means that the technique of interspecific somatic cell hybridisation would not be a suitable method by which to assign the structural gene for this enzyme. More sophisticated methods for locating gene loci such as *in situ* hybridisation of specific DNA probes to metaphase chromosomes would require the hardly justifiable expenditure of a considerable amount of time and resources. In the light of these arguments, conventional linkage studies are believed to be the most economical approach towards mapping the 'pentosuria gene', particularly with the discovery of more and more restriction fragment length polymorphisms.

So far, no strong evidence in favour of linkage between the 'pentosuria gene' locus and any of the twenty-one other gene loci investigated, has been found by this approach. The highest lod score obtained was 0,795 (at a θ value of 0,05) in favour of linkage with the 6-phosphogluconate dehydrogenase (*PGD*) locus which is situated on the short arm of chromosome 1. Positive lod scores in favour of linkage with the phosphoglucomutase-1 (PGM_1) locus which is also on the short arm of chromosome 1 were also obtained. The *PGD* and PGM_1 loci lie fairly far apart (the approximate recombination fraction in males is 0,39 and that in females 0,65 (Cook and

Hamerton, 1979)), and since negative scores were obtained for linkage between the L-xylulose reductase locus and the Rh locus which lies between *PGD* and PGM_1, it is unlikely that the L-xylulose reductase locus is linked to either of these. Furthermore, a lod score of 0,795 is far short of 3,0, the value at which the odds in favour of linkage are considered to be significant (Morton, 1955). Consistently negative lod scores were obtained for possible linkage with the Rh, Duffy and Kell blood group determining genes, the serum protein system, *Hp* and the red cell enzyme systems, ACP_1 and AK_1. Lod scores which are lower than -2,0, a value which is considered to be significant evidence against linkage (Morton, 1955) were found for the *Rh* locus at θ values of 0,05 and 0,10, as well as for the GPT_1 locus at a θ value of 0,05. Another gene which does not appear to be closely linked to the major L-xylulose reductase locus is that whose mutants cause autosomal recessive retinitis pigmentosa. Khachadurian (1962) encountered both pentosuria and retinitis pigmentosa in a Lebanese family but found that the genes for these conditions had segregated independently in various family members.

4.3. Conclusions

1. Normal individuals have at least two L-xylulose reductases in their liver tissue and red blood cells. Pentosurics lack one of these isozymes.
2. It is likely (but not proven) that the two main L-xylulose reductases of normal individuals (the major and minor isozymes) are coded for by genes at separate loci.
3. The inheritance of pentosuria in the South African Lebanese family appears to be erecssive. This finding contradicts a previous suggestion

(Politzer and Fleischman, 1962) that the condition is dominantly inherited in this family.

4. The frequency of the pentosuria allele in a sample drawn from the local Ashkenazi-Jewish population is estimated to be 0,0127.

5. No close linkage between the pentosuria allele and any of twenty-one other polymorphic gene loci was found.

4.4 Further studies

1. The purification of the major and minor isozymes and an investigation of their molecular weights and possible subunit compositions.

2. A comparison of electrophoretic mobilities, substrate affinities and susceptibility to inhibition of the mitochondrial, the major and minor isozymes.

3. A search for electrophoretically distinct variants in various populations and an extension of the linkage study to other loci such as the HLA system and those of arbitrary restriction fragment length polymorphisms.

4. An investigation of the faint band seen in electrophoresed samples from pentosurics in order to determine whether it is due to an altered form of the major isozyme.

5. A possible ultimate comparison of the Lebanese and Ashkenazi alleles at the DNA level and the chromosome assignment of the major L-xylulose reductase structural gene, by *in situ* hybridisation to metaphase chromosomes.

REFERENCES

Akedo, H., Nishihara, H., Shinkai, K., Komatsu, K. and Ishikawa, K. (1972). Multiple forms of human adenosine deaminase. I. Purification and characterization of two molecular species. Biochim. Biophys. Acta. 276, 257-271.

Alper, C.A. and Johnson, A.M. (1969). Immunofixation electrophoresis : a technique for the study of protein polymorphism. Vox Sang. 17, 445-452.

Arsenis, C. and Touster, O. (1969). Nicotinamide adenine dicucleotide phosphate-linked xylitol dehydrogenase in guinea pig liver cytosol. J. Biol. Chem. 244, 3895-3899.

Ashwell, G., Kanfer, J., Smiley, J.D. and Burns, J.J. (1960). Metabolism of ascorbic acid and related uronic acids, aldonic acids and pentoses. Ann. N.Y. Acad. Sci. 92, 105-114.

Barnes, H.D. and Bloomberg, B.M. (1953). Paper chromatography of the urinary sugar in essential pentosuria. S. Afr. J. med. Sci. 18, 93-98.

Bässler, K.H. and Reimond, W.V. (1965). Lactatbildung aus Zuckern und Zuckeralkoholen in Erythrocyten. Klin. Wschr. 43, 169-171.

Beutler, E. (1975). A manual of biochemical methods. Second edition. Grune and Stratton, New York, San Francisco, London.

Beutler, E., Duron, O. and Kelly, B.M. (1963). Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61, 882-890.

Biochemica information I (1973). Published by Boehringer Mannheim GmbH, Biochemica, 136-137.

Blakley, R.L. (1951). The metabolism and antiketogenic effects of sorbitol. Sorbitol dehydrogenase. Biochem. J. 49, 257-271.

Bloomberg, B.M., Alldis, D. and Epstein, M. (1946). Chronic essential pentosuria with report of a case. S. Afr. med. J. 20, 271-274.

Bozian, R.C. and Touster, O. (1959). Essential pentosuria : Renal or enzymatic disorder. Nature, 184, 463-464.

Burns, J.J. and Evans, C.J. (1956). The synthesis of L-ascorbic acid in the rat from D-glucuronolactone and L-gulonolactone. J. Biol. Chem. 223, 897-905.

Burns, J.J. and Kanfer, J. (1957). Formation of L-xylulose from gulonolactone in rat kidney. J. Amer. Chem. Soc. 79, 3604-3605.

Caporali, R. (1896). Pentosuria in tre morfiomani e pentosuria sperimentale. Riv. clin. e. terap. 18, 13-17.

Chakravarti, A. and Chakraborty, R. (1978). Elevated frequency of Tay-Sachs Disease among Ashkenazi Jews unlikely by genetic drift alone. Am. J. Hum. Genet. 30, 256-261.

Charalampous, F.C. (1960). Biochemical studies on inositol. IV. Mechanism of cleavage of inositol to D-glucuronic acid. J. Biol. Chem. 235, 1286-1291.

Charalampous, F.C. and Lyras, C. (1957). Biochemical studies on inositol. VI. Conversion of inositol to glucuronic acid by rat kidney extracts. J. Biol. Chem. 228, 1-13.

Chase, G.A, and McKusick, V.A. (1972). Founder effect in Tay-Sachs disease. Am. J. Hum. Genet. 24, 339-340.

Chen, S-H., Giblett, E.R., Anderson, J.E. and Fossum, B.L.G. (1972). Genetics of glutamic-pyruvic transaminase : its inheritance, common and rare variants, population distribution, and differences in catalytic activity. Ann. Hum. Genet. Lond. 35, 401-409.

Cohen, B.E., Bodonyi, E. and Szeiberg, A. (1961). Phenylketonuria in Jews. Lancet, i, 344-345.

Cook, P.J.L. and Hamerton, J.L. (1979). Report of the committee on the genetic constitution of chromosome 1. In: Evans, J.H., Hamerton, J.L., Klinger, H.P. and McKusick, V.A. eds. Human gene mapping 5 : Edinburgh Conference (1979), Fifth International Workshop on human gene mapping. Cytogenet. Cell Genet. 25, 9-20.

Dean, J. (1963). The porphyrias, a story of inheritance and environment. Pitman, London.

Dayton, P.G., Eisenberg, F. Jr. and Burns, J.J. (1958). Conversion of D-glucuronolactone, L-gulonolactone and L-ascorbic acid to glucose. Fed. Proc. 17, 209.

Eisenberg, F. J. and Gurin, S. (1952). The biosynthesis of glucuronic acid from 1-C^{14}-glucose. J. Biol. Chem. 195, 317-325.

Eisenberg, F.J., Dayton, P.G. and Burns, J.J. (1959). Studies on the glucuronic acid pathway of glucose metabolism. J. Biol. Chem. 234, 250-253.

Elston, R.C. and Stewart, J. (1971). A general model for the analysis of pedigree data. Hum. Hered. 21, 523-542.

Enklewitz, M. and Lasker, M. (1933). Studies in pentosuria. A report of 12 cases. Am. J. Med. Sci. 186, 539-548.

Enklewitz, M. and Lasker, M. (1935). The origin of *l*-xyloketose (urine pentose). J. Biol. Chem. 110, 443-456.

Dixon, M. and Webb, C. (1964). Enzymes. Second edition. Spottiswoode, Ballantyne and Co. Ltd. London and Colchester.

Fildes, R.A. and Parr, C.W. (1963). Human red cell phosphogluconate dehydrogenase. Nature 200, 890-891.

Freedberg, I.M., Feingold, D.S. and Hiatt, H.H. (1959). Serum and urine L-xylulose in pentosuric and normal subjects and in individuals with pentosuria trait. Biochem. Biophys. Res. Commun. 1, 328-332.

Garrod, A.E. (1908a). The Croonian lectures on inborn errors of metabolism. Lecture I. Lancet, ii, 1-7.

Garrod, A.E. (1908b). The Croonian lectures on inborn errors of metabolism. Lecture IV. Lancet, ii, 214-220.

Giblett, E.R. (1969). Genetic markers in human blood. Blackwell Scientific Publications, Oxford and Edinburgh.

Ginsburg, V., Weissbach, A. and Maxwell, E.S. (1958). Formation of glucuronic acid from uridine diphosphate glucuronic acid. Biochem. et. biophys. acta. 28, 649-650.

Goodman, R.M. (1979). Genetic Disorders among the Jewish People. The Johns Hopkins University Press, Baltimore and London.

Hankes, L.V., Politzer, W.M., Touster, O. and Anderson, L. (1969). Myo-inositol catabolism in human pentosurics : The predominant role of the glucuronate-xylulose-pentose phosphate pathway. Ann. N.Y. Acad. Sci. 165, 564-576.

Harris, H. and Hopkinson, D.A. (1976). Handbook of enzyme electrophoresis in human genetics. North-Holland Publishing Company. Amsterdam.

Hassan, M.U. and Lehninger, A.L. (1956). Enzymatic formation of ascorbic acid in rat liver extracts. J. Biol. Chem. 223, 123-138.

Hiatt, H.H. (1958a). Studies of ribose metabolism. IV. The metabolism of D-glucuronolactone in normal and pentosuric subjects. Biochim. biophys. Acta. 28, 645-647.

Hiatt, H.H. (1958b). Studies of ribose metabolism. VI. Pathways of ribose synthesis in man. J. Clin. Invest. 37, 1461-1464.

Hickman, J. and Ashwell, G. (1958). Purification and properties of D-xylulokinase in liver. J. Biol. Chem. 232, 737-748.

Hickman, J. and Ashwell, G. (1959). A sensitive and stereospecific enzymatic assay for xylulose. J. Biol. Chem. 234, 758-761.

Hirschhorn, R. (1975). Conversion of human erythrocyte-adenosine deaminase activity to different tissue specific isozymes. Evidence for a common catalytic unit. J. Clin. Invest. 55, 661-672.

Hollmann, S. (1960). Trennung, Reinigung und Eigenschaften der mitochondrialen Xylit-Dehydrogenasen der Meerschweinchenleber. Hoppe-Seyler's Zeitschrift Für Physiologische Chemie 317, 193-216.

Hollmann, S. and Touster, O. (1956). An enzymatic pathway from L-xylulose to D-xylulose. J. Amer. Chem. Soc. 78, 3544-3545.

Hollmann, S. and Touster, O.(1962). Alterations in tissue levels of uridine diphosphate glucose dehydrogenase, uridine diphosphate glucuronic acid pyrophosphatase, and glucuronyl transferase induced by substances influencing the production of ascorbic acid. Biochim. Biophys. Acta. 62, 338-352.

Horowitz, H.H., Doerschuk, A.P. and King, C.G. (1952). The origin of L-ascorbic acid in the albino rat. J. Biol. Chem. 199, 193-198.

Horowitz, H.H. and King, C.G. (1953). Glucuronic acid as a precursor of ascorbic acid in the albino rat. J. Biol. Chem. 205, 815-821.

Human Gene Map (1983). Am. J. Hum. Genet. 35, 134-156.

Isherwood, F.A., Chen, Y.T. and Mapson, L.W. (1953). Synthesis of L-ascorbic acid in plants and animals. Nature 171, 348-349.

Janeway, T.C. (1906). Essential pentosuria in two brothers. Am. J. Med. Sci. 132, 423-428.

Kanfer, J., Burns, J.J. and Ashwell, G. (1959). L-ascorbic acid synthesis in a soluble enzyme system from rat-liver microsomes. Biochim. Biophys. Acta. 31, 556-558.

Khachadurian, A.K. (1962). Essential pentosuria. Amer. J. Hum. Genet. 14, 249-255.

Knox, W.E. (1958). Sir Archibald Garrods "Inborn errors of metabolism". IV. Pentosuria. Amer. J. Hum. Genet. 10, 385-397.

Koestler, A. (1976). The Thirteenth Tribe. The Khazar Empire and its heritage. Hutchinson, London.

Kornberg, A. (1955). Nucleotide pyrophosphatase. In: Colowick, S.P. and Kaplan, B.O. eds. Methods in Enzymol. II, 665-659. Academic Press, New York.

Kühnl, P., Spielmann, W. and Loa, M. (1978). An improved method for the identification of Gc' substypes (Group-specific component) by isoelectric focusing. Vox Sang. 35, 401-404.

Kumahara, Y., Feingold, D.S., Freedberg, I.M. and Hiatt, H.H. (1961). Studies of pentose metabolism in normal subjects and in patients with pentosuria and pentosuria trait. J. Clin. Endocr. 21, 887-894.

Larson, H.W., Blatherwick, N.R., Bradshaw, P.J., Ewing, M.E. and Sayer, S.D. (1941). The metabolism of L-xylulose. J. Biol. Chem. 138, 353-360.

Lasker, M. (1952). The geographic distribution of heterozygotes for essential pentosuria. Am. J. Phys. Anthropol. 10, 258.

Lasker, M. (1955). Mortality of persons with xyloketosuria. A follow-up study of a rare metabolic anomaly. Hum. Biol. 27, 294-300.

Lasker, M., Enklewitz, M. and Lasker, G.W. (1936). The inheritance of l-xyloketosuria (essential pentosuria). Hum. Biol. 8, 243-255.

Levene, P.A. and La Forge, F.B. (1914). Note on a case of pentosuria. J. Biol. Chem. 18, 319-327.

Longenecker, H.E., Fricke, H.H. and King, C.G. (1940). The effect of organic compounds upon vitamin C synthesis in the rat. J. Biol. Chem. 135, 497-510.

Margolis, J.I. (1929). Chronic pentosuria and migraine. Am. J. Med. Sci. 177, 348-371.

Meals, R.A. (1971). Paradoxical frequencies of recessive disorders in Ashkenazi Jews. J. Chronic Dis. 23, 547-558.

Mizrahi, O. and Ser, I. (1963). Essential pentosuria. In: Goldschmidt, E. ed. Genetics of migrant and isolate populations p 300. Williams and Wilkins, Baltimore.

Morton, N.E. (1955). Sequential tests for the detection of linkage. Am. J. Hum. Genet. 7. 277-318.

Mosbach, E.H. and King, C.G. (1950). Tracer studies of glucuronic acid biosynthesis. J. Biol. Chem. 185, 491-498.

Mourant, A.E. and Kopeć, A.C. (1976). The distribution of the human blood groups and other polymorphisms. Second Edition. London Oxford University Press, New York Toronto.

Mourant, A.E., Kopeć, A.C. and Domaniewska-Sobczak, K. (1978). The genetics of the Jews. Clarendon Press. Oxford.

Musulin, R.R., Tully, R.H., Longenecker, H.E. and King, C.G. (1939). Vitamin C synthesis and excretion by the rat. J. Biol. Chem. 129, 437-453.

Myrianthopoulos, N.C. and Aronson, S.M. (1966). Population dynamics of Tay-Sachs disease. I : Reproductive fitness and selection. Am. J. Hum. Genet. 18, 313-327.

McCorkindale, J. and Edson, N.L. (1954). Polyol dehydrogenases. I. The specificity of rat-liver polyol dehydrogenase. Biochem. J. 57, 518-523.

Neuberg, C. (1900). Ueber die Harnpentose ein optisch inatives naturlich vorkomnendos Kohlehydrat. Ber. Deutsch. Chem. Ges. 33, 2243-2254.

Ott, J. (1974). Estimation of the recombination fraction in human pedigrees : efficient computation of the likelihood for human linkage studies. Am. J. Hum. Genet. 26, 588-597.

Packham, M.A. and Butler, G.C. (1954). The fate of injected sodium glucuronate and glucurone in the rat. J. Biol. Chem. 207, 639-646.

Paigen, K. (1979). Acid hydrolases as models of genetic control. Ann. Rev. Genet. 13, 417-466.

Patai, R. and Patai Wing, J. (1975). The myth of the Jewish race. Charles Scribner's Sons, New York.

Politzer, W.M. and Fleischmann, H. (1962). L-xylulosuria in a Lebanese family. Amer. J. Hum. Genet. 14, 256-260.

Price, R.G. and Dance, N. (1972). The demonstration of multiple heat stable forms of N-acetylglucosaminidase in normal human serum. Biochim. Biophys. Acta. 271, 145-153.

Race, R.R and Sanger, R. (1968). Blood groups in Man. Fifth edition. Blackwell Scientific publications. Oxford and Edinburgh.

Reale, E. (1894). Second case of pentosuria in a morphine addict. Riv. clin. e terap. 16, 118-130.

Salkowski, E. and Jastrowitz, M. (1892). Uber eine bisher nich beobachtete Zuckerart im Harn. Centralbl. med. Wissensch. 30, 337-339.

Saron, G. (1965). In: Feldberg, G. ed. South African Jewry 1965. Alex White.

Schultz, J.C. (1938). Onderzoekingen over Pentosurie. Kosmos, Amsterdam.

Strominger, J.L., Kalckar, H.M., Axelrod, J. and Maxwell, E.S. (1954). Enzymatic oxidation of uridine diphosphate glucose to uridine diphosphate glucuronic acid. J. Am. Chem. Soc. 76, 6411-6412.

Teisberg, P. (1970). High voltage agarose gel electrophoresis in the study of C3 polymorphism. Vox Sang. 19, 47-56.

Touster, O. (1959). Pentose metabolism and pentosuria. Amer. J. Med. 26, 724-739.

Touster, O. (1962). D- and L-*threo*-Pentulose (D- and L-xylulose). Pyrimidine-catalysed epimerization of xylose. In: Whistler, R.L. and Wolfrom, M.L. eds. Methods of carbohydrate chemistry, I. 98-101. Academic Press, New York.

Touster, O., Hutcheson, R.M. and Reynolds, V.H. (1954). The formation of L-xylulose in mammals and its utilization by liver preparations. J. Amer. Chem. Soc. 76, 5005-5006.

Touster, O., Hutcheson, R.M. and Rice, L. (1955). The influence of D-glucuronolactone on the excretion of L-xylulose by humans and guinea pigs. J. Biol. Chem. 215, 677-684.

Touster, O., Mayberry, R.H. and McCormick, D.B. (1957). The conversion of 1-^{13}C-D-glucuronolactone to 5-^{13}C-L-xylulose in a pentosuric human. Biochem et biophys. Acta. 25, 196-198.

Touster, O., Reynolds, V.H. and Hutcheson, R.M. (1956). The reduction of L-xylulose to xylitol by guinea pig liver mitochondria. J. Biol. Chem. 221, 697-709.

Touster, O. and Shaw, D.R.D. (1962). Biochemistry of the acyclic polyols. Pysiol. Rev. 42, 181-225.

Wagener, D., Cavalli-Sforza, L.L. and Barakat, R. (1978). Ethnic variation of genetic disease : Roles of drift for recessive lethal genes. Am. J. Hum. Genet. 30, 262-270.

Wang, Y.M. and van Eys, J. (1970). The enzymatic defect in essential pentosuria. New Engl. J. Med. 282, 892-896.

Wing, J.P. (1974). Blood protein polymorphism in Jewish populations. Hum. Hered. 24, 323-344.

Winkelman, J. and Ashwell, G. (1961). Enzymatic formation of L-xylulose from β-keto-L-gulonic acid. Biochim. Biophys. Acta. 52, 170-175.

Wright, W.T. (1961). Incidence of pentosuria. N. Engl. J. Med. 265, 1154.

Zerner, E. and Waltuch, R. (1913). Ein beitrag zur kenntnis der Pentosurie von chemischen standpunkt. Mschr. Chem. 34, 1639-1652.

Zerner, E. and Waltuch, R. (1914). Zur Frage des Pentosuriezuckers. Biochem. Z. 58, 410-412.